STEROIDS AND THE BRAIN

EDITED BY: Takayoshi Ubuka, Ishwar Parhar and Vance L. Trudeau
PUBLISHED IN: Frontiers in Endocrinology and Frontiers in Neuroscience

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88963-851-2
DOI 10.3389/978-2-88963-851-2

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STEROIDS AND THE BRAIN

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Citation: Ubuka, T., Parhar, I., Trudeau, V. L., eds. (2020). Steroids and the Brain. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-851-2

Table of Contents

frontiers in Endocrinology

EDITORIAL
published: 03 June 2020
doi: 10.3389/fendo.2020.00366

Editorial: Steroids and the Brain

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Keywords: neurosteroids, sexual dimorphism, sexual behavior, memory, neuropsychiatric disorders, Alzheimer's disease, stress, endocrine disrupting chemicals

Editorial on the Research Topic

Steroids and the Brain

Steroids contain the perhydrocyclopentanophenanthrene ring in their chemical nuclei. In vertebrates, steroids are synthesized in gonads, adrenal, and other endocrine glands and secreted into general circulation as hormones. Steroids and their receptors play significant roles in broad functions of the brain, such as regulation of socio-sexual behavior, aggression, neurogenesis, learning and memory, stress, cognition, mood and emotion. However, the brain is not only a target of steroids action but may also be the site of *de novo* synthesis from cholesterol or their precursors entering the brain. Malfunctions of steroid synthesis and signaling are related to a variety of human disorders such as gender dysphoria, anxiety, depression, autism spectrum disorder, and aging related diseases notably Alzheimer's, among others. Therefore, this Research Topic aimed to collect knowledge in all aspects of steroid function in the brain from an evolutionary to physiological and pathological standpoints, which may bring new insights into steroid actions. The subtopics include neurosteroids, sex steroids and sexual dimorphism, learning, memory, various neuropsychiatric disorders, stress and steroids, and endocrine disrupting chemicals.

NEUROSTEROIDS

Neurosteroids are metabolic steroids synthesized from cholesterol in the central and the peripheral nervous systems (1, 2). The first article, a perspective of Steroids and Brain, is written by Baulieu who was the first to discover local synthesis of steroids in the brain (3). This perspective specifically reports on MAP4343, a synthetic pregnenolone-derivative. Additionally discussed is FKBP52, a key protein component of hetero-oligomeric steroid receptors, which interacts with Tau protein, thus playing important roles in Alzheimer's disease and other dementias.

The second article by Diotel et al. reviews neurosteroidogenesis and signaling of estrogen, progestogen, and androgen in the brain of fish, birds, and mammals and discusses the roles of sex steroids in neurogenesis, neuroprotection, and sexual behavior (4). This review further probes how steroids and lipoproteins are transported between the periphery and the brain. The authors emphasize the beneficial effects of steroids and lipoproteins against ischemic stroke, also highlighting their potential anti-inflammatory, antioxidant, and neuroprotective properties.

The third article by Da Fonte et al. is an original research article that reports on secretoneurin A (SNa) and regulation of goldfish radial glial cells (RGCs). The neuropeptide SNa is derived from proteolytic processing of the secretogranin-2 in magnocellular cells within the RGC rich preoptic nucleus. Radial glial cells are the main macroglia and have established roles in neuroesgtogen synthesis as the only site for aromatase B expression in the teleost brain, a characteristic linked to neurogenesis (5). Their previous study indicated that SNa inhibits the expression of aromatase B that converts estrogens from androgen in RGCs (6). The new results based on transcriptomic analysis suggest additional roles for SNa in the control of cell proliferation and neurogenesis.

OPEN ACCESS

Edited and reviewed by:
Hubert Vaudry, Université de Rouen, France

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *14 April 2020*
Accepted: *11 May 2020*
Published: *03 June 2020*

Citation:
Ubuka T, Trudeau VL and Parhar I (2020) Editorial: Steroids and the Brain. Front. Endocrinol. 11:366. doi: 10.3389/fendo.2020.00366

The next original research article by Ulhaq and Kishida investigated the role of aromatase B (7) in the development of serotonergic neurons. Aromatase B is highly expressed during early development of the zebrafish brain. Early development of serotonergic neurons is also considered to play important roles in neurogenesis. In this article, they tested the effect of estradiol administration and morpholino mediated aromatase B knockdown in zebrafish embryos and larvae. Their results suggest that neuroestrogen synthesis sustains early development of serotonergic neurons.

Brain-derived steroids also act locally to affect socio-sexual behaviors (8) and locomotor movements associated with migration (9). Wingfield et al. discuss how and why neurosteroid production evolved and why peripherally produced steroids do not always fulfill central roles. Their investigations on free-living animals suggest that neurosteroids may have evolved to regulate specific behavior throughout the year independently of different life history stages. They highlight two examples. The first is the control of territorial aggression of songbirds in autumn by sex steroid production from circulating precursors such as dehydroepiandrosterone (DHEA) or *de novo* in the brain. The second example is the production of 7α-hydroxypregnenolone within the brain that appears to affect locomotor behavior in several contexts.

SEX STEROIDS AND SEXUAL DIMORPHISM

Sex steroids coordinate the development and maintenance of the central nervous system. In the first article of this subtopic, Larson discusses the relationship between sex steroids and neuroinflammation, and the impact on neuropsychiatric and neurodegenerative disorders (10). She highlights the complex interactions between sex steroids, neuroinflammation, and regeneration of the central nervous system through adult neurogenesis.

Sex steroids also play key roles in the regulation of social recognition, reproductive behavior and parental care, which are highly sexually dimorphic. However, contribution of sex steroids in modulating adult neurogenesis in the forebrain ventricular-subventricular zone that continuously generates new neurons throughout life is underestimated (11). Ponti et al. review the literature describing sexual dimorphism and sexual differences across the physiological phases.

Steroids play important roles in sexually dimorphic brain development during perinatal and pubertal periods. It was previously demonstrated that estrogen receptor α and aromatase genes are essential to sexual differentiation of the anteroventral periventricular nucleus (AVPV) and the principal nucleus of the bed nucleus of the stria terminalis (BNSTp) in mammals (12, 13). Androgen receptor gene is also essential to sexual differentiation of the BNSTp. Kanaya et al. studied if these genes are sexually differentially expressed in the AVPV and BNSTp during puberty. Their results suggest that testicular testosterone may affect the formation of male BNSTp during puberty *via* androgen receptor and estrogen receptor α after conversion to estradiol by aromatase.

Cao et al. review sex differences in glutamatergic synaptic inputs and intrinsic excitability of rat medium spiny neurons, the output neurons of the striatum (14). They also review evidence for estradiol-mediated sexual differentiation in the nucleus accumbens core (15). The striatal brain regions including the caudate-putamen, nucleus accumbens core and shell are interesting because they express membrane-associated but not nuclear estrogen receptors. The authors conclude that striatal brain regions exhibit heterogeneity in sex differences in electrophysiological properties.

Although estrogens play important roles in sexual dimorphism of the brain, whether and how estrogens regulate the cerebral cortex are not fully understood. Denley et al. review evidence that estrogens regulate the molecular machinery required for fine-tuning the processes central to the cortex (16). The authors also discuss how estrogens regulate the function of the key molecules and signaling pathways involved in corticogenesis and highlight whether these processes are sexually dimorphic.

Funabashi et al. hypothesize that transsexual humans produce different gonadotropin levels in response to sex steroids stimulation, because the bed nucleus of the stria terminalis was suggested to be involved in gender identity and this brain area is involved in gonadotropin secretion (17). The authors examined if estrogen combined with progesterone leads a change in gonadotropin secretion in female-to-male, male-to-female transsexual, and control subjects. Their results suggest that the brain area related to gender identity may also be involved in gonadotropin secretion in humans.

The next original research article by Sano et al. investigated the role of estrogen receptor β in the dorsal raphe nucleus on female sexual behavior in mice. Previously, the authors showed that estrogen receptor β may have an inhibitory role in lordosis behavior of female mice (18). This study focused on the dorsal raphe nucleus that expresses estrogen receptor β in higher density than estrogen receptor α (19). Specific knock down of estrogen receptor β in the dorsal raphe nucleus showed the inhibitory role of estrogen receptor β in this nucleus on sexual behavior on the day after estrous in cycling female mice.

The last article of this subtopic discusses retinal disorders by Nuzzi et al. Epidemiological studies and research articles indicate a correlation between many retinopathies and sex due to potential effects of sex steroids against the development of certain disorders (20). For example, macular holes are more common in women than men, particularly in post-menopausal women. The course of retinitis pigmentosa appears to be ameliorated by progestin therapy. Diabetic retinopathy appears to be more common among men than women. The authors conclude that sex steroids may be useful for the treatment of eye diseases, particularly retinal disorders.

LEARNING, MEMORY, AND VARIOUS NEUROPSYCHIATRIC EFFECTS

Original research by Jakob et al. concerns the interactions of estrogen and the genotype of dopamine transporter (DAT1) in reinforcement learning in humans (21). The authors assessed how the natural rise of 17β-estradiol (E2) in the late follicular phase and the 40 base-pair variable number tandem repeat polymorphism of DAT1 affects reinforcement learning capacity. Their data suggest an interaction of DAT1 genotype and the transient hormonal state. They found that carriers of the 9-repeat allele experienced a significant decrease from early to late follicular phase in the ability to avoid punishment.

Ratner et al. extend their discovery of positive and negative neurosteroid allosteric modulators of GABA type-A, NMDA, and non-NMDA type glutamate receptors (22, 23) toward a state-of-the art view of how modulation of neural circuitry may affect memory and memory deficits. They conclude that the effects of neurosteroids on neural networks across the life span of males and females point to an underlying pharmacological connectome that may modulate memory across diverse altered states of mind.

The article by Hojo and Kawato reviews the local production of sex steroids in the hippocampus, a center for learning and memory in adult rodents. Hippocampal principal neurons have a complete system for sex steroids biosynthesis in males. Another recent study from the same group clarified that the levels of hippocampal steroids fluctuate across the estrous cycle in adult female rats (24). They also introduce a direct evidence of the role of hippocampal neurosteroids in hippocampal function including neurogenesis, long-term potentiation, and memory consolidation (25).

Hippocampal sex steroids including 5α-dihydrotestosterone (DHT), testosterone (T), and E2 rapidly modulate dendritic spines, which is essential for synaptic plasticity and memory (26). Soma et al. investigated the possible involvement of Src tyrosine kinase in the rapid changes of dendritic spines in response to DHT, T, and E2 using hippocampal slices of adult male rats. DHT, T, and E2 increased the total density of spines, and differentially modified the morphology of spines. However, a Src tyrosine kinase inhibitor completely blocked the increases in spine numbers induced by these steroids, indicating that Src kinase is essentially involved in non-genomic modulation of spine density and morphology induced by sex steroids.

Domonkos et al. reviewed the effects of T on anxiety during development in rodents (27). It was found that females are less anxious than males from puberty to middle age. Early organizational effects of T may influence anxiety-like behavior of females and males. However, it may be modified by activational effects of T and its metabolites. They conclude that the effects of sex steroids leading to anxiogenesis or anxiolysis depend on factors that affect hormonal status, such as age (28).

Aggression is an essential social behavior that increases survival and reproductive fitness. Munley et al. discuss the neuroendocrine mechanism of aggression in Siberian hamsters which display robust neural, physiological, and behavioral changes across seasons. The authors showed considerable evidence that DHEA, an adrenal hormone precursor, is important in maintaining aggression during the non-breeding season both in male and female hamsters (29). They conclude that adrenal DHEA likely serves as an essential precursor for neural androgen synthesis during non-breeding season (30).

Previously, it was found that E2 replacement in ovariectomized female rats reduced seizure related damage in the sensitive hilar region of hippocampal dentate gyrus (31). Iacobas et al. determine the protective effects of E2 against kainic acid-induced status epilepticus associated transcriptome alterations in the dentate gyrus of ovariectomized female rats. Their results suggest that the estrogen signaling pathway acts like a buffer against status epilepticus induced alteration of neurotransmission, which possibly contributes to E2 mediated maintenance of brain function after status epilepticus in post-menopausal women.

Tobiansky et al. highlight how androgens alter behavioral flexibility, decision making, and risk taking in their review article. After reviewing the neuroanatomy of the mesocorticolimbic system, they present evidence that androgen and other steroid receptors are present in the mesocorticolimbic system (32). They then describe evidence for local androgen synthesis in mesocorticolimbic regions (33). This review also describes how androgens modulate the neurochemistry and structure of the mesocorticolimbic system, especially the dopaminergic system. Finally, they discuss how androgens influence executive functions.

It has been observed that pervasive age-related dysfunction in hypothalamic-pituitary-gonadal axis is associated with cognitive impairments in aging and age-related neurodegenerative diseases such as Alzheimer's disease. Although estrogen modulates cognition, the effect of estrogen replacement therapy on cognition and disease diminishes with advancing age. Bhatta et al. highlight the important role for luteinizing hormone in brain function (34, 35).

STRESS AND STEROIDS

Pinna explains that the endocannabinoid system and the biosynthesis of neuroactive steroids are involved in the neuropathology of post-traumatic stress disorder (PTSD) and major depressive disorders. The author suggests that establishing a biomarker axis for PTSD is useful to define the disorder (36). Allopregnanolone biosynthesis is downregulated in PTSD patients and stimulation of neurosteroidogenesis may be a useful strategy to treat PTSD. The author claims that peroxisome-proliferator activated receptor-α can be a target of the endocannabinoid system to enhance neurosteroidogenesis.

The article by Frost et al. is an original research on the effect of childhood emotional abuse on the associations of corticomotor white matter structure and stress neuromodulators in women with and without depression. Although experience

of adversity alters the activity of the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis, the underlying neural pathways are not understood well (37). The authors investigated 74 women who exhibit depression severity and/or childhood emotional abuse. They used diffusion tensor imaging to examine if the structure of white matter predicts differences in the interaction of the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis as a function of early adversity. Their findings suggest that corticomotor projections may be a key to altered neural circuitry in adults with history of childhood emotional abuse.

van Campen et al. studied if stress and corticosteroids aggravate morphological changes in the dentate gyrus of the hippocampus after early-life febrile seizures in mice. It is suggested that stress is a seizure precipitant in patients with epilepsy (38). The authors investigated the consequences of ear corticosteroid exposure for epileptogenesis in mice. They investigated structural and functional plasticity in the dentate gyrus, such as changes in neurogenesis, morphology, mossy fiber sprouting, glutamatergic postsynaptic currents, and long-term potentiation. The results show that corticosterone exposure during early epileptogenesis elicited by experimental febrile seizures aggravates morphological but not functional changes in dentate gyrus.

Metabolism of glucocorticoids occurs in the brain by the actions of 11β-hydroxysteroid dehydrogenases (11β-HSD1, 11β-HSD2) (39). Rensel et al. measured 11β-HSD1, 11β-HSD2, glucocorticoid, and mineralocorticoid receptor (GR, MR) expressions in the songbird brain. 11β-HSD2, GR, and MR mRNAs were expressed throughout the adult brain. 11β-HSD2 expression covaried with GR and MR mRNAs in several brain regions. Although 11β-HSD1 mRNA was undetectable in the adult brain, the brain of developing bird expressed low levels of 11β-HSD1 mRNA. These results suggest that 11β-HSD2 protects the adult songbird brain by rapid metabolism of glucocorticoids.

ENDOCRINE DISRUPTING CHEMICALS

Bisphenol A (BPA) is a xenoestrogen, which is widely used in plastic products and considered an environmental endocrine disruptor. It is thought that BPA affects normal brain development by interfering with neuronal differentiation because steroids play significant roles in brain development. Fujiwara et al. investigated the effects of BPA and bisphenol F [BPF, (40)], an alternative chemical of BPA, on neural differentiation using a human fetus-derived neural progenitor cell-line. Their results showed that BPA but not BPF decreased β III-tubulin mRNA and β III-tubulin, suggesting that BPA potentially disrupts human brain development.

The last article by Ubuka et al. searched for BPA responsive genes in the rat brain to understand modifications to neurodevelopmental processes and behavior in later life. They used transgenic rats carrying enhanced green fluorescent protein tagged to gonadotropin-inhibitory hormone (GnIH) promotor (41). GnIH is a hypothalamic neuropeptide that has inhibitory effects on gonadotropin secretion and behavior (42, 43). They found upregulation of transmembrane protease serine 2 (Tmprss2) and downregulation of Forkhead box A1. Tmprss2 immunoreactivity was observed in 26.5% of GnIH neurons in the hypothalamus of 3-day-old male rat. Their results suggest that BPA disturbs the neurodevelopmental process and behavior by modifying Tmprss2 and Foxa1 expressions in the brain.

AUTHOR CONTRIBUTIONS

TU wrote the manuscript. VT and IP edited the manuscript.

ACKNOWLEDGMENTS

We thank the authors who contributed their articles to this Research Topic. We also thank the reviewers who kindly accepted to review the manuscripts submitted to this Research Topic and gave important comments to improve the manuscripts.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 26 January 2018
doi: 10.3389/fendo.2018.00003

Stress and Corticosteroids Aggravate Morphological Changes in the Dentate Gyrus after Early-Life Experimental Febrile Seizures in Mice

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: 19 November 2017
Accepted: 05 January 2018
Published: 26 January 2018

Citation:
van Campen JS, Hessel EVS, Bohmbach K, Rizzi G, Lucassen PJ, Lakshmi Turimella S, Umeoka EHL, Meerhoff GF, Braun KPJ, de Graan PNE and Joëls M (2018) Stress and Corticosteroids Aggravate Morphological Changes in the Dentate Gyrus after Early-Life Experimental Febrile Seizures in Mice. Front. Endocrinol. 9:3. doi: 10.3389/fendo.2018.00003

Stress is the most frequently self-reported seizure precipitant in patients with epilepsy. Moreover, a relation between ear stress and epilepsy has been suggested. Although ear stress and stress hormones are known to influence seizure threshold in rodents, effects on the development of epilepsy (epileptogenesis) are still unclear. Therefore, we studied the consequences of ear corticosteroid exposure for epileptogenesis, under highly controlled conditions in an animal model. Experimental febrile seizures (eFS) were elicited in 10-day-old mice by warm-air induced hyperthermia, while a control group was exposed to a normothermic condition. In the following 2 weeks, mice received either seven corticosterone or vehicle injections or were left undisturbed. Specific measures indicative for epileptogenesis were examined at 25 days of age and compared with vehicle injected or untreated mice. We examined structural [neurogenesis, dendritic morphology, and mossy fiber sprouting (MFS)] and functional (glutamatergic postsynaptic currents and long-term potentiation) plasticity in the dentate gyrus (DG). We found that differences in DG morphology induced by eFS were aggravated by repetitive (mildly stressful) vehicle injections and corticosterone exposure. In the injected groups, eFS were associated with decreases in neurogenesis, and increases in cell proliferation, dendritic length, and spine density. No group differences were found in MFS. Despite these changes in DG morphology, no effects of eFS were found on functional plasticity. We conclude that corticosterone exposure during early epileptogenesis elicited by eFS aggravates morphological, but not functional, changes in the DG, which partly supports the hypothesis that ear stress stimulates epileptogenesis.

Keywords: stress, corticosteroids, epilepsy, epileptogenesis, febrile seizures, hyperthermia, early-life

Abbreviations: DG, dentate gyrus; eFS, experimental febrile seizures; HT, hyperthermia; NT, normothermia.

INTRODUCTION

Epilepsy is a common neurological disorder, especially in childhood where its prevalence is as high as 0.5–1.0% (1). An important factor influencing epilepsy and epileptic seizures is stress, which is the most frequently self-reported seizure precipitant in patients with epilepsy [reviewed in Ref. (2)]. The seizure precipitating effects of stress are also confirmed by prospective studies (3–7). Besides direct effects on seizure susceptibility, stress can also influence the risk of being diagnosed with epilepsy later in life (8–10). Thus, associations between stress and epilepsy exist on multiple levels. However, the mechanisms behind these relations are so far poorly understood.

Animal models can provide more insight into the exact mechanisms by which stress influences epilepsy. In various preclinical epilepsy models, stress has been shown to lower the threshold for the induction of epileptic seizures and to increase seizure severity (11–16). The effects of stress on epilepsy are largely attributed to neuronal exposure to stress hormones. Especially stress hormone exposure early in life can have profound effects on later brain morphology and function and predispose to the development of brain diseases [as reviewed in Ref. (17–21)]. Stress hormones have been shown to directly affect neuronal excitability [reviewed in Ref. (22, 23)]. Despite these effects of stress on seizures on the one hand, and on brain development on the other, effects of stress and stress hormones on the *development* of epilepsy (i.e., epileptogenesis) are currently unknown.

To improve insight into the impact of stress hormones on epileptogenesis, we studied the effects of corticosterone, an important stress hormone, during early-life epileptogenesis on neuronal morphology and functional plasticity in the rodent brain. Using a controlled design, we elicited experimental febrile seizures (eFS) in young mouse pups by warm-air induced hyperthermia (HT) and subsequently exposed them to repetitive (1) high concentrations of corticosterone, (2) vehicle injections (a control condition that is also a mild stressor), or (3) no injections. We next examined alterations in morphological and functional parameters in the dentate gyrus (DG), a hippocampal subarea that is affected by eFS (24–28) as well as stress hormones (29). To assess morphological changes, we investigated neurogenesis, cell proliferation, dendritic morphology, spine density, and mossy fiber sprouting (MFS). Functional plasticity was assessed measuring glutamatergic transmission and long-term potentiation (LTP) in the DG. We hypothesize that corticosterone aggravates the epileptogenic changes after eFS.

MATERIALS AND METHODS

Animals

Breeding pairs of C57BL6/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and subsequently bred in-house. Litters used in this experiment were derived from multiple breeding pairs ($n = 99$). On postnatal day (P) 1, litters were culled to four to six pups consisting of both males and females. The pups were not weaned during the experiment. Animals were kept in a controlled 12-h light–dark cycle (light on 7 a.m. to 7 p.m.) with a temperature of 22 ± 1°C. Food and water were available *ad libitum* (2111 RMH-TM diet; Hope Farms, Woerden, the Netherlands). All animals were housed in transparent Plexiglas cages (Macrolon type II) with sawdust bedding and paper tissues for nest building. Cages were cleaned at P7 and at P17/18 (in between injection days) by replacing half of the sawdust bedding. All experimental procedures were performed according to the institutional guidelines of the University Medical Center Utrecht and approved by the committee on ethical considerations in animal experiments of Utrecht University (DEC Utrecht, permit number 2012.I.03.047). All efforts were made to minimize suffering of the animals. Pups were assigned to multiple treatment groups per litter. A maximum of two pups per litter was used per treatment group per method of analysis to minimize litter effects on outcome measures. All animal experiments were performed within a period of 6 months and animals in all treatment groups were tested across the whole period to control for environmental or seasonal variation. For the purpose of this study, experiments were only performed on male animals.

Corticosterone Levels after Injection

To evaluate corticosterone levels after injection with corticosterone or vehicle, naïve P12 mice ($n = 4$–6 per time point per treatment group) were injected intraperitoneally with corticosterone (corticosterone-HBC complex 3 mg/kg dissolved in saline, total injection volume of 10 µl/g body weight) or vehicle (both obtained from Sigma-Aldrich, the Netherlands) between 8.30 and 9.00 a.m. Immediately before, or at 15, 30, 60, 120, 180, or 240 min after injection, mice were decapitated and trunk blood was collected. Between injection and decapitation, pups were returned to their home cage and left undisturbed. A separate group of non-injected mice was decapitated at the same time points to control for diurnal corticosterone variability.

Epileptogenesis

Epileptogenesis was induced using the HT-induced eFS model, a very subtle epilepsy model with close resemblance to the human situation in which children who experience complex febrile seizures are at increased risk to develop temporal lobe epilepsy later in life (30, 31). A unique aspect of this model is the relatively long-lasting latent phase of epileptogenesis, making it easier to study effects of additional risk factors prior to the actual onset of epilepsy, and irrespective of the damage and compensatory mechanisms induced by spontaneous seizures.

Prolonged eFS were induced in P10/11 mice by heated-air induced HT using a previously described paradigm in rats (32), which we adapted to mice ((33)). A temperature-sensitive transponder (IPTT-300 BioMedic Data Systems, Plexx BV, Elst, the Netherlands) was implanted subcutaneously on P9/10 in pups with a bodyweight between 5.0 and 6.5 g. One day after transponder implantation, body weight was determined and mice were placed in a preheated cylindrical chamber and exposed to a warm air stream of 41–48°C. To prevent skin burn and adverse effects on behavior, the temperature of the chamber floor was maintained at 39°C. Core body temperature was measured at least every 2.5 min period using a wireless temperature reader (WRS-6007; Plexx BV). To provoke prolonged seizures, air

temperature was adjusted to maintain the core body temperature between 41.5 and 42°C. The presence of tonic–clonic convulsions was monitored by observation. These behavioral seizures correlate closely with electroencephalographic seizures, i.e., spike-wave discharges in the hippocampus, as shown by previous experiments in our lab (27, 34). After 30 min of HT (defined as core temperature ≥ 39°C) pups were rapidly cooled in a water bath at room temperature, gently dried with paper and returned to the dam. This procedure is known to induce epileptogenesis, as spontaneous (encephalographic) seizures are observed after a latent period of approximately 3 months in 35–68% of animals (26, 35, 36), and epileptiform interictal discharges in 88% (35). normothermia (NT) controls were treated as HT pups, except that the temperature of the air stream was kept at 30–32°C, resulting in a constant body temperature. All eFS experiments were performed between 10.00 a.m. and 3.00 p.m.

Injections and Decapitation

Animals were weighed at 2, 4, 6, 8, 10, 12, and 14 days after exposure to HT/NT and injected intraperitoneally with corticosterone or vehicle between 8.00 and 9.30 a.m., during the circadian trough. A separate group of animals was left undisturbed after HT/NT. One day (~24 h) after the last injection, mice were weighed and decapitated or perfused (see **Figure 1**). Decapitation was performed between 8.00 and 9.30 a.m.

Endocrinology

Trunk blood samples were collected immediately after decapitation at P25/26 from all animals subjected to HT/NT except those receiving perfusion fixation. Directly after decapitation, thymus and both adrenals were resected and weighed. Blood samples were centrifuged for 10 min at 4,000 rpm at 4°C. Plasma was stored at −80°C until assayed with an I^{125}-corticosterone radioimmunoassay for mice (MP Biomedicals, Inc., Aberdeen, UK) according to the manufacturer's instructions. All samples were processed in the same assay to exclude inter-assay variability.

Morphology and Functional Plasticity

All morphological and functional outcome parameters were examined in the DG. This hippocampal area was selected based on its functional and anatomical characteristics. First, the (epileptogenic) changes induced by eFS are located in the hippocampus, including the DG (24–28, 36, 37). Second, the DG has an important function in filtering excitation and seizure propagation to the other parts of the hippocampus (38, 39). Third, it is one of the few sites where neurogenesis continues in later life, a process that can be stimulated by seizures and has been implicated in epileptogenesis (40–44). Finally, the DG exhibits abundant receptors for corticosterone and therefore its morphology and function are largely influenced by early-life stress (29).

All following experimental procedures were performed by experimenters unaware of the treatment groups. Per outcome measure, tissue of animals belonging to different treatment groups was ordered in a semi-randomized way to control for environmental or experimenter effects on recording, staining and/or quantification over the total experimental period.

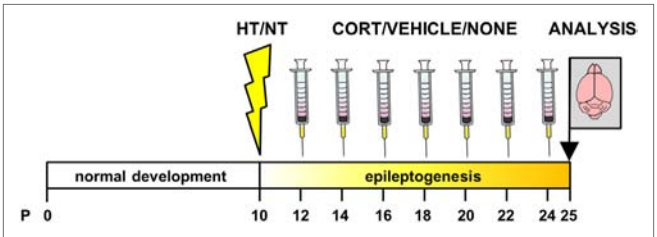

FIGURE 1 | Experimental design. Animals were exposed to hyperthermia (HT) or normothermia (NT) at postnatal day (P) 10 or 11 and subsequently received injections with corticosterone, vehicle or no injection every other day in the latent phase of epileptogenesis. Brains were dissected 24 h after the last injection, before the onset of spontaneous seizure activity.

Neurogenesis

Male mice ($n = 6$ per group) were decapitated between 9.00 and 10.30 a.m. Brains were dissected, post fixed at 4°C in 4% formaldehyde for 4 h, transferred to 30% sucrose for 24 h, frozen on powdered dry ice and stored at −80°C. Cryostat sections (20 μm) were cut in the coronal plane, collected in series of 15 on Superfrost slides and stored at −80°C until further use.

As a measure of neurogenesis, tissue was stained for Doublecortin (DCX), a marker for neuronal precursor cells and immature neurons, and Ki67, a marker for proliferating cells using modified protocols. Mounted sections were postfixed in acetone-methanol (1:1) at −20°C for 7.5 min, washed in 0.05 M Tris HCl 0.9% saline (TBS) pH 7.6 and heated in 0.01 M citrate buffer (pH 6.0) in a microwave oven for 10 min at 800 W followed by 5 min at 480 W and 5 min at 260 W. After a cool down period of 20 min, sections were washed in TBS. Endogenous peroxidase activity was blocked with 0.5% (for DCX) or 1.5% (for Ki67) H_2O_2 in TBS for 15 min. Sections were washed and, after incubation in 2% milk powder in TBS for 30 min, incubated with the primary antibody [DCX (polyclonal goat anti-DCX, SantaCruz; 1:800) or Ki67 (polyclonal rabbit anti-Ki67p, Novocastra, 1:5,000)], diluted in supermix (0.25% gelatine and 0.1% Triton in TBS) at room temperature for 1 h and then incubated overnight at 4°C. The next morning, sections were washed and incubated for 2 h with donkey antigoat biotinylated (for DCX, Jackson; 1:500) or goat antirabbit biotinylated (for Ki67, Vector; 1:200) secondary antibody diluted in supermix at room temperature. Sections were washed, incubated in avidin–biotin complex (ABC) (ABC Elite, Vector Laboratories; 1:800 in TBS) for 2 h (for DCX) or 1.5 h (for Ki67) at room temperature, washed again and incubated with biotinylated tyramide (1:500) in 0.01% H_2O_2 in TBS for 30 min. Sections were washed and incubated in ABC (1:800 in TBS) for 1.5 h at room temperature and washed in TBS. After washing in 0.05 M Tris HCl pH 7.6 (TB), chromogen development was performed with diaminobenzidine (DAB; 50 mg/100 ml Tris buffer, pH 7.6, 0.01% H_2O_2, 0.05% Nickel) for 40 min. Sections were washed in TB and stored overnight at 4°C. The next day, sections were washed in distilled water, counterstained with Hematoxylin and shortly rinsed in distilled water. After washing with running tap water, sections were dehydrated using a grading series of ethanol, cleared in xylene and coverslipped using Entallan.

DCX$^+$ and Ki67$^+$ cells in the granule cell layer and the subgranular zone of the DG were quantified unilaterally in every 15th section in a total of five sections per animal within Bregma range −1.46 to −2.80 (coronal) without a left/right preference within or between animals. DCX$^+$ cells were quantified stereologically using a StereoInvestigator system (Microbright field, USA) with a ×100 oil-immersion objective of a Zeiss Axiophot microscope and StereoInvestigator software, according to the optical fractionator method. The number of DCX$^+$ cells was estimated using a 25 μm × 25 μm counting frame, with a grid size of 70 μm × 80 μm. Section thickness was 10.5 μm. The estimated total of DCX$^+$ cells within the studied range was determined using the optical fractionator method and multiplied by two to correct for unilateral counting. The mean Gundersen coefficient of error of stereological quantification ($m = 1$) was 0.076 (range 0.06–0.10). Ki67$^+$ cells were counted manually using a light microscope (Olympus BH-2) with ×40 magnification and multiplied by the inverse of the sampling fraction and by two to correct for unilateral counting. The total estimated number of DCX$^+$ or Ki67$^+$ cells per animal was used for analysis.

Dendritic Morphology

Male mice ($n = 6$ per group) were decapitated between 9.00 and 10.30 a.m. Directly after decapitation, brains were dissected. Rapid Golgi staining (FD rapid-Golgi staining, Neurotechnologies) was performed according to the manufacturer's instructions with an impregnation time of 9 days. Vibratome sections (200 μm) were cut in the transversal plane (Leica VT 1000S; Leica Microsystems, Nussloch, Germany). Images were obtained using Zen 2011 (Carl Zeiss) in combination with an automated stage and focus control connected to the microscope. Golgi-impregnated dentate granule cells, fulfilling the following criteria, were randomly selected: (1) localization in the middle part (relative to the DG curvature and start of the CA3 region) of the suprapyramidal blade of the DG, within Bregma range −2.16 and −3.16 (transversal), (2) consistent and dark impregnation along the entire extent for all dendrites, and (3) relative isolation from neighboring impregnated neurons to avoid interference with analysis.

For morphological quantification, eight neurons from each animal in each treatment group were traced. Image stacks of 0.5 μm thickness were automatically acquired and combined. Neurons were traced using NeuroLucida software (MicroBrightField, Inc., Colchester, VT, USA) to obtain a 3D representation of each cell. Numerical analysis and graphical processing were performed with NeuroExplorer (MicroBrightField). Traced dendritic trees were evaluated by two investigators unaware of the treatment, on completeness of staining/tracing and on whether they belonged to a single cell, followed (if required) by a consensus meeting. Dendritic trees that were considered not completely stained/traced or belonging to multiple neurons were excluded from analysis [$n = 50$ (17%), 5–10 neurons per group, evenly distributed over the groups]. Spines were counted in two segments of ±20 μm per neuron, located on different dendrites. Segments were randomly chosen based on the following criteria: (1) localization at approximately 100 μm (80–120 μm) radial distance from the cell soma; (2) secondary or higher order dendritic branches; and (3) straight and remaining in a single focal-plane. Of each cell, dendritic properties were evaluated by analyzing the total dendritic length and maximum dendritic reach (radial distance), and the dendritic complexity index [(Σ branch tip orders + # branch tips) × (total dendritic length/total number of primary dendrites) (45)]. If dendritic length significantly differed between treatment groups, *post hoc* Sholl plots (46) were constructed by plotting the dendritic length as a function of radial distance from the soma center in 18 μm intervals. To normalize the distribution of the dendritic complexity index, neurons with a dendritic complexity index > 2 SD from the group mean were considered outliers and removed from the respective analysis [$n = 12$ (5%), 1–3 neurons per group, evenly distributed over the groups]. The remaining cells were subdivided into cells located in the inner-most part of the granule cell layer versus cells located in the middle or outer part of the granule cell layer (see Results). This subdivision was performed independently by two investigators blind to the treatment groups and compared afterward. In 96% of the cases the subdivision made by the two investigators was in agreement. In the remaining cases consensus was reached after in-depth investigation of the location.

Mossy Fiber Sprouting

Male mice ($n = 6$ per group) were killed between 9 and 12 a.m. under deep pentobarbital anesthesia (200 mg/kg body weight, i.p.) by transcardial perfusion with 0.1% sodium sulfide for 5 min, followed by 4% formaldehyde for 5 min (each in 0.01 phosphate-buffered saline, pH 7.4). Brains were removed from the skull, postfixed at 4°C in 4% formaldehyde/15% sucrose overnight, immersed at 4°C in 30% sucrose in phosphate-buffered saline until they sank and frozen on powered dry ice. Cryostat sections (30 μm) were cut in the coronal plane, mounted on superfrost slides in series of 15 and stored at −80°C until further use. Mossy fibers were stained with Timm histochemistry, according to Danscher (47). Staining was performed in two batches, each containing one slide of every animal to avoid staining-based variation between treatment groups. Sections were developed in the dark for 180 min in a freshly prepared 90/45/15/0.75 (volume/volume) solution of 50% arabic gum, 51% hydroquinone, 25.5% citric acid/23.5% sodium citrate, and 17% silver nitrate. After washing with running tap water, sections were dehydrated using a grading series of ethanol, cleared in xylene and coverslipped using malinol.

Mossy fiber staining in the hippocampal CA3 area and infrapyramidal blade of the DG (the main areas of possible MFS) was scored according to the scoring system described by Holmes et al. (48), ranging from 0 (no staining) to 5 (maximum staining). Eight sections of the septal hippocampus per animal (four sections for both staining batches), within Bregma range −1.46 to −2.80 (coronal), were scored by two independent observers, followed by a consensus meeting. Consensus scores of these eight sections were pooled per animal and the average was used for statistical analysis.

Slice Preparation for Electrophysiology

Male mice ($n = 6$–9 per group) were decapitated between 8.15 and 9.15 a.m., a few minutes after taking the animal out of its

home cage. Only the first two mice of each litter were used for electrophysiological analysis to avoid confounding effects of a rise in plasma corticosterone due to acute stress. After rapid dissection, the brain was chilled in ice-cold, carbogenated (95% O_2:5% CO_2) artificial cerebrospinal fluid (aCSF) containing in mM: NaCl 120, KCl 3.5, $MgSO_4$ 5.0, NaH_2PO_4 1.25, $CaCl_2$ 0.2, $NaHCO_3$ 25.0, and D-glucose 10.0. After removing frontal lobes and cerebellum, 350 μm transversal hippocampal sections were prepared using a vibratome (Leica VT 1000S; Leica Microsystems, Nussloch, Germany). Both hemispheres were separated and all sections were incubated at room temperature in continuously carbogenated aCSF for at least 1 h.

Patch-Clamp Recording of Spontaneous Excitatory Postsynaptic Currents (sEPSCs)

Patching of DG neurons was performed using an upright microscope (Nicon Eclipse E600FN) with differential interference contrast and a water immersion objective (×40) to visually identify the cells. The sections were continuously perfused with carbogenated aCSF containing in mM NaCl 120, KCl 3.5, $MgSO_4$ 5.0, NaH_2PO_4 1.25, $CaCl_2$ 0.2, $NaHCO_3$ 25.0, and D-glucose 10 and 20 μM biccuculine to block γ-aminobutyric acid $(GABA)_A$-receptor mediated transmission (flow rate 2.0 ml/min, temperature 32°C, pH 7.4). Cell patching was performed as described by Pasricha et al. (49). Briefly, patch electrodes were pulled from a Sutter Instruments Micropipette puller and had a tip resistance of 4–6 MΩ when filled with the pipette (intracellular) solution containing in mM; Cs-methane sulfonate 120, CsCl 17.5, HEPES 10, BAPTA 5, MgATP 2, NaGTP 0.1, pH 7.3 (adjusted with CsOH). An Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) was used for whole cell recordings, operating in the voltage-clamp mode. The patch-clamp amplifier was interfaced to a computer *via* a Digidata (type 1322A; Axon Instruments) analog-to-digital converter. Data acquisition was performed with Clampex, version 8.2 (Axon Instruments) at a sampling rate of 50 μs and a 5 kHz Bessel filter. The surface of the section was cleaned to have better vision of the cells in the deeper layers of DG. After establishing a gigaseal, the membrane patch was ruptured and the cell was clamped at a holding potential of −70 mV to allow measurement of currents mediated by the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, as at this potential, the *N*-methyl-D-aspartic acid or *N*-methyl-D-aspartate receptor is blocked by Mg^{2+}. sEPSCs were recorded for 5 min, starting 5–10 min after membrane rupture. After administration of tetrodotoxin (TTX, 0.5 μM, Bioconnect services) for 5 min to block voltage-gated Na channels and consequently also the development of action potentials, miniature EPSCs (mEPSCs) were measured for 5 min. Only one cell was recorded per slice and no more than two recordings were obtained per animal. Only recordings with an uncompensated series resistance of <2.5 times the pipette resistance, <20% variation during the recording period, and with frequencies <2 SD from the mean, were accepted for analysis. For each event, the area under the curve (AUC) and inter-event interval was analyzed using Clampfit version 9.2 (Axon Instruments). The median AUC and inter-event interval per cell were used for statistical analysis.

Field Potential Recordings

Sections were transferred to a submersion type recording chamber and continuously perfused with carbogenated aCSF containing in mM: NaCl 124, KCl 2.5, $MgSO_4$ 4.0, NaH_2PO_4 1.2, $CaCl_2$ 4.0, $NaHCO_3$ 26.0, D-glucose 10.0, and 20 μM bicuculline (flow rate 2 ml/min, temperature 32°C, pH 7.4). Field excitatory postsynaptic potentials (fEPSPs) were recorded in the DG, using glass microelectrodes filled with aCSF, positioned in the medial perforant pathway over the suprapyramidal blade of the DG, as confirmed by paired pulse stimulation elicited paired pulse inhibition. Minimum and maximum stimulation intensities were identified. After an incubation period of 20 min, an input–output response curve was generated by gradually increasing the stimulus intensity to define the stimulus intensity that generated the half-maximal response in peak-amplitude; this intensity was used for the remainder of the experiment. To measure paired pulse depression, paired pulses were delivered at an inter stimulus interval of 50, 100, and 200 ms. After 15–20 min of stable baseline recordings, sections were tetanized with 4 trains of 50 pulses of supramaximal intensity at 100 Hz (30 s inter train interval) to induce LTP. Field potentials were recorded for 1 h posttetanus at 30 s intervals. At the end of this period, the post-tetanic paired pulse depression and final input–output curve were determined. LTP was quantified by calculating the ratio between fEPSP slopes recorded post tetanization and pre tetanization. Data were acquired and analyzed with Signal 2.0 software (Cambridge Electronic Design, UK).

Statistical Analysis

The effect of eFS and injection type on outcome measures was analyzed with a general linear mixed model in a 2 × 3 design, examining the main effects of HT (HT versus NT) and injection type (corticosterone versus vehicle versus none), as well as their interaction. *A priori* we hypothesized that group differences would be caused by HT treatment or injection type. Therefore, not all possible group comparisons were included in statistical analysis. More specifically, in case of a significant main effect of injection type, *post hoc* tests were performed comparing injection types with Bonferroni correction for multiple comparison. Additionally, differences between HT and NT animals were analyzed per injection group with an independent samples *t*-test, Bonferroni corrected for multiple comparison. When data from multiple neurons per animal, or multiple segments per neuron, were analyzed, a linear mixed model was used, including the animal (for dendritic complexity and glutamatergic transmission) or neuron (for spine density) as subject variables. Effects of HT, injection type and their interaction on Sholl distribution were tested with a repeated measure general linear model. Correlation between parameters was assessed with Pearson correlation coefficient. Mean fEPSP slopes pre- and posttetanization were compared with a paired samples *t*-test.

Normality of residues was evaluated with Q–Q plots, variance of residues was evaluated with error plots. Differences were considered statistically significant at $p < 0.05$ (two-tailed) after correction for multiple comparison. Differences that were significant before, but not after correction for multiple comparison ($0.05 \leq p < 0.15$), were considered trends. Data were analyzed

using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA). Unless stated otherwise, data are presented as mean ± SEM.

RESULTS

Epileptic Seizures and Neuroendocrine Changes

Experimental seizures were elicited in all mice exposed to the HT protocol and the average seizure duration was 26.4 ± 2.0 min. Corticosterone injection resulted in a high peak concentration of corticosterone (1,043.3 ± 28.0 ng/ml at 15 min after injection) with a fast return to baseline in approximately 180 min, while vehicle injection elicited a much smaller (94.6 ± 8.2 ng/ml) and more short-termed increase in corticosterone levels (**Figure 2A**).

Weight increase between HT/NT treatment and decapitation, a 15 days interval, was significantly lower in HT animals compared to controls [main effect HT, $F_{(1,280)} = 10.73$, $p = 0.001$], but was not influenced by subsequent injections (**Figure 2B**). No group differences were observed in adrenal and thymus weights (data not shown). Although there were less than 2 min between successive decapitations of animals belonging to the same litter, later decapitation was associated with increased corticosterone levels (**Figure 2C**, left panel). While no group differences were observed in corticosterone levels in animals decapitated first of their litter (**Figure 2C**, middle panel), injection type did significantly influence corticosterone levels in animals decapitated as

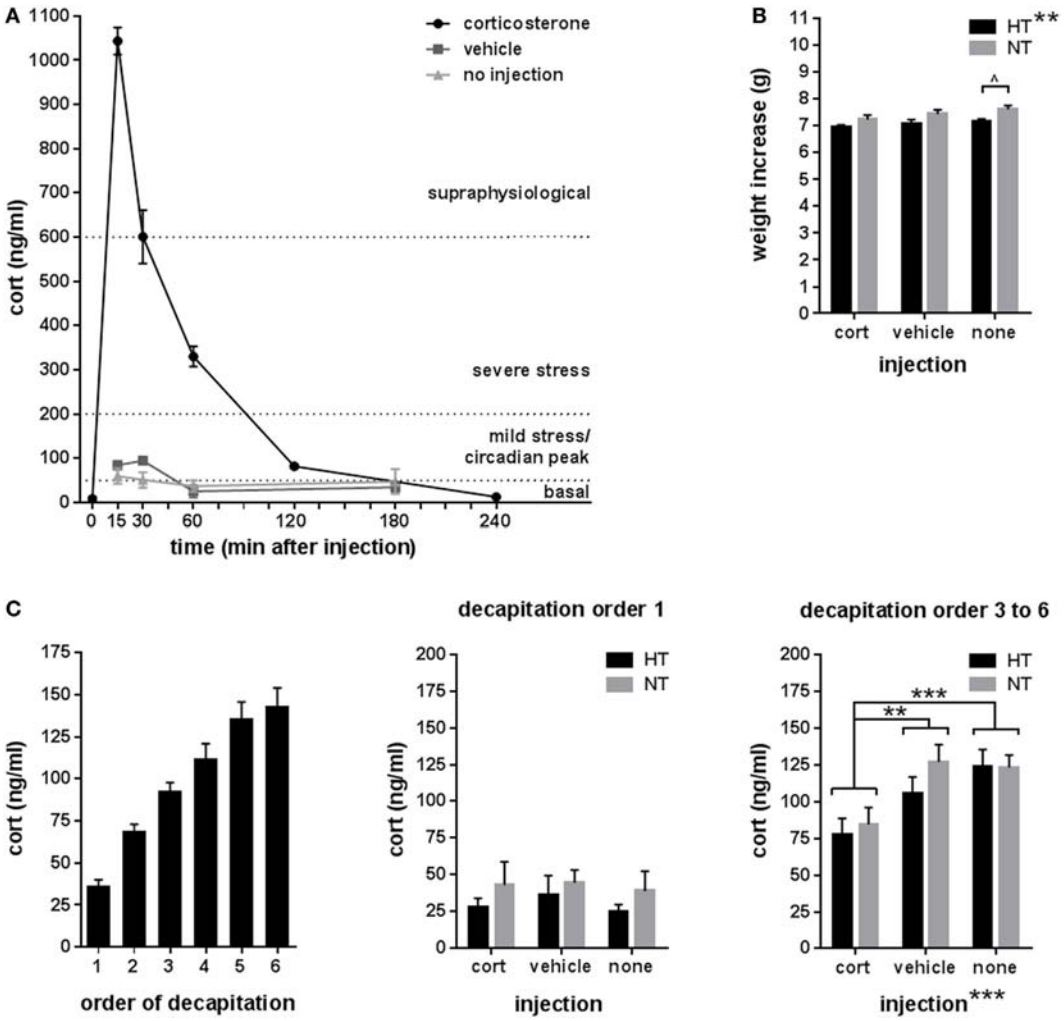

FIGURE 2 | Endocrinology. **(A)** Corticosterone (cort) levels at several time points after single intraperitoneal injection with corticosterone, vehicle, or no injection at 9.00 a.m. Reference corticosterone levels: basal < ±50 ng/ml, circadian peak or mild stress ±50–200 ng/ml, severe stress ±200–600 ng/ml, and supraphysiological > ±600 ng/ml (50–52). **(B)** Weight increase was significantly lower in hyperthermia (HT)- compared to normothermia (NT)-treated animals overall, while analyses per injection type only showed a trend difference in the not injected groups. **(C)** Corticosterone levels after decapitation increased with the order of decapitation within the litter (left panel). In animals decapitated as first of their litter (middle panel), corticosterone levels did not differ between treatment group, while in animals decapitated third to sixth of their litter (right panel), corticosterone levels differed between injection types—with significantly lower levels in the corticosterone injected animals—but not between HT- and NT-treated animals. Data represented as mean ± SEM. ***$p < 0.001$, **$p < 0.01$, and ^$p < 0.05$ before correction for multiple comparison (trend).

third to sixth of their litter [**Figure 2C**, right panel, main effect injection, $F_{(2,68)} = 8.7$, $p < 0.001$; corticosterone versus vehicle $p = 0.009$, corticosterone versus no injection $p < 0.001$, vehicle versus no injection $p = 0.36$], indicating that repetitive corticosterone injection indeed affected stress hormone regulation, which confirms successful manipulation.

Morphology

Neurogenesis

Neurogenesis significantly differed between HT and NT animals. HT was associated with a decrease in the number of immature DCX-positive neurons [main effect HT, $F_{(1,30)} = 6.21$, $p = 0.019$] (**Figure 3A**), and an increase in the number of proliferating cells in the DG [main effect HT, $F_{(1,30)} = 4.94$, $p = 0.034$] compared to NT (**Figure 3B**). Accordingly, the number of immature neurons and proliferating cells per animal were negatively correlated ($r = -0.55$, $p = 0.001$). Analyses per injection type revealed that the effects of HT on both DCX- and Ki67-staining were significant in the vehicle injected animals [$F_{(1,10)} = 12.16$, $p = 0.018$, respectively, $F_{(1,10)} = 15.89$, $p = 0.009$], while a similar effect was observed at trend level after corticosterone injection [$F_{(1,10)} = 5.71$, $p = 0.12$, respectively, $F_{(1,10)} = 6.88$, $p = 0.09$]. Non-injected HT and NT groups did not differ at all.

Dendritic Morphology

To assess the effects of eFS and injection type on dendritic morphology, we evaluated total dendritic length, dendritic reach, dendritic complexity and spine density. As dendritic complexity differed between dentate granule cells with a cell body located in the inner part of the granular cell layer, bordering the subgranular layer (referred to as "inner layer," $n = 15$–27 per group), versus the middle or outer part of the granule cell layer (referred to as "outer layer," $n = 11$–25 per group) (**Figure 4A**, A1), these cells were analyzed separately. In inner layer neurons, dendritic complexity index was increased in HT animals compared to NT controls [main effect HT, $F_{(1,111)} = 6.23$, $p = 0.014$], an effect that was only significant in animals receiving no subsequent injections [$F_{(1,7)} = 6.64$, $p = 0.04$] and was less pronounced after vehicle or corticosterone injection (**Figure 4A**, A2, left panel). Also the total dendritic length of inner layer neurons was increased in HT animals [main effect HT, $F_{(1,29)} = 4.47$, $p = 0.04$], an effect that aggravated with injection type, although analyses per injection type only revealed a difference in the corticosterone-injected group at trend level (**Figure 4A**, A2, right panel). No main nor interaction effects were observed on total dendritic reach. Sholl analyses per neuron revealed a main effect of HT [$F_{(1,120)} = 6.18$, $p = 0.01$] and radial distance from the soma [$F_{(3,358)} = 560.71$, $p < 0.001$] on dendrite length, as well as a

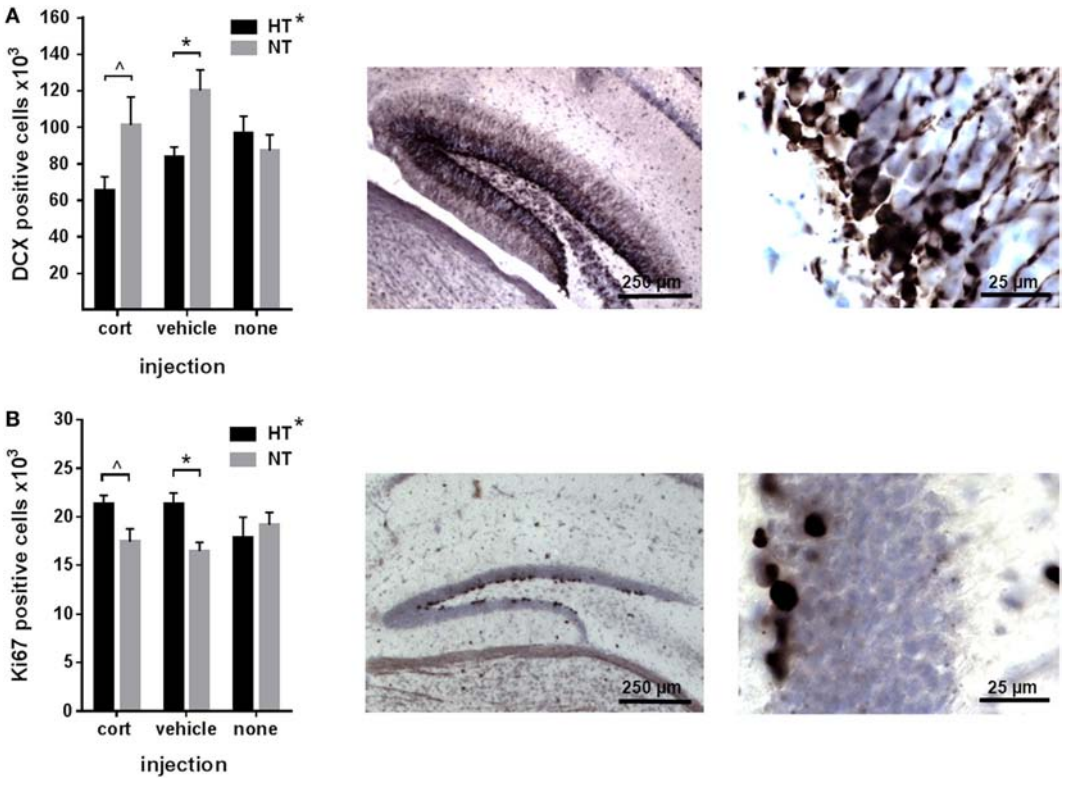

FIGURE 3 | Neurogenesis. **(A)** Left: the number of immature neurons in the dentate gyrus was decreased in male animals exposed to hyperthermia (HT) compared to normothermia (NT) after injection. Middle/right: representative example of DCX staining at lower and higher magnification. **(B)** Left: the number of proliferating cells in the dentate gyrus was increased in males exposed to HT. Middle/right: representative example of Ki67 staining at lower and higher magnification. Data represented as mean ± SEM. *$p < 0.05$ and ^$p < 0.05$ before correction for multiple comparison (trend).

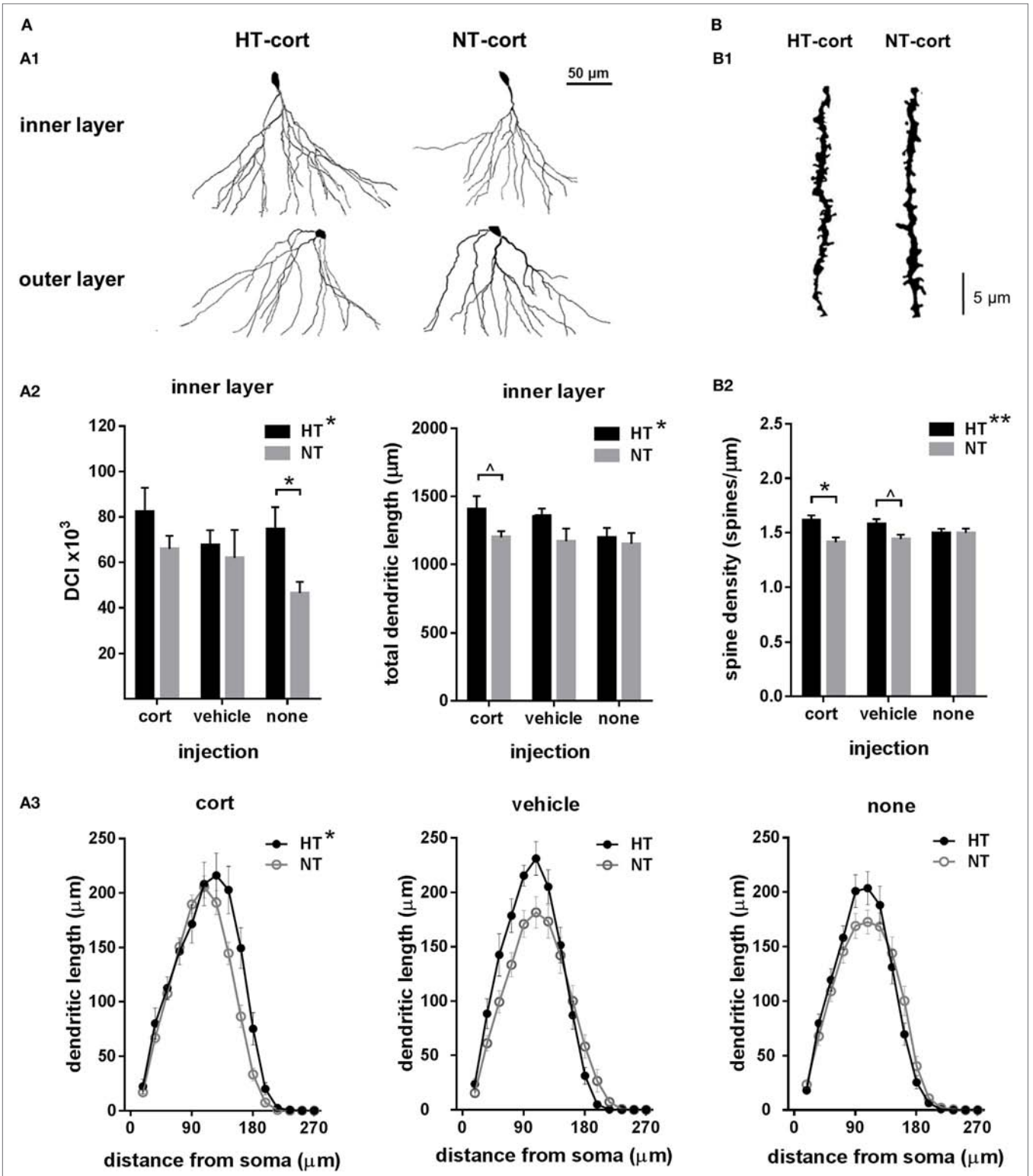

FIGURE 4 | Dendritic morphology. **(A)** Dendritic complexity. (A1) Dendritic complexity differed between dentate granule cells with a cell body located in the granular cell layer or inner part of the molecular layer (inner layer) versus the middle or outer part of the molecular cell layer (outer layer), representative examples of corticosterone (cort) injected animals after hyperthermia (HT) and normothermia (NT). (A2) Left: dendritic complexity index (DCI) in inner layer neurons was increased after HT compared to NT. Right: total dendritic length of inner layer neurons was increased after HT, an effect that increased with injection type. (A3) HT significantly influenced Sholl distribution. **(B)** Spine density. (B1) Representative example of HT-cort and NT-cort animal. (B2) Spine density was significantly higher in animals exposed to HT compared to NT, an effect that increased with injection type. Data represented as mean ± SEM. $^*p < 0.05$ and $^\wedge p < 0.05$ before correction for multiple comparison (trend).

HT × injection type × radial distance interaction [$F_{(6,358)} = 3.18$, $p = 0.005$] (**Figure 4A**, A3). In outer layer neurons, no main or interaction effects of HT or injection type were observed on dendritic complexity (data not shown).

Spine density was significantly higher in animals exposed to HT compared to NT [main effect HT, $F_{(1,165)} = 7.28$, $p = 0.008$]. This effect was significant after corticosterone injection [$F_{(1,58)} = 8.80$, $p = 0.01$], at trend level after vehicle injection [$F_{(1,55)} = 4.67$, $p = 0.11$], while it was not observed in animals receiving no injections [$F_{(1,52)} = 0.00$, $p = 1.00$] (**Figure 4B**, B1,B2).

Mossy Fiber Sprouting

Hippocampal MFS was assessed in the DG and the CA3 area of the hippocampus. As expected, intense Timm staining was observed in the hilus of the DG and in the stratum lucidum of the CA3 area, the main projection sites of mossy fibers. The amount of infrapyramidal Timm staining, characteristic for MFS, was very low in all treatment groups (mean MFS score DG 0.76 ± 0.09, CA3 1.07 ± 0.08, on a scale ranging from 0 to 5). HT or injection type did not significantly affect MFS score in both areas, although there was a trend towards an increased MFS score in the CA3 area after HT in animals receiving corticosterone injections [$F_{(1,10)} = 7.56$, $p = 0.06$, **Figure 5**].

Functional Plasticity

The eFS-induced changes in morphology and particularly the increased spine density may affect glutamatergic transmission in the DG, which could be reflected at the level of single cells as well as field potentials. To test this, we examined effects of HT and corticosterone injection on single cell spontaneous synaptic events (which are among other things determined by the number of synaptic contacts) and field excitatory potentials.

Single Cell Glutamatergic Transmission

Whole-cell voltage-clamp recordings of AMPA receptor mediated EPSCs in the DG were analyzed in 9–11 cells per treatment group. Input resistance and capacitance did not differ between groups (see **Table 1**). For both sEPSCs and mEPSCs, no HT × injection interaction or main effect of HT was observed. Interestingly, injection type did influence sEPSC and mEPSC properties (**Figures 6A,B**). A significant main effect of injection type existed on the interval between consecutive events for both sEPSC [main effect injection, $F_{(2,32)} = 3.48$, $p = 0.03$; corticosterone versus vehicle $p = 0.63$; corticosterone versus no injections $p = 0.06$, vehicle versus no injections $p = 0.01$] (**Figure 6A**, right panel) and mEPSC [main effect injection, $F_{(2,32)} = 3.95$, $p = 0.03$; corticosterone versus vehicle $p = 0.65$; corticosterone versus no injections $p = 0.07$, vehicle versus no injections $p = 0.01$] (**Figure 6B**, right panel), with a lower inter-event interval in the vehicle injection group compared to the non-injection group; a similar difference, although only at trend level, was observed for the corticosterone-injection versus non-injection groups. Also the AUC of the sEPSCs was influenced by injection type [$F_{(2,36)} = 4.39$, $p = 0.02$; corticosterone versus vehicle $p = 0.04$, vehicle versus no injections $p = 0.004$, corticosterone versus no injections not significant] with a smaller AUC after vehicle injection compared to the other groups (**Figure 6A**, middle panel), while the AUC of mEPSC did not significantly differ between groups (**Figure 6B**, middle panel).

Synaptic Plasticity

Changes in glutamatergic transmission at the single cell level may alter circuit properties and the ability to induce synaptic plasticity. This was tested with field potential recording and application of high-frequency stimulation. Treatment groups did not differ with respect to baseline slopes or stimulation intensity necessary to produce the half-maximal fEPSP, suggesting that HT and injection type did not influence baseline-evoked transmission (see **Table 1**). No significant differences were observed in paired pulse facilitation before and after high-frequency stimulation (**Figure 7A**). The input-output curve was increased after high frequency, and did not differ significantly between treatment groups (**Figure 7B**). A significant increase in fEPSP slope post tetanization (LTP) was elicited in 71% of all animals (38–88%

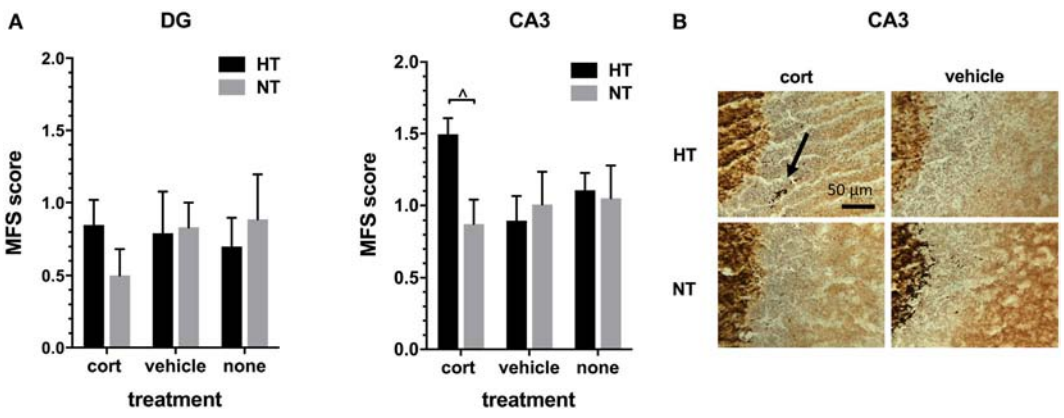

FIGURE 5 | Mossy fiber sprouting (MFS). **(A)** Left: the MFS score in dentate gyrus (DG) did not differ significantly between treatment groups. Right: in the CA3 area of the hippocampus only a trend was seen toward an increase in MFS score after hyperthermia (HT) in animals receiving corticosterone injections. **(B)** Representative example of MFS in the CA3 area in HT and normothermia (NT) animals with corticosterone versus vehicle injection. Arrow: MFS. Data represented as mean ± SEM. ^$p < 0.05$ before correction for multiple comparison (trend).

per group). Synaptic plasticity at half-maximal or maximal stimulation was not significantly influenced by the HT × injection interaction or by HT, but only by injection type [main effect injection, $F_{(2,38)} = 4.20$, $p = 0.02$; corticosterone versus vehicle $p = 0.02$, corticosterone versus no injections $p = 0.44$, vehicle versus no injections $p = 0.01$, other group comparisons not significant] (**Figure 7C**). At maximum stimulation intensities, no group differences were observed.

TABLE 1 | Baseline measurements of single cell glutamatergic transmission and synaptic plasticity.

Treatment group	Single cell transmission		Synaptic plasticity	
	Input resistance (mΩ)	Capacitance (pF)	Baseline slope (V/s)	Stimulation intensity (mA)
HT-cort	336 ± 49	9.8 ± 0.7	−0.13 ± 0.02	0.63 ± 0.05
NT-cort	338 ± 38	9.0 ± 0.8	−0.20 ± 0.02	0.71 ± 0.03
HT-vehicle	303 ± 33	9.0 ± 0.7	−0.19 ± 0.02	0.66 ± 0.02
NT-vehicle	320 ± 38	9.2 ± 0.7	−0.14 ± 0.03	0.77 ± 0.08
HT-none	285 ± 35	9.4 ± 0.8	−0.22 ± 0.02	0.80 ± 0.12
NT-none	302 ± 37	9.6 ± 1.1	−0.22 ± 0.04	0.84 ± 0.14

Baseline measurements of functional plasticity were not significantly influenced by the HT × injection type interaction, HT, or injection type. Data represented as mean ± SEM.

DISCUSSION

To increase understanding of the effects of stress and stress hormones on early-life epileptogenesis, we induced epileptogenesis in young mice using the HT-induced eFS model and subsequently exposed them to (1) corticosterone, (2) vehicle injection (a mild stressor), or (3) no injections, and evaluated morphological and functional parameters in the DG. In this latent phase of early epileptogenesis, few effects of eFS were observed in animals that were not subsequently injected. However, in mice that received repetitive corticosterone or vehicle injection (mild stress), eFS induced epileptogenesis was associated with changes in DG morphology, namely a lower number of immature cells, an increased cell proliferation and an increased dendritic length and spine density. These

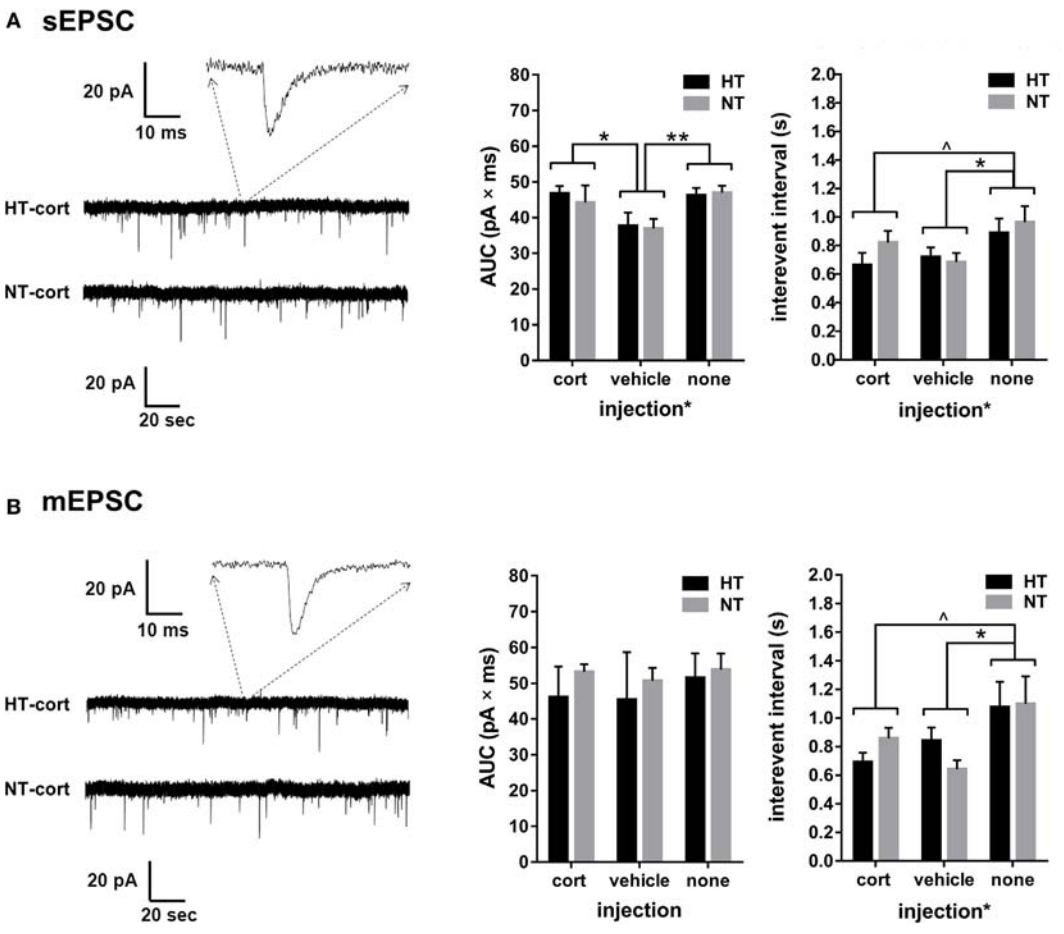

FIGURE 6 | Single cell glutamatergic transmission. **(A)** Left: example showing a spontaneous excitatory postsynaptic current (sEPSC) recording. Middle and right: a main effect of injection type, but not HT, is observed on sEPSC area under the curve (AUC) and interevent interval. **(B)** Left: example showing a miniature excitatory postsynaptic current (mEPSC) recording. Middle: no group differences are observed in mEPSC AUC. Right: a main effect of injection type, but not HT, is observed on mEPSC interevent interval. Data represented as mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$, and $\hat{}p < 0.05$ before correction for multiple comparison (trend).

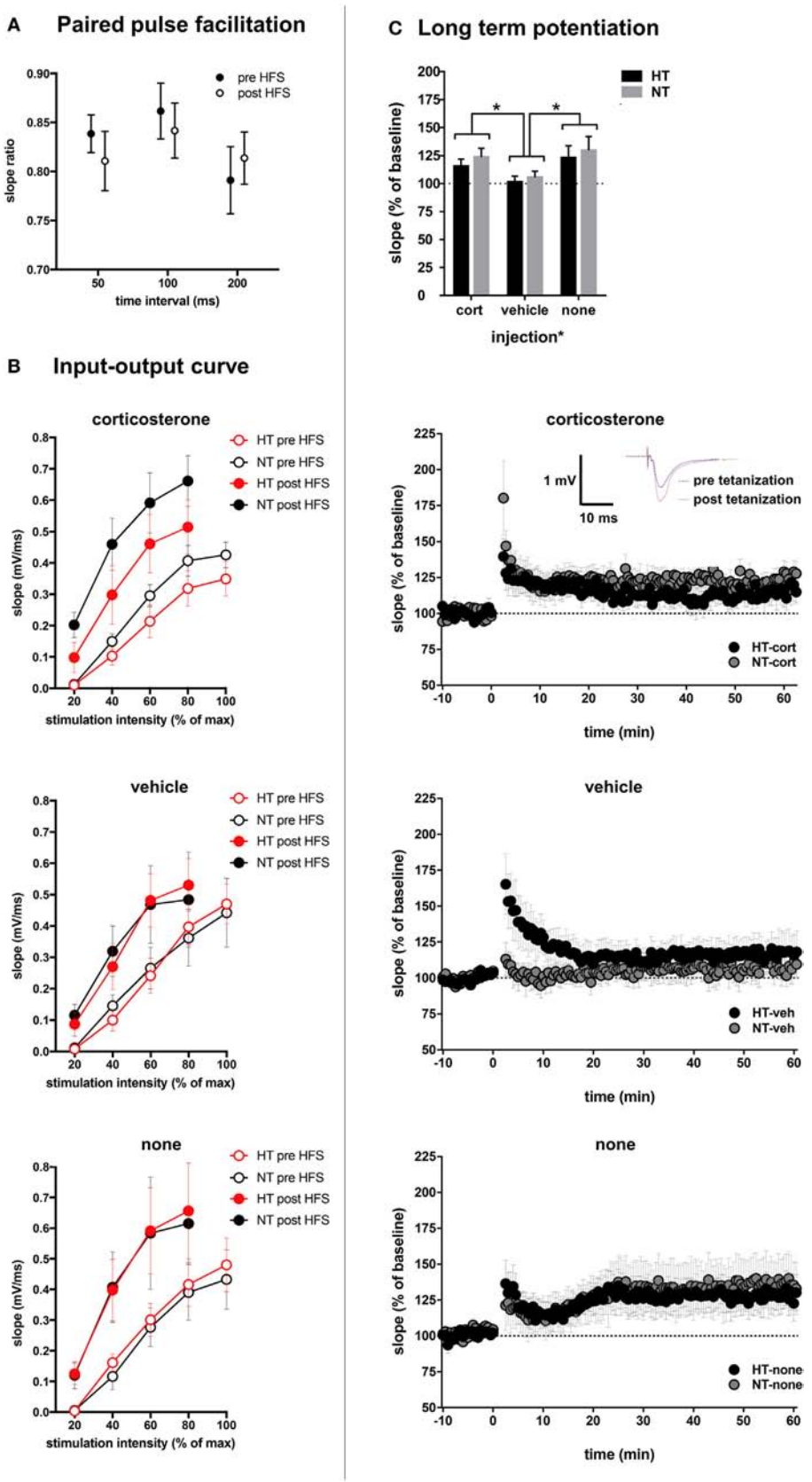

FIGURE 7 | Continued

FIGURE 7 | Synaptic plasticity. **(A)** Paired pulse facilitation before and after high-frequency stimulation (HFS). **(B)** The input–output curve is increased after HFS, but did not differ significantly between treatment groups. **(C)** Top: injection type, but not HT, influenced field-evoked postsynaptic potentials (fEPSP). Upper middle: LTP recordings of fEPSP in hyperthermia (HT) vs. normothermia (NT) animals after repetitive corticosterone injection (tetanization started at $t = 0$). Inset: example showing increased slope and amplitude of fEPSP after tetanization. Lower middle: fEPSP in HT vs. NT animals after repetitive vehicle injection. Bottom: fEPSP in HT vs. NT animals without subsequent injections. Data represented as mean ± SEM, $^*p < 0.05$.

morphological changes associated with epileptogenesis did not translate into differences in glutamatergic functional plasticity.

Experimental Model

The eFS model is a model of early-life prolonged febrile seizures with close resemblance to the human situation (30, 31). The relatively long-lasting latent phase of epileptogenesis in this model provides the unique opportunity to study epileptogenesis in the absence of spontaneous seizures and relatively apart from the damage and compensatory mechanisms that may arise due to recurrent electrographical or clinical seizure activity. However, non-epileptogenic effects of the initial eFS cannot be fully excluded. As we were interested in epileptogenic changes, outcome measures were assessed before the onset of spontaneous seizures. Based on earlier literature, a subset of animals exposed to eFS is expected not to develop epilepsy (26, 35, 36). However, the normal distribution of our outcome measures suggests a gradual distribution of epileptogenesis among animals. Further studies measuring long-term (seizure) outcome are required to determine whether the morphological and functional measures assessed are stable during the course of epileptogenesis, as well as their relevance for epilepsy outcome.

We hypothesized the mild epileptogenic changes in the latent phase after eFS to be aggravated by stress (hormone) exposure. As different stressors might differentially affect seizure-susceptibility [reviewed in Ref. (2, 23, 53)], we decided to use a clean design of injections with corticosterone—the end product of the stress response, exerting large effects on brain structure and function, including neuronal excitability (22)-, and control groups of mild injection stress and undisturbed animals. Stress paradigms in rodents are usually associated with a reduction in bodyweight and effects on adrenals and thymus. In our experiment, we did not observe such changes, possibly due to the mild nature of the injection stress. However, the reduced corticosterone levels in corticosterone-injected animals that were decapitated after prior handling of littermates, which can be considered an acute stressor, indicate that corticosterone injections *did* downregulate responsiveness of the hypothalamic–pituitary–adrenal axis. The effects of these supraphysiological doses of corticosterone were smaller than expected, which might relate to the young age of the animals, just after the stress hyporesponsive period. Also, the transient increases in corticosterone levels in the morning may not have interfered with the ultradian pulsatility of endogenous hormone levels (54), benefiting its comparability to real life stress exposure.

Effects of Corticosteroids on Structural Plasticity during Epileptogenesis

In animals that were *not* subsequently injected, HT only affected dendritic complexity, but none of the other outcome measures. The negative results during this early phase of epileptogenesis are in line with previous studies that also reported no differences in MFS (24, 27) or DG neurogenesis (24) around this age. Similarly, in non-injected mice we observed no effects of HT on DG spine density.

Differences between animals exposed to HT and NT only became manifest after repetitive corticosterone or vehicle injections. The increase of HT-associated changes after stress (hormone) exposure is in line with the vast amount of previous studies reporting stress exposure *before seizure induction* to increase seizure-susceptibility and seizure-severity [reviewed in Ref. (55)]. The effects of HT combined with corticosteroids or mild stress on proliferation are similar to those reported at a later stage of epileptogenesis after HT only (25, 26), suggesting that corticosterone and stress *accelerate* epileptogenesis-related structural plasticity. We observed an inverse relation between the number of proliferating cells and the amount of immature neurons, suggesting that the increased proliferation mainly occurs in non-neuronal (e.g., glial) cells, consistent with the gliosis prominent in many epilepsy models (56, 57).

Dendritic length, complexity and spine density are generally reported to decrease after seizures (58–63), which is considered a compensatory mechanism in response to the excess of excitatory input. Dendritic complexity was previously shown to be increased after eFS (28). The enhanced dendritic length and spine density that we found during the latent phase of early epileptogenesis in combination with corticosterone exposure and mild stress is therefore likely to be part of the epileptogenic process.

Clearly, the parameters that we investigated might not only be altered by epileptogenesis but also by corticosterone and stress themselves. This is shown for instance by the increased number of DCX^+ cells in the vehicle injected compared to non-injected NT group, that is consistent with the literature (21, 64).

Effects of Corticosteroids on Functional Plasticity during Epileptogenesis

In non-injected animals, glutamatergic transmission and LTP were unaffected by HT. The latter is somewhat surprising, as around this age HT was shown to affect GABAergic transmission in DG (65) and both glutamateric and GABA-ergic transmission in CA1 (27, 66–69). Since we did not record GABAergic signals, we cannot exclude that in our model GABAergic transmission in the DG might have been altered.

In view of the increased dendritic length and spine density observed after HT when followed by corticosterone (and to a lesser degree vehicle) injection, the latent phase of early epileptogenesis may be accompanied by an expanded postsynaptic

"potential" for synaptic transfer of signals. We tested whether this translated to the functional level; i.e., a higher spine density may result in increased mEPSC frequency and this, in turn, may enhance the ability to induce LTP (70). However, this appeared not to be the case. Rather than HT, the condition of mild stress related to vehicle injection resulted in a higher frequency of both sEPSCs and mEPSCs, and a smaller AUC of sEPSCs; the latter could explain the reduced ability to induce LTP in these groups. The discrepancy between structural changes in dendrites and spines versus sEPSC frequency is unexpected, but emphasizes that presynaptic changes, e.g., after mild injection stress, are important for the overall outcome in functional terms. Presynaptic effects of stress or corticosterone are indeed well documented (71, 72), also in the mouse DG (49), although these were never investigated with this particular paradigm and at this age. The data furthermore illustrate that the functional effect of mild injection stress cannot be extrapolated to effects of a high dose of corticosterone. This may relate to a bell-shaped dose-dependency for corticosterone, as indeed often observed (73, 74) or, importantly, the fact that mild (injection) stress causes the release of several stress-related hormones in addition to corticosterone.

Potential Implications for Epileptogenesis

The increase in morphological differences after HT when followed by corticosteroid and mild stress exposure suggests that stress (hormones) aggravates or accelerates epileptogenesis. Epileptogenesis, i.e., the neurobiological processes leading to epilepsy, is not limited to the time before the onset of spontaneous seizures, but continues during the course of epilepsy, contributing to the progression of the disease (75, 76). As none of the currently available anti-epileptic drugs can favorably modify the disease process, prevention or reduction of epileptogenesis remains one of the main challenges in the field of epilepsy (77–79) and could be beneficial for all patients, irrespective of the underlying pathology. Children with complex febrile seizures are of special interest, as they have a high risk to develop epilepsy—ranging from 21% after prolonged febrile seizures (80) to as much as 49% after prolonged seizures with focal semiology that reoccur within 24 h (81)—that is already identified at the very beginning of epileptogenesis, making them very suitable for early intervention. Large population-based studies, prospectively following children after complex febrile seizures and systematically documenting stress exposure, as well as epilepsy outcome, can provide more insight into the effects of stress on epileptogenesis in this human population.

In conclusion, our results suggest that stress and stress hormones modulate epileptogenesis, indicating that stress reduction strategies and possibly even medication targeting the stress system may have a potential role in reducing epileptogenesis. As studying epileptogenesis apart from seizure frequency is difficult, especially in humans, animal studies could provide valuable information on the effects of stress reduction on epileptogenesis, for example, by studying effects of an enriched environment during the latent phase of epileptogenesis on seizure outcome.

ETHICS STATEMENT

All experimental procedures were performed according to the institutional guidelines of the University Medical Center Utrecht and approved by the committee on ethical considerations in animal experiments of Utrecht University (DEC Utrecht, permit number 2012.I.03.047).

AUTHOR CONTRIBUTIONS

JC, KPJB, PG and MJ contributed conception and design of the study. JC, EH, KB, GR, ST, EU, and GM contributed to data acquisition. JC organized the database, performed the statistical analysis, and wrote the first draft of the manuscript. JC, KB, GR, PL, PG, and MJ contributed to data interpretation. All authors contributed to manuscript revision, read, and approved the submitted version.

ACKNOWLEDGMENTS

We acknowledge the expert help and technical assistance of H. Karst, R. A. Sarabdjitsingh, and B. Jongbloets. Moreover, we thank the practical assistance of B. Visser, V. Bonapersona, and T. Kliest in neuronal reconstructions for dendritic complexity or spine analyses, S. Vugts in optimizing neurogenesis stainings, M. Sep in Ki67 staining and quantification, and M. A. Pet in DCX quantification. This work was supported by a grant of the UMC Utrecht Alexandre Suerman Stipendium. This manuscript includes content that previously appeared in the first author's thesis (82).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 09 February 2018
doi: 10.3389/fendo.2018.00024

The Effects of Low-Dose Bisphenol A and Bisphenol F on Neural Differentiation of a Fetal Brain-Derived Neural Progenitor Cell Line

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Environmental chemicals are known to disrupt the endocrine system in humans and to have adverse effects on several organs including the developing brain. Recent studies indicate that exposure to environmental chemicals during gestation can interfere with neuronal differentiation, subsequently affecting normal brain development in newborns. Xenoestrogen, bisphenol A (BPA), which is widely used in plastic products, is one such chemical. Adverse effects of exposure to BPA during pre- and postnatal periods include the disruption of brain function. However, the effect of BPA on neural differentiation remains unclear. In this study, we explored the effects of BPA or bisphenol F (BPF), an alternative compound for BPA, on neural differentiation using ReNcell, a human fetus-derived neural progenitor cell line. Maintenance in growth factor-free medium initiated the differentiation of ReNcell to neuronal cells including neurons, astrocytes, and oligodendrocytes. We exposed the cells to BPA or BPF for 3 days from the period of initiation and performed real-time PCR for neural markers such as β III-tubulin and glial fibrillary acidic protein (GFAP), and Olig2. The β III-tubulin mRNA level decreased in response to BPA, but not BPF, exposure. We also observed that the number of β III-tubulin-positive cells in the BPA-exposed group was less than that of the control group. On the other hand, there were no changes in the MAP2 mRNA level. These results indicate that BPA disrupts neural differentiation in human-derived neural progenitor cells, potentially disrupting brain development.

Keywords: neural differentiation, bisphenol A, neuron, estrogen, environmental chemicals, brain development, bisphenol F

OPEN ACCESS

Edited by:
Ishwar Parhar, Monash University Malaysia, Malaysia

Reviewed by:
Fumihiko Maekawa, National Institute for Environmental Studies, Japan
Shoji F. Nakayama, National Institute for Environmental Studies, Japan

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *24 November 2017*
Accepted: *19 January 2018*
Published: *09 February 2018*

Citation:
Fujiwara Y, Miyazaki W, Koibuchi N and Katoh T (2018) The Effects of Low-Dose Bisphenol A and Bisphenol F on Neural Differentiation of a Fetal Brain-Derived Neural Progenitor Cell Line. Front. Endocrinol. 9:24. doi: 10.3389/fendo.2018.00024

INTRODUCTION

A variety of environmental chemicals are known to induce adverse effects in animals and humans including general toxicity (e.g., organ damage), carcinogenesis, mutagenesis, and reproductive toxicity. The risk assessment of these toxic substances is done according to OECD guidelines. Recently, several animal studies have shown that gestational exposure to low-dose toxicants, such as dioxin, may cause adverse effects including neurodevelopmental alterations without affecting

Abbreviations: BPA, bisphenol A; BPF, bisphenol F; NPCs, neural stem/progenitor cells; DA, dopaminergic, VM, ventral mesencephalon.

the dams (1, 2). These reports suggested that the developing brain is vulnerable to exposure to environmental chemicals, even at low doses. Many studies have demonstrated neurodevelopmental and behavioral disorders attributable to gestational and/or lactational exposure to environmental chemicals. We have previously shown that during the maternal and postnatal periods, dioxin can cross the developing blood–brain barrier and cause learning deficits, emotional abnormalities, and changes in social behavior (3–5).

During brain development, neural cells, including neurons and glial cells, are differentiated from neural stem/progenitor cells (NPCs) (6). The differentiation process is precisely and tightly controlled by many factors, including hormones and receptors. Estrogen is an important factor for neural differentiation, protection, and function. Estrogen acts by binding to nuclear estrogen receptors (ERs). The differentiation of dopaminergic (DA) neurons in the ventral mesencephalon (VM) (7) is also regulated by the estrogen–ER pathway. Several environmental chemicals, such as bisphenol A (BPA), isoflavones, and phthalates, which are also called phytoestrogen or xenoestrogen, are known to disrupt such pathways because of their estrogenic actions (8, 9). Among these, the toxicity of BPA is considered one of problems as disruptors on estrogenic pathway.

Large volumes of BPA are used to make toys, plastic containers, and dental pastes (10–12). BPA is readily eluted from these products and delivered to the human body through foods and drinks. Consequently, BPA has been detected in the tissue including the brain of human fetuses, babies, and pregnant mothers (13). The distribution of BPA in both fetuses and babies reflects that BPAs are delivered through placenta and breast milk. In fact, BPA was detected in fetal cord blood, placental tissues, and breast milk (14, 15). Surprisingly, the metabolites of BPA can also cross the placenta, and such metabolites are deconjugated to BPA in fetuses (16). Transplacental and lactational exposure may cause different adverse effect. In addition, the differences of these adverse effects may depend on not only the timing of exposure to BPA in transplacental (fetal) or lactational but also sex in mice and rat (17). This hypothesis is evidenced further by a human epidemiological study, showing that gestational BPA exposure induced sex-dependent associations with aggressive behaviors in children and adolescents (18). Thus, we consider it important to investigate the effects of BPA on brain development, especially neural differentiation. Recent studies also showed that low-dose BPA exposure causes toxicological effects in the brain (19). BPA induces estrogen-like effects *via* ERs (20–22), disrupting sexual differentiation in the developing brain and changing sex-dependent behaviors by perinatal exposure. Past studies revealed sex-based behavioral differences following maternal exposure to BPA in learning and memory, novel exploration, and emotional behavior (23–25). BPA exposure increases locomotor activity and anxiety-like behavior (26, 27), decreases impulsive behavior, and changes social behaviors (24, 28). Although the precise mechanisms that cause these behavioral alterations are unclear, some studies have indicated that BPA exposure suppresses synapse formation (29), disrupts neural migration (30, 31), increases the number of glial cells (32, 33), and upregulates neural cytoskeletal proteins (32, 34). *In vitro* studies using cell lines showed the inhibition of dopamine release (35) and the augmentation of the microtubule-associated protein 2 (MAP2) mRNA expression. MAP2 is a cytoskeleton-related protein in neurons, and its expression is changed in the presence of neurodegenerative diseases such as schizophrenia (19, 20). These changes may cause abnormal brain development and behavioral alterations. BPA may exert adverse effects on normal brain development by disrupting neural differentiation, including DA neurons in the VM. However, the mechanisms of BPA action have not yet been fully clarified, especially in humans.

To avoid BPA exposure, several alternative substances are produced. Bisphenol F (BPF) is one such compound that is used in epoxy resins and coatings. Although the exposure levels of BPF in the environment, humans, and wild animals are lower than those of BPA, BPF may also affect human health, including brain development. However, to our knowledge, the effects of BPF on neural differentiation from NPCs in humans have not yet been reported.

In this study, to examine the effects of BPA and BPF exposure during neural differentiation, we used a human fetal VM-derived NPC cell line, ReNcell VM cell line. This cell line is appropriate to investigate the effects on the neural differentiation of human, because this was derived from a 10-week human VM brain tissue and established as an NPC cell line with immortalization. Moreover, as stated above, BPA and BPF may disrupt the differentiation through ERs because of the potencies of their estrogen-like effects. We then examined the changes in neuronal differentiation attributable to BPA or BPF exposure.

MATERIALS AND METHODS

All experiments in this study were performed under the restrictions in biosecurity and safety procedures of Gunma University.

Chemicals

Bisphenol A (99% purity) and BPF (99% purity) were purchased from Sigma-Aldrich (MO, USA). BPA and BPF were dissolved in ethanol and DMSO, respectively. β-estradiol (E2), 98% pure and dissolved in ethanol (99.5% purity), was also obtained from Sigma-Aldrich.

Cell Culture

ReNcell VM (Millipore, MA, USA) is an immortalized NPC line derived from the VM of a 10-week-old human fetal brain. The cells were cultured as previously described (36). The passage of all cells used in this investigation was lower than 31, because previous work has shown that these cells maintain a stable karyotype up to 45 passages. Briefly, ReNcell VM cells were expanded in an expansion medium (ReNcell NSC Maintenance Medium, Millipore, MA, USA), supplemented with 20 ng/ml of epidermal growth factor (Millipore, MA, USA) and 20 ng/ml of basic fibroblast growth factor (Millipore, MA, USA) on laminin-coated (Wako Pure Chemical Industries, Ltd., Osaka, Japan) 1.7-μg/cm^2 TC-treated culture flasks at 37°C in a 5% CO_2 humidifier incubator. The medium was renewed every 2 days during proliferation, and the cells were subcultured approximately every 5 days (90% confluence) by detaching them using Accutase

(Millipore, MA, USA). After each passage, cell concentration and viability were determined by counting with a hemocytometer (Hausser Scientific, Horsham, England) using the trypan blue dye (Invitrogen, CA, USA) exclusion test. After this, the cells were again seeded at 5×10^4 cells/ml in freshly laminin-coated flasks. Differentiation of the cells was accomplished by adding fresh differentiation medium (ReNcell NSC Maintenance Medium without growth factors) to confluent monolayers of cells. Unless otherwise indicated, cells were incubated for 3 days in a differentiation medium, and the medium was changed every 2 days.

At the onset of differentiation, the cells were exposed to BPA, BPF, or E2 and incubated for 3 days. Control cells were exposed to solvents following the same protocol. E2-exposed group was used for positive control, because BPA and BPF have the potency to induce estrogen-like effects through ERs.

Cell Cytotoxicity Assay

Cell cytotoxicity was measured using the Cell Counting Kit-8 (CCK-8; Dojindo, Osaka, Japan) according to the manufacturer's protocol. Briefly, ReNcell VM cells (1×10^5 cells/well) were seeded onto laminin-coated TC-treated culture 96-well plates and cultured with an expansion medium at 37°C in a 5% CO_2 humidifier incubator. After 24 h, cells were incubated with a BPA-containing expansion medium for another 24 h. At the end of the culturing period, we added 10 µl of CCK-8 reagent to the cells and incubated them for 4 h at 37°C.

A water-soluble tetrazolium salt (WST-8) containing a CCK-8 reagent reacts with dehydrogenase from living cells and turns into WST-8 formazan, an orange dye. The fluorescence intensity correlates with the number of living cells in the sample. After incubation with CCK-8 reagents for 4 h, we measured the absorbance of light at 450 nm with a Synergy HTX plate reader (Biotek Instruments, Inc., VT, USA).

Quantitative Real-time PCR

Total RNA was isolated using the miTotal™ RNA Extraction Miniprep System (VIOGENE, New Taipei City, Taiwan), and 2.0 µg of total RNA was reverse-transcribed into cDNA using ReverTraAce qPCR RT Master Mix (TOYOBO, Osaka, Japan). Quantitative real-time PCR was performed with specific primers (**Table 1**) in the StepOne thermal cycler (Applied Biosystems/ Life Technologies, CA, USA) using THUNDERBIRD SYBER qPCR Mix (TOYOBO Japan) for 40 cycles according to the following program: a denaturation step at 95°C for 30 s followed by an annealing/extension step at 95°C for 30 s. The data were analyzed using the delta–delta Ct method. GAPDH was used for normalization.

TABLE 1 | Primer sequences.

Primer	Forward	Reverse
β III-tubulin	CATGGACGAGATGGAGTTCA	TTCGTACATCTCGCCCTCTT
MAP2	CAGAAGTTCAGGCCCACTCT	GGTTTTCCGCTTAACACAGG
GFAP	GAGATCGCCACCTACAGGAA	CAGGCTGGTTTCTCGAATCT
S100β	GGGAGACAAGCACAAGCTG	TCCACAACCTCCTGCTCTTT
Olig2	GACAAGCTAGGAGGCAGTGG	GGCTCTGTCATTTGCTTCTTG
Nestin	GACTTCCCTCAGCTTTCAGG	TCAGGACTGGGAGCAAAGAT
Dcx	TCCCCAACACCTCAGAAGAC	GCGTAGAGATGGGAGACTGC
NCAM1	GAACCAGCAAGGAAAATCCA	CAGGACGAAGATGACGATGA
MCT1	TTGGTTGGCTCAGCTCTGTA	CAGCATTCCACAATGGTCAC
ESR1	GATGAATCTGCAGGGAGAGG	TCCAGAGACTTCAGGGTGCT
ESR2	TCAGGCATGCGAGTAACAAG	TCCAGCAGCAGGTCATACAC
Rtp1	GGTTGGAAGCAGTACCTGGA	GGTCCAGGAACATGTGGAAG
GAPDH	GATCATCAGCAATGCCTCCT	TGAGTCCTTCCACGATACCA

Immunocytochemistry

Following growth and differentiation on laminin-coated chamber slides, cells were washed with PBS and fixed with 3% paraformaldehyde solution in PBS for 30 min. After washing twice with PBS, cells were incubated with 0.1% triton X-100 solution and were blocked in PBS containing 10% BSA for 1 h at room temperature. Subsequently, cells were incubated with the following antibodies: β III-tubulin (Sigma-Aldrich, MO, USA) (1:500 dilution) and glial fibrillary acidic protein (GFAP) (Abcam, Cambridge, England) (1:500 dilution) overnight at room temperature. After washing with PBS, cells were incubated with the following secondary antibodies for 1 h in the dark at room temperature. Cells were then washed with PBS again, and the nuclei were counterstained with 1 µg/ml Hoechst 33342 (Sigma-Aldrich, MO, USA) for 10 min, in the dark. Stained cells were washed with PBS and were observed with a fluorescence microscope (EVOS fl: Thermo Fisher Scientific, MA, USA).

Statistical Analysis

Experimental data were analyzed using GraphPad Prism (GraphPad Software, CA, USA). Results are represented as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Tukey test. In **Figures 2** and **6B**, Student's *t*-test was used to compare two groups. For all comparisons, the results were considered to be significant if the *p*-value was <0.05.

RESULTS

BPA Did Not Induce ReNcell VM Cell Death

First, to examine whether BPA exposure induced ReNcell VM cell death, we investigated the cytotoxicity of BPA using a CCK-8 (DOJINDO LABORATORIES, Kumamoto, Kyushu, Japan). The amount of formazan dye, measured as the absorbance of light at 450 nm, is proportional to the number of living cells. There was no difference in the absorbance, indicating that BPA exposure did not induce cell death at concentrations between 10^{-16} and 10^{-10} M (**Figure 1**).

BPA Induced a Dose-Dependent Decrease in Both β III-Tubulin and S100 β mRNA Levels

Next, we investigated the ratio of differentiated cell types using a real-time PCR method with various neural markers, such as βIII-tubulin for neurons during differentiation, MAP2 for mature neuron, GFAP and S100β for astrocytes, Olig2 for oligodendrocytes, and nestin, Dcx, NCAM 1, and MCT 1 for NPCs. The expression of both ESR1 (ERα) and ESR2 (ERβ) was also

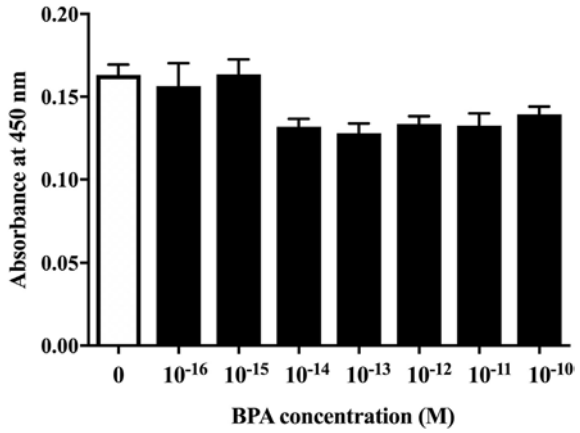

FIGURE 1 | The cell viability of ReNcell ventral mesencephalon cells was assessed after a 24-h exposure to BPA or a control reagent. The cell viability assay was performed using a Cell Counting Kit-8 kit (see Materials and Methods section for details). Data are represented as mean ± SEM (n = 7/group).

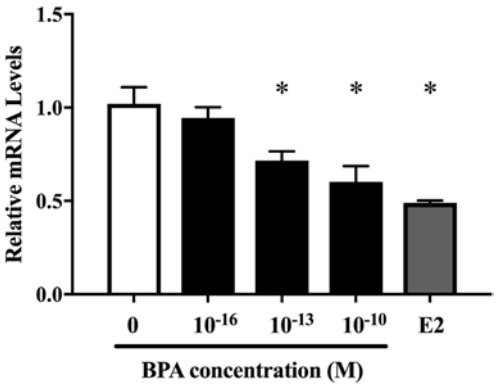

FIGURE 3 | Dose-dependent differences in mRNA levels of β III-tubulin in differentiated ReNcell ventral mesencephalon cells following exposure to 10^{-16} and 10^{-10} M bisphenol A (BPA) and 10^{-10} M β-estradiol. Expression levels of β III-tubulin were analyzed by a real-time quantitative PCR assay. Data are represented as mean ± SEM, n = 3.

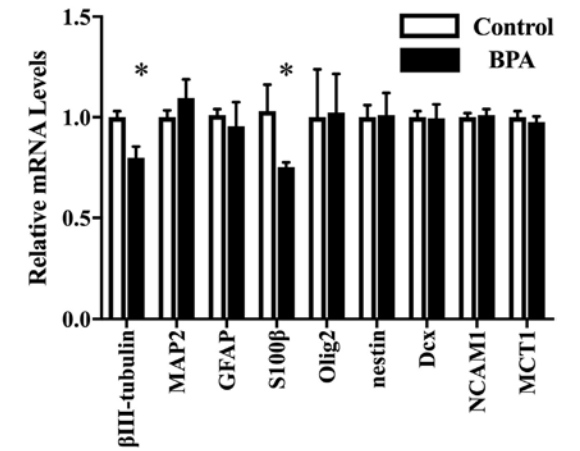

FIGURE 2 | mRNA levels of several genes in differentiated-ventral mesencephalon (VM) cells. mRNA levels of β III-tubulin, glial fibrillary acidic protein (GFAP), Olig2, nestin, Dcx, NCAM1, and MCT in differentiated ReNcell VM cells after exposure to 10^{-10} M bisphenol A (BPA). Data are represented as mean ± SEM, n = 3. The control group values were defined as 1. *$p < 0.05$.

confirmed in ReNcell VM cells. We observed a decrease in β III-tubulin and S100β mRNAs induced by 10^{-10} M BPA treatment (**Figure 2**). The expression of other markers was not altered. ESR1 and ESR2 mRNA levels were not altered by the exposure to BPA (Figure S1 in Supplementary Material). Their expression was not altered by BPF or E2 treatment. To verify the dose dependency of the effect of BPA on β III-tubulin, cells were exposed to various concentrations of BPA. E2 (10^{-10} M) was used as a positive control. BPA at both 10^{-13} and 10^{-10} M significantly decreased β III-tubulin levels (**Figure 3**). Analysis by ANOVA showed a significant dose-dependent effect [$F(4, 17) = 9.688$; $p = 0.0003$]. E2 also decreased β III-tubulin levels.

BPA Reduced the Number of β III-Tubulin-Positive Cells

We hypothesized that the decrease of β III-tubulin mRNA levels by BPA may be due to the decrease of the number of differentiated neurons. To test this hypothesis, we performed immunofluorescence staining with both anti-β III-tubulin and anti-GFAP antibodies, along with Hoechst staining for nucleus. The number of β III-tubulin-positive cells was counted and expressed as a percentage of the total number of cells (the number of Hoechst-positive cells) (**Figure 4A**). We observed that the percentage of β III-tubulin-positive cells decreased significantly in the BPA-exposed group compared to that in controls (**Figure 4B**). In addition, E2 also decreased the percentage of β III-tubulin-positive cells.

BPF Did Not Affect β III-Tubulin Levels

We also investigated whether BPF induced changes similar to BPA using RT-PCR and immunofluorescence staining for β III-tubulin. To compare the effects of BPF and BPA exposure, we used the same concentration of BPF (10^{-10} M) as was used for BPA. RT-PCR (**Figure 5**) and immunofluorescence staining (**Figures 6A,B**) showed no significant alteration in response to BPF exposure.

DISCUSSION

In this study, we investigated the effects of BPA exposure on neural differentiation from NPCs. Exposure to BPA for 3 days from the initiation of neural differentiation decreased both β III-tubulin and S100 β mRNAs without inducing cell death. The number of β III-tubulin-positive cells was decreased significantly following BPA exposure; by contrast, BPF, a substitute for BPA, did not alter β III-tubulin mRNA levels or the number of β III-tubulin-positive cells. Moreover, we observed similar changes in β III-tubulin mRNA and β III-tubulin-positive cells following E2 treatment. Together with previous studies showing that BPA may act as a

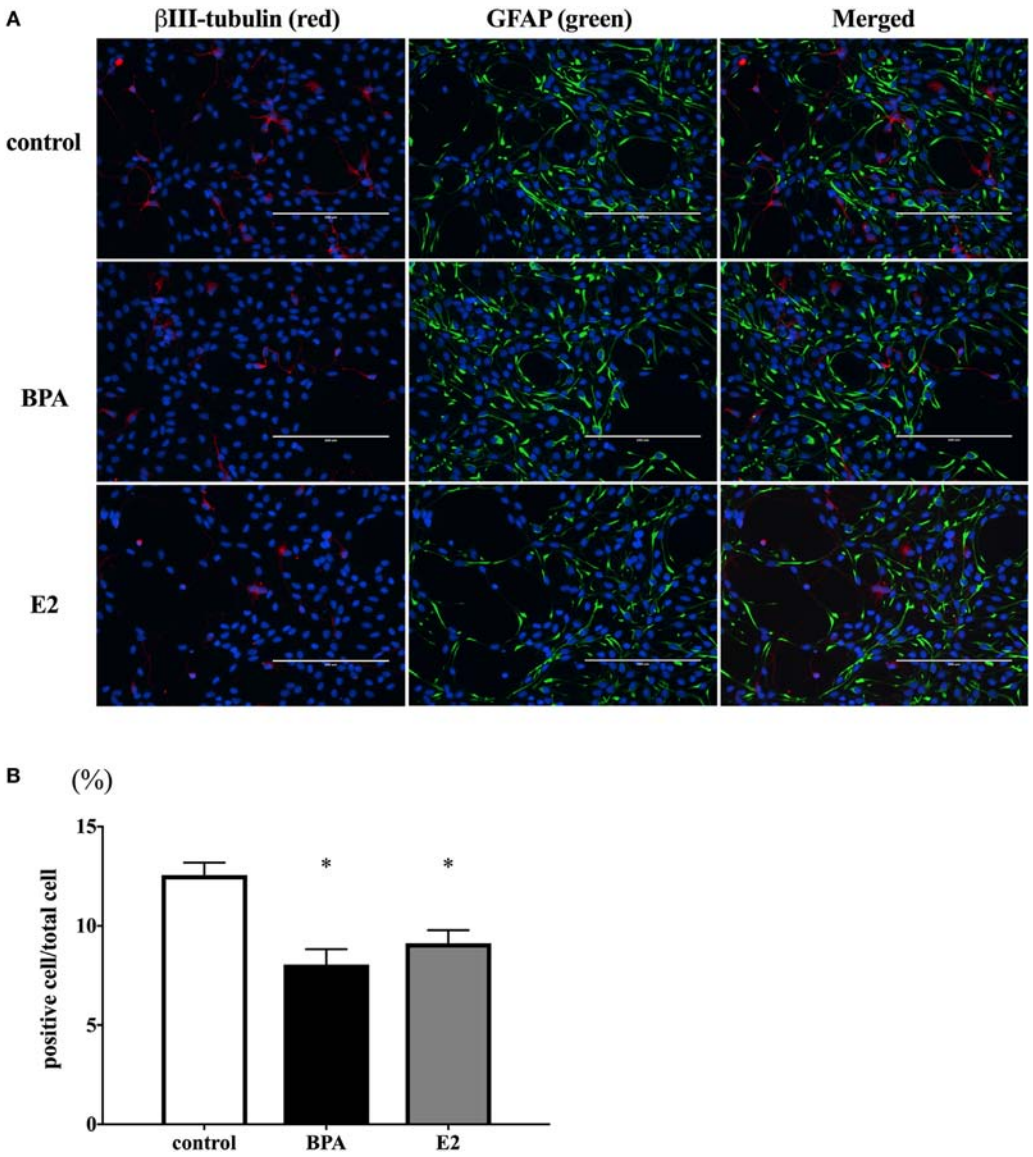

FIGURE 4 | Number of β III-tubulin-positive cells. **(A)** ReNcell ventral mesencephalon cells treated with bisphenol A (BPA), β-estradiol (E2), or control were immune-stained with antibodies for β III-tubulin (red) and glial fibrillary acidic protein (GFAP) (green). Nuclei were identified by Hoechst 33342 staining. Panel **(B)** shows the ratio of β III-tubulin-positive cells per total cells. The number of β III-tubulin-positive cells was counted with four arbitrarily selected fields per well (scale bar: 200 μm). Data are represented as mean ± SEM, $n = 47$.

"xenoestrogen," these results suggest that BPA exposure affects neural differentiation through estrogen-like and/or ER-mediated pathways. In addition, BPF may be a useful substitute for BPA and may avoid the adverse effects of BPA on neural differentiation.

The effects of BPA on neural differentiation using primary cell culture methods using NPCs of the telencephalon or hippocampus have been reported. Using NPCs of the rat hippocampus, Agarwal et al. showed that BPA caused oxidative stress in mitochondria of the NPCs. Such mitochondrial dysfunction inhibited the proliferation and differentiation to β-III-tubulin-positive neuronal cells (37). We also showed the decrease of the number of β-III-tubulin-positive neuronal cells by BPA without alteration of the expression level of MAP2 mRNA. These results indicate that BPA accelerated the differentiation in the later stage from β-III-tubulin-positive neuronal cells to MAP2-positive neuron but not in the earlier stage from NPCs to β-III-tubulin-positive neuronal cells. On the other hand, there were no effects in oligodendrocytes differentiation in our study. Okada et al. reported that exposure to BPA increased the ratio of oligodendrocytes differentiated from NPCs of the rat telencephalon (38). However, other report showed that BPA reduced the differentiation to oligodendrocytes from NPCs of the rat hippocampus (39). Moreover, there are no

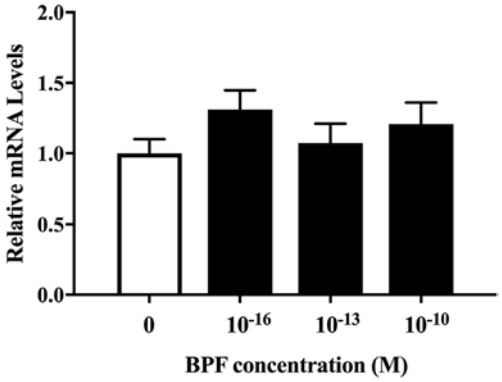

FIGURE 5 | mRNA levels of β III-tubulin in differentiated ReNcell ventral mesencephalon cells exposed to bisphenol A. Cells were harvested after 3 days exposure to 10^{-16}, 10^{-13}, and 10^{-10} M bisphenol F (BPF) and extracted total RNA. Real-time quantitative PCR analyses were performed using a primer set for β III-tubulin. Data are represented as mean ± SEM, $n = 3$.

reports showing the change in astrocyte differentiation by BPA. These results, including our study, suggest that BPA may disrupt neural differentiation from NPC to neuron, whereas further study may be required to confirm its effect on differentiation to oligodendrocyte or astrocyte.

Estrogen and its nuclear receptors, ERs, regulate brain development during differentiation, growth, and the acquisition of brain function (40, 41). Moreover, protection from several toxicants, cellular survival, and neuronal peptides synthesis are also induced by estrogen. In the neural differentiation process, E2 induces the expression of neurotrophic factors such as brain-derived neurotrophic factor, neural growth factor, and neurotropin-3. Such increases in neurotrophic factor expression may affect the proliferation of NPCs, the ratio of neurons to glial cells, and neurite outgrowth. BPA may also bind to ERs and exert agonistic and/or antagonistic activities in humans and animals, potentially causing neurotoxicity, reproductive toxicity, and immune toxicity. The binding affinity of BPA to ERs is 1/500–1/15,000 smaller than that of E2 (21, 42, 43) in humans

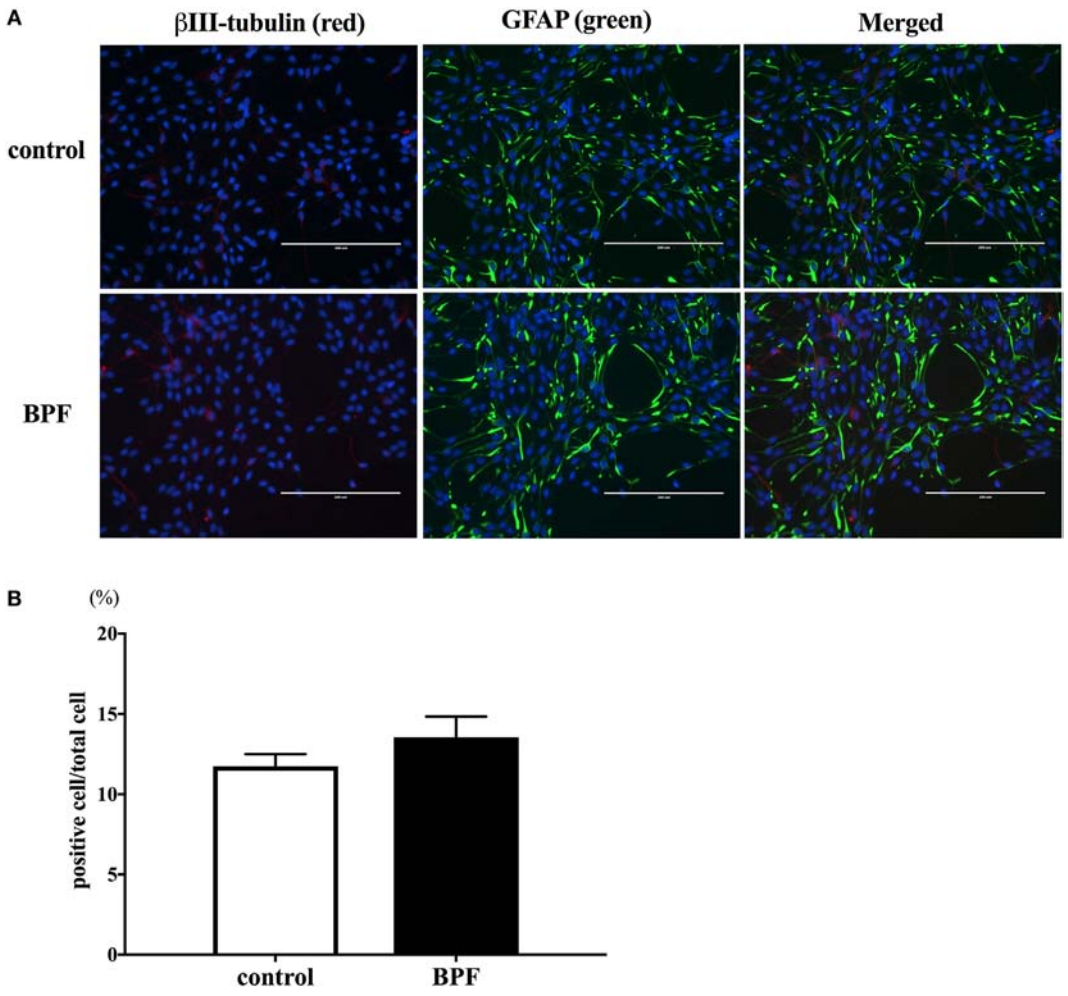

FIGURE 6 | The effects of bisphenol F (BPF) on the number of β III-tubulin-positive cells. **(A)** We performed immunostaining of ReNcell ventral mesencephalon cells treated with BPF or control reagents with antibodies for β III-tubulin (red) and glial fibrillary acidic protein (GFAP) (green), and Hoechst 33342 solution was used for nuclei staining (scale bar: 200 μm). **(B)** The ratio of β III-tubulin-positive cells to total cells was measured. Data are represented as mean ± SEM, $n = 3$.

and rats. Nevertheless, BPA can strongly activate ER-mediated transcription in transient transfection-based reporter gene assay (20, 42, 44, 45). In the present study, to examine whether the effect of BPA is induced through ER, it would be useful to examine the change in estrogen-responsive genes in differentiating human brain cells. However, such gene has not yet been identified. A previous report showed that the exposure to BPA or BPF induced the expression of estrogen-sensitive markers, cyp19a1b, in developing zebra fish brain (46). Unfortunately, this gene is not expressed in mammalian brain. Other candidate estrogen-responsive genes are Nlrp3 and Rtp1, which are expressed in adult mice cerebral cortex (47). Thus, we examined the change in these mRNA levels during differentiation. However, we could not observe any changes of the expression of Rtp1 even by the exposure to E2 (Figure S2 in Supplementary Material), and Nlrp3 was not expressed in the differentiating cells. These results indicate that the marker that we used may not be appropriate to examine the effect of E2 and BPA in differentiating cells. To further investigate the involvement of ER-mediated pathway on BPA effect during neural differentiation, additional study including the identification of estrogen-responsive genes in differentiating cells may be necessary. Nevertheless, by considering the strong BPA action on ER-mediated transcription, the possibility that the BPA action seen in the present study is exerted through ER cannot be excluded.

Although BPA can activate ER-mediated transcription, its effect on ER-mediated action in neural differentiation is controversial. This is partly because E2 plays different roles in this process. ERs are expressed and may be involved in the differentiation of DA neurons (48). However, while E2 may mediate the proliferation and survival of NPCs (49), long-term E2 exposure induces the suppression of cell proliferation in the dentate gyrus (50). Thus, even if BPA acts through ERs, its action may be affected by the dose, length, and period of exposure (39). Huang et al. reported that BPA suppressed DA neuron differentiation in human embryonic stem cells by suppressing E2-activated insulin-like growth factor pathways (51). In the VM region, Elsworth et al. reported that prenatal BPA exposure decreased the number of DA neurons in the fetal VM and spine synapses in the hippocampus (52). In the present study, both BPA and E2 suppressed neural differentiation. Further study is required to differentiate the effects of BPA on ER-mediated regulation of DA neuron differentiation.

We also observed a decrease in S100β mRNA induced by BPA. S100β is often used as an astrocyte marker because it is specifically synthesized and secreted from astrocytes. Unfortunately, we could not find a good antibody to stain S100β protein for immunocytochemistry. Thus, we could not count the number of S100β-positive cells. On the other hand, mRNA levels of GFAP, another astrocyte marker, were not altered by BPA. These results indicate that BPA may specifically affect the expression or the secretion of S100β without changing the number of astrocytes. However, although we were able to immunostain GFAP, we could not obtain a stable count of the number of astrocytes, because astrocytes have long processes and relatively small stomata, both of which were strongly GFAP-positive. Thus, in the present study, we were unable to fully examine the effect of BPA on astrocyte differentiation. Wise et al. reported that BPA did not alter the number of astrocytes in the rat prefrontal cortex (53). On the other hand, S100β is known as a neurotrophic factor (54) that regulates neuronal development processes such as neurite outgrowth. Thus, abnormal neuronal differentiation resulting from BPA exposure may be partly due to decreases in S100β. The decrease in β III-tubulin-positive cells may be partly induced by a decreased secretion of S100β.

To avoid the potential adverse effects of BPA, BPF is sometimes used as a substitute. BPF has also been detected in various foods and in the human body. Prenatal exposure to BPF, like BPA, caused several behavioral changes, including increased anxiogenic behaviors and depression in mice (55, 56). The expression of several neuropeptide-related genes has also been shown to be affected by BPF in zebra fish (57). The binding capacity of BPF to ERs was equivalent to that of BPA and BPF-activated ERs. These reports indicate that BPF may disrupt ER-mediated processes, potentially having adverse effects on the brain. In the present study, we did not observe any changes in neural differentiation following BPF treatment. Thus, at least regarding neural differentiation, BPF may have less adverse effects than BPA.

In conclusion, BPA may disrupt the neural differentiation of human-derived NPCs. Such alterations may cause abnormal brain development following gestational exposure.

AUTHOR CONTRIBUTIONS

YF and WM conducted the complete experiment and prepared the data and manuscript. WM, TK, and NK had the responsibility for the whole experiment, earned the grant, made the strategy, and prepared the manuscript. YF and WM contributed equally to this work.

ACKNOWLEDGMENTS

The authors thank Dr. Izuki Amano, Dr. Yusuke Takatsuru, Dr. Asahi Haijima, Mr. Hiroyuki Yajima, Mr. Michifumi Kokubo, Ms. Miski Aghnia, Ms. Winda Ariyani, and Ms. Noriko Sekie for their technical assistance and advice. The study was supported in part by Grant in Aid for Scientific Research (Nos. 22681008, 26870445, and 25281024) to WM and NK from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fendo.2018.00024/full#supplementary-material.

FIGURE S1 | mRNA levels of ESR1 and ESR2 in differentiated ReNcell VM cells exposed to BPA, BPF, and E2. Cells were harvested after 3 days exposure to 10^{-10} M of these compounds and extracted total RNA. Real-time quantitative PCR analyses were performed using a primer set for ESR1 or ESR2. Data are represented as mean ± SEM, $n = 3$.

FIGURE S2 | mRNA levels of Rtp1 in differentiated ReNcell VM cells exposed to BPA, BPF, and E2. Cells were harvested after 3 days exposure to 10^{-10} M of these compounds and extracted total RNA. Real-time quantitative PCR analyses were performed using a primer set for Rtp1. Data are represented as mean ± SEM, n = 3.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 20 February 2018
doi: 10.3389/fnins.2018.00084

Steroid Transport, Local Synthesis, and Signaling within the Brain: Roles in Neurogenesis, Neuroprotection, and Sexual Behaviors

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience

Received: *01 December 2017*
Accepted: *02 February 2018*
Published: *20 February 2018*

Citation:
Diotel N, Charlier TD, Lefebvre d'Hellencourt C, Couret D, Trudeau VL, Nicolau JC, Meilhac O, Kah O and Pellegrini E (2018) Steroid Transport, Local Synthesis, and Signaling within the Brain: Roles in Neurogenesis, Neuroprotection, and Sexual Behaviors. Front. Neurosci. 12:84. doi: 10.3389/fnins.2018.00084

Sex steroid hormones are synthesized from cholesterol and exert pleiotropic effects notably in the central nervous system. Pioneering studies from Baulieu and colleagues have suggested that steroids are also locally-synthesized in the brain. Such steroids, called neurosteroids, can rapidly modulate neuronal excitability and functions, brain plasticity, and behavior. Accumulating data obtained on a wide variety of species demonstrate that neurosteroidogenesis is an evolutionary conserved feature across fish, birds, and mammals. In this review, we will first document neurosteroidogenesis and steroid signaling for estrogens, progestagens, and androgens in the brain of teleost fish, birds, and mammals. We will next consider the effects of sex steroids in homeostatic and regenerative neurogenesis, in neuroprotection, and in sexual behaviors. In a last part, we will discuss the transport of steroids and lipoproteins from the periphery within the brain (and vice-versa) and document their effects on the blood-brain barrier (BBB) permeability and on neuroprotection. We will emphasize the potential interaction between lipoproteins and sex steroids, addressing the beneficial effects of steroids and lipoproteins, particularly HDL-cholesterol, against the breakdown of the BBB reported to occur during brain ischemic stroke. We will consequently highlight the potential anti-inflammatory, anti-oxidant, and neuroprotective properties of sex steroid and lipoproteins, these latest improving cholesterol and steroid ester transport within the brain after insults.

Keywords: aromatase, cholesterol, blood-brain barrier, estrogens, HDL, lipoproteins, stroke, progestins

INTRODUCTION

Steroid hormones display important physiological functions and exert pleiotropic effects on many target organs including among others the gonads, the liver, and the nervous system. Neurosteroids are produced in the central nervous system (CNS), either via *de novo* synthesis from cholesterol or from local metabolism of steroid intermediate produced in the periphery. The shift from systemic to local synthesis and regulation of steroid action within target tissues, such as the brain, was referred to "Balkanization" of the endocrine system, and could allow the

tissue to autonomously synthesize and modulate local steroid signaling (Schmidt et al., 2008). Neurosteroids and peripherally produced steroids have pleiotropic effects and can modulate both brain homeostasis and cerebral functions. The aim of the present review is to summarize the current knowledge regarding the activity and the expression of the steroidogenic enzymes and the targets of steroids, produced in the periphery as well as locally in the brain. We will further consider the roles of androgens, estrogens, and progestagens on physiology and behavior, focusing our discussion on constitutive and regenerative neurogenesis, notably in stroke conditions, as well as the impact of these locally-produced sex steroids on sexual behavior. These points will be discussed from studies performed in fish, birds, and mammals from a comparative point of view. Furthermore, we will discuss the transport of peripheral steroids through the blood-brain barrier (BBB) and their effects on its permeability. We will emphasize the role of this transport by lipoproteins in the functioning of the BBB and during CNS insults, raising the question of the potential roles of cholesterol/steroid transport in neuroprotection and reactive neurogenesis.

NEUROSTEROIDOGENESIS IN THE BRAIN: AN OVERVIEW IN FISH, BIRDS, AND MAMMALS

Steroidogenesis

Steroidogenesis is the enzymatic process by which cholesterol is converted to biologically active steroid hormones. The steroidogenic acute regulatory protein (StAR), and translocator protein (TSPO), in a complex with various proteins including VDAC and ATAD3A, are involved in the transport of cholesterol to the inner membrane of the mitochondria. The first and rate-limiting enzymatic step of the steroidogenic process is the conversion of cholesterol into pregnenolone by P450 side chain cleavage ($P450_{scc}$; CYP11A1). As shown in the steroidogenic pathway illustrated in **Figure 1**, pregnenolone can subsequently be converted into progesterone (P) by 3-beta-hydroxysteroid dehydrogenase (3β-HSD) or into 17-hydroxypregnenolone by 17-alpha-hydroxylase/17,20 lyase (CYP17). Then, CYP17 can convert progesterone into 17-hydroxyprogesterone, while 3β-HSD can convert 17-hydroxypregnenolone into 17-hydroxyprogesterone. In a next step, 17-hydroxypregnenolone and 17-hydroxyprogesterone can be converted by CYP17 into dehydroepiandrosterone (DHEA) and androstenedione, respectively. In turn, the activity of different 17β-HSD enzymes catalyzes the synthesis of androstenediol from DHEA and testosterone (T) from androstenedione. Aromatase converts androstenedione and testosterone into estrone (E1) and estradiol (E2), respectively, while 5α-reductase converts testosterone into 5α-dihydrotestosterone (DHT). Considering glucocorticoids, the synthesis of 11-deoxycortisol and 11-deoxycorticosterone from 17-hydroxyprogesterone and progesterone, respectively, are catalyzed by 21-hydroxylase (CYP21A2), followed by the P450C11 (Cyp11, cytochrome P450 11β-hydroxylase B1 and/or B2) activity leading to the synthesis of cortisol and corticosterone. Cortisone can also be converted into cortisol by 11β-HSD.

Steroidogenic Activities in the Brains of Fish

In the 80s, pioneering studies performed in the goldfish (*Carassius auratus*) and the toadfish (*Opsanus tau*) documented very high brain aromatase and 5α-reductase activities (Pasmanik and Callard, 1985, 1988). High aromatase expression and activity in the brain is a common feature of teleost fish (Kah et al., 2009; Diotel et al., 2010), as reported in the African catfish (*Clarias gariepinus*) (Timmers and Lambert, 1987), the Atlantic Salmon (*Salmo salar*) (Andersson et al., 1988), the three-spined stickleback (*Gasterosteus aculeatus*) (Borg et al., 1989), the European sea bass (*Dicentrarchus labrax*) (Gonzalez and Piferrer, 2003), and the zebrafish (*Danio rerio*) (Goto-Kazeto et al., 2004; Diotel et al., 2011b). The high aromatase activity in the brain of fish is due to the sustained expression of the *cyp19a1b* gene coding for the Cyp19a1b/Aromatase B protein (AroB), while the *cyp19a1a* isoform coding for Cyp19a1a/Aromatase A protein is mainly expressed in gonads. These two isoforms likely resulted from a third round of genomic duplication event occurring ∼320–350 million years ago in fish (Ravi and Venkatesh, 2008).

Biochemical studies using RP-HPLC analysis showed that the brains of adult zebrafish is able to convert [^{3}H]-pregnenolone into [^{3}H]-progesterone, documenting subsequently 3β-HSD activity in the brain of this teleost. These data were reinforced by the absence of progesterone synthesis following treatment with trilostane, a specific inhibitor of 3β-HSD (Sakamoto et al., 2001a). Ten years later, using similar methods, it was shown that the adult zebrafish brain can convert [^{3}H]-pregnenolone into a wide variety of radiolabeled steroids including 17OH-pregnenolone, progesterone (P), and tetrahydro-P, DHEA, androstenedione, testosterone (T), dihydrotestosterone (DHT), 17β-estradiol (17β-E2), and also estrone (E1) (Diotel et al., 2011b). Expression and activity studies in the brain of adult zebrafish were documented for 3α-Hsd, 3β-Hsd, 17β-Hsd, Cyp17, AroB, and 5α-reductase (Diotel et al., 2011b). The recent reanalysis of the RP-HPLC profiles previously reported by Diotel et al. (2011a) indicated that [^{3}H]-pregnenolone can also be converted into cortisol and tetrahydrodeoxycorticosterone (THDOC), further highlighting Cyp21A2 and Cyp11C1 activities within the brain of adult zebrafish (Weger et al., accepted).

Taken together, these data demonstrate that the brain of adult fish is able to *de novo* synthesize a wide variety of steroids from pregnenolone, suggesting that the substrates available for steroidogenesis can originate from local synthesis within the brain, and also from the conversion of peripherally produced precursors.

Steroidogenic Enzyme Expression in the Brains of Fish

The first attempts to localize aromatase-expressing cells in the brain of fish were performed in goldfish using antibodies raised against human placental aromatase (Gelinas and Callard,

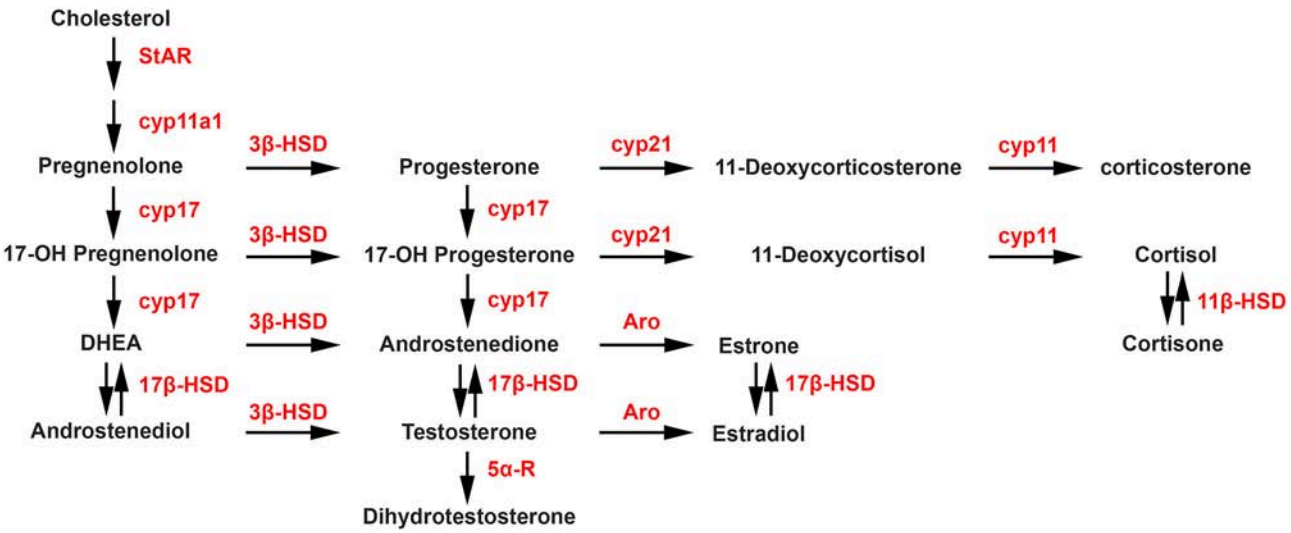

FIGURE 1 | Biosynthetic pathways of neurosteroid synthesis. Cyp11a1, cytochrome P450 family 11 subfamily A member 1 (P450scc), cytochrome P450 side-chain cleavage; Cyp17, cytochrome P450 17α-hydroxylase/C17, 20-lyase; Aro, aromatase; 3β-HSD, 3β-hydroxysteroid dehydrogenase D5–D4 isomerase; 5α-R, 5α-reductase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; Cyp21, cytochrome P450 21 hydroxylase; Cyp11, cytochrome P450 11β-hydroxylase B1 and/or B2. StAR, steroidogenic acute regulatory protein.

1997). In this work, neuron-like cells were labeled in the olfactory bulb, the telencephalon, the preoptic area, and the hypothalamus. However, *cyp19a1b in situ* hybridization and double immunohistochemistry using specific AroB antibodies and glial (GFAP, S100β, and BLBP) or neuronal markers (HuC/D or NeuN) clearly demonstrated the exclusive glial nature of AroB$^+$ cells in others teleost species (Forlano et al., 2001; Goto-Kazeto et al., 2004; Forlano and Bass, 2005; Strobl-Mazzulla et al., 2005; Pellegrini et al., 2007; Mouriec et al., 2008; Tong et al., 2009; März et al., 2010; Coumailleau et al., 2015; Diotel et al., 2016). These data were further reinforced by the development of a *cyp19a1b*-GFP transgenic zebrafish line, showing the exclusive GFP co-expression with glial markers (Tong et al., 2009). Interestingly, AroB$^+$ cells correspond to radial glial cells (RGCs), a particular type of cells with a characteristic morphology. Radial glial cells display a soma localized in the vicinity of the ventricular layer and exhibit two cytoplasmic processes, a short one extending to the ventricle and a longer one running through the brain parenchyma toward the pial surface. In mammals, RGCs behave as neural stem cells (NSCs) during embryonic development and transform into astrocytes at the perinatal stage (Noctor et al., 2001; Weissman et al., 2003; Kriegstein and Alvarez-Buylla, 2009). In fish, they persist during adulthood and maintain neural progenitor properties (Adolf et al., 2006; Pellegrini et al., 2007, 2013, 2015; Tong et al., 2009; März et al., 2010; Rothenaigner et al., 2011; Kizil et al., 2012; Diotel et al., 2016).

Other studies performed in zebrafish documented the expression of 3β-Hsd in neuronal soma and fibers throughout the brain (i.e., dorsal telencephalon, thalamus, preoptic area, paraventricular organ), the cerebellum and the spinal cord (Sakamoto et al., 2001a). In the African lungfish (*Protopterus annectens*), 3β-Hsd was also shown to be expressed in neurons while 5α-reductase was detected in both glia and neurons (Mathieu et al., 2001). Furthermore, numerous experiments documented the expression of the steroidogenic enzymes (*P450scc*, *3β-hsd*, *cyp17,* and *cyp19a1b*) in the brains of fish including the Tongue sole (*Cynoglossus semilaevis*) and the Black porgy (*Acanthopagrus schlegeli*) (Tomy et al., 2007; Chen et al., 2010b). In adult zebrafish, *in situ* hybridization experiments indicate that there is a wide and overlapping distribution of *P450scc*, *3β-hsd*, *cyp17,* and *cyp19a1b* throughout the brain particularly in the telencephalon, the preoptic area, the hypothalamus, and the cerebellum (Diotel et al., 2011b). The distributions of *P450scc*, *3β-hsd,* and *cyp17* suggest a potential expression in neurons as well as in neural progenitors, as shown by co-expression with AroB, a RGC marker. From 2011 until now, new isoforms for these steroidogenic enzymes have been identified: *3-βhsd1*, *3-βhsd2, cyp11a1*, *cyp11a2, cyp17a1,* and *cyp17a2*. Recent work confirmed the sites of expression of these steroidogenic enzymes in the brain of adult zebrafish and further described the distribution of their respective isoforms (Weger et al., accepted). It appears that all these steroidogenic enzymes display overlapping distributions. In addition, expression of *cyp21a2*, *cyp11c1,* and *fdx1/fdx1b* (co-factors of glucocorticoid synthesis) was recently described in adult zebrafish brain (Weger et al., accepted), and patterns appeared similar to other steroidogenic enzymes. Furthermore, isolation and culture of goldfish RGCs was performed (Xing et al., 2014), and deep RNA sequencing of these RGCs indicates expression of steroidogenic acute regulatory protein (*star*), *cyp11a1, cyp17a1*, *fdx1, hsd17b10* in addition to *cyp19a1b* and *5α-reductase* (Da Fonte et al., 2017). Proteomic

analyses also revealed the production of 20β-Hsd (Xing et al., 2016).

Altogether, these studies suggest that the brains of adult fish widely express the biologically active steroidogenic enzymes leading to sex steroid synthesis. They also suggest that RGCs in fish may be capable of *de novo* steroid production from cholesterol and could be consequently envisioned as true steroidogenic cells (**Figure 2A**).

Steroidogenic Enzyme Activities and Expression in the Brains of Birds

Birds are excellent models for understanding the impact of peripherally and locally produced steroids on brain functions, including behavior. Pioneering studies from the groups of Tsutsui and Schlinger have shown that the brains of the quail (*Coturnix japonica*) and zebra finch (*Taeniopygia guttata*) are capable of *de novo* steroidogenesis. They showed that the avian brain expresses the active P450scc, 3β-HSD, CYP17, 17β-HSD, and aromatase enzymes, leading to the production of a wide variety of neurosteroids including pregnenolone, progesterone, androstenedione, testosterone, and estradiol from cholesterol (Tsutsui and Yamazaki, 1995; Tsutsui et al., 1999, 2006, 2009; Matsunaga et al., 2001; London et al., 2006, 2009; London and Schlinger, 2007; Tsutsui, 2011; Schlinger and Remage-Healey, 2012). Western-Blot and immunohistochemistry experiments allow the detection of P450scc immunoreactive neurons through the preoptic area, the thalamus, the hypothalamus, and the cerebellum in Purkinje cells (Tsutsui, 2011). 3β-HSD expression was highlighted based on enzymatic activity in the brain of the adult quail, with a strong presence in the telencephalon and the diencephalon and a lower one in the mesencephalon (Ukena et al., 1999). In quail, *Cyp17* gene expression is detected in the preoptic area, the thalamus, the hypothalamus, and the optic tectum as well as in Purkinje cells (Matsunaga et al., 2001).

Aromatase expression was thoroughly studied in several brain regions particularly, in the hypothalamus and the preoptic area in quail (Balthazart and Foidart, 1993; Balthazart et al., 1996; Balthazart and Ball, 1998; Tsutsui, 2011) and in the telencephalon in zebra finches (Schlinger, 1997). Interestingly, data showed overlapping distribution of *StAR*, *CYP11A1*, *3β-HSD*, and *CYP17 mRNA* in the ventricular proliferative zone of the avian brain during support (London and Schlinger, 2007), suggesting potential roles of neurosteroids in neurogenesis and CNS development. It should be noted that 3β-HSD is also present throughout the telencephalon in adult song birds (Soma et al., 2004; Schlinger et al., 2008). Furthermore, aromatase expression was also studied throughout the avian brain during support and *de novo* expression was documented in RGCs following brain injury in adult birds (Peterson et al., 2004). These data surface a potential role of aromatase and estrogen synthesis in neuroprotection and brain repair mechanisms.

These different studies highlight the capacity of the avian brain to synthesize its own steroids and suggest key roles of neurosteroids in reproductive behavior and neurogenesis.

Steroidogenic Enzyme Activities and Expression in the Brains of Mammals

$P450_{scc}$ gene expression was detected in numerous brain regions of the rodent (i.e., cerebral cortex) and human brain (i.e., cerebral cortex, olfactory bulbs, hippocampus, cerebellum) (Pelletier, 2010). Cells immunoreactive for $P450_{scc}$ were observed in the white matter, the pyramidal and granular neurons of the developing and adult hippocampus (a brain region known for maintaining active neurogenesis during adulthood), and also in Purkinje cells from the cerebellum (Le Goascogne et al., 1987; Tsutsui and Ukena, 1999; Kimoto et al., 2001).

In the 80s, 3β-HSD activity was reported in rat brain homogenates through the conversion of pregnenolone into progesterone (Weidenfeld et al., 1980). More generally, 3β-HSD gene and protein expressions were documented in primary cultures of rodent oligodendrocytes and neurons, and distributed especially in the olfactory bulbs, the thalamus, the cerebral cortex, the hypothalamus, and the cerebellum (Dupont et al., 1994; Guennoun et al., 1995; Meffre et al., 2007; Pelletier, 2010). In humans, *3β-HSD* gene expression was detected in neuronal, astroglial, and oligodendrocyte cell lines (Brown et al., 2000) and had a similar distribution compared to rodents, notably in the thalamus, the hippocampus, and the cerebellum (Yu et al., 2002).

The presence of Cyp17 in the mammalian central nervous system (CNS) is currently not clear. Indeed, some work suggested the absence of *Cyp17* gene expression while other studies reported *Cyp17* gene expression in the cerebellum and the brainstem of rat, and showed Cyp17 immuno-positive cells in the hippocampus, the hypothalamus, and in the Purkinje cells (Mellon and Deschepper, 1993; Strömstedt and Waterman, 1995; Hojo et al., 2004; Pelletier, 2010). During development, the expression of *Cyp17* in the rat brain is present but decreases postnatally (Compagnone and Mellon, 2000). Hence, the question of Cyp17 expression and activity in the mammalian brain remains open.

In rodents, aromatase expression (mRNA and protein) and activity were documented in numerous brain regions including the cerebral cortex, the preoptic nucleus, the hypothalamus as well as the hippocampus; in humans, it was also detected in the pons, the thalamus, the hypothalamus, and the hippocampus (Lephart et al., 1992; Lephart, 1996; Sasano et al., 1998; Garcia-Segura et al., 2003; Hojo et al., 2004; Yague et al., 2006; Pelletier, 2010). In rodents, aromatase expression was mainly detected in neurons and not in glial cells, except following brain injury that results in a *de novo* aromatase expression by astrocytes, suggesting a role of estrogens in neuroprotection (Garcia-Segura et al., 1999; Azcoitia et al., 2001; Garcia-Segura, 2008). In contrast to rodents, in some primates including humans, the estrogen-synthesizing enzyme expression was also reported in some subpopulations of astrocytes in addition to neurons (Yague et al., 2006; Roselli, 2007). As reviewed by Pelletier (2010), numerous studies demonstrate aromatase activity in the brain of mammals including primates.

Consequently, *de novo* steroid synthesis occurs in the brains of mammals (**Figure 2B**). Neurosteroidogenesis is active in

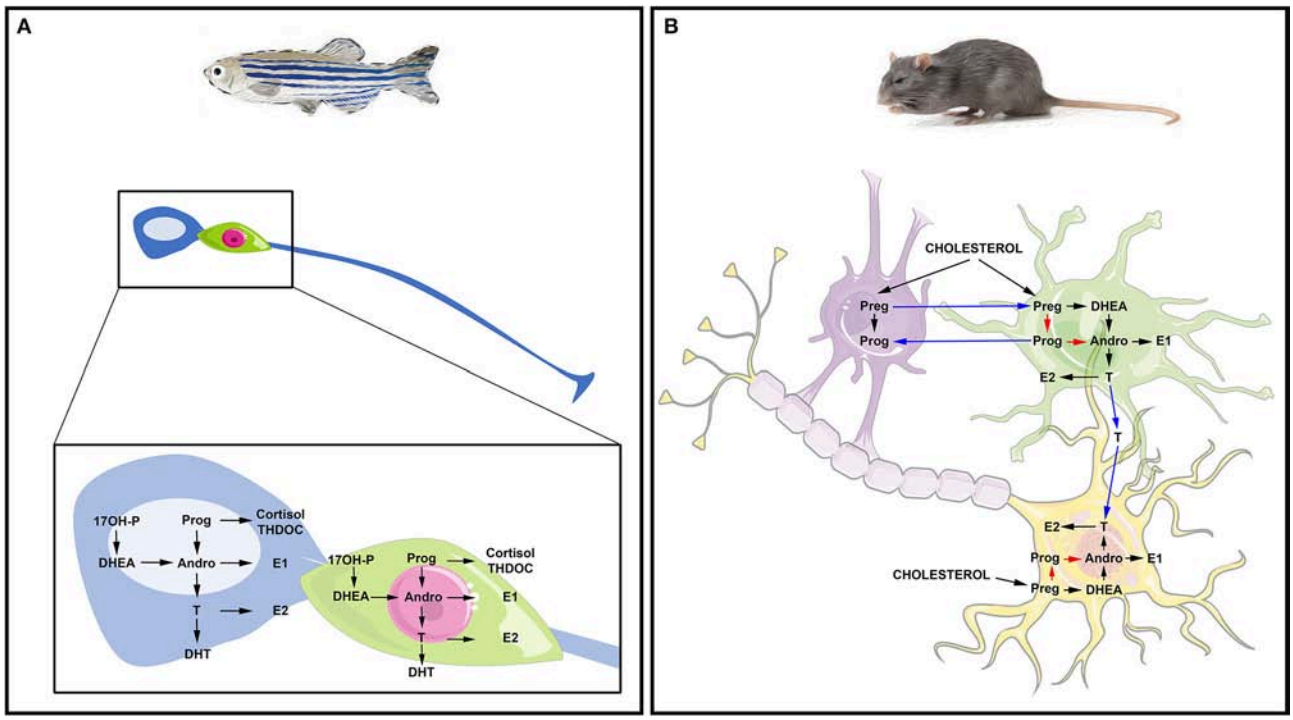

FIGURE 2 | Steroidogenesis in the brain of fish and mammals. **(A)** Steroidogenesis in the brain of adult fish. The blue cell corresponds to a radial glia cell acting as neural stem cell, while the green cell corresponds to a neuron. Data obtained by *in situ* hybridization, RNA sequencing and/or proteomics show neuronal and radial glial expression of steroidogenic enzymes involved in the synthesis of 17OH-Pregnenolone (17OH-P), Progesterone (P), dehydroepiandrosterone (DHEA), Androstenedione (Andro), Estrone (E1), 17β-estradiol (17β-E2, called here E2), testosterone (T) and dihydro-testosterone (DHT), cortisol and THDOC (tetrahydrodeoxycorticosterone). **(B)** Steroidogenesis in the brain of rat. The purple cell corresponds to an oligodendrocyte that participates in the myelin sheath genesis of axons; the green cell is an astrocyte and the yellow one is a neuron whose axon is myelinated by the oligodendrocyte. This schematic view adapted from Zwain and Yen (1999) highlights potential interaction of oligodendrocytes, astrocytes, and neurons in neurosteroidogenesis. The main steroidogenic pathway is shown by black arrows, the minor ones by red arrows and the suggested ones by blue arrows referring to the work of Zwain and Yen (1999). Note that arrows do not necessarily document the direct conversion of the one steroid into another, but can include several enzymatic processes.

numerous regions including the hippocampus, known for maintaining an intense neurogenic activity during adulthood. Interestingly, this region was shown to synthesize 17β-E2 from pregnenolone (Hojo et al., 2004), reinforcing the idea that steroids could impact neurogenesis.

In conclusion, neurosteroidogenesis appears to be a conserved process well-documented in fish, birds and mammals (**Figure 2**). It occurs in numerous evolutionary conserved regions such as the telencephalon, the diencephalon (i.e., preoptic area and hypothalamus), and also the cerebellum. This sustained synthesis of steroids may regulate several brain functions and participate to brain homeostasis, neurotransmission, neurogenesis, and brain plasticity by targeting different cell types.

STEROID SIGNALING IN THE BRAIN: FOCUS ON THE BRAINS OF FISH, BIRDS, AND MAMMALS

Peripherally and locally produced steroids can target different brain regions and cell-types expressing their respective and specific receptors. Here, we will describe the main sites of expression of steroid receptors, with a special focus on estrogen, progesterone, and androgen receptors. We will first discuss receptor distributions in the brain of fish and birds before highlighting the situation in mammals.

Expression of Nuclear and Membrane-Associated Steroid Receptors in the Brains of Fish

Nuclear Estrogen Receptors (ER)

Unique among vertebrates, fish have three nuclear estrogen receptors resulting from an ancient genome duplication: one ERα and two ERβ. The ERα and ERβ probably arose from a first duplication event prior the emergence of ray-finned species. A second duplication led to the emergence of two *ERβ* and *ERα* genes but the second copy of the *ERα* probably disappeared (Thornton, 2001; Bardet et al., 2002; Nagler et al., 2007).

Nuclear estrogen receptors genes [*ERα* (*esr1*), *ERβ1* (*esr2b*), and *ERβ2* (*esr2a*)] were identified in many teleost species, including the rainbow trout (*Oncorhynchus mykiss*), tilapia (*Oreochromis niloticus*), medaka (*Oryzias latipes*), goldfish, and zebrafish among others (Pakdel et al., 1990; Tan et al., 1995;

Muñoz-Cueto et al., 1999; Tchoudakova et al., 1999; Xia et al., 1999; Hawkins et al., 2000; Kawahara et al., 2000; Ma et al., 2000; Socorro et al., 2000; Bardet et al., 2002; Lassiter et al., 2002; Menuet et al., 2002; Andreassen et al., 2003; Choi and Habibi, 2003; Halm et al., 2004; Sabo-Attwood et al., 2004; Filby and Tyler, 2005; Forlano et al., 2005; Nagler et al., 2007; Strobl-Mazzulla et al., 2008; Chakraborty et al., 2011; Zhang et al., 2012; Katsu et al., 2013).

Most studies carried out so far reported the presence of *esr* transcripts in the brain of adult fish. *In situ* hybridization and immunohistochemistry experiments used to localize ER-expressing cells in the brain revealed distinct but partly overlapping patterns of expression in many fish species including medaka, Atlantic croaker (*Micropogonias undulatus*), pejerrey (*Odontesthes argentinensis*), trout and zebrafish (Salbert et al., 1991; Anglade et al., 1994; Hawkins et al., 2000, 2005; Menuet et al., 2002; Pellegrini et al., 2005; Nagler et al., 2007; Strobl-Mazzulla et al., 2008; Zempo et al., 2013; Coumailleau et al., 2015). In general, transcripts for ERs have a broad distribution and were detected in many brain regions known to regulate sexual, reproductive, social behaviors, and sensory-motor activities such as the telencephalon, the preoptic area, the hypothalamus, and the cerebellum. Additionally, ER transcripts have been reported in cells lining the ventricles, where RGCs are localized, and also in cells localized more deeply in the brain parenchyma. Interestingly, there are clear evidences that ERα is expressed in dopaminergic, GABAergic, kisspeptin- and neuropeptide B-positive neurons in several species (Linard et al., 1996; Anglade et al., 1999; Mitani et al., 2010; Escobar et al., 2013; Zempo et al., 2013; Hiraki et al., 2014). Similarly to rodents, ERs were not reported in neurons expressing gonadotrophin-releasing hormone (Navas et al., 1995).

In midshipman (*Porichthys notatus*), pejerrey, and zebrafish, the pattern of expression of the nuclear estrogen receptors and AroB (Cyp19a1b) are closely related (Forlano et al., 2005; Strobl-Mazzulla et al., 2008; Diotel et al., 2011a). Careful examination of adjacent sections hybridized with *cyp19a1b* and *esr1* riboprobes in trout and zebrafish failed to evidence any co-localization (Menuet et al., 2003, 2005). However, *esr2b* was recently shown to be expressed in AroB-expressing RGCs (Pellegrini et al., 2015), and RT-PCR analysis performed in glial cells enriched cultures from adult trout or goldfish suggested that a weak expression of ERα could not be excluded (Menuet et al., 2003; Xing et al., 2015). A recent study designed to characterize the transcriptome of cultured goldfish RGCs and reported *esr1*, *esr2b,* and *esr2a* expression in these neural progenitors (Da Fonte et al., 2017).

Knowledge about estrogen signaling during early development in fish is very limited and the zebrafish is the only species in which expression of the three ERs during embryogenesis was monitored. Quantitative-PCR experiments established that *esr1*, *esr2a,* and especially *esr2b* mRNA were maternally inherited and expressed in eggs before dropping down to 24 h post-fertilization (hpf) (Bardet et al., 2002; Lassiter et al., 2002; Mouriec et al., 2009; Bondesson et al., 2015). Nuclear estrogen receptor expression then started to increase at 24 h, when the onset of zygotic transcription is activated (Bardet et al., 2002; Lassiter et al., 2002; Mouriec et al., 2009). Whole mount *in situ* hybridization showed that *esr2a* and *esr2b* mRNA were detectable at 48 hpf in the ventral telencephalon and in the presumptive preoptic area as well as in the hypothalamus, in line with what was described in the adult brain (Mouriec et al., 2009). A clear expression for *esr1* is described in these regions but at later stages, between 14 days post-fertilization (dpf) and 21 dpf (Kallivretaki et al., 2007; Mouriec et al., 2009). Using *cyp19a1b*-GFP zebrafish embryos, GFP expression was shown to be inducible in RGCs at 24 h after 17β-E2 treatment, an effect blocked by the ER antagonist ICI 182,780, indicating that ERs were fully functional in the brain early during development (Mouriec et al., 2009). A morpholino approach confirmed the specific role of *esr2b* in the induction of the *cyp19a1b* gene (Griffin et al., 2013). Temporary disruption of *esr2a* expression in zebrafish with the same technological approach was associated with a developmental defect of sensory hair cells (Froehlicher et al., 2009). Knockdown of both maternally inherited and zygotic *esr2a* was associated with an increase in apoptotic cells particularly in the brain leading to severe brain defects (Celeghin et al., 2011).

Membrane-Associated Estrogen Receptors (mER)

Membrane receptors responding to 17β-E2 stimulation via rapid non-genomic signaling have been characterized in fish. G protein-coupled estrogen receptor 1 (GPER or GPR30) is a member of this receptor family. A partial or full-length *gper* was cloned, characterized, and detected in the brain of adult zebrafish, Atlantic croaker, and goldfish (Pang et al., 2008; Liu et al., 2009; Pang and Thomas, 2009; Mangiamele et al., 2017). In zebrafish, *gper in situ* hybridization on adult brain sections showed a specific pattern of expression in the olfactory bulbs, the telencephalon, the hypothalamus, the optic tectum, the cerebellum, and the medulla oblongata (Liu et al., 2009). In goldfish, *gper* was also expressed in the forebrain and the suprachiasmatic nucleus, the preoptic area, and the optic tectum (Mangiamele et al., 2017). Originally, it was proposed that goldfish RGCs did not express the G-protein coupled estrogen receptor because attempts to amplify a specific cDNA from cultured cells failed (Xing et al., 2015). However, whole RNA sequencing of separate primary cultures of these cells revealed expression of *gper1*, suggesting that some subpopulations of RGCs express the membrane ER (Da Fonte et al., 2017).

The spatiotemporal distribution of *gper* was investigated in zebrafish using PCR. Its expression was observed in the anterior diencephalon, the midbrain and the hindbrain (Jayasinghe and Volz, 2012; Shi et al., 2013). Both *gper* and *esr2b* mRNA are expressed in neuromasts, suggesting a role of estrogens and a close interaction between these receptors in the developmental regulation of this mecanoreceptive organ (Froehlicher et al., 2009). Knock-down of *gper* resulted in morphological defects in the zebrafish brain at 24 hpf (Shi et al., 2013). At 30 hpf, these morphants displayed an increased number of apoptotic cells, and a decreased brain cell proliferation (Shi et al., 2013). A slight reduction in *otx2* gene expression, which is required for sensory organs development and brain function, was also observed in *gper* knock-down zebrafish. Taken together, these data show the

potential involvement of both nuclear and membrane-associated estrogen receptors in brain development and functions.

Nuclear Progestin Receptors (PR)

Nuclear progestin receptor (PR) genes have been characterized in a few fish species (Ikeuchi et al., 2002; Morini et al., 2017). In European eel (*Anguilla Anguilla*), two PR genes, *pgr1* and *pgr2*, were differentially expressed in the brain (olfactory bulb, telencephalon, diencephalon, cerebellum) and in the pituitary of immatures males and females (Morini et al., 2017). Two genes were also identified in the Japanese eel (*Anguilla japonica*) but only *pgr2* mRNA was detected in the brain (Ikeuchi et al., 2002). In zebrafish, a single locus encoding PR (Pgr) was identified (Chen et al., 2010a; Hanna et al., 2010). Ontogenic expression analysis determined by PCR during early embryogenesis showed that *pgr* mRNA was not maternally inherited and became detectable in embryos at 8 hpf (Chen et al., 2010a). Immunohistochemistry and *in situ* hybridization revealed a widespread distribution of zebrafish Pgr receptor (protein and mRNA) in the brain especially neuroendocrine regions (olfactory bulbs, preoptic area, telencephalon, thalamus, hypothalamus, optic tectum, torus longitudinalis, valvula cerebelli) (Hanna et al., 2010; Diotel et al., 2011c). It should be noted that Pgr-positive cells were observed along the ventricular cavities and also more deeply in the brain parenchyma. In this work, it was shown that periventricular Pgr-positive cells displayed a stronger staining compared to Pgr-positive cells located deeply in the parenchyma (Hanna et al., 2010; Diotel et al., 2011c). By performing double immunostainings, it was shown that a large portion of Pgr-positive cells located in the cerebral parenchyma correspond to acetylated-tubulin positive neurons (Diotel et al., 2011c). In contrast, immunohistochemistry on *cyp19a1b*-GFP transgenic zebrafish clearly showed Pgr-expressing cells along the ventricle correspond to AroB^{+} RGCs (Diotel et al., 2011c). These data were confirmed by a transcriptomic analysis showing *pgr* mRNA expression in cultured goldfish RGCs (Da Fonte et al., 2017). Pgr expression is consequently high in the estrogen-synthesizing RGCs. This suggests a relationship between locally-produced estrogens and Pgr expression. Indeed, treatments with the aromatase inhibitor ATD or with 17β-E2 respectively decrease or increase *prg* expression in the brains of adult zebrafish and also in larvae (Diotel et al., 2011c).

Gene knockout using the TALENs strategy allowed to generate zebrafish lines with null Pgr expression to assess the *in vivo* function of this receptor. Unfortunately, all studies performed with these models have focused at the gonadal levels and none of them looked at the effect of Pgr expression disruption in the brain (Zhu et al., 2015; Tang et al., 2016; Wang et al., 2016).

Membrane-Associated Progestin Receptors (mPR)

In mammals, many progestins exert non-genomic effects through rapid activation of intracellular signaling pathways mediated by two groups of membrane progestin receptors: mPRs (belonging to the PARQ family) and PGRMC1 and PGRMC2 (Petersen et al., 2013). However, there are only scarce data on membrane-associated progestin receptors existence and sites of expression in the brain and the pituitary of fish (Thomas, 2008).

The *mPRα* was identified and detected in the brain of adult spotted seatrout (*Cynoscion nebulosus*) (Zhu et al., 2003). It was also recently cloned in medaka but no data about its brain expression are available (Roy et al., 2017). In goldfish, *mPRα*, *mPRβ*, *mPRγ1,* and *mPRγ2* were also detected in the brain (Tokumoto et al., 2006, 2012). Other studies documented the expression of different mPRs and/or PGMRCs in the brain and the pituitary of the European eel (*Anguilla anguilla*), the channel catfish (*Ictalurus punctatus*) and also the rainbow trout (Kazeto et al., 2005; Mourot et al., 2006; Morini et al., 2017). To date, mPRα and mPRβ were the only form to be identified in zebrafish (Zhu et al., 2003). Using specific antibodies for zebrafish mPRα and mPRβ, Hanna and Zhu demonstrated their expression in brain samples (Hanna and Zhu, 2009). Currently, the distributions and functions of mPR in the brain of fish, if any, are completely unknown.

Nuclear Androgen Receptors (AR)

Two *ar* genes (*ar*1 and *ar*2) were isolated and characterized in several fish species including the Japanese eel (*Anguilla japonica*), the rainbow trout, the kelp bass (*Paralabrax clathratus*), the Atlantic croaker, and cichlids (Ikeuchi et al., 1999; Sperry and Thomas, 1999a,b; Takeo and Yamashita, 1999, 2000; Harbott et al., 2007). More recently, only one *ar* isoform was described in the stickleback (Olsson et al., 2005), the wrasse (*Halichoeres trimaculatus*) (Kim et al., 2002), the zebrafish (Jorgensen et al., 2007; de Waal et al., 2008; Gorelick et al., 2008; Hossain et al., 2008), and in the plainfish midshipman (Forlano et al., 2010).

The first immunohistochemistry studies carried out on the brains of adult goldfish with an heterologous antibody revealed AR-positive cells with neuronal appearance in neuroendocrine regions such as the preoptic area and the hypothalamus, as well as in the olfactory bulbs, the telencephalon and the optic tectum (Gelinas and Callard, 1997). More detailed results were obtained in the brain of the plainfin midshipman, a fish that express mating behavior triggered by auditory signal. These data showed mRNA expression in auditory-related nuclei of the telencephalon, hypothalamus, midbrain, and in the vocal prepacemaker and vocal motor nuclei (Forlano et al., 2010). Numerous studies showed AR expression in the brain of fish including among others wrasse and zebrafish (Kim et al., 2002; Harbott et al., 2007; Hossain et al., 2008; Pouso et al., 2010).

In adult zebrafish, *ar* is strongly expressed along the ventricle and within the brain parenchyma of the telencephalon, the preoptic area, and the hypothalamus (Gorelick et al., 2008). In embryos, *ar* trancripts are maternally deposited and expression levels start to increase substantially at 24 hpf (Hossain et al., 2008). The *ar* gene expression is detected at 24 hpf in the olfactory placodes and in the midbrain, at 48 hpf in the medial diencephalon and at 3 dpf in the pineal organ (Gorelick et al., 2008). All neuroanatomical studies undertaken to date show that ARs are expressed in ventricular margins, where RGC are located (Harbott et al., 2007; Gorelick et al., 2008; Forlano et al., 2010; Pouso et al., 2010). Recently, transcriptomic analysis showed the expression of *ar* in goldfish RGCs (Da Fonte et al., 2017).

As mentioned above, it was shown that goldfish RGCs express progesterone receptor (Pgr), androgen receptor (*ar*), estrogen

receptor α (*esr1*), estrogen receptor β1 (*esr2b*), and estrogen receptor β2 (*esr2a*) (Da Fonte et al., 2017). This data supports other reports in zebrafish showing that RGCs express Pgr protein (Diotel et al., 2011c) and observations for the expression of *ar*, *esr1*, *esr2a*, and *esr2b* mRNAs within neurons and/or AroB$^+$ RGCs (Diotel et al., 2011a; Pellegrini et al., 2015). All this suggests that RGCs are both a source and a target of neurosteroids (Diotel et al., 2011a; Pellegrini et al., 2015; Xing et al., 2016), arguing for key roles of steroids in RGC activity.

Expression of Nuclear and Membrane-Associated Steroid Receptors in the Brains of Birds

Nuclear and Membrane-Associated Estrogen Receptors

Early data on the distribution of sex hormone receptors in the brain of birds was extensively reviewed (Gahr, 2001). Similar to mammals, there are two different avian genes coding for ERα and ERβ (Bernard et al., 1999; Jacobs et al., 1999). In birds, ERα is expressed in the brain of all the species studies so far, including members of Apodiformes, Passeriformes, Galliformes, Columbiformes, and Psittaciformes. These ERs are distributed in various brain territories including the telencephalon, the diencephalon, and the rhombencephalon, with more noticeable expression in the preoptic area and the hippocampus (Gahr, 2001). For instance, in the brain of zebra finch, ERα mRNAs was observed in the nidopallium, the arcopallium, the hippocampus, the diencephalon, the midbrain and within the vocal control circuitry (Jacobs et al., 1999). Interestingly, ERα mRNAs expression overlap with that of aromatase (Jacobs et al., 1999). ERβ was first described in the brain of the Japanese quail, the European starling (*Sturnus vulgaris*), and the canary (Bernard et al., 1999; Foidart et al., 1999; Gahr, 2001). In general, ERβ was reported in the preoptic area, the hypothalamus, the thalamus and different midbrain nuclei. In the European starling, *in situ* hybridization experiments allowed the detection of ERβ mRNA in the nidopallium and the preoptic area in a pattern reminiscent of aromatase (Bernard et al., 1999; Axelsson et al., 2007). Consequently, ERα, ERβ and aromatase distributions strongly overlap. As reported in oscine birds, ER labeling was detected in different forebrain regions while it was not found in non-oscine ones, highlighting discrete differences in ER expression in songbird species and families (Gahr, 2001). It should be noted that, while histological studies have focused on the distribution of the nuclear isoforms, numerous functional studies suggest that estrogen receptors act at the level of the membrane, probably via interaction with other receptors such as glutamate receptors (Pawlisch and Remage-Healey, 2015; Seredynski et al., 2015).

Nuclear and Membrane-Associated Progesterone Receptors

Concerning PR expression, most studies in birds have focused on the hypothalamic region, because of the potential role of PR signaling in reproductive and egg-laying behaviors. Similarly to ER and AR, PR expression was investigated and documented by *in situ* hybridization and immunohistochemistry in the preoptic area, the hypothalamus, the thalamus, the hippocampus of the zebra finch, and the hen (Sterling et al., 1987; Lubischer and Arnold, 1990).

Androgen Receptors

The first studies documenting AR distribution in the brain of birds were performed by *in vivo* autoradiography with radio-labeled steroids (Arnold, 1980; Gahr, 2001). In zebra finch, these autoradiographic studies demonstrated the binding of tritiated testosterone in the HVC (used as the proper term, but previously known as hyperstriatum ventrale pars caudalis or and high vocal center), the magnocellular nucleus of the anterior nidopallium, the robust nucleus of the arcoplallium, the nucleus intercollicularis of the midbrain, and the periventricular magnocellular nucleus of the anterior hypothalamus (Arnold, 1980). In the golden-collared manakins (*Manacus vitellinus*), *in situ* hybridization for AR revealed expression in the forebrain, in the nucleus taeniae of the amygdala and in the arcopallium (Fusani et al., 2014). In general, ARs are detected in the hypothalamus, the preoptic area, the midbrain, and hindbrain in all species studied to date.

Taken together, it appears that ER, AR, and PR display almost similar distribution in the avian brain, with higher expression in the preoptic area, hypothalamus, thalamus, and hippocampus.

Expression of Nuclear and Membrane-Associated Steroid Receptor in the Brains of Mammals

Estrogen Receptors

Data concerning ERs mRNA and protein expression mostly originate from rat studies showing a strong expression of ERα and ERβ throughout the brain (Li et al., 1997; Shughrue et al., 1997). *In situ* hybridization and immunohistochemistry experiments revealed that ERα and ERβ are expressed in neurons and glia from many CNS regions including the olfactory bulbs, the preoptic area, the hypothalamus, the zona incerta, the ventral tegmentum as well as the cerebellum in Purkinje cells (Li et al., 1997; Shughrue et al., 1997; Mitra et al., 2003; Frick et al., 2015). Both ERs were also detected in the cerebral cortex and in the hippocampus, with higher ERβ expression compared to ERα (Shughrue et al., 1997). In the brain of mouse, immunohistochemistry against ERβ allows the detection of immunoreactive cells in regions similar to the rat. However, ERα expression appears stronger in the hippocampus and the hypothalamus, and lower in the cerebral cortex and cerebellum (Mitra et al., 2003), suggesting some differences in ER signaling between rat and mouse. Importantly, ERs are widely and strongly expressed in the brain of mouse at postnatal day 7, but their respective expression declines the two following days with different kinetics (Sugiyama et al., 2009). In humans, ERα is detected in the cortex at 9 gestational weeks in proliferating zones and in the cortical plate. Its expression subsequently decreases until birth before increasing gain during adulthood. At 15 gestational weeks, ERβ was also detected in proliferating zones and in the cortical plate with expression persisting in the adult cortex (González et al., 2007). Interestingly, from 15

gestational weeks to adulthood, ERα and ERβ are detected in the hippocampus including the dentate gyrus, known to be a neurogenic region (González et al., 2007). In the same line, ERs have been detected in embryonic/adult NSCs in rodents and humans, suggesting a roles of estrogen signaling in NSC activity (Brännvall et al., 2002; Kishi et al., 2005; Hajszan et al., 2007; Suzuki et al., 2007; Okada et al., 2010). The spatiotemporally regulated expression of ERs in the embryonic brain of mouse and human, notably in the cerebral cortex and the hippocampus, argue in favor of different roles of estradiol during development (González et al., 2007; Sugiyama et al., 2009). Furthermore, a plasma membrane-associated ER (ER-X, GPER, GPR30, GPER1) was identified in the brain, mediating estrogen activation of MAPK/ERK (Toran-Allerand, 2005). This receptor is functionally distinct from ERα and ERβ and is up-regulated in the adult brain after ischemic stroke (Toran-Allerand et al., 2002). Its expression in the hippocampus is likely to play a role in synaptic plasticity (Waters et al., 2015).

Progesterone Receptors

In mammals, progesterone receptors include the classic nuclear PRA and PRB receptors, splice variants, and membrane-associated receptors mPR (mPRα, mPRβ, and mPRγ) (Brinton et al., 2008). Nuclear PRs are expressed through the brain in neurons and glial cells of the hippocampus, the cortex and the hypothalamus (Hagihara et al., 1992a,b; Brinton et al., 2008), and their expression is up-regulated by estrogens in a region specific manner (Guerra-Araiza et al., 2003). Among the three mPR subtypes described, mPRα is the best characterized pharmacologically. *In situ* hybridization and immunohistochemistry experiments have shown a wide mPRα neuronal expression in the olfactory bulbs, the cortex, the hypothalamus, the hippocampus, and the cerebellum (Meffre et al., 2013). Very interestingly, after traumatic brain injury, mPRα is *de novo* expressed in microglia, astrocytes and oligodendrocytes, highlighting a potential role of this receptor in inflammation and brain repair mechanisms (Meffre et al., 2013). In rat, mPRβ-expressing neurons are detected in the forebrain and the midbrain (Zuloaga et al., 2012). In human, mPR mRNAs are distributed in the forebrain, the hypothalamus and also the hippocampus (Pang et al., 2013).

Androgen Receptors

In adult rodents, AR mRNA and protein are detected in neurons and glia of the medial amygdala, the preoptic area, the hypothalamus, the cerebellum, and the dentate gyrus of the hippocampus (Commins and Yahr, 1985; Sar et al., 1990; Hajszan et al., 2007; Feng et al., 2010; Pelletier, 2010; Mhaouty-Kodja, 2017). Interestingly, AR protein level is correlated with circulating levels of estradiol and testosterone across the estrous cycle, suggesting an estrogenic regulation of its expression (Feng et al., 2010).

To conclude, across vertebrate species, the distribution of ER, PR, and AR in the brain are evolutionary conserved, especially in the hypothalamus, the preoptic area, and some striatal regions. They are also expressed in neurogenic brain regions, arguing in favor of a role sex steroids in the regulation of NSC activity, neuronal differentiation, and cell survival.

ROLE OF STEROIDS IN THE BRAINS OF FISH, MAMMALS, AND BIRDS

In this third part, we will discuss the action of estrogens, progestagens and androgens on constitutive neurogenesis and their effects on CNS following insults such as stroke and traumatic brain injury, with a focus on the impact of sex on brain injury. Then, we will discuss the impact of neurosteroids on behavior in fish, birds, and rodents, focusing only on sexual behavior.

Constitutive and Regenerative Neurogenesis

The actions of steroids in the brain are complex and depend of many factors such as the strain, the species, the timing, the concentration, and the rhythm of secretion, as well as the regions studied (Duarte-Guterman et al., 2015; Heberden, 2017). Sex steroids are well-documented for impacting neuronal plasticity including synaptogenesis/spinogenesis such as shown for estrogens (Fester et al., 2012; Brandt et al., 2013; Sellers et al., 2015; Sager et al., 2017), progesterone (Sakamoto et al., 2001b, 2002; Zhao et al., 2011), and testosterone (Manolides and Baloyannis, 1984; Devoogd et al., 1985). They have been also well-characterized for their role on learning/memory processes (Frye and Walf, 2008, 2010; Frye et al., 2008; Phan et al., 2012; Celec et al., 2015; Frick et al., 2015). In addition, estrogens, progestins, and androgens display neuroprotective effects through genomic and non-genomic mechanisms (López-Rodríguez et al., 2015). These neuroprotective effects involved the upregulation of anti-apoptotic factors (i.e., Bcl2) and antioxidant enzymes (i.e., SOD and GPx), as well as the down-regulation of pro-inflammatory cytokines (López-Rodríguez et al., 2015). Due to limited space and to avoid redundancy with recently published reviews (Shahrokhi et al., 2012; Heberden, 2017; McEwen and Milner, 2017), we will mainly focus on the roles of these sex steroids on neurogenesis under homeostatic and regenerative conditions (i.e., stroke, traumatic brain injury).

Effects of Estrogens

In the brain, estrogens modulate synaptic plasticity, NSC proliferation, newborn neuron migration, differentiation, and survival, as well as neuroprotection (McEwen and Woolley, 1994; McEwen et al., 1995; Azcoitia et al., 2001; McCullough et al., 2003; Murashov et al., 2004; Mukai et al., 2010; Frick et al., 2015).

For instance, 17β-E2 treatment of rat embryonic NSCs increases cell proliferation and neuronal differentiation (Brännvall et al., 2002), and promotes human embryonic NSC differentiation into dopaminergic neurons (Kishi et al., 2005). In addition, ERβ knock-out mice display a thinner cortex at E18.5 due to defects in neuronal migration and increased cell-death arguing for a role of ERβ in newborn neuronal differentiation and survival during the late phase of corticogenesis (Wang et al., 2003).

During adulthood, rat hippocampal cell genesis in female is modulated by ovarian hormone levels and is higher during pro-estrus, when 17β-E2 concentration is elevated (Tanapat et al., 1999, 2005). In the same line of evidence, ovariectomy leads to decreased hippocampal proliferation that is restored by 17β-E2 treatments through ER dependent mechanism as demonstrated by the use of specific ER agonists (Mazzucco et al., 2006). The impact of other estrogens such as estradiol-benzoate, 17α-E2, and estrone have been shown to also increase cell proliferation in ovariectomized rats suggesting that the positive neurogenic effects of estrogens are a general feature of female sex hormones (Frick et al., 2015). In meadow voles (*Microtus pennsylvanicus*), the reproductive status also influences dentate gyrus cell proliferation and/or surviving (higher in inactive females; higher in active males) (Ormerod and Galea, 2001, 2003). However, hippocampal cell proliferation in squirrels (*Sciurus carolinensis*) was not shown to be sexually dimorphic and to be modulated across the reproductive cycle (Lavenex et al., 2000). Furthermore, in striking contrast with most studies, an acute 17β-E2 treatment decreases cell proliferation in the subventricular zone (SVZ) and leads to a decrease in newborn cells in the olfactory bulbs (Brock et al., 2010). In addition, chronic 17β-E2 treatments in zebrafish impair NSC proliferation, cell survival, and the newborn cell migration (Diotel et al., 2013).

Estrogens also display neuroprotective properties and promote neural regeneration following traumatic brain injury and cerebral ischemia by decreasing apoptotic signaling, neuroinflammation, and oxidatative stress and by normalizing glutamate concentrations (Petrone et al., 2015). These neuroprotective effects of estrogens on brain ischemia have been well-established in ovariectomized rodents, and result in a significang decrease in the size of the lesion and in the infarct volume (Gibson et al., 2006; Petrone et al., 2015).

Effects of Progesterone and Allopregnenolone

In the brain, progesterone is known to regulate spinogenesis, synaptogenesis, neuronal survival, and dendritic growth (McEwen and Woolley, 1994; Brinton et al., 2008; Tsutsui, 2008; Zhang et al., 2010; Rossetti et al., 2016). Following cerebral ischemia in rat, progesterone promotes neurogenesis in the SVZ of the lateral ventricles and favors cell survival in the peri-infarct region several days post-stroke (Jiang et al., 2016). Interestingly, numerous studies documented estrogen and progesterone effects on learning and memory across the estrous cycle as reviewed in Duarte-Guterman et al. (2015). Allopregnanolone, a progesterone metabolite considered as one of the most important neuroactive steroid in the CNS, also plays key roles by increasing neurogenesis, neuronal cell survival, and by reducing cell-death in the hippocampus and the midbrain (Charalampopoulos et al., 2008; Zhang et al., 2015; Rossetti et al., 2016). It also exerts major neuroprotective roles in neurodegenerative diseases (Rossetti et al., 2016).

Effects of Androgens

Concerning androgens, a pioneer study in songbird suggested that testosterone could favor neurogenesis (Louissaint et al., 2002), further corroborated by mice castration studies resulting in decreased hippocampal neurogenesis (Spritzer and Galea, 2007; Heberden, 2017). In songbirds, androgens and estrogens are well-known to induce seasonal-like growth of song nuclei in the adult (Tramontin et al., 2003; Balthazart and Ball, 2016). Additional experiments in rodents document the positive effect of testosterone on SVZ cell proliferation (Tramontin et al., 2003; Farinetti et al., 2015; Balthazart and Ball, 2016), and those of testosterone and dihydrotestosterone on newborn neuron survival (Spritzer and Galea, 2007). In the same line, testosterone increases neurogenesis in wild-type males but not in androgen-insensitive ones (Hamson et al., 2013). This effect is blocked by AR antagonist (flutamide), demonstrating that androgens act through AR and not through ER after conversion into estrogens (Hamson et al., 2013). However, the reality appears more complex as treatment with nandrolone, a synthetic androgen, decreases hippocampal neurogenesis in male and female (Brännvall et al., 2005). Experiments performed in post-ischemic mice established that endogenous androgens do not alter injury-induced neurogenesis, while supra-physiological levels of testosterone and dihydrotestosterone strongly inhibits post-stroke neurogenesis in the dentate gyrus, reducing brain repair (Zhang et al., 2014).

Sex Difference after Brain Injuries

Interestingly, clinical and experimental findings are abundant and highlight important sex differences after stroke (Roof and Hall, 2000; Girijala et al., 2017). Clinical studies showed that aging women display worse outcomes following ischemic stroke than men, and higher mortality after hemorrhagic stroke (Sohrabji, 2015). In addition, ischemic stroke model in rodents document that young female have smaller infarcted area than young males (Alkayed et al., 1998), and also exhibit less severe stroke consequences during pro-estrus (high 17β-E2 concentration) than during metestrus (low 17β-E2 concentration) (Liao et al., 2001). However, these studies only correlate between levels of 17β-E2 and stroke severity without taking into consideration other steroid hormone such as progesterone. During aging, mortality is higher in female than in male, and in the case of hemorrhagic stroke, males display greater bleeding and mortality (Sohrabji, 2015). It was also shown that estrogen treatments improve outcomes in young females and males after ischemic and hemorrhagic stroke, while their effects are controversial on aging females during ischemia (Sohrabji, 2015). Other clinical and experimental findings also document the impact of gender and of sex steroids on other CNS insults. For instance, after traumatic brain injury, men displayed greater levels of injury severity as indicated by the Glasgow Coma Scale score, but such an effect is not always found (Slewa-Younan et al., 2004; Davis et al., 2006). Taken together, these data argue in favor of an impact of sex and circulating steroids on the consequences of CNS insults.

In conclusion, sex steroids, peripherally or centrally produced, exert numerous actions on constitutive and regenerative neurogenesis, on brain plasticity, learning, and behaviors (**Figure 3**). For more details on sex hormone modulation of hippocampal neurogenesis, see (Galea et al., 2006; Frick et al., 2015).

Sexual Behavior

As introduced earlier, sex steroids module in a wide array of behaviors, including aggression, parental behavior, sexual behavior, but also more cognitive aspects, including attention and visual and verbal memory. Although original studies investigated the role of gonadally-produced steroids, it is now clear that the local synthesis of these steroids directly in the central nervous system play a significant role as well. The shift from systemic to local synthesis and regulation of steroid action within target tissues, such as the brain, was called "Balkanization" of the endocrine system (Schmidt et al., 2008). This process would allow the brain to produce specific steroids required for the activation of defined behavior, while leaving other tissues unaffected. The central synthesis of estrogens in the male brain is probably the best example, as high peripheral production of these steroids would have major feminizing effects. Amongst the many behavioral effects of steroids, we chose to discuss in more detail the steroid-dependent modulation of sexual behavior. Indeed, the neuroanatomy and physiology underlying this reproductive behavior have been under study for decades, and sexual behavior provide an excellent model to further investigate the respective role of locally- vs. peripherally- produced sex steroids.

Importance of Neuroestrogens

The role for aromatase on male sexual behavior was hypothesized over 50 years ago (Clemens, 2013; Södersten, 2013) and a very large number of studies using pharmacological tools have demonstrated that testosterone action on male sexual behavior in various species requires its aromatization in the brain (Christensen and Clemens, 1975; Beyer et al., 1976; Morali et al., 1977; Balthazart and Foidart, 1993; Roselli et al., 2003). These observations were further confirmed by the use of various aromatase knock-out mice (Fisher et al., 1998; Honda et al., 1998; Matsumoto et al., 2003; Bakker et al., 2004). Furthermore, the importance of the central production of estrogens was highlighted by stereotaxic experiments affecting estrogen synthesis within the preoptic area. This region is a key brain center involved the control of male sexual behavior in most vertebrate species and, importantly, contains a very high concentration of aromatase, especially in birds (Balthazart et al., 2004; Perkins and Roselli, 2007; McCarthy, 2011). Numerous studies have shown that preoptic aromatase activity is critical for the activation of male sexual differentiation and male sexual behavior. For example, chronic inhibition of aromatase activity by specific inhibitors delivered to the preoptic area results in the complete suppression of copulatory behavior within a few days (Christensen and Clemens, 1975; Watson and Adkins-Regan, 1989a; Balthazart and Surlemont, 1990; Balthazart et al., 1990). In castrates, the male sexual behavior can be restored by the local administration of testosterone as well as estrogens (Christensen and Clemens, 1974; Watson and Adkins-Regan, 1989b). These results indicate that the expression of male sexual behavior is tied to changes in aromatase concentration and activity in the preoptic area (POA). Interestingly, these change in aromatase expression and male sexual behavior are also linked to an important local neuroplasticity (Aste et al., 1993; Panzica et al., 1998; Charlier et al., 2008). It should be noted that most of these effects were taking place after several days of steroid treatment, clearly suggesting direct genomic effects via the activation of nuclear receptors (Panzica et al., 1998; Scordalakes et al., 2002; Balthazart et al., 2004).

In addition to the long-term effects of estrogens, estrogens also display rapid effects on several aspects of male sexual behavior. For example, research in mouse and Japanese quail showed that acute modulation of estrogen concentrations rapidly affect male sexual behavior (Cornil et al., 2006; Taziaux et al., 2007; Seredynski et al., 2013, 2015). Many of these rapid effects require doses of estradiol that surpass systemic estradiol concentrations in the blood, but local estradiol levels could reach high concentrations quickly, because aromatase and other steroidogenic enzymes can be rapidly regulated in the brain (see below) and are enriched in subcellular compartments such as the presynaptic bouton (Peterson et al., 2005; Saldanha et al., 2011; Cornil et al., 2012). These rapid and local effects of steroids have led to the hypothesis that neuroestrogens act as neurotransmitters (Balthazart and Ball, 2006). Moreover, exposure to sexual stimuli will in turn affect aromatase activity in several regions. Indeed, copulation rapidly and significantly reduced aromatase activity in hypothalamic nuclei and in median preoptic nucleus or tuberal hypothalamus isolated using the Palkovits punch technique (Cornil et al., 2005; de Bournonville et al., 2013). Conversely, exposure to stress upregulates aromatase activity within 5 min in a sex- and brain region-dependent manner in quail (Dickens et al., 2011, 2013), but this increase was counteracted by copulation with a female (Dickens et al., 2012). The mechanisms leading to rapid changes of aromatase activity seem to rely on phosphorylation-dephosphorylation processes but the exact mechanisms are far from being understood (Balthazart et al., 2005, 2006; Charlier et al., 2011a).

It should be noted that the measurements of estrogens do not correlate with aromatase activity in most cases and future work should tackle this discrepancy (Charlier et al., 2011b; Dickens et al., 2014; de Bournonville et al., 2017b). Interestingly, central aromatase is not only important for male sexual behavior but was also shown recently to be involved in female sexual behavior in Japanese quail (de Bournonville et al., 2016, 2017a), see also recent discussion in Cornil (2017). The very high concentration of aromatase in birds make them a very suitable model to investigate its function in modulating sexual behavior but it is likely that rapid change in aromatase activity are also important in mammals and fish, as rapid effects of estrogens were demonstrated on sexual motivation in rat and Goldfish (Cross and Roselli, 1999). However, little is known about the behavioral role of aromatase in fish. As stated above, fish in general possess a very wide distribution of aromatase but the presence of high levels of aromatase, androgen, and estrogen receptors, in brain regions involved in the detection and processing of visual stimuli, suggests that visual processes related to reproduction may be influenced by sex steroids (Gelinas and Callard, 1993, 1997). Testosterone is necessary and sufficient for the enhancement of male approach responses toward a female stimulus to occur and androgen treatments in female goldfish also induce selective approach responses toward female visual stimuli (Thompson et al., 2004). Injections of 17β-E2 had the same behavioral effect

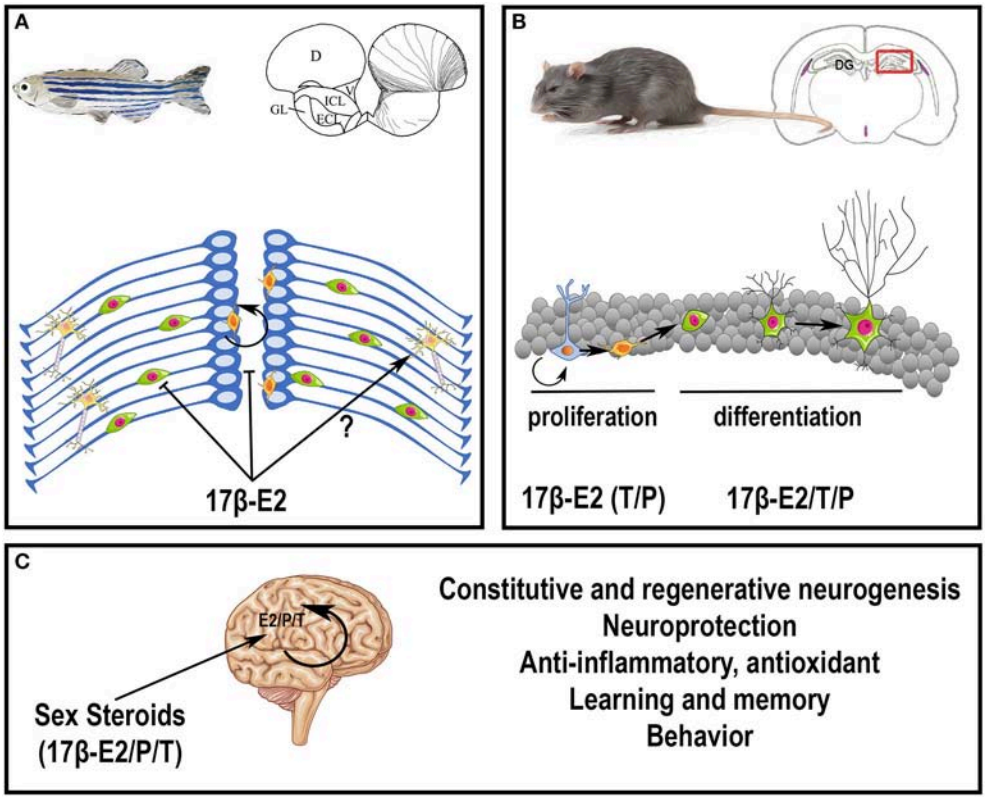

FIGURE 3 | Potential roles of steroids in the brain. **(A)** Effects of 17β-estradiol on neurogenesis in adult zebrafish. At the top, the scheme represents a zebrafish and a transversal brain section through the olfactory bulbs, taken from Menuet et al. (2005). The lines on the right hemisphere correspond to radial glial cells, that behave as neural stem cells. At the bottom, the scheme illustrates neurogenesis events occurring on the zebrafish brain. Radial glial cells (blue) give rise to new neurons (green) that migrate and differentiate into mature and functional neurons (yellow). The orange cells close to the ventricle vicinity are further committed progenitors. It was shown that 17β-estradiol impairs neural progenitor proliferation and new born cell migration; the data obtained for neural differentiation and survival are not clear (Diotel et al., 2013; Pellegrini et al., 2015). **(B)** Effects of sex steroids on neurogenesis in adult rodents. At the top, the scheme represents a rodent and a transversal brain section through the dentate gyrus (DG) of the hippocampus (framed box), where neurogenesis is maintained during adulthood. At the bottom, the scheme illustrates neurogenesis events occurring in the granular cell layer of the dentate gyrus. Radial glial cells (blue light) give rise to neural progenitors (orange) that give birth to new neurons (green with a small neuritic arborescence) that migrate and differentiate into mature and functional neurons (green with a huge neuritic arborescence). In general, 17β-estradiol (17β-E2) favors neural stem cell/progenitor proliferation; to a lesser extent, testosterone (T) and progesterone (P) also exert positive role on proliferation; 17β-estradiol, testosterone and progesterone also promote neuronal migration and differentiation (Heberden, 2017). In addition, these three sex steroids promote new born cell survival in constitutive and regenerative neurogenesis. Note, that the effects of sex steroids on neurogenesis could be dependent of the timing, the concentration, the rhythm of exposure and the regions targeted. For instance, estradiol treatment has been shown to decrease neurogenesis in the SVZ (Brock et al., 2010). **(C)** Peripheral steroids and neurosteroids impact brain functions and homeostasis, by modulating neurogenesis under homeostatic and regenerative conditions, by promoting neuroprotection, learning, and memory, and by exerting anti-inflammatory and antioxidant properties. They also modulate sexual behavior.

as testosterone, and pretreatment with the aromatase inhibitor (fadrozole) 15 min prior to testosterone injections significantly reduced the male's ability to detect and/or orient toward potential mates. Local 17β-E2 synthesis in neural pathways involved in visual processing could therefore sensitize the males to some visual features of females and modulate orientation responses in the context of reproduction (Lord et al., 2009).

Importance of Neuroprogestins: Neuroprogesterone

In most species, the synchrony between ovulation and female sexual behavior is obviously fundamental for efficient reproductive output. Both processes require a sequential elevation of estradiol and progesterone (Mahesh and Brann, 1998; Stephens et al., 2015). Although preliminary observations suggested that progesterone originating from the periphery (Buckingham et al., 1978; Putnam et al., 1991; Mahesh and Brann, 1992), ovariectomized and adrenalectomized rats continue to produce a LH surge in response to estradiol priming, suggesting a role for the central production of progesterone (Ferin et al., 1969; Micevych et al., 2003). This central production of neuroprogesterone is linked to astrocytic activity as astrocytes possess transport proteins (TSPO, StAR) and steroidogenic enzymes (P450ssc, 3βHSD, etc.) required to transform cholesterol into progesterone. Importantly, the synthesis of progesterone in female hypothalamic astrocytes is regulated by estradiol. This estradiol, from gonadal origin will likely affect the transcription and/or activity of multiple transporters and enzymes, including StAR, TSPO, P450ssc,

and 3β-HSD, as observed *in vitro* and *in vivo* (Sinchak et al., 2003; Soma et al., 2005; Micevych et al., 2007; Micevych and Sinchak, 2008a; Kuo et al., 2010; Chen et al., 2014). To our knowledge, the role of neuroprogesterone on the display of sexual behavior is currently not known, although the very fast effect of progesterone receptor antisense oligonucleotides on lordosis behavior could suggest a local synthesis of progesterone (Mani et al., 1994). Although the role of neuroprogesterone on female sexual behavior has not been extensively studied, the function of its metabolite allopregnanolone has been investigated in more detail (Frye, 2011).

Importance of Neuroprogestins: Allopregnanolone

Allopregnanolone, also known as 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP), is a neurosteroid produced in several regions of the central nervous system by local conversion of progesterone via 5α-reductase. After paced mating 3α,5α-THP rapidly increases in the midbrain ventral tegmental area (VTA), a dopaminergic region involved in reproductive behaviors of female rodents. The rapidity of the rise in midbrain 3α,5α-THP levels, and independence of ovaries and/or adrenals as sources of steroids, suggest that biosynthesis underlies mating-induced increases (Frye and Vongher, 1999). Paced mating also increases 3α,5α-THP concentrations in other structures of the mesocorticolimbic circuit, such as hippocampus, and cortex (Frye et al., 2013). Central infusions with an inhibitor of TSPO (PK-11195), or with an inhibitor of 3α-HSD within the midbrain VTA significantly attenuated 3α,5α-THP levels of pro-estrus rats and led to a significant reduction in sexual behavior (Frye and Paris, 2011). In order to assess whether central 3α,5α-THP is necessary and sufficient for these effects, pro-estrus rats were infused with 3α,5α-THP subsequent to inhibitor infusions and results indicated a reinstatement of sexual behavior (Frye and Paris, 2011). In hamsters, infusions of a TSPO agonist, to the VTA increased sexual responsiveness and lordosis of cycling, estradiol and progesterone-primed hamsters, compared to vehicle. In contrast, and similarly to what was observed in rat, the TSPO antagonist (PK-11195) injection to the VTA attenuated sexual responsiveness of naturally receptive or estradiol benzoate (EB) + progesterone -primed hamsters compared to vehicle. In parallel, these treatments respectively led to increased and decreased midbrain levels of allopregnenolone (Petralia and Frye, 2005). Future work should define in more detail the precise relationship between localized allopregnanolone synthesis and specific sexual behaviors.

Neurosteroid synthesis is pronounced during early development. Numerous steroidogenic enzymes are expressed at very high levels in the developing avian brain (Goodson et al., 2005; Tsutsui et al., 2006; London and Schlinger, 2007). In rodent brain, most steroidogenic enzymes are typically expressed at much lower levels compared to fish and birds and thus more difficult to quantify but the developmental expression pattern was described for a few steroidogenic enzymes. For example, in rats, brain expression of P450c17, the enzyme responsible for the transformation of pregnenolone to DHEA, is elevated in the embryo and decreases postnatally (Compagnone and Mellon, 2000). Brain 3β-HSD mRNA in rodents is highest during early development (Ibanez et al., 2003). Although steroids from gonadal origin are known to be a significant parameter in sex differentiation in bird, neurosteroids may regulate sexual differentiation in songbird species. In nestling European starlings, neural metabolism of DHEA and 17β-E2 can be greater in males than females at specific ages (Chin et al., 2008; Shah et al., 2011). In zebra finches, 17β-E2 synthesized *de novo* in cultured male brain slices triggers development of an important projection in the song control circuit (Holloway and Clayton, 2001). Female brain slices developed this projection only when co-cultured with male slices, suggesting a sex difference in neural 17β-E2 synthesis during development (Holloway and Clayton, 2001; Schlinger et al., 2001). Progesterone is also synthesized in the brain during development (Zwain and Yen, 1999; Micevych and Sinchak, 2008a,b) and promotes myelination (Koenig et al., 1995; Schumacher et al., 2007).

STEROIDS AND LIPOPROTEINS: FROM PERIPHERY TO THE BRAIN

Transport and Actions of Steroids on the Blood-Brain Barrier

Although neurosteroids play key roles on brain functions, peripheral steroid hormones have the main effects on the CNS, either by crossing the BBB or by targeting the BBB cells, which in turn impact the brain parenchyma by modulating inflammatory and oxidative signals. Some of these possibilities were recently discussed in a review for other hormones called adipokines (Parimisetty et al., 2016). In this part, we will first describe the structure of the BBB in fish, birds, and mammals, then document the transport of steroid through the BBB before discussing the potential roles of steroids on the BBB.

Blood-Brain Barrier in Fish, Bird, and Mammals

The BBB is a specialized layer of cells that controls molecular trafficking between blood and brain, and contributes to the regulation (homeostasis) of the brain microenvironment. In mammalian species, BBB is composed of adjacent endothelial cells bound to each other by tight junctions whose major components are transmembrane and cytoplasmic anchoring protein such as occludin, claudin, and zona occludens (ZO-1). To complete the barrier, endothelial cells are wrapped by pericytes and surrounded by astrocytic cytoplasmic processes called end-feet. This cellular association is important for proper brain homeostasis, neuronal activity but also for protecting nervous tissue pathogens and harmful molecules transported by blood flow. The BBB, by separating the peripheral blood circulation from the brain parenchyma, filters the entrance of many molecules but also the removal of molecules from the brain (Banks, 2012).

Very few studies have focused on anatomical and functional organization of the BBB in birds and fish. Electron microscopy performed in adult and embryonic brains of quail and chicken showed that cerebral endothelial cells of both species are bound by tight junctions and that BBB is probably functional very early during embryonic development (Wakai and Hirokawa, 1978;

Stewart and Wiley, 1981; Roncali et al., 1986). In embryonic chick, aquaporin 4, a molecular water channels identified in mammalian BBB, was described in astroglia (Nico et al., 2001). In zebrafish, anatomical analysis and transmission electron microscopy revealed that adult endothelial cells display physical barrier properties and express Claudin-5 and ZO-1 proteins (Jeong et al., 2008; Eliceiri et al., 2011; Li et al., 2017). Complete maturation of the zebrafish BBB occurs between 3 and 10 days when expression of Claudin-5 and ZO-1 proteins are detected in cerebral endothelial vascular cells (Jeong et al., 2008; Eliceiri et al., 2011; Fleming et al., 2013; Li et al., 2017). Although some studies have highlighted the presence of stellate astrocytes in some fish (Kawai et al., 2001; Alunni et al., 2005; Strobl-Mazzulla et al., 2010), immunohistochemistry against GFAP or S100β protein has generally failed to demonstrate the presence of star-shaped cells resembling mammalian astrocytes in brain of many fish, including trout, carp, and zebrafish (Arochena et al., 2004; Pellegrini et al., 2005; Grupp et al., 2010; März et al., 2010). These findings raise the possibility that specialized functions of mammalian astrocytes could be supported by the GFAP, BLBP, and S100β-positive RGCs that remains numerous in adults. RNA sequencing analysis performed on goldfish RGC revealed the presence of many receptors and signaling molecules known to be expressed by astrocytes in mammals, a finding that strongly suggests that RGCs share functional similarities with mammalian astrocytes (Grupp et al., 2010; Fleming et al., 2013; Da Fonte et al., 2017). Very interestingly, in the zebrafish brain, blood vessels are wrapped by AroB^{+} processes, in a way similar to astrocytic end-feet in mammals, further reinforcing the idea that RGCs in fish perform some, if not all, functions of astrocytes (**Figures 4A,B**).

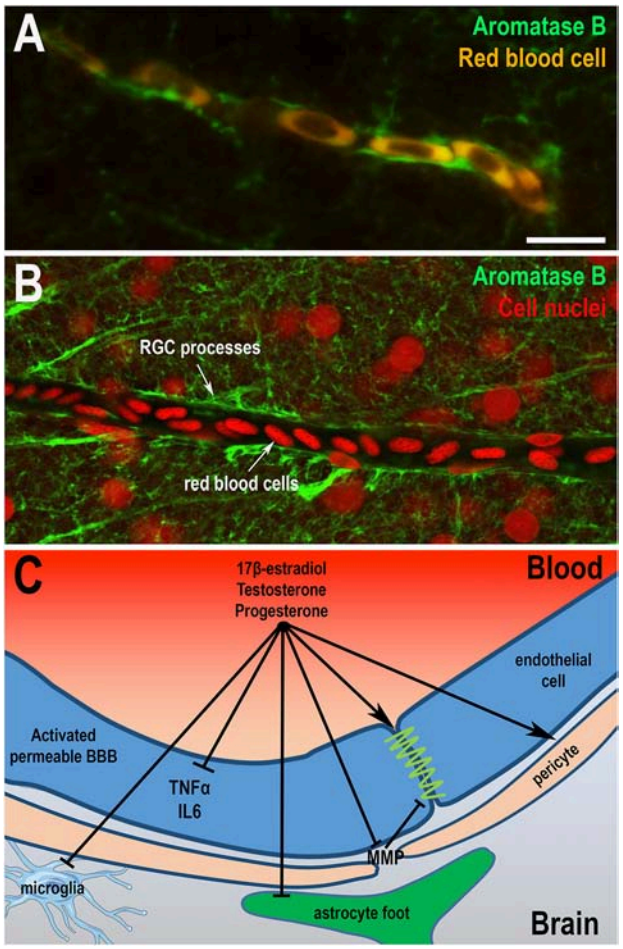

FIGURE 4 | Sex hormones restore blood-brain barrier physiology and integrity. **(A,B)** Aromatase B immunohistochemistry (green) on zebrafish brain highlights that radial glial processes envelop blood vessels as shown by the auto-fluorescence of red blood cells **(A)** or by nuclear staining **(B)**. These data suggest that radial glial cells could endorse the role of astrocytes in the establishment of the BBB, given the absence of astrocytic cell-type in the brain of fish. It also raises the question of the potential role of locally-produced estrogens on the BBB physiology. Scale bar: 7 μm. **(C)** Sex steroids (17β-E2, progesterone, and testosterone) display direct and indirect effects on the BBB through the restauration of thigh junctions, the inhibition of inflammatory cytokine expression and metalloproteinases production, the regulation of pericytes contraction, and consequently the modulation of cerebral blood flow. They also limit reactive gliosis through the inhibition of glial activation under pathological conditions (astrocytes and microglia). Sex steroids participate in the maintenance of a functional BBB, reducing neuroinflammation and promoting neuroprotection.

Steroid Transport through the Blood-Brain Barrier

Steroid hormones are lipophilic and thus may cross the BBB by simple diffusion (Witt and Sandoval, 2014). Hormones can also enter the brain via specific transporters (Banks, 2012). In mammals, the transporters that help the passage from the blood circulation to the brain parenchyma are named influx transporters and include organic anion transporting polypeptides (OATPs), proton-coupled monocarboxylate transporters (MCTs), and peptide transporter. In the case of simple diffusion through the BBB, steroid concentrations will tend to equilibrate between the plasma and the brain. However, most steroid hormones are transported in the blood by albumin, sex hormone-binding globulin, or by corticosteroid-binding globulin, changing their availability and the possibility of passive diffusion through the BBB, as shown in most vertebrates, including fish, birds, and mammals (Hammond, 2011, 2016; Rosner, 2015). In rats, radiolabeled steroids (i.e., progesterone, testosterone, estradiol and corticosterone, and cortisol), intravenously injected have been shown to diffuse through the BBB, but this diffusion was significantly slowed down by the presence of binding globulin (Pardridge and Mietus, 1979).In addition, one must take into account that the concentration of carrier proteins may be different in the peripheral circulation vs. the cerebrospinal fluid (CSF). For example, the albumin concentration is one hundred time less important in the CSF than in the plasma in humans (Alafuzoff et al., 1983), potentially modulating availability and stability of the steroids.

In addition, hormones may be transported back into the blood circulation by efflux transporters. In mammals, these transporters, belonging to the ABC (ATP Binding Cassette) family, play a key role in brain homeostasis and mediate active efflux of many potential toxicants including lipophilic compounds. They include the P-glycoprotein, also known as

MDR1, ABCB1), multidrug resistance-associated protein, and breast cancer-related protein (Witt and Sandoval, 2014). Organic ion transporting polypeptides (OATPs) and the organic ion transporters, which belong to the solute-linked carrier (SLC) class, could be involved in uptake and/or efflux (Kusuhara and Sugiyama, 2005). For example, metabolites of estrogens can be transported back in the blood circulation from the brain such as demonstrated in male rats (Sugiyama et al., 2001). The same was also shown in rats for the neuroactive androgenic steroid dehydroepiandrosterone sulfate (DHEAS) that is transported back through a member of the SLC class (Asaba et al., 2000). However, the role of efflux transporter in the regulation of the concentration of steroids in the brain is not well-known and remains controversial.

Effects of Steroids on the Blood-Brain Barrier

Steroids could act directly or indirectly on the BBB, notably by binding to receptors present in the cells composing the BBB. The effects of glucocorticoids on the BBB are well-described and notably include decrease in inflammation (cytokines, chemokines, metalloproteinases) and increase in protective or reparative effects on tight junctions (Witt and Sandoval, 2014). Sex steroids were also shown to exert protective effects on the BBB, using microvessel endothelial bEnd.3 cells (Na et al., 2015). Considering estrogens, treatment with 17β-E2 restores the BBB integrity and its permeability in a model of stroke or in a lipopolysaccharides-induced inflammation in rodents (Maggioli et al., 2016; Xiao et al., 2017). Of interest, the BBB permeability is increased when circulating estrogens decrease with aging (Bake and Sohrabji, 2004). With respect to androgens, when gonadal testosterone is depleted from the body, glial cells (microglia and astrocytes) are activated and BBB permeability and inflammation are increased. Testosterone treatments restore these parameters (Atallah et al., 2017). Finally, it also seems that progesterone exerts positive effects on the BBB physiology after stroke and TBI (Ishrat et al., 2010; Si et al., 2014). The effects (direct and indirect) of steroids on the BBB is described in **Figure 4C**.

Interestingly, it was also demonstrated that sex steroids (17β-E2, testosterone, and progesterone) regulate both cerebrovascular tone, endothelial function, oxidative stress, and inflammation as well as brain functions under normal and/or pathological conditions (i.e., middle cerebral artery occlusion: MCAO) (Krause et al., 2011; Gonzales, 2013; Si et al., 2014).

Lipoproteins in the Brain

Lipid metabolism, high density lipoprotein (HDL)-, and low density lipoprotein (LDL)-cholesterol were studied in a wide variety of groups including rodents, lagomorph, birds, and fish (Liu and Wu, 2004). Lipoprotein metabolism, HDL-, and LDL-cholesterol in zebrafish appear similar to humans, making zebrafish an appropriate model for studying lipoproteins, given that zebrafish express the main set of lipid transporters, apolipoproteins, and enzymes involved in lipoprotein metabolism (Fang et al., 2014). However, to our knowledge, except in rodents, not much is known concerning the effects of HDL-cholesterol in the transport of steroids, in the maintenance of the BBB integrity and in neuroprotection. We consequently emphasize these points in this last part.

The brain is a cholesterol-rich organ, accounting for about 25% of the total amount present in humans. In the CNS, cholesterol is mainly synthesized by astrocytes, oligodendrocytes, microglia and to a lesser extent by neurons, where it is essentially present in its unesterified form (Björkhem and Meaney, 2004; Dietschy and Turley, 2004; Zhang and Liu, 2015). Brain cholesterol is involved in myelin sheath genesis, in synaptogenesis, and neurotransmission as well as in neurosteroidogenesis (Mauch et al., 2001; Do Rego et al., 2009; Linetti et al., 2010; Liu et al., 2010; Zhang and Liu, 2015). In adults, sterols are excreted from the brain to the plasma, and cholesterol is converted into 24S-hydroxycholesterol, which is more soluble and may diffuse across the BBB to reach the plasma where it is metabolized to bile acids after being picked up by circulating lipoproteins (Quan et al., 2003; Mahley, 2016). Almost no cholesterol enters the brain from the peripheral circulation. However, small high density lipoprotein (HDL) particles may cross the BBB and transport cholesterol within the brain (Ladu et al., 2000; Koch et al., 2001).

Spherical lipoproteins similar to HDLs have been isolated from the CSF. They are composed of ApoE and ApoJ for the largest ones whereas smaller particles contain ApoAI and ApoAII. Astrocytes are the main producers of both ApoE (with microglial cells) and ApoJ (with neurons and ependymal cells) (Vance and Hayashi, 2010). Different isoforms of ApoE have been described in brain HDL particles (ApoE2, ApoE3, and ApoE4). ApoE is the main cholesterol carrier in the brain and is associated with Alzheimer's disease and other neurodegenerative disorders (Liu et al., 2013). Interestingly, cultured neurons and astrocytes expressing the ApoE4 isoforms display a reduced secretion of cholesterol and phospholipids, as well as a blunted lipid-binding capacity (Mahley, 2016). In addition, estrogens were shown to promote ApoE expression in microglia and astrocytes (Stone et al., 1997). Other brain apolipoproteins (i.e., ApoA-I, Apo-J, and Apo-D) may participate in cholesterol distribution via different receptors including LDL receptor, ABC (ATP-binding cassette receptor) -A1 and -A2 transporters, LDL Receptor-related protein), VLDL receptor, ApoE receptor 2, and megalin (Herz and Bock, 2002; Björkhem and Meaney, 2004).

In pathological conditions that lead to BBB loss of integrity, such as ischemic stroke, exogenous lipoproteins may enter the cerebral parenchyma and produce different effects. Functional HDL particles (HDLs displaying anti-inflammatory and antioxidant properties, see **Figure 5A**) are taken up by endothelial cells of the BBB in a thrombo-embolic stroke model of rat. Injection of fluorescent lipoproteins post-stroke are taken up by endothelial cells and astrocytes, but not by neurons (Lapergue et al., 2010). In this model and in the MCAO (middle-cerebral artery occlusion), one intravenous injection of HDLs isolated from healthy subjects reduced the mortality, the infarct volume and the hemorrhagic transformation associated with rtPA (recombinant tissue plasminogen activator) treatment (Lapergue et al., 2013). It is not known whether the cholesterol or steroids contained within these HDL particles are involved in the protective effects. It is indeed possible that either ester of steroids

or cholesterol delivery for local steroid synthesis could participate in neurogenesis and post-stroke remodeling. In this line, there is no study showing any beneficial effect of reconstituted HDL particles in CNS disorders. Noteworthy, these HDLs are composed of ApoA-I and phospholipids, but are devoid of cholesterol (Darabi et al., 2016). Ortiz-Munoz et al. recently demonstrated that at the acute phase of stroke, HDL particles are dysfunctional (HDLs displaying defective anti-inflammatory and antioxidant properties) and larger than in controls (Ortiz-Muñoz et al., 2016). These qualitative abnormalities blunted their ability to protect the BBB during acute brain injury. In fact, in addition to their action on reverse transport of cholesterol, HDLs also display anti-oxidant, anti-inflammatory, and anti-protease activities (Tran-Dinh et al., 2013). These pleiotropic effects could be due to different proteins associated with HDL particles such as paraoxonase-1 (PON-1), but also to the lipids that they transport, and to steroids. In type 2 diabetes mellitus conditions (T2DM), HDL anti-inflammatory capacity is impaired due to decreased PON-1 activity (Ebtehaj et al., 2017). Another study investigated the association between plasma cholesterol/lipoproteins and BBB permeability in a condition of CNS inflammation such as multiple sclerosis (Fellows et al., 2015). HDL-Cholesterol was associated with lower levels of BBB injury and low CSF total protein concentration. The authors suggest that the BBB structural integrity is associated with plasma membrane subdomains involved in cholesterol homeostasis. HDL particles are larger in stroke patients than in controls and they display a reduced protective effect for the BBB (Ortiz-Muñoz et al., 2016). Small HDL particles enter the CNS via SR-B1-mediated uptake and transcytosis (Balazs et al., 2004; Vitali et al., 2014). Morphological abnormality of HDLs in brain injury condition like stroke could be responsible for their reduced capacity to cross the blood-cerebrospinal fluid barrier. HDL supplementation could represent an interesting and innovative protective therapy for brain injury.

Interestingly, Leszczynski and Schafer (1991) demonstrated the interaction between lipoproteins and steroid hormones (androstenediol, 17β-E2, DHEA, DHT, pregnenolone, and progesterone), highlighting a new pathway in steroid hormone processing in plasma and/or steroid hormone delivery to cells (Leszczynski and Schafer, 1991). Other reports also suggest that 17β-E2 could be incorporated into HDL particles by lecithin:cholesterol acyltransferase under the form of 17β-E2 ester, that could be in turn transferred into LDL particles by cholesteryl ester transfer protein LDL particles as reviewed in Tikkanen et al. (2002) and Höckerstedt et al. (2004). The **Figure 5B** illustrates such a transport of 17β-E2 ester in HDLs and LDLs. These data would argue in favor of potential antioxidant, anti-inflammatory, and neuroprotective effects of HDLs implying cholesterol and other steroids (or their esterified forms) associated to lipoprotein particles. Indeed, estrogen derivatives with an unsubstituted A-ring phenolic hydroxyl group confers stronger antioxidant protection to LDL and HDL (Badeau et al., 2005). Such data could suggest a potential therapeutic use of HDL particles in order to improve their neuroprotective properties through their enrichment with steroids.

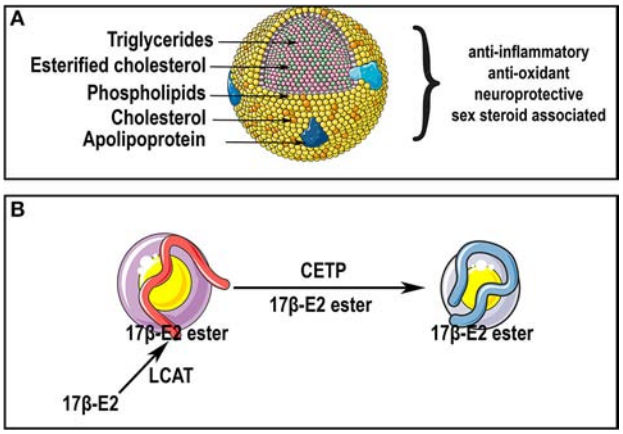

FIGURE 5 | HDL particles and sex steroids. **(A)** Lipoprotein particles display a central core containing cholesterol, cholesterol esters and triglycerides, that is enveloped by free cholesterol, phospholipids, and apolipoproteins. HDL particles display pleiotropic effects partly supported by their anti-inflammatory, anti-oxidant and neuroprotective properties. HDLs have been shown to display metabolic interaction with sex steroids (i.e., androstenediol, estradiol, DHEA, dihydrotestosterone, pregnenolone, and progesterone). **(B)** As suggested by Tikkanen et al. (2002), 17β-E2 could be esterified with fatty acids by lecithin:cholesterol acyltransferase (LCAT) in HDL. Esterified 17β-E2 could be subsequently transferred from HDLs to LDLs by cholesteryl ester transfer protein (CETP). Thus, HDL particles could be a source of cholesterol and steroids in the brain notably after CNS insults, and could be used as a carrier for improving steroids delivery in therapeutically approaches.

CONCLUSION

In this review, we reported that the brains of fish, birds, and mammals are able to *de novo* synthesize a wide variety of sex steroids demonstrating that neurosteroidogenesis is an evolutionary conserved feature shared by common ancestors. We also emphasize that neurogenic regions are closely related to neurosteroid production. In particular, RGCs, acting as neural progenitors in the brain of adult fish, strongly express Aromatase B and some other steroidogenic enzymes, suggesting that RGCs are true steroidogenic cells. Such an expression of aromatase in RGCs is not restricted to fish as it was also observed in mammals during embryonic development (Martínez-Cerdeño et al., 2006) and in birds after mechanical lesion of the brain (Peterson et al., 2001).

Peripheral, we also highlighted the fact that the brains of fish, birds, and mammals are targeted by peripheral and locally-produced steroids namely estrogens, progestins, and androgens. It appears that nuclear and membrane-associated sex steroid receptors are widely expressed in the brains of vertebrates particularly in neuroendocrine regions (i.e., preoptic area and hypothalamus) as well as in neurogenic niches and neural progenitors, suggesting key roles of steroids in sexual behaviors and neurogenesis. Indeed, we notably depicted the roles of estrogens, progestins and androgens in constitutive and regenerative neurogenesis and in sexual behaviors. Due to their anti-inflammatory, antioxidant, and anti-apoptotic properties, sex steroids also exert neuroprotective properties in the brain, particularly after CNS insults such as stroke and traumatic brain

injury. However, the effects of steroids are not trivial to study and understand given that their actions are largely dependent on numerous variables (i.e., the timing, the concentration, the rhythm of exposure,...).

Peripheral steroids may diffuse or cross the BBB through transporters, raising the question of the respective roles of peripherally vs. centrally-produced steroids in brain homeostasis and functions. Peripheral sex steroid and neurosteroids can also act on the BBB permeability by modulating inflammatory and oxidative signals and consequently regulate brain homeostasis.

Of interest, in this review, we also discussed the potential hypothesis that the protective effects of HDL particles on stroke could be partly attributed to their interactions with sex-steroids. Thus, we raised the question of the contribution of cholesterol and sex-steroids transported by lipoproteins in the neuroprotection at the acute phase of stroke. Steroids are considered as good therapeutic candidates for CNS insults (i.e., stroke, traumatic brain injury), aging, and neurodegenerative diseases. It opens a door for therapeutic research through the use of steroids alone, in cocktail, or transported to the injured site by HDLs.

AUTHOR CONTRIBUTIONS

ND and EP designed the manuscript and supervised the work. ND, EP, TC, OM, CL, DC, VT, JN, and OK participated in the writing of the review. ND and CL made the figures.

FUNDING

This work was supported by grants from La Réunion University (Bonus Qualité Recherche, Dispositifs incitatifs), Conseil Régional de La Réunion, European Union (CPER/FEDER), Philancia association and Fondation de France (for UMR1188 DéTROI), as well as by grants from the Region Bretagne (SAD) and Rennes Metropole (awarded to TC).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 28 February 2018
doi: 10.3389/fendo.2018.00060

DAT1-Genotype and Menstrual Cycle, but Not Hormonal Contraception, Modulate Reinforcement Learning: Preliminary Evidence

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *13 October 2017*
Accepted: *09 February 2018*
Published: *28 February 2018*

Citation:
Jakob K, Ehrentreich H, Holtfrerich SKC, Reimers L and Diekhof EK (2018) DAT1-Genotype and Menstrual Cycle, but Not Hormonal Contraception, Modulate Reinforcement Learning: Preliminary Evidence. Front. Endocrinol. 9:60. doi: 10.3389/fendo.2018.00060

Hormone by genotype interactions have been widely ignored by cognitive neuroscience. Yet, the dependence of cognitive performance on both baseline dopamine (DA) and current 17ß-estradiol (E2) level argues for their combined effect also in the context of reinforcement learning. Here, we assessed how the interaction between the natural rise of E2 in the late follicular phase (FP) and the 40 base-pair variable number tandem repeat polymorphism of the dopamine transporter (DAT1) affects reinforcement learning capacity. 30 women with a regular menstrual cycle performed a probabilistic feedback learning task twice during the early and late FP. In addition, 39 women, who took hormonal contraceptives (HC) to suppress natural ovulation, were tested during the "pill break" and the intake phase of HC. The present data show that DAT1-genotype may interact with transient hormonal state, but only in women with a natural menstrual cycle. We found that carriers of the 9-repeat allele (9RP) experienced a significant decrease in the ability to avoid punishment from early to late FP. Neither homozygote subjects of the 10RP allele, nor subjects from the HC group showed a change in behavior between phases. These data are consistent with neurobiological studies that found that rising E2 may reverse DA transporter function and could enhance DA efflux, which would in turn reduce punishment sensitivity particularly in subjects with a higher transporter density to begin with. Taken together, the present results, although based on a small sample, add to the growing understanding of the complex interplay between different physiological modulators of dopaminergic transmission. They may not only point out the necessity to control for hormonal state in behavioral genetic research, but may offer new starting points for studies in clinical settings.

Keywords: estrogen, dopamine transporter, reinforcement learning, gender, steroid hormone, hormonal contraception

INTRODUCTION

The sex steroid 17ß-estradiol (E2) increases dopaminergic transmission in the reward system (1) and may enhance behavioral responses to reward and drugs of abuse [e.g., Ref. (2)]. In the animal model, E2 has been found to amplify the dopaminergic response in the striatum by (1) promoting stimulated dopamine (DA) release (3), (2) increasing DA synthesis capacity (4), and (3) decreasing

the affinity of inhibitory D2-receptors (DRD2) [(5–7); but also see Ref. (8), who indicate a possible regional specificity of this effect in the striatum that might also lead to an increase]. Recent neuroimaging evidence in humans points in a similar direction. Diekhof and Ratnayake (9) showed that—similar to DA-agonistic drugs (e.g., L-DOPA)—the increase of E2 level during the late follicular phase (FP) of the menstrual cycle had the potential to enhance reward learning capacity at the expense of the ability to learn from negative feedback [see also Ref. (10)]. Previous pharmacological and behavioral genetic evidence already suggested that both intra- and inter-individual variations in dopaminergic capacity may drive differences in reward learning and punishment avoidance capacity [e.g., Ref. (11, 12)]. According to theory, the differential action of DA at two subgroups of DA receptors (DRD1 and DRD2) that are located at the direct and the indirect pathways of the basal ganglia, respectively, may determine the extent to which a person is sensitive to the rewarding as opposed to the punishing outcomes of one's action. Phasic DA release after a rewarded action is thereby assumed to promote learning *via* long-term potentiation at the corticostriatal synapses of the direct "Go-pathway" that mainly expresses DRD1. At the same time, avoidance learning capacity is suppressed through DRD2-dependent long-term depression of the indirect "NoGo-pathway." Conversely, when an action is followed by a dip in tonic DA, such as the one following a negative action outcome or reward omission, the NoGo-pathway is strengthened and avoidance responses are facilitated at the expense of the reward learning capacity (13). Diekhof and Ratnayake (9) found that activation of the dorsal anterior cingulate cortex (dACC) was reduced during processing of negative feedback in the late FP. Assuming that the dACC may particularly optimize action selection during avoidance learning (14), a reduced response of this brain region in a state of heightened E2 may fit well with the proposed properties of E2 in the regulation of DA release (1, 6), which should have strengthened reward learning through weakening DRD2-mediated punishment avoidance capacity.

In addition to that, there is further behavioral evidence for an inverted U-shaped relationship between baseline dopaminergic capacity, cycle-dependent changes in endogenous E2 from the early to the late FP, and reward-related decision making (15, 16) as well as working memory performance in humans (17). Smith et al. (15) assessed the influence of the COMT Met158Val polymorphism (rs4680) as a proxy of prefrontal DA on temporal discounting across the menstrual cycle. Against expectation, they found a reduction of temporal discounting (i.e., the "Now Bias"), when E2 level was increased in the late FP. Diekhof (16) used the Barratt Impulsiveness Scale (BIS) as an indirect proxy of mesolimbic DA. Using a temporal response time adaptation paradigm that is sensitive for differences in striatal DA transmission (18), Diekhof (16) assessed the ability to speed up in order to maximize reward (i.e., a measure of reward sensitivity) and compared it to the capacity to slow down for higher reward (i.e., an indicator of punishment sensitivity). Similar to Smith et al. (15), they found a paradoxical decline in the ability to speed up for higher reward from the early to the late FP, while the capacity to wait for increasing reward value improved from the low to high E2 state. Yet, when looking at inter-individual differences in hormone concentration, they also found a positive correlation between E2 concentration and an enhancement of reward sensitivity as reflected by an improved ability to speed up for higher reward. Interestingly, this correlation only existed at the lower end of the E2 distribution (i.e., in the early FP) and was further specific for subjects with low "trait impulsiveness" in the BIS, who may be characterized by a habitually low baseline DA synthesis capacity (19). In sum, these data are consistent with an inverted U-shaped relationship by showing that only subjects at the lower end of the DA distribution were affected by changes in E2, while subjects with DA levels near the point of optimality, i.e., at the center of the DA distribution, may not show such strong perturbations. Moreover, given the assumption that trait impulsiveness may to some extent mirror one's genetic underpinnings (20–22), this finding points to a possible hormone–genotype interaction in the domain of reinforcement learning.

The present study aimed at elucidating the link between genetic predisposition and the degree to which natural variations in E2 level affect reinforcement learning. It has already been shown that genetic differences can modulate central dopaminergic transmission and cognitive functions (23). Further, individual predisposition may incline the individual to an either more reward- or punishment-sensitive learning style. For example, carriers of genetic polymorphisms, that may be associated with reduced DA autoregulation by DRD2 or an increased DA synthesis capacity, were compromised in their ability to avoid punishment in a probabilistic feedback learning task [e.g., Ref. (12, 24)]. Here, we assessed the interaction between cycle-dependent variations in E2 level and a genetic polymorphism that has been implicated in the reuptake of DA in the striatum. The 40 base-pair (40-bp) variable number tandem repeat polymorphism of the dopamine transporter (DAT1), which has been described in the 3′ untranslated region of the gene SLC6A3, has two common alleles with either 9 or 10 repeats (RP) of the 40-bp sequence The two variants may differentially affect the expression of the DAT in the striatum [(25–28), but see (29) for a null finding], and can also modulate reward-related processing (30–32), although evidence is mixed and it has not been determined, which variant may actually predispose for a higher DAT density. E2 can induce a reversal of DAT function *in vitro*, which is achieved by rapid alterations in several signaling pathways that cause efflux of DA from the DAT instead of DA reuptake (33–35). Given this evidence, the closer examination of a possible E2 by DAT1-genotype interaction and its relation to the model of the inverted U-shape of DA content and reinforcement learning capacity might add to the growing understanding of the baseline dependency of a neurocognitive function that is mediated by DA. When E2 level rises from the early to the late FP, we would predict a reversal of normal DAT function that should result in increased DA transmission in the striatum. By subdividing our sample in carriers of the 9RP allele and homozygotes of the 10RP, we should further be able to identify the genotype of the DAT1-polymorphism (9RP carriers versus subjects that are homozygote for the 10RP variant [10H]) that predisposes a person for a higher DAT density. This is because we would expect that the genetic variant that predisposes one for a higher DAT density and thus a lower DA

baseline to begin with, should be more affected by the proposed E2-induced DAT reversal that is expected to happen in the high E2 state of the late FP. For one thing, in the late FP this genotype should become relatively more reward sensitive and should be less able to learn from negative feedback when heightened E2 level might induce a reversal of normal transporter function, which would promote a rise in DA level. Conversely, in the early FP when E2 is still at its nadir, we would expect the same genotype to be relatively more punishment sensitive, because the habitually higher density of DAT and thus more effective DA reuptake, that is unaffected by E2 at this point in time, would promote avoidance learning ability.

In order to test our hypothesis of a possible cycle phase by genotype interaction during reinforcement learning, our subjects performed a probabilistic feedback learning task. This task has been shown to be a sensitive measure for genetic variations in baseline DA (12, 24) and transient variations in DA concentration that were induced by pharmacological intervention [e.g., Ref. (11, 36)]. Two groups of healthy young women were genotyped for the DAT1-polymorphism. The first one had a natural menstrual cycle, while the second group took hormonal contraceptives (HC) on a regular basis. These latter women did not experience natural fluctuations of E2 level over the course of the menstrual cycle, but were influenced by synthetic ethinylestradiol and progestines, which might also affect reinforcement learning capacity. Both groups were tested twice, either in a low versus high E2 phase of the menstrual cycle (i.e., in the early and late FP) or during the "pill break" and the intake phase of HC, respectively.

MATERIALS AND METHODS

Subjects

In total, 85 women were recruited for this study (i.e., 45 participants in the cycle group and 40 subjects in the HC group). Of these, five participants had to be excluded because of either technical problems with the response box or the test protocol, lack of compliance, or drop-out after the first test. Another subject had an exceedingly high E2 level, indicating a possible hormonal disturbance, which also led to exclusion from all further analyses. The remaining 79 healthy women [age (mean ± SEM) = 25.5 ± 0.4 years] had no current or previous psychiatric or neurological diagnosis, reported to have no history of drug abuse or gynecological problems (e.g., endometriosis) and did not have any chronic disorder related to the hormone system (e.g., diabetes, Hashimoto's thyroiditis, PCO). Subjects were of Middle European origin as determined by the place of birth of their parents and grandparents. All subjects gave written informed consent and were paid for participation. The present study was approved by the local ethics committee (*Ethikkommission der Ärztekammer Hamburg*).

The Cycle Group

The cycle group consisted of 39 women, who were free of any medication including hormonal contraception for at least three menstrual cycles in the past and reported to have regular menstrual cycles in the normal range of 24–36 days. Following the procedure applied by former neurobehavioral studies on the effects of E2 across the menstrual cycle (9, 15, 16), subjects from this group were only included in our final analyses if they showed an increase in E2 level from the early to the late FP. This was done because we wanted to assess the effect of rising E2 in the late FP. Ovulation is hidden in humans and the actual extent of follicular development can only be ascertained by vaginal ultrasound. Yet, a rising E2 level during the FP may be an indirect indicator of normal follicular growth. Therefore, an E2 rise from early to late FP in combination with a negative lutropin (LH) ovulation test before testing (see below) was a necessary prerequisite for the late FP test to take place, which left 31 subjects.

Since the goal of the study was to compare DAT1 9-repeat allele carriers and 10-repeat allele homozygotes in their reaction to a natural rise in E2 level and its subsequent impact on reinforcement learning capacity, the participants were also genotyped. In the present sample, 14 subjects were carriers of the 9RP allele (2 of them were homozygote for the 9RP), while the remaining 16 subjects were homozygous for the 10RP variant. There was also one person that carried the rare 11-repeat allele variant in combination with the 9RP (11/9), who was subsequently excluded, leaving the final number of 30 participants in the cycle group [age (mean ± SEM) = 27.5 ± 0.7 years; age range = 21–35 years]. About 38% of the cycle participants were currently in a relationship with a male partner and the majority considered themselves as heterosexual (two bisexual subjects).

The women were tested twice, once during the first 3 days of menstruation (early FP) and once two days before expected ovulation (late FP). The date of expected ovulation was calculated from the expected cycle length individually for each participant. For this we asked the participants to state their expected cycle length and then, upon the onset of menstruation, used the last expected cycle day to determine the optimal test day with a common counting method: for all subjects with a cycle length shorter than 28 days, we subtracted 15 days from the expected cycle end. For subjects with an expected length of 28–31 days, 16 days were subtracted, and for cycle lengths longer than 31 days, 17 days were subtracted to schedule the test in the late FP. Our subjects also determined the daily concentration of LH with a common *in vitro* urine test (One Step® by AIDE Diagnostic Co., Ltd.), starting 2 days before the test date. In case of a positive result before the test day, the test was postponed to the subsequent cycle.

The test design was counterbalanced for cycle phase. Half of the participants started the test protocol in the early FP (i.e., seven carriers of the 9RP variant; eight were 10H). The remaining subjects started in the late FP (same genotype proportion).

The HC Group

The 40 participants of the HC group were recruited when the data collection for the cycle group was almost finished. The HC group allowed us to compare the data from a phase of low hormone availability (pill break) with a phase, during which participants were under the influence of synthetic hormones (intake phase), while the impact of natural hormones was blocked. Participants

from the HC group took HC for at least 12 months (mean duration of HC intake = 7.77 ± 0.61 years), but were otherwise free of medication. HC contained ethinylestradiol in the range of 0.015–0.03 mg and different amounts of progestin compounds (e.g., levonorgestrel, dienogest), which are used to suppress follicular growth and ovulation (37). Therefore, we expected no significant change in hormone level between the two test phases, except from a numerical E2 increase or a fall in E2 during the intake phase (38).

The HC group consisted of 20 carriers of the 9RP variant (one of them was homozygote) and 18 subjects were genotyped as 10H. One person in this group also had the rare 11-repeat allele variant combined with the 10RP allele (11/10) and was therefore excluded, leaving a final number of 38 subjects in the HC group [age (mean ± SEM) = 23.5 ± 0.5 years; age range = 19–32 years]. About 87% of the HC participants, i.e., almost three times as many as in the cycle group, were currently in a relationship with a male partner and the majority considered themselves as heterosexual (one bisexual subject).

The HC group had an analogous counterbalanced test design to the cycle group. Twenty participants started the test protocol in the OFF-phase ($n_{9RP} = 10$; $n_{10H} = 10$). The term "OFF-phase" refers to the so-called pill break of 7 days. The test was scheduled for the third or fourth day of the pill break. The second test phase, the "ON-phase," required an intake of HC for at least five consecutive days. In that way, the OFF-phase resembles the early FP in terms of low E2 availability, while the ON-phase might be rather characterized by a slight influence of synthetic hormone content (in this case, the mixture of ethinyestradiol and progestin compounds contained in contraceptive medication). In order to achieve a comparable repeated test schedule to the naturally cycling women, subjects who started in the OFF-phase had the subsequent ON-test approximately 7 days later (mean ± SEM = 7 ± 1.6 days), while the other group had a gap of 18 days (mean ± SEM = 18.5 ± 0.12 days).

Test Procedure

Participants performed a probabilistic feedback task, described in more detail by Diekhof and Ratnayake (9), which is a well-established test of reinforcement learning capacity [see also Ref. (11, 12) for description of the task]. In this task, subjects learned to associate certain stimuli with a higher probability of positive or negative feedback (i.e., a smiley or a grumpy face). During the initial learning phase (session 1), they were confronted with three fixed stimulus pairs of different hiragana and kanji symbols (i.e., pairs AB, EF, CD) from which they had to choose the one symbol that allowed them to maximize reward (positive feedback in form of a smiley) and to avoid frequent punishment (negative feedback of a grumpy face). The task goal was to receive as many smileys as possible. Unbeknownst to the participants, the reward contingencies differed between pairs and stimuli. Among all symbols, A of the pair AB was the best option (80% positive feedback upon selection), while B was the worst option (only 20% positive feedback). The reward contingencies of the remaining symbols lay in between these contingencies (i.e., pair CD: C = 70%, D = 30%; pair EF: E = 60%, F = 50%). After learning, subjects were tested in a transfer phase (session 2) to check whether they had been able to maximize positive outcome through either selection of the better option (i.e., reward learning) or by more effective avoidance of negative feedback (i.e., punishment learning). The individual learning preference cannot be dissociated from the fixed stimulus pairs of session 1, which always combined the same good and bad stimuli (e.g., A and B). This means that in session 1 the preference for the better option (e.g., for the best stimulus A in pair AB) can be equally well driven by approach of the good option A or avoidance of the bad one B. So in the transfer phase, new stimulus pairings (like AC, BD) were presented next to the original ones (AB, CD, EF). These new pairs enabled us to find out whether a subject showed a preference for the best option A (i.e., Choose A performance), which would indicate an enhanced reward learning capacity. Conversely, a participant was classified as having a high punishment learning capacity when she showed more effective avoidance of B in the new stimulus pairs (i.e., Avoid B performance) [see also Ref. (11)]. In the transfer task direct feedback was no longer provided, so that no further learning could take place. The percentage of selections of A from the old pair AB further showed the combined effect of Choose A and Avoid B performance and was thus an indicator of overall learning capacity.

Subjects were tested twice in the two phases, but with different hiragana and kanji symbols for the stimuli A, B, C, D, E, and F on each test day. Tests were scheduled in a counterbalanced sequence that was equally distributed across the two study groups and genotypes (see above).

Genotyping

Genotyping was performed by a commercial laboratory (Bioglobe, Hamburg, Germany). DNA was extracted from buccal swabs and purified with a standard commercial extraction kit. The DAT1-polymorphism was characterized by fragment length determination of PCR products across the variable region. Assignment of genotypes was performed with the software GeneMarker v1.75 (Softgenetics). The PCR amplification procedure used the following primers: AAATAAAACTCCTTGAAACCAGC (forward primer), TGTTGTTATTGATGTGGCACG (backward primer). The distribution of genotypes was in Hardy-Weinberg equilibrium, either when calculated separately for each study group or when looking at the whole group of genotyped subjects.

Collection and Analysis of Salivary E2

Samples of morning saliva were collected on each test day. Subjects started collection at their normal wake-up time and collected five samples in 2 ml Eppendorf tubes at regular intervals over the course of 2 h. No consumption of food or beverages other than water was allowed during this time. This collection method avoided the contamination of samples and controlled for the episodic secretion pattern of steroid hormones, thus providing a representative sample of the free E2 level on the test day. Saliva samples were frozen at −20°C for further analyses. When all samples were collected, aliquots were obtained and processed with a 17beta-Estradiol Luminescence Immunoassay (IBL International, Hamburg, Germany) following the procedure described by Diekhof and Ratnayake (9).

Statistical Analysis

The analysis of the behavioral data was done with the software package IBM SPSS Statistics for Windows (version 22.0; IBM Corp.). Repeated-measures general linear models (GLMs) were performed on the percentage of selections of the better option made in sessions 1 and 2. The specific factors included in the GLMs are further specified in the Section "Results." In order to examine the effect of the cycle phase during which participants entered the study on behavioral outcome in the probabilistic feedback task [see Ref. (39) for a similar procedure], we also re-calculated the GLMs as specified in the Section "Results." For *post hoc* comparisons, either paired or independent *t*-tests were used. Pearson correlations tested for the association between individual differences in hormone level and behavioral preferences. A *p*-value smaller than 0.05 (two-tailed) was considered significant in all tests. If not otherwise indicated, we report the arithmetic mean ± SEM in the text, tables, and figures.

A more fine-grained analysis assessed the more difficult decisions to be made in session 2, which comprised the so-called "WIN-WIN trials" (i.e., pairs "AC," "AE," and "CE") and the "LOSE-LOSE trials" (i.e., "BD," "BF," and "DF"). In the "WIN-WIN trials," the better options A, C, and E were paired with each other and the percentage of selections of the relatively better option was measured. In the "LOSE-LOSE trials," the worse options (B, D, F) were combined with each other and we calculated the percentage of effective avoidance of the relatively worse option. Processing of these options required a finer representation of actual stimulus value and thus indicated the individual sensitivity for more detailed value representations that subjects could have learned from the nature of positive and negative feedback in session 1 (9, 40).

RESULTS

Analysis of E2 Concentration

The cycle group exhibited a significant increase in E2 level from the early to the late FP [$n = 30$; $E2_{early} = 2.42 \pm 0.36$ pg/ml; $E2_{late} = 4.35 \pm 0.48$ pg/ml; $t(29) = -6.16$, $p < 0.001$], while the HC group did not [$n = 38$; $E2_{OFF} = 2.28 \pm 0.24$ pg/ml; $E2_{ON} = 2.63 \pm 0.25$ pg/ml; $t(37) = -1.53$, $p = 0.134$]. This was also reflected by a significant group difference in the phase-related change of E2 level [Delta of $E2_{late-early}$ cycle group $= 1.93 \pm 0.31$ pg/ml; Delta of $E2_{ON-OFF}$ HC group $= 0.35 \pm 0.23$ pg/ml; $t(56) = 4.15$, $p < 0.001$].

Behavioral Data of the Cycle Group

We tested the influence of menstrual cycle phase and thus of the natural rise of follicular E2 on behavior in the probabilistic feedback learning task separately for the learning phase (session 1) and the transfer phase (session2). We found that both overall learning (in session 1) and the capacity for learning from positive as opposed to negative feedback as demonstrated in the transfer phase were affected by the factors "*phase*" and "*DAT1-genotype*" (see **Table 1** for an overview of all main effects and interactions).

For session 1, the GLM with the two within-subject factors "*pair*" (AB, CD, EF) and "*phase*" (early FP versus late FP) and the between-subject factor "*DAT1-genotype*" (9R versus 10H) revealed significant main effects of "*phase*" [$F(1,28) = 6.76$, $p = 0.015$, partial eta squared = 0.19] and "*pair*" [$F(2,56) = 20.53$, $p < 0.001$, partial eta squared = 0.42] as well as a significant two-way interaction between "*phase*" and "*DAT1-genotype*" [$F(1,28) = 6.14$, $p = 0.020$, partial eta squared = 0.18]. All other main effects and interactions in session 1 did not reach the statistical criterion of $p < 0.05$ (see **Table 1**).

Post hoc t-tests showed that subjects exhibited enhanced learning during the early FP [overall selection of the better option across pairs: early FP = 66.69 ± 2.91%; late FP = 59.53 ± 2.45%; $t(29) = 2.25$, $p = 0.032$] and learning success followed the expected pattern of A > C > E, which was in line with the associated reward contingencies (selection of A = 70.1 ± 2.65%; selection of C = 63.46 ± 2.79%; selection of E = 55.77 ± 2.14%; significance of all *post hoc* comparisons: $p < 0.05$).

Finally, carriers of the 9RP variant showed a significant decline in the ability to select the better option during learning in the comparison of the early and late FP [$t(13) = 3.3$, $p = 0.006$], while the 10H were not [$t(15) = 0.09$, $p = 0.927$]. Moreover, during the early FP, the 9RP allele carriers were also significantly better in selecting the better option than 10H [$t(28) = 2.56$, $p = 0.016$], and this difference vanished in the late FP [$t(28) = 0.85$, $p = 0.854$] (see **Table 2** for the descriptive statistics).

For session 2, the GLM with the two within-subject factors "*learning capacity*" (Choose A, Avoid B, old pair AB) and "*phase*" (early versus late FP) and the between-subject factor "*DAT1-genotype*" (9RP versus 10H) revealed a significant main effect of "*learning capacity*" [$F(1.42,39.69) = 5.97$, $p = 0.011$, partial eta squared = 0.18], the two-way interactions of "*phase*" and "*DAT1-genotype*" [$F(1,28) = 4.88$, $p = 0.035$, partial

TABLE 1 | Main effects and interactions in the cycle group ($n = 30$).

Main effect or interaction	*F*-value	Degrees of freedom	*p*-value	Partial eta squared
Learning phase (session 1)				
Phase*	**6.76**	**1, 28**	**0.015**	**0.19**
Phase × DAT1-genotype*	**6.14**	**1, 28**	**0.020**	**0.18**
Pair*	**20.53**	**2, 56**	**<0.001**	**0.42**
Pair × DAT1-genotype	3.10	2, 56	0.053	0.10
Phase × pair	1.49	2, 56	0.235	0.05
Phase × pair × Dat1-genotype	1.25	2, 56	0.295	0.04
DAT1-genotype	2.25	1, 28	0.145	0.07
Transfer phase (session 2)				
Phase	0.49	1, 28	0.488	0.02
Phase × DAT1-genotype*	**4.89**	**1, 28**	**0.035**	**0.15**
Learning capacity*	**5.97**	**1.4, 39.7**	**0.011**	**0.18**
Learning capacity × DAT1-genotype	2.074	1.4, 39.7	0.151	0.07
Phase × learning capacity*	**8.03**	**1.4, 40.1**	**0.003**	**0.22**
Phase × learning capacity × DAT1-genotype*	**3.66**	**1.4, 40.1**	**0.049**	**0.12**
DAT1-genotype	0.13	1, 28	0.723	0.01

**Effects that are significant at $p < 0.05$ are highlighted in bold and are marked with an asterisk. If required violations of sphericity were corrected with Greenhouse-Geisser.*

TABLE 2 | Descriptive statistics (arithmetic mean ± SEM) of choices during reinforcement learning (session 1) and in the transfer phase (session 2), as well as current E2 level subdivided by study group, DAT1-genotype, and phase.

	Cycle group (n = 30)				HC group (n = 38)			
	9-repeat allele carriers (9/9, 9/10)		10-repeat allele homozygotes (10/10)		9-repeat allele carriers (9/9, 9/10)		10-repeat allele homozygotes (10/10)	
	Early FP	Late FP	Early FP	Late FP	OFF_{HC}	ON_{HC}	OFF_{HC}	ON_{HC}
Learning phase (session 1)								
Choose better option (%)	73.97 ± 4.47	59.04 ± 3.01	60.32 ± 3.11	59.96 ± 3.84	63.51 ± 3.36	63.65 ± 2.72	58.54 ± 2.98	58.93 ± 2.65
Transfer phase (session 2)								
Choose A from new stimulus pairs (%)	50.66 ± 9.10	64.94 ± 6.27	63.12 ± 5.33	75.26 ± 6.37	66.89 ± 5.45	70.39 ± 4.68	63.56 ± 5.29	65.43 ± 4.39
Avoid B in new stimulus pairs (%)	78.32 ± 5.66	52.27 ± 6.70	62.91 ± 4.79	60.96 ± 5.98	68.28 ± 4.72	70.98 ± 5.46	65.56 ± 4.13	59.83 ± 3.26
E2 level (pg/ml)	2.38 ± 0.56	4.24 ± 0.56	2.45 ± 0.48	4.45 ± 0.76	2.27 ± 0.34	2.49 ± 0.31	2.28 ± 0.34	2.78 ± 0.42

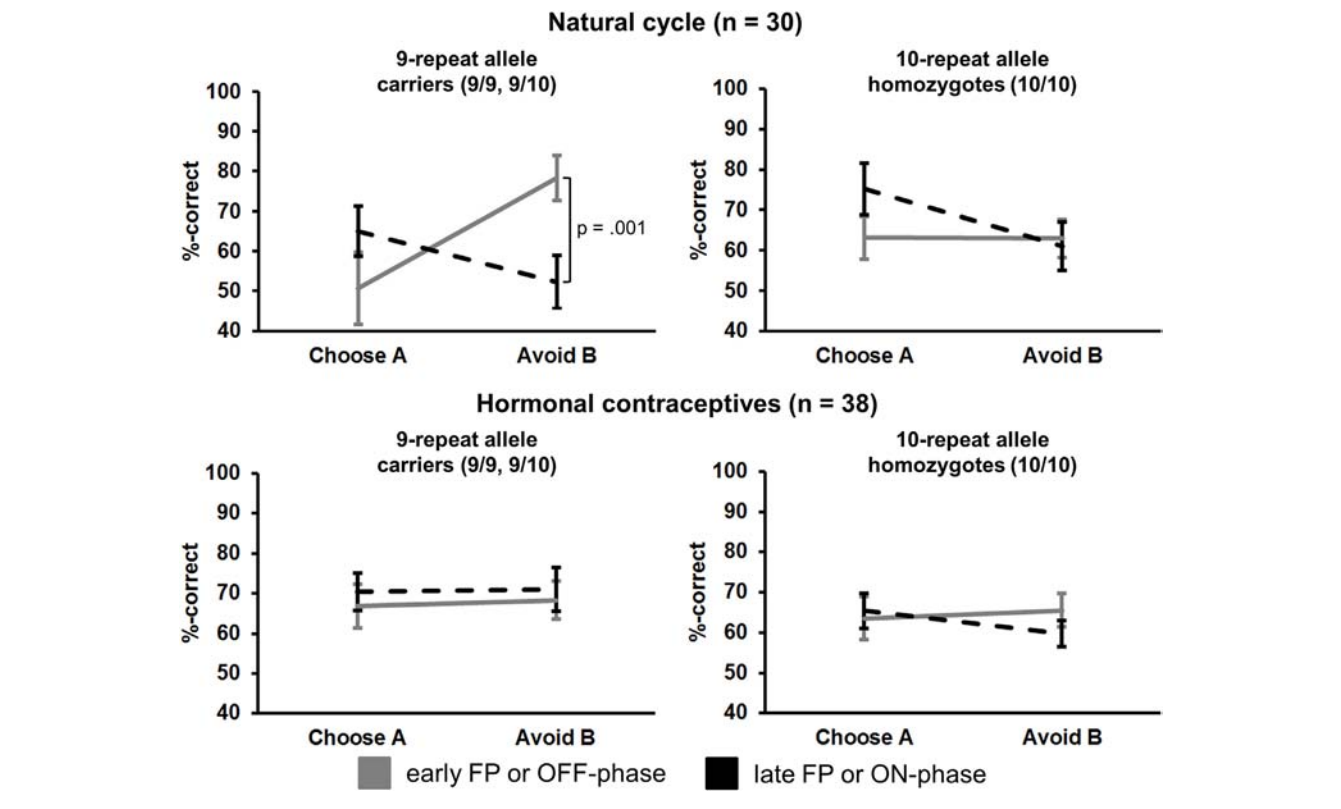

FIGURE 1 | Interaction of "*phase*" × "*preference*" × "*study group*" × "*DAT1-genotype*" in the transfer phase (session 2). Carriers of the 9RP in the cycle group experienced a significant decline in the ability to avoid punishment from the early to the late FP (top left graph). Neither the 10H from the cycle group nor 9RP carriers or 10H from the HC group showed this difference between phases.

eta squared = 0.15], and of "*phase*" and "*learning capacity*" [$F(1.43,40.08) = 8.032$, $p = 0.003$, partial eta squared = 0.22]. The respective *post hoc t*-tests showed that the participants of the cycle group were better at choosing symbol A in the old stimulus pair AB compared to the new pairings ($p < 0.05$ in all *post hoc* comparisons). They were also better at choosing the best option A from all other options in the new stimulus pairs when being in the high E2 state [Choose A performance: early FP = 57.3 ± 5.14%; late FP = 70.44 ± 4.51%; $t(29) = -2.05$; $p = 0.049$], and became significantly worse during avoidance of B in the new stimulus pairs [Avoid B performance: early FP = 70.1 ± 3.88%; late FP = 56.90 ± 4.46%; $t(29) = 2.39$; $p = 0.024$].

Most notably, we also found the hypothesized three-way interaction of "*phase*" by "*learning capacity*" by "*DAT1-genotype*" [$F(1.43,40.08) = 3.66$, $p = 0.049$, partial eta squared = 0.12]. Carriers of the 9RP allele in the cycle group experienced a significant decline in the ability to avoid punishment (i.e., Avoid B performance) from the early to the late FP [$t(13) = 4.53$, $p = 0.001$], while the 10H from the cycle group did not show this difference between phases [$t(15) = 0.24$, $p = 0.816$] (**Table 2**; **Figure 1**). Further, in the direct comparison of genotypes, it

became obvious that the difference in learning capacity was not only evident in the Delta between early and late FP, which was significantly bigger in the carriers of the 9RP [Delta Avoid B: $t(28) = -2.34$; $p = 0.024$; Delta Old pair AB: $t(28) = -2.87$; $p = 0.008$], but there was also a significant group difference in the early FP with the 9RP variant carriers outperforming the 10H in avoidance learning [Avoid B performance: $t(28) = 2.10$, $p = 0.045$] and in the Old pair AB [$t(28) = 2.32$, $p = 0.024$] (see also **Figure 2**). These findings suggested that the original two-way interaction of "*phase*" by "*learning capacity*" was most likely driven by the pronounced behavioral change observed in the 9RP allele carriers.

Based on this assumption a more detailed analysis of the value representations in "WIN-WIN-" and "LOSE-LOSE-trials" was performed with the 9RP variant carriers and 10H. In line with the observation of a reduced punishment sensitivity in the late as opposed to the early FP in 9RP allele carriers, we found a reduced ability to avoid the least rewarded symbols in the more difficult "LOSE-LOSE trials" for this genotype [avoidance of worst option from difficult pairs: early FP = 82.48 ± 4.14%; late FP = 64.33 ± 4.30%; $t(13) = 3.95$, $p = 0.002$], but not for the 10H [early FP = 71.76 ± 4.36%; late FP = 72.06 ± 4.14%; $t(15) = -0.43$, $p = 0.966$]. No significant difference emerged in the "WIN-WIN trials" for the 9RP carriers [choice of better option from difficult pairs: early FP = 28.66 ± 6.31%; late FP = 39.74 ± 5.81%; $t(13) = -1.17$, $p = 0.263$], nor the 10H [early FP = 40.52 ± 3.96%; late FP = 49.71 ± 4.33%; $t(13) = -2.09$, $p = 0.055$].

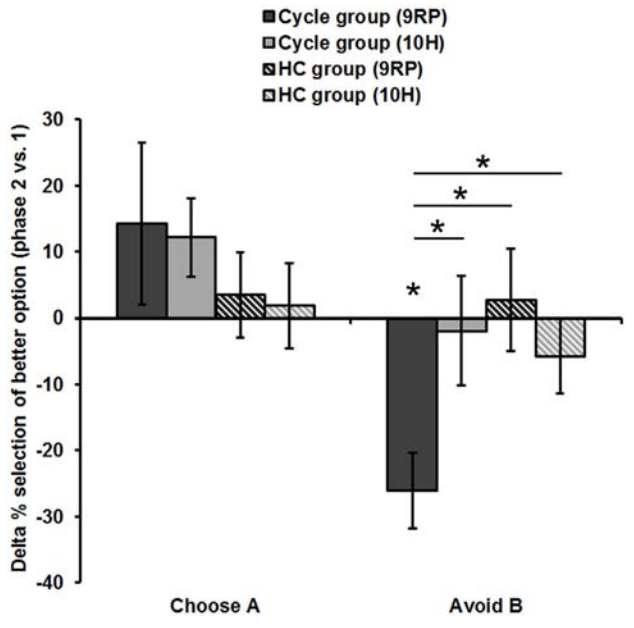

FIGURE 2 | Difference in reinforcement learning capacity between phases (session 2). Displayed is the Delta of the late minus early FP and of the ON- minus OFF-phase, respectively. Only the 9RP genotype showed a significant phase-related change in punishment avoidance capacity. Significant differences ($p < 0.05$, two-tailed) from zero and between genotypes and groups, respectively, are marked with an asterisk. Phase 1, early FP or OFF-phase; phase 2, late FP or ON-phase.

Behavioral Data of the HC Group

In order to find out whether the intake of HC (i.e., synthetic estrogens) may evoke similar changes in reinforcement learning capacity as natural fluctuations in E2 during the FP, we tested this second group of subjects once during the pill break and once during the intake phase of HC. In contrast to the cycle group, we were unable to document a phase-related change in learning performance in session 1 nor was there an effect of HC intake on Choose A and Avoid B performance in session 2. Further, genotype also did not affect performance, whether considered alone or in the interaction with test phase (see **Table 3**).

Group Comparison

For the group comparison (cycle versus HC group), we used the phase-related Deltas of learning performance in the two groups (i.e., Delta of "late–early FP" and Delta of "ON–OFF-phase," respectively). We thereby intended to assess the impact of the between-subject factors "*study group*" (cycle versus HC group) and "*DAT1-genotype*" (9RP allele carriers versus 10H) on the within-subject factor "*Delta learning capacity*," which contained the three Deltas of Choose A, Avoid B, and Old pair AB performance from session 2. The GLM revealed a trend-wise three-way interaction of "*Delta learning capacity*" by "*study group*," and "*DAT1-genotype*," [$F(1.52, 97.23) = 2.93$, $p = 0.072$, partial eta squared = 0.04], a significant two-way interaction of "*Delta learning capacity*" and "*study group*" [$F(1.52, 97.23) = 3.62$, $p = 0.042$, partial eta squared = 0.05] and a significant main effect of "*Delta learning capacity*" [$F(1.52, 97.23) = 6.68$, $p = 0.004$, partial eta squared = 0.10].

Accordingly, 9RP allele carriers of the cycle group showed a stronger phase-related decline in avoidance learning from the low to the high E2 phase [Delta of Avoid B performance in 9RP: cycle group = −26.06 ± 5.74%; HC group = 2.70 ± 7.7%; $t(32) = -2.76$, $p = 0.005$; Delta of Old Pair AB performance in

TABLE 3 | Main effects and interactions in the HC group ($n = 39$).

Main effect or interaction	*F*-value	Degrees of freedom	*p*-value	Partial eta squared
Learning phase (session 1)				
Phase	0.02	1, 36	0.894	<0.01
Phase × DAT1-genotype	0.004	1, 36	0.950	<0.01
Pair*	**24.34**	**2, 72**	**<0.001**	**0.40**
Pair × DAT1-genotype	1.27	2, 72	0.288	0.03
Phase × pair	0.50	2, 72	0.609	0.01
Phase × pair × Dat1-genotype	0.18	2, 72	0.833	<0.01
DAT1-genotype	1,73	1, 36	0.197	0.05
Transfer phase (session 2)				
Phase	0.01	1, 36	0.944	<0.01
Phase × DAT1-genotype	0.59	1, 36	0.447	0.02
Learning capacity*	**9.84**	**1.7, 60.6**	**<0.001**	**0.22**
Learning capacity × DAT1-genotype	0.29	1.7, 60.6	0.713	0.01
Phase × learning capacity	0.29	1.6, 57.1	0.697	0.01
Phase × learning capacity × DAT1-genotype	0.18	1.6, 57.1	0.788	0.01
DAT1-genotype	2.72	1, 36	0.108	0.07

Effects that are significant at $p < 0.05$ are highlighted in bold and are marked with an asterisk. If required violations of sphericity were corrected with Greenhouse-Geisser.

9RP: cycle group = −26.22 ± 6.31%; HC group = 2.15 ± 7.7%; $t(32) = -2.7$, $p = 0.011$] (see also **Figure 2**). Further, in the late FP, 9RP allele carriers of the cycle group were also more compromised in the ability to avoid negative feedback and to choose symbol A from the old stimulus pairs, when being compared to the same genotype in the HC group and the ON-phase [Avoid B performance in 9RP: $t(32) = -2.18$, $p = 0.037$; Old Pair AB performance in 9RP: $t(32) = -2.31$, $p = 0.027$; see also **Table 2** for descriptive statistics].

In contrast, there were no phase-related differences to be found between the 10RP allele homozygotes from the cycle and the HC group. The two *post hoc t*-tests for the main effect and the two-way interaction yielded only weak results. For one thing, in the comparison of the groups there was a non-significant, numerical difference between the negative Delta of Avoid B performance [Cycle group = −13.2 ± 5.53%; HC group = −1.29 ± 4.85%, $t(66) = -1.62$, $p = 0.11$] and the positive Delta of Choose A performance [Cycle group = 13.14 ± 6.41%; HC group = 2.73 ± 4.46%, $t(67) = 1.37$, $p = 0.18$], which were both less pronounced in the HC group. Further, when considering all subjects together, there was a significant difference between the mean Delta of Choose A performance (7.32 ± 3.79%) and Avoid B performance (−6.55 ± 3.69%) [$t(67) = 2.46$, $p = 0.016$]. Yet, we presume that any group-related differences had their origin in the strong decline of avoidance learning capacity from the early to the late FP that was rather specific for 9RP variant carriers of the cycle group.

Effect of Initial Test Phase

We also wanted to examine whether the test order may have had a significant influence on reinforcement learning capacity as demonstrated by previous studies. Following the procedure described by Wallen and Rupp (39), we performed two GLMs on the data of the initial test day, when subjects were still naïve with regard to performing the probabilistic feedback task.

For session 1, the GLM included the within-subject factor "*pair*" (AB, CD, EF) and the between-subject factors "*test phase*" (early FP, late FP, OFF-phase, and ON-phase) and "*DAT1-genotype*" (9RP versus 10H). No significant effects emerged. Only the interaction between "*pair*" and "*DAT1-genotype*" reached statistical trend level [$F(2, 120) = 2.54$, $p = 0.083$, partial eta squared = 0.04].

For the transfer phase, the within-subject factor "*learning capacity*" (Choose A, Avoid B, old pair AB) and the two between-subject factors "*test phase*" (early FP, late FP, OFF-phase, and ON-phase) and "*DAT1-genotype*" (9RP versus 10H) we included in the GLM. Here, we found a significant main effect of "*learning capacity*" [$F(1.43, 85.42) = 13.84$, $p < 0.001$, partial eta squared = 0.19] as well as a significant two-way interaction of "*learning capacity*" and "*test phase*" [$F(4.27, 85.42) = 2.66$, $p = 0.018$, partial eta squared = 0.12] and a significant three-way interaction that also included the factor "*DAT1-genotype*" [$F(4.27, 85.42) = 2.93$, $p = 0.023$, partial eta squared = 0.13]. The *post hoc t*-tests confirmed that only the 9RP allele carriers were significantly worse in the avoidance of negative feedback in the LF as opposed to the EF [9RP—Avoid B performance: EF = 87.82% ± 5.59%; LF = 39.56% ± 8.37%; $t(12) = 4.80$, $p < 0.001$], while the 10H were not [10H—Avoid B performance: EF = 64.24% ± 7.47%; LF = 67.28% ± 8.36%; $t(12) = -0.27$, $p = 0.790$]. Moreover, performance was significantly different between genotypes in both the EF [$t(13) = -2.46$, $p = 0.028$] and the LF [$t(13) = 2.33$, $p = 0.036$].

In addition, the women with a natural cycle and the 9RP variant also showed a significant performance decline in the LF (Avoid B = 39.56% ± 8.37%) in comparison to the ON-phase (Avoid B = 79.59 ± 6.51%) [$t(15) = -3.83$, $p = 0.002$]. In contrast, the comparisons of the EF and the OFF-phase in the same genotype and those in the 10H did not yield significant differences in avoidance learning between study groups and phases.

Pearson Correlations between Phase-Dependent E2 Concentration and Performance in the Transfer Phase

To better understand the association between the individual variation in E2 concentration within the different groups and phases and reinforcement learning capacity, we also explored the respective hormone-behavior correlations in the transfer phase [see Ref. (16) for a similar approach]. First, we found a positive correlation between early follicular E2 level and reward sensitivity (i.e., Choose A performance) across genotypes in the cycle group ($n = 30$, $r = 0.39$, $p = 0.034$, two-tailed). No significant correlation emerged in the late FP or in the HC group.

Second, when subdividing the two samples by genotype, only the 9RP carriers of the cycle group exhibited a trend-wise positive correlation in the early FP ($n = 14$, $r = 0.50$, $p = 0.07$, two-tailed), suggesting that this genotype may have primarily driven the positive correlation in the complete cycle group. In contrast, the carriers of the 9RP from the HC group showed the reverse correlation in the OFF-phase ($n = 20$, $r = -0.49$, $p = 0.027$, two-tailed), and the two correlation coefficients were also significantly different (Fisher *r*-to-*z* transformation: $z = 2.8$, $p = 0.0051$, two-tailed).

DISCUSSION

Interactions of genotype and hormonal state have been widely ignored by cognitive neuroscience [but see Ref. (15, 17)]. The present study is the first to systematically assess how the natural rise of E2 level in the late FP may interact with DAT1-genotype during reinforcement learning. Our data show that the effect of DAT1-genotype on reinforcement learning may indeed interact with transient hormonal state, but only in women with a natural menstrual cycle. Notably, we found that carriers of the 9RP variant experienced a significant decrease in the ability to avoid punishment from the early to the late FP. No such plasticity emerged in the HC group or in the 10H from either group.

DAT1-Genotype and Avoidance Learning Capacity

Our main finding of a reduced punishment learning capacity in 9RP allele carriers during the high E2 state (i.e., the late FP) conforms to the idea that physiological E2 may act as an endogenous DA-agonist that amplifies central dopaminergic

transmission (1, 34, 35). Previous studies have already shown that synthetic drugs that increase striatal DA level may reduce the ability to avoid punishment and may in turn increase reward learning ability (11, 40). A similar effect has also been documented for changes in endogenous hormone level over the course of the menstrual cycle (9, 10, 16). Finally, behavioral genetic studies showed that the sensitivity for negative feedback may be reduced in carriers of polymorphisms associated with a higher DA synthesis capacity and reduced DRD2 density [e.g., the minor A+ allele of the Taq1A polymorphism (12, 24)]. The present study is the first to demonstrate the interactive effect of a state of physiologically increased E2 level (i.e., the late FP) and a genetic polymorphism that might be related to lower striatal DA transmission on intra-individual variations in reinforcement learning capacity. Most previous neuroimaging studies on the DAT1-polymorphism used mixed sex samples and failed to control for menstrual cycle phase in women. It currently remains unclear whether in comparison to the 10RP allele the 9RP variant of the DAT1-polymorphism is associated with a lower (25) or higher density of striatal DAT (26–28), if there is an association with DAT expression at all (29). Behavioral evidence is also mixed, with some studies suggesting that homozygosity of the 10RP allele may enhance reward-related responses [e.g., Ref. (32)], while others found evidence for the opposite with increased activation in 9RP allele carriers [e.g., (30, 31)]. The present data may provide preliminary evidence for the assumption that the 9RP variant may be related to a higher DAT density. If the state of enhanced E2 indeed induced a reversal of normal DAT function that promoted increased DA efflux as suggested by Watson et al. (35), then subjects with the genetic predisposition for a higher density of the DAT should be more strongly affected by this effect of rising E2 in the late FP. This is because the strongest E2-induced DA efflux through the DAT might be expected in individuals carrying the highest density of transporters, which should then also lead to a pronounced behavioral change from the early to the late FP. In addition to that, the same genotype should also have a higher punishment avoidance capacity in comparison to the other variant at cycle onset, when E2 level is at its nadir. Carriers of the 9RP allele of the cycle group fulfilled these two prerequisites, when being compared to homozygotes of the 10RP variant (see **Figure 1**; **Table 2**). Following this logic, the present results may also conform to the model of the inverted U-shape relationship between behavioral performance, baseline DA level, and DA-agonistic substances (41). More specifically, according to this model only subjects with a low baseline DA concentration, who according to our data might be the carriers of the 9RP allele, should be significantly influenced by a physiological rise in E2 level [see also Ref. (16)]. In contrast, the E2 rise in the late FP should have no or only a small effect on punishment learning capacity in subjects with a supposedly higher baseline DA capacity to begin with (as demonstrated here for the 10H).

However, the present observations do not conform to the results of a recent behavioral genetic study that assessed social reward learning capacity in healthy young men. Eisenegger et al. (42) used pharmacological intervention with L-DOPA, which transiently enhances striatal DA levels and assessed the interaction with DAT1-genotype (comparison of carriers of the 9RP allele versus 10H) on learning from economic interactions with either prosocial or antisocial partners. They found an increase of social learning success resulting in enhanced interactions with prosocial partners and higher pay-off under L-DOPA treatment of male 10H. In contrast, male 9RP allele carriers appeared to be impaired in learning from prosocial interactions following treatment with L-DOPA. Yet, there are several differences between the two studies that may explain the divergent findings. First, Eisenegger et al. (42) used a between-subjects design, in which both the pharmacological intervention (L-DOPA versus placebo) and the pairing with a prosocial versus antisocial partner varied between participants. This did not only result in relatively small samples for comparison (e.g., of the group confronted with a prosocial partner, only 16 of the 43 male 9RP allele carriers were treated with L-DOPA), but may have also increased the influence of inter-individual variance. For example, Eisenegger et al. (42) did not control for other genetic polymorphisms in the DA system nor in related neurotransmitter systems (e.g., the serotonin system) that could have significantly affected reinforcement learning capacity or social cognition thus contributing to group differences independent of the pharmacological intervention. When we assessed whether the effects identified in the present study were already evident during the initial, naïve test day, we were also required to use a between-subjects approach. Yet, our finding from the initial test day was in line with the finding that emerged in the within-subject design, which allowed us to rule out these potential confounds related to intersubject variation. Further, the L-DOPA treatment in healthy young men may have induced a supraphysiological DA level. The risk of dopaminergic overstimulation is always immanent when using an effective agent like L-DOPA in healthy young adults. It has already been demonstrated that the effects of supraphysiological stimulation on the DA system are not necessarily comparable to those achieved by stimulation in the physiological range and could even reverse the expected behavioral effects (3), which might explain the discrepant findings of Eisenegger et al. (42). Finally, and most importantly, Eisenegger et al. (42) assessed young men, while the present study was restricted to a female sample. Sex differences in the neural correlates of reward processing have repeatedly been demonstrated [e.g., Ref. (43)]. Further, one may speculate that a high endogenous testosterone concentration in men might have a similar effect on DAT function as rising E2 in women. The DA-agonistic effects of testosterone have repeatedly been documented (44), and testosterone may exert some of its central effect through conversion to E2. It is therefore possible that the DA response of young men, who were at their point of peak fertility, may have also been influenced by their current testosterone level, which may have further contributed to inter-individual differences between subjects. Again, this renders the risk of overstimulation by L-DOPA even more likely and might further explain discrepant findings between the two studies.

It has recently been argued that test order effects may render the interpretation of the results from previous studies on the menstrual cycle difficult (39, 45). For this reason, we analyzed the data from the initial test day separately and found that the

results from the analysis of the influence of cycle phase during this naïve test replicated the effects of the repeated-measures design. For this reason, we are confident to assume that the effects identified in this preliminary study could indeed be a consequence of the hypothesized cycle phase by genotype interaction.

Differences between the Cycle and the HC Group

Another important finding of the present study was that the decrease in avoidance learning capacity was restricted to the women from the cycle group who experienced the effect of a natural rise in E2 in the late FP. The HC group showed no behavioral variations between the pill break and the intake phase. Since the intake of HC suppressed the physiological rise in E2 to such an extent that only a slight numerical increase of E2 concentration remained at the group level (**Table 2**), the lack of effect of natural E2 on reinforcement learning ability was not unexpected. Yet, our data also suggest that the women of the HC group remained unaffected by the intake of synthetic hormones. This was in so far surprising as more general differences have been hypothesized to exist between women with a natural menstrual cycle and those that take HC on a regular basis [e.g., Ref. (46)]. The HC sample consisted of women that took HC for at least 1 year, with an average intake duration of more than 7 years. This might have induced profound adaptations to the constant hormonal treatment, such as a compensatory reorganization of neuroanatomy or function that has been demonstrated elsewhere and may also promote behavioral differences (37). When considered as deflections from homeostasis, compensatory long-term adaptations should become particularly evident when the pharmacological agent is with-held (i.e., during the pill break) and may then show up as deflections from homeostasis. Such a mechanism might be comparable to the long-term drug effects on the DA system in substance abuse disorders (47). Yet, the HC subjects from the present study remained quite stable across phases when considering the mean performance in the probabilistic feedback learning task (**Table 2**), which could indicate that long-term HC might have rendered the brain rather unresponsive to the change induced by a short OFF-period. Only the observation of a negative correlation between E2 level and reward sensitivity in the OFF-phase, which contrasted the positive correlation documented in the cycle group, may hint us to the possibility of compensatory long-term adaptations, a speculation, which however needs to be replicated in a bigger sample.

Only a limited number of studies have so far assessed the influence of HC on brain function and anatomy. Bonenberger et al. (48) examined the influence of HC in the context of reward processing in a modified version of the monetary incentive delay task. They demonstrated that the regular intake of HC may slightly alter activation in the anterior insula during reward expectation, but not in the striatum nor in other regions of the mesolimbic DA system. In addition, another two studies suggested HC-related changes in brain regions that are important for various fundamental aspects of cognition (46), which may in part depend on the subtype of HC used [for example in relation to face recognition performance (49)]. In the present sample, 17 women ($n_{9RP} = 9$) used HC containing androgenic progestins, while the remaining 21 subjects ($n_{9RP} = 11$) took HC with anti-androgenic progestins. Exploratory *t*-tests revealed no significant behavioral differences between the HC subtypes at $p < 0.05$ (two-tailed), also not when accounting for the influence of genotype and pill phase. Yet, the present sample was quite small. Since the DA-agonistic properties of androgens have already been demonstrated (44), it might be valuable to readdress the potential impact of HC subtype (androgenic versus anti-androgenic progestins) on reinforcement learning in a bigger sample.

Alternatively, it might also be possible that any changes in reinforcement learning capacity from the OFF- to the ON-phase were masked by a rise in progestin content that accompanied the synthetic estrogen administration. If that was the case, the DA-antagonistic properties of progesterone and its metabolites [e.g., Ref. (50–52); see also Ref. (9)] would have neutralized any E2-related effect on dopaminergic transmission in the ON-phase. Since we did not measure salivary progesterone level, we are unable to rule out this latter possibility.

Finally, other group differences, like the fact that most HC subjects were in a committed relationship, while less then half of the participants from the cycle group indicated to have a partner, could have also contributed to differences in the underlying neurofunctional structure, since partnership has been shown to affect the hormone system, which could indirectly influence brain physiology [e.g., Ref. (53)].

No Evidence of a Cycle Phase by Genotype Interaction in the Context of Reward Learning Capacity—A Possible Relation to Tonic DA?

But why did the 9RP allele carriers become compromised in avoidance learning ability without experiencing an increase in reward sensitivity from the early to the late FP? The functional opponency of reward and punishment learning capacity has been demonstrated repeatedly and can be induced by both variations in central DA transmission [high versus low baseline DA, respectively, e.g., Ref. (11, 13)] and endogenous E2 level (9, 10, 16). The present data indicated a slight rise of the "Choose A" performance from the early to the late FP in both 9RP variant carriers and in 10H in the transfer phase (**Table 2**; **Figure 1**), which, against the background of a decline in "Avoid B" performance, would be in line with the assumption of functionally opponent processes. Yet this increase was not significant. This latter observation may fit with previous evidence suggesting that if tonic DA level is low, the transient upregulation of the phasic DA response can still be observed, which may preserve a normal responsiveness to reward (54). Therefore, even relatively lower levels of DA (e.g., those expected during the early FP) may be sufficient for effective learning from reward through a positive reward prediction error (55). This might have been one explanation for the observation that the rise in E2 level in the late FP had no further enhancing effect on reward

learning capacity in the probabilistic feedback task. Diekhof and Ratnayake (9) documented a similar finding, when comparing the late FP and the luteal phase of the menstrual cycle and also found no evidence for a change in striatal processing of positive feedback. In contrast to that, the ability to avoid negative feedback appeared to be more sensitive to variations in tonic DA level. In order to realize effective learning from the negative outcome of an action, a significant depression of tonic DA is required (55, 56). Moreover, the dip in dopaminergic tone has to be strong enough for engaging the respective corticostriatal connections of the indirect NoGo-pathway of the basal ganglia to realize effective punishment learning in the probabilistic feedback task (13). One may assume that in the E2-dominated state of the late FP and the supposedly higher DA content in the striatum, the suppression of dopaminergic tone following a negative feedback would become more difficult. This would decrease the signal-to-noise ratio and thus render the negative prediction error signal less likely, which would ultimately result in a significant decline in punishment learning capacity. Diekhof and Ratnayake (9) demonstrated a significant decline in "Avoid B" performance in the late FP, which was also accompanied by a reduced activation of the dACC by negative feedback. In the present study, the highest sensitivity for more detailed value representations in "LOSE-LOSE trials" was evident in the 9RP allele carriers in the early FP, and this sensitivity declined by 18% when E2 level rose in the late FP. Moreover, carriers of the 9RP variant also showed a cycle phase-related difference in learning performance in session 1. Subjects became particularly worse when learning from pair AB [early FP = 79.3 ± 4.6%; late FP = 62.8 ± 4.8%; $t(13) = 2.77$, $p = 0.016$]. This suggests a reduced ability to identify the least rewarded option already in the fixed stimulus pairs.

Nevertheless, the prediction error theory of dopamine may provide only one possible explanation for the current and previous findings [e.g., Ref. (9)] observed in the probabilistic feedback task. Alternatively, variations in the motivation to act or to engage in goal-directed action might have equally well contributed to the observed differences between cycle phase-related variations in punishment and reward learning capacity [see Ref. (57) for review].

Limitations

First, in this initial study, we tested a relatively small sample of women [in the range of previous behavioral genetic studies; e.g., Ref. (17)] and looked at a single polymorphism that may affect DAT density in the striatum. For these reasons, the present results can only be considered as preliminary and require further replication.

Second, the cycle phase by genotype interaction had a relatively small influence on variations in reinforcement learning capacity, as indicated by the small effect size of the interaction when the factor "*study group*" (HC versus cycle group) was also taken into account (partial eta squared < 0.10). However, the present study used a rather conservative approach by comparing phase-related changes in both a cycle group and a sample including only women that took HC. Most previous studies that assessed the effect of menstrual cycle phase on reward processing did not include such a control sample [e.g., Ref. (15)]. Accordingly, when the cycle group was considered alone in our study, the effect size for the interaction of "*phase*" × "*learning capacity*" × "*DAT1-genotype*" increased from 0.04 to 0.12 (see **Table 1**). Nevertheless, even the smaller effect size of 0.04 is in the range of effect sizes reported by pharmacological intervention studies published in the field. For example, none of the effect sizes reported by Eisenegger et al. (42) for interactions involving the factor "*DA intervention*" and "*DAT1-genotype*" in relation to social reward learning were higher than a partial eta squared of 0.098. When considering the fact that we assessed the impact of the physiological rise in E2 level, our findings indicate that endogenous E2 may be a quite potent modulator of DA transmission with effect sizes comparable to pharmacological agents like L-DOPA. In addition to that, like many of the previous studies on the role of the DAT1-polymorphism in reward processing, our study also did not control for other, potentially relevant genetic polymorphisms that could equally well affect reinforcement learning capacity. Future studies with bigger samples ($n > 200$) should use more advanced behavioral genetic methods like genome-wide association or at least haplotype analysis in order to draw a more comprehensive picture of cycle phase by genotype interactions relevant for inter- and intra-individual variations in reinforcement learning capacity.

Another limitation might be the use of a social feedback (smiley versus grumpy face) in the probabilistic feedback task. This was done to create a design that was comparable to Frank et al. (11), Klein et al. (12), and Diekhof and Ratnayake (9). However, a social feedback may have a lower salience than a monetary reward or loss and may thus lead to a reduced dopaminergic response, i.e., a less effective DA burst or dip, which could have made it easier for variations in E2 to actually tip the balance in favor of reward at the expense of punishment sensitivity. Even though, this thought is currently mere speculation, future studies have to address this potential confound and should test, whether learning from a monetary loss during action selection is equally affected by follicular E2.

Finally, the present study assessed the influence of the rise in endogenous E2 concentration, which precludes any solid inferences on causality. Therefore, placebo-controlled E2 administration studies in young women or a comparison between menopausal women who receive a hormone-replacement therapy or not will be important in that context [e.g., Ref. (58)]. Yet, pharmacological E2 may also have certain disadvantages like the possibility of supraphysiological stimulation in young women, as already outlined above, or the stimulation of a neural structure that might be already compromised by biological aging effects. As an initial step, the understanding of physiological E2 and its role in reinforcement learning may thus be crucial to provide an informed basis for future studies that use pharmacological intervention.

CONCLUSION

Taken together, the present study adds to the growing awareness of the complex interplay between various physiological

determinants of dopaminergic transmission. The observed effects on reinforcement learning capacity cannot simply be attributed to cycle phase or genotype alone, but may be a result of their interaction. Furthermore, the present data may provide preliminary evidence for a differential effect of natural and synthetic hormones on reinforcement learning capacity. In that way, they may not only point out the necessity to control for hormonal state and biological sex in behavioral genetics research, but may also offer new ideas for studies in clinical settings.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the local ethics committee "Ethikkommission der Ärztekammer Hamburg (Germany)" with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the "Ethikkommission der Ärztekammer Hamburg (Germany)."

AUTHOR CONTRIBUTIONS

ED designed the research, supervised data collection, analyzed the data, and wrote the paper. KJ collected the data, analyzed the data, and reviewed the manuscript. HE collected the data and reviewed the manuscript. SH supervised data collection and reviewed the manuscript. LR supervised data collection and reviewed the manuscript.

ACKNOWLEDGMENTS

We would like to thank A. Kroll for performing the hormone analysis, M. Langbehn for programming the test protocol and the batches for initial data extraction, F. Starke, J. Schümann, and V. Groenewold for help in data acquisition, as well as all the anonymous participants for their participation. This research did not receive any specific grant from funding agencies in the public, commercial, or non-profit sectors. It was conducted with regular financial resources provided by the Biology Department at the Universität Hamburg.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 02 March 2018
doi: 10.3389/fendo.2018.00066

Gonadal Hormones and Retinal Disorders: A Review

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Aim: Gonadal hormones are essential for reproductive function, but can act on neural and other organ systems, and are probably the cause of the large majority of known sex differences in function and disease. The aim of this review is to provide evidence for this hypothesis in relation to eye disorders and to retinopathies in particular.

Methods: Epidemiological studies and research articles were reviewed.

Results: Analysis of the biological basis for a relationship between eye diseases and hormones showed that estrogen, androgen, and progesterone receptors are present throughout the eye and that these steroids are locally produced in ocular tissues. Sex hormones can have a neuroprotective action on the retina and modulate ocular blood flow. There are differences between the male and the female retina; moreover, sex hormones can influence the development (or not) of certain disorders. For example, exposure to endogenous estrogens, depending on age at menarche and menopause and number of pregnancies, and exposure to exogenous estrogens, as in hormone replacement therapy and use of oral contraceptives, appear to protect against age-related macular degeneration (both drusenoid and neurovascular types), whereas exogenous testosterone therapy is a risk factor for central serous chorioretinopathy. Macular hole is more common among women than men, particularly in postmenopausal women probably owing to the sudden drop in estrogen production in later middle age. Progestin therapy appears to ameliorate the course of retinitis pigmentosa. Diabetic retinopathy, a complication of diabetes, may be more common among men than women.

Conclusion: We observed a correlation between many retinopathies and sex, probably as a result of the protective effect some gonadal hormones may exert against the development of certain disorders. This may have ramifications for the use of hormone therapy in the treatment of eye disease and of retinal disorders in particular.

Keywords: gonadal hormones, estrogens, hormone therapy, eye disorders, retinopathies, optic nerve, age-related macular degeneration, sex-related differences

OPEN ACCESS

Edited by:
Takayoshi Ubuka, Monash University Malaysia, Malaysia

Reviewed by:
Barney A. Schlinger, University of California, Los Angeles, United States
María Miranda, Universidad CEU Cardenal Herrera, Spain

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *28 November 2017*
Accepted: *14 February 2018*
Published: *02 March 2018*

Citation:
Nuzzi R, Scalabrin S, Becco A and Panzica G (2018) Gonadal Hormones and Retinal Disorders: A Review. Front. Endocrinol. 9:66. doi: 10.3389/fendo.2018.00066

INTRODUCTION

There is a growing body of evidence for the importance of gonadal hormone action in the function of the reproductive and other systems (1), including bone (2) and cardiovascular system. Sex hormones (androgenic, estrogenic, and progestinic) are produced by both sexes, though the quantity and mode differ by sex and age. Moreover, they are produced, not only by the gonads, but also by

other organs (3, 4), including the central nervous system (CNS) in which estrogens are thought to exert a neuroprotective role (5, 6).

Historically, interactions between gonadal hormones and the eye have received scarce attention; however, recent research into sex-related differences has begun to reveal possible links between estrogens and eye diseases, i.e., glaucoma, age-related macular degeneration (AMD), and cataracts. This has carried over into the evaluation of the implications that postmenopausal hormone replacement therapy (HRT) and anti-estrogenic therapy in breast cancer could have for concomitant eye disorders (7).

Since, research in this area is still at its beginning, the available studies are few and often limited in sample size; this does not allow to reach a univocal and definitive answer about the relationship between sex, sex hormones, and ocular pathologies. The purpose of this review is, therefore, to summarize the results currently present in the literature.

BASICS OF BIOLOGY AND EPIDEMIOLOGY OF INTERACTION BETWEEN GONADAL HORMONES AND THE EYE

Presence of Hormone Receptors in the Human Eye

The eye was long considered a "sexually neutral" structure, meaning that it was believed that there were no differences in ocular physiology and pathology between the sexes. Today, however, we know that differences among sexes exist both in the physiology and in the pathology of the eye. In fact, the eye is a target for sex steroid hormones as demonstrated by the large presence of sex steroid hormone receptors (SSHRs). The SSHRs' mRNAs are present everywhere in the eye (8): cornea, lens, iris, ciliary body, retina, lacrimal gland, meibomian gland, conjunctiva [for a complete list of citations see Ref. (9)]. In all these locations, estrogen receptor α (ERα), estrogen receptor β (ERβ), progesterone receptor, and androgen receptor (AR) mRNAs have been detected.

The distribution of SSHRs in the eye varies by sex and age, which partly explains the difference in the epidemiology of certain eye diseases (9). PCR assay, Western blot, and immunohistochemical analysis have demonstrated the presence of ER-α protein in the retina and RPE of young women, but not in postmenopausal women or men (10).

In addition to AR mRNA (8), the AR protein is present in the lachrymal and meibomian glands, the cornea, the bulbar conjunctiva, the lens, and the RPE, together with 5α-reductase (the enzyme converting testosterone into the more powerful dihydrotestosterone, DHT) type 1 and 2 mRNA (8).

Synthesis of Steroids in the Retina

The mammalian retina has the ability of synthetize neurosteroids (pregnenolone, progesterone, dehydroepiandrosterone, desoxycorticosterone, 3 alpha, 5 alpha-tetrahydrodesoxycorticosterone, 3 alpha-hydroxy-5 alpha-dihydro-progesterone, 17-hydroxyprogesterone, and 17-hydroxypregnenolone) from cholesterol, as demonstrated by using retinal explants, thus excluding interferences from circulating steroids (11).

Following studies demonstrated also the presence of steroidogenic enzymes (mRNA and protein) in the retina (12): cytochrome CYP11A1 (CYP450scc) that converts cholesterol in pregnenolone; 3-β-hydroxysteroid dehydrogenase which converts pregnenolone in progesterone; cytochrome CYP17A1 (P450c17) involved in the production of 17-α-hydroxymetabolites; and CYP19A1 (P450 aromatase) which converts testosterone in 17-β-estradiol [(11, 13, 14), **Figure 1**]. Cholesterol, which activates the metabolic cascade leading to the production of E_2, is also produced in the retina: HMG-CoA reductase, the main enzyme for cholesterol synthesis, is present in the RPE, photoreceptors, and Müller cells. Exogenous cholesterol, on the other hand, is derived from high-density and low-density lipoproteins (HDL and LDL, respectively) that bind to specific receptors of the retinal cells (15). The principal limiting steps for estrogen production in the retina are the regulation of CYP450scc and aromatase enzymatic activities (14) (**Figure 1**). The steroidogenic enzymes are found in retinal neurons, glial cells, and photoreceptors in amounts similar to those observed in other part of the CNS (16). Enzymes' concentration is greatest in the internal nuclear layer, which is considered the principal site of retinal steroids' synthesis (14).

Finally, it is important to note that estrogens and androgens are also produced outside the retina (gonads, adrenals) and, through the blood flow, they can reach the eye where, in males, circulating testosterone may be locally metabolized in 17-β-estradiol. Diseases or functional alterations linked to steroid hormones may, therefore, be due to both retinal steroidogenic enzyme failure (short-term effects?), alterations in the gonadal and adrenal hormone supply (long-term effects?), or both conditions.

Differences in Retinal Function between Men and Women

There are marked sex-related differences in ocular anatomy and pathophysiology, particularly for the retina. Studies on mice have identified differences in retinal structure between males and females and found that, as measured by multifocal electroretinography (mfERG), retinal function is better in females of reproductive age than in males and older females (17). Similar mfERG studies on humans found a statistically significant difference in neuroretinal function between men and women below 50 years, but not after this age; in addition, neuroretinal function was lowest in women who received a hysterectomy during reproductive age, with subsequent iatrogenic-induced menopause. These findings suggest that the estrogenic cycle has a beneficial effect on neuroretinal function and that estrogens may have a protective role (18).

Sex-Related Differences in the Prevalence of Eye Disorders

Sex-related differences in eye anatomy and physiology are reflected in disease processes (19). Cataracts, for example, are far more prevalent among women than men: the prevalence of lens opacities in women aged between 65 and 74 years is 24–27%, but only 14–20% in their male counterparts (20–23). Estrogen levels, besides other risk factors, appear to play a role. Numerous studies have shown that HRT is a protective factor in postmenopausal

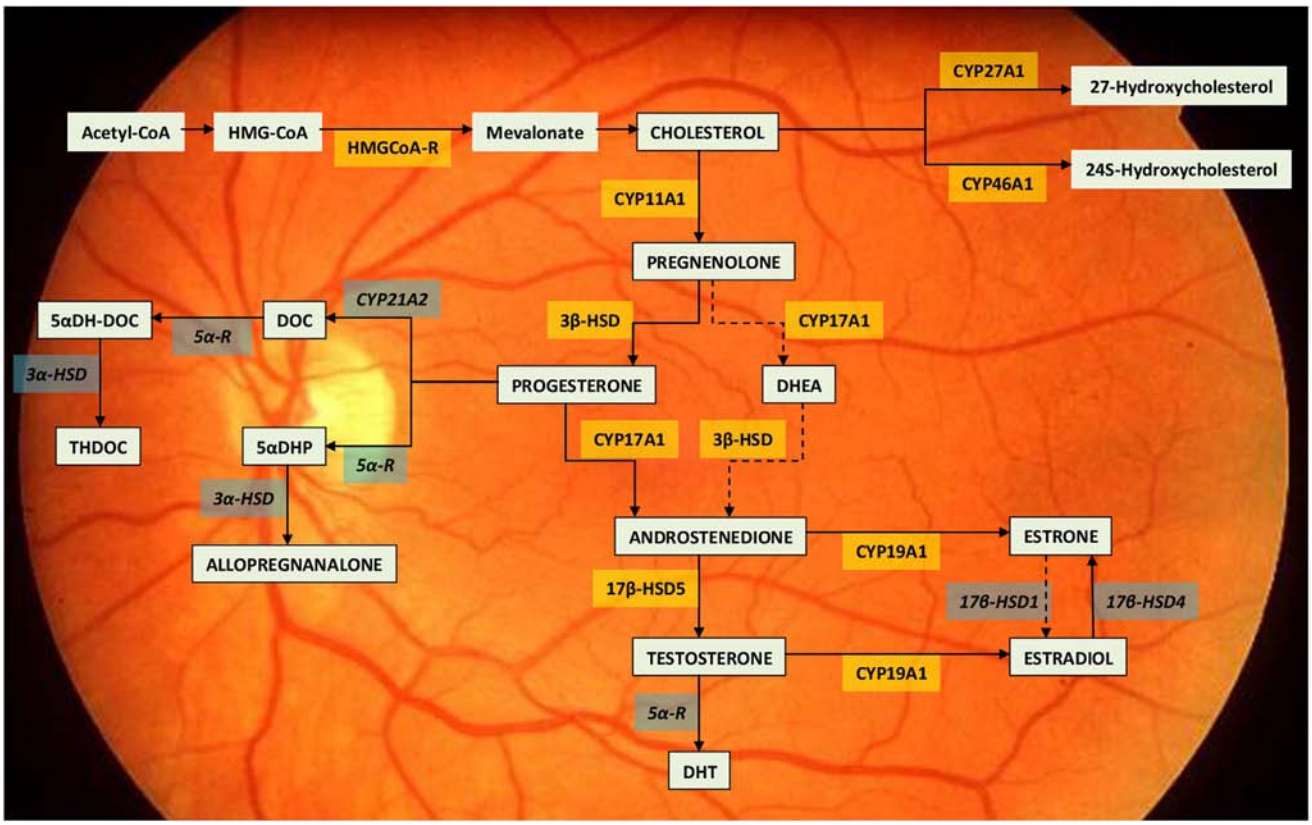

FIGURE 1 | Neurosteroid synthesis in the retina. The drawing report of the metabolic pathway leading to estradiol synthesis within the retina (11, 14). The steroidogenic enzymes already identified for their mRNA, activity or immunolocalization are indicated within yellow boxes. The enzymes still lacking of identification are indicated within the grey boxes. Dotted lines indicate so far unclear enzymatic activities. Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase; 3α-HSD, 3α-hydroxysteroid dehydrogenase; 5αDH-DOC, 5 alpha-dihydrodeoxycorticosterone; 5αDHP, 5α-dihydroprogesterone; 5α-R, 5α-Reductase; 17β-HSD1, 17β-hydroxysteroid dehydrogenases 1; 17β-HSD4, 17β-hydroxysteroid dehydrogenases 4; 17β-HSD5, 17β-hydroxysteroid dehydrogenases 5; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; DOC, deoxycorticosterone; HMG-CoA, hydroxymethylglutaryl-CoA; HMGCoA-R, hydroxymethylglutaryl-CoA reductase.

women and that late menopause or early menarche, both of which augment estrogen exposure, lower the risk of cataracts in advanced age (24–27). The main protective action of estrogens on the lens is probably due to their antioxidant properties (28). With the onset of menopause, the drop in estrogen levels increases the risk of cataracts in older women. Differently, the levels of estrogen converted by aromatase from testosterone in men do not seem to be age dependent, affording men greater protection against the development of cataracts (29). This sex-related difference in eye disorders is not always so distinct. For example, sex is not considered a factor in AMD (30, 31), though some studies have suggested its higher prevalence among women (32, 33). When distinguished by type of AMD, neovascular (34) and drusenoid (32) forms are more prevalent among women. As reported for cataracts, longer exposure to estrogens or HRT confers a lower risk of developing AMD in later age (35, 36), suggesting that this is due to the antioxidant and anti-inflammatory actions of estrogen (37, 38).

Glaucoma is also more frequent in women (39), though this is more likely linked to the longer life expectancy of women and as such is an age-related risk factor (40). Nonetheless, sex-related differences have been associated with different types of glaucoma: a study conducted on an Asian population (39) reported that angle-closure glaucoma is more prevalent among women; other studies reported the same findings for white and black populations (41, 42), but other studies found that, after adjusting for age and population, angle-closure glaucoma is more common among men (40, 43). Sex-related differences in the prevalence of diabetic retinopathy are associated with the difference in the prevalence of diabetes. Though more men than women are affected by type 1 diabetes (44) no study to date has found a significant difference in its prevalence (45, 46). Similarly, no statistically significant sex-related differences in the prevalence of diabetic retinopathy associated with type 2 diabetes have been established, though some studies have suggested that it is more frequent among men (47, 48).

Various other risk factors besides sex alone need to be taken into account (49). For example, in a study on a rural south Indian population, Nirmalan et al. (50) tried to understand if female reproductive factors (age at menarche and menopause, number of pregnancies, etc.) were related with eye diseases. The study found no association with cataracts, open-angle glaucoma, macular

degeneration, or myopia; nonetheless, the study presented several limitations, including the fact that the data on reproductive factors were gleaned from self-report questionnaires and that eye disorders were diagnosed in most subjects during the course of the study.

NEUROPROTECTIVE EFFECT OF SEX HORMONES

Preclinical Studies

Several studies have investigated whether estrogens have a neuroprotective role and, if so, through which mechanisms they exert such action. Nixon et al. (51) examined the role of hormones in elevated levels of glutamate, which has a neurotoxic effect. High glutamate concentrations inhibit the cysteine/glutamate transporter, which reduces the production of glutathione, an antioxidant. This decrease leads to the augmented production of reactive oxygen species (52), which, owing to oxidative damage, promote the development of eye diseases, such as AMD (53) and retinitis pigmentosa (54). The experiment was carried out *in vitro* on 661 W cells, i.e., a mouse cone photoreceptor cell line, and demonstrated that E_2 and the non-feminizing estrogen analogs ZYC-26 and ZYC-3 exert a protective action against the damage induced by 5 μM of glutamate. It was also observed that the protective effect of ZYC-26 and ZYC-3 is not exerted *via* the classic estrogen receptors ERα and ERβ, as demonstrated by the persistent protective action despite the use of an estrogen receptor pan-antagonist (ICI182780) and the lack of a protective effect after the use of the two agonists of ERα and ERβ. Based on these results, it was hypothesized that non-feminizing hormones could be used in the treatment of neurodegenerative eye disorders, and thus avert the side effects of prolonged estrogen therapy. Mo et al. (55) investigated intracellular neuroprotective mechanisms in ovariectomized mice, as measured by electroretinography of light-induced apoptosis in retinal cells. Following intravitreal administration of 17β-estradiol, retinal function was preserved due to the reduction of neuronal apoptosis. The involved pathway is PI3k/Akt activation: administration of a PI3K inhibitor (LY294002) increases retinal neuronal apoptosis, while the administration of estrogens leads to the translocation of NF-kB p65 from the cytosol to the nucleus, which is inhibited in the presence of LY294002. These results demonstrated that the protective action of estrogen on the retina is exerted *via* activation of the PI3K/Akt cascade and concludes with the nuclear translocation of NF-kB (55). The mechanism of action reported in this study is not the only one through which sex hormones exert their neuroprotective effect.

Estrogens also have a protective effect on intraretinal synapses. Kaja et al. (56) used a mouse model in which mild retinal ischemia was induced by transient occlusion of the middle cerebral artery. This experimental condition is ideal for examining the earliest stages of retinal damage that precede the development of neurodegenerative processes. Synaptic activity was measured using an immunoreactive technique based on the detection of Vesl-1L7Homer 1c (V-1L), a neuronal cytosolic protein involved in receptor clustering for neurotransmitters and in neuronal development and plasticity. V-1L is also a good marker to evaluate the changes in synaptic connectivity during the early stages of apoptosis of retinal ganglion cells. The study (56) found that retinal ischemia, though mild, can significantly reduce the number of V-1L-positive synapses in the internal plexiform layer of the retina, and increase the number of neuronal apoptotic cells in the ganglion cell layer. Estrogen administration exerts a protective effect by reducing the percentage of cells undergoing apoptosis and by preventing early ischemia-induced changes preceding apoptosis in the synaptic connections.

The neuroprotective action of estrogens appears to be closely linked to their antioxidant activity. Among the studies investigating this property, the early study by Moosmann et al. (57) merits mention as it demonstrated that the antioxidant-neuroprotective action of the hormones is not due to their genomic property, i.e., their ability to influence the transcription of specific genes, but rather because of their chemical properties as hydrophobic phenolic molecules. Estrogens were compared with other phenol molecules: the results showed that the protective effect against glutamate-induced oxidative toxicity in neuronal mouse cells was present in all the compounds studied and that the dose to obtain this effect was also the same. The study also showed that there is no correlation between estrogen strength and its antioxidant properties. The discovery of the dissociation between the hormonal effect and the neuroprotective effect could open the way to the development of new therapies using molecules that possess the same protective properties as estrogens, but without the unwanted effects associated with their activities as sex hormones.

Nakazawa et al. (58) investigated differences in neuroprotective activity between "endogenous" estrogens (produced in ovary) and "exogenous" estrogens (administrated through intravitreal injection) in a mouse model by comparing their neuroprotective effects against retinal ganglion cell (RGC) death following axotomy of the optic nerve, which mimics glaucoma-induced RGC death. To evaluate endogenous estrogen activity, samples of retinal tissue from female mice, ovariectomized or not, served as controls in which RGC density was measured. While ovariectomy had no effect on RGC density, the density was significantly reduced in mice that received ovariectomy and axotomy as compared to those that received only axotomy. These findings underscored the neuroprotective role of endogenous estrogens. In the evaluation of exogenous estrogens, ovariectomized mice administered intravitreal 17β-estradiol had a reduction in RGC death correlated with axotomy. The neuroprotective role of estrogens in this experimental condition indicates the potential for estrogen therapy in persons with etiologically similar eye diseases, such as glaucoma. In the second part of the study, the researchers wanted to identify the mechanism by which the exogenous estrogens exert their neuroprotective action. Immunoblot assay and immunohistochemical analysis showed that following intravitreal administration of E_2, activation of the ERK signal transduction pathway, and c-Fos was augmented, whereas no change in PI3K/Act activation was observed. This finding was then confirmed in an experiment using U1026, an ERK inhibitor. Administration of U0126 before administration of E_2 inhibited the neuroprotective effect of the estrogens (58).

Another molecular mechanism by which estrogens exert their neuroprotective effect involves upregulation of stromal cell-derived factor 1 (SDF-1). SDF-1 protects against retinal ischemia *via* its powerful chemotactic effect that promote tissue repair and the migration of stem cells produced in bone marrow to the site of damage (59). To demonstrate this factor's mechanism of action, transient retinal ischemia was induced by increasing intraocular pressure (IOP) to 110 mm Hg in a mouse model. Following reperfusion, the expected increase in mRNA and SDF-1 was measured *via* real-time PCR and Western blot. To evaluate the role of estrogens, E_2 was administered peripherally before inducing ischemia and SDF-1 was measured. The increased quantity of SDF-1 indicated that also in this condition estrogens play a neuroprotective role in reducing retinal damage (60).

Several studies aimed to determine whether only estrogens exerted neuroprotective action or other sex hormones, such as progesterone possessed similar properties (61). Progesterone was administered by peripheral infusion to one half of a population of male rats that had undergone photostress-induced retinal degeneration. Electroretinography showed no statistically significant differences between the two cohorts. The study findings suggested that progesterone has no protective effects similar to those of estrogens.

Clinical Studies

In addition to preclinical animal studies, also clinical studies have been performed, including a population-based study in postmenopausal Korean women (62) in which the women were administered a questionnaire investigating their gynecological characteristics and whether they were taking estrogens as HRT. The women also received an eye examination, which revealed a higher prevalence of eye diseases, including anterior polar cataracts and other retinal disorders in the women not receiving HRT, suggesting its neuroprotective action.

EFFECT OF SEX HORMONES ON OCULAR BLOOD FLOW

Besides acting directly on retinal neuronal cells, sex hormones can also influence tissue perfusion by modulating retinal and choroid blood flow. A recent review of the literature (63) on sex differences in ocular blood flow sought to determine the possible role of sex hormones. The rationale for the study was derived from the fact that the eye diseases in which reduced blood flow is considered a causal or contributing factor, including AMD (64–66), glaucoma (67, 68), and diabetic retinopathy (69–71) are also those for which a sex-related correlation with prevalence has been found, suggesting that sex hormones may be implicated in the development of these diseases. Estrogens appear to play a protective role probably because of their vasodilatory action in reducing vascular resistance. This finding is shared by several studies that compared blood flow velocity and resistance of the ophthalmic artery and the central retinal artery in pre- and postmenopausal women. Blood flow velocity was higher and vascular resistance indices were lower in the premenopausal women (72), whereas vascular resistance of the central retinal artery was reduced after estrogen administration as compared to placebo (73). Retinal blood flow was higher in women receiving HRT than in those naïve to HRT (74); however, the evidence was not sufficiently strong to recommend HRT in the treatment of these conditions.

While estrogens exert a vasodilatory effect on retinal perfusion, testosterone, like progesterone, has the opposite effect (72). Progesterone exerts a vasoconstrictive effect on ocular blood flow. As demonstrated by color Doppler imaging (75), progesterone increases the resistance of ophthalmic and retinal arteries. In women of reproductive age, progesterone was found to antagonize the vasodilatory effect of estrogen during the menstrual cycle, as measured with the pulsatility index of the central retinal artery (76).

The role of HRT has been extensively studied because of the implications it can have for postmenopausal women with eye diseases. Postmenopausal women receiving HRT or not were compared with regards to blood flow in the inferotemporal retinal artery (ITRA), the peripapillary retina, and the margin of the optic nerve head, as measured using stereometric parameters and electroretinography (73). Blood flow in the ITRA was significantly higher and trophism of the optic nerve head and surrounding area was better in those receiving HRT. The effect of estradiol on retinal perfusion was investigated using ovariectomized mice; in this model E_2 treatment improved retinal perfusion mainly through the increase in blood flow. Both HRT and administration of E_2 exert a protective effect on the retina and the retinal nerve fiber layer by modulating tissue perfusion.

Harris-Yitzhak et al. (77) compared blood flow velocity in the retrobulbar arteries of postmenopausal women receiving HRT or not and young women of reproductive age. Hemodynamic resistance in the ophthalmic artery was lower in the young women and those receiving HRT than in the postmenopausal women not receiving HRT, whereas central retinal artery blood flow was similar for all three groups. Blood flow in the posterior ciliary arteries was better in the young women than in the two groups of postmenopausal women in which blood flow was similar. These findings suggested that HRT may modulate resistance in the ophthalmic artery, whereas its effect on other arteries is less pronounced, since changes in blood perfusion in these areas seem to be related to age.

Other studies have compared the effect of estradiol and testosterone on ocular hemodynamics by measuring the serum levels of the two hormones in pre- and postmenopausal women not receiving HRT, in addition to evaluating *via* color Doppler blood flow velocity and vascular resistance in the ophthalmic and central retinal arteries (72). The findings showed that peak systolic blood flow velocity in the ophthalmic artery correlated with serum estradiol levels, whereas vascular resistance of the central retinal artery decreased with increasing levels of estrogens in both groups of women. Peak systolic blood flow velocity correlated negatively with serum testosterone levels in the premenopausal women, whereas vascular resistance increased with higher testosterone levels. The two hormones were found to exert opposite effects: testosterone seemed to exert an antagonist effect as compared to estrogen.

The action of testosterone on ocular hemodynamics has also been studied in men, in which testosterone levels are naturally

higher than in women. Low testosterone levels correlated with hypertension and higher cardiovascular risk (78, 79). There are also population-based differences. In fact, Malan et al. (80) described differences for the cardiometabolic prognosis and intraocular perfusion pressure in two cohorts of black and white men aged between 28 and 68 years. Only in white men there was a positive correlation between free testosterone levels and retinal vessel diameter (except for the central retinal artery in which the vessel diameter was inversely proportional to testosterone levels). The findings suggested a population-based protective effect of testosterone on vascularization and retinal perfusion, probably linked to the vasodilatory effect in the microvasculature.

DISEASES OF THE RETINA, OPTIC NERVE, AND POSSIBLE SEX-RELATED EFFECTS

In this section of the review we tried, for each pathology, to collect the evidence in favor of the correlation with sex hormones and those against (**Table 1**).

Age-Related Macular Degeneration

Evidence for the Protective Effect of Estrogens

Age-related macular degeneration is a progressive multifactorial eye disease that leads to deterioration of vision, loss of spatial and color vision, and adaptation to darkness (81–85). According to the World Health Organization, AMD is the cause of blindness in 10% of cases. Histological hallmarks are degeneration of the RPE, Bruch's membrane, and the choriocapillaris, resulting in photoreceptor damage and death (86).

The pathogenesis of AMD is multifactorial: lipofuscin accumulation in the lysosomes of the RPE; extracellular drusen deposits between the RPE and the internal collagen layer of Bruch's membrane; oxidative damage; and chronic inflammation. Owing to their antioxidant and anti-inflammatory properties, estrogens might play a protective role in AMD (38). Beside these pathogenic factors there are genetic and environmental risk factors: age is perhaps the most important, in addition to smoking, obesity, atherosclerosis, hypertensions, hypercholesterolemia, unhealthy diet, and history of cataract surgery (81). Female sex is a weak risk factor (32, 87, 88) though exudative AMD is more common among women (34). These risk factors have been investigated in various studies. A case-control study (33) involved the participants in the Age-Related Eye Disease Study (AREDS) and used as controls subjects with fewer than 15 small drusen. Preliminary analysis showed that age was the main risk factor; subsequent analyses were accordingly adjusted for age to minimize its confounding with other factors. Smoking and hypertension both resulted as risk factors. Other correlated characteristics were Caucasian race, body-mass index, low educational level, hypermetropia, lens opacities, and female sex. A population-based study (89) identified alcohol abuse as another risk factor. Exposure to exogenous estrogens was a weakly protective factor against drusenoid deposit in AMD.

Spurred by the prospects of a protective action by estrogens against the development of AMD, researchers investigated the effect of HRT in postmenopausal women. Haan et al. (90) sought to determine whether HRT had a beneficial effect and whether different HRTs achieved different effects. They compared the efficacy of therapy based on conjugated equine estrogens (CEE) with CEE therapy combined with a progestinic. No association was found between the use of either therapy and the early development of AMD, suggesting that the early stages of AMD are not influenced by HRT. In contrast, conjugate therapy was more effective than CEE therapy alone in reducing the risk of developing both the drusenoid and neovascular forms of AMD.

In addition to investigating the protective effect of HRT on the development of certain types of AMD, Feskanich et al. (91) examined the potential role of estrogens as oral contraceptives during reproductive age. They observed a lower risk for the development of neovascular AMD in women receiving HRT. The risk was further reduced in those who, in addition to HRT, had also taken oral contraceptives. An unexpected result was the correlation between the risk of early AMD and HRT. The risk of early AMD was higher in the women who had received HRT; this contrasted with other studies that reported no correlation between the two factors. When the gynecological characteristics (age at menarche and menopause, number of pregnancies) were analyzed, none of these reproductive factors significantly modified the risk of developing AMD, except for a slight reduction associated with multiple pregnancies.

Velez Edwards et al. (92) investigated whether genetic factors could interact with HRT in modulating the risk of AMD and found that postmenopausal HRT and use of estrogen oral contraceptives during reproductive age had a protective effect against AMD. When, however, the study population was stratified by AMD severity, i.e., distinguishing between early AMD characterized by geographic atrophy and neovascular AMD, the lower risk remained only for neovascular AMD. During the second part of the study, genetic analyses were carried out on peripheral blood samples to determine whether single nucleotide polymorphisms (SNPs) in genes thought to increase the risk of AMD could modulate the risk of AMD in association with HRT or previous exposure to oral contraceptives. The study findings showed that two SNPs of the *ARMS2* gene (AMD 2) located on chromosome 10 enhanced the positive effect of HRT in preventing AMD.

The hypothesis for a link between HRT and lower AMD incidence was investigated by other studies which showed that estrogen exerted a protective effect only against certain types of AMD, like drusenoid AMD, demonstrating that this type of AMD is more prevalent among women with multiple pregnancies, whereas the correlation was not statistically significant when comparing early AMD and late AMD (93).

Other studies focusing attention on the role reproductive factors can have in the incidence of maculopathies. Blasiak et al. (37) reported a statistically significant reduction in the incidence of advanced AMD in women who had received HRT and a significantly higher risk of developing advanced AMD in women who began menarche late. These findings demonstrated that estrogen exposure, including exogenous estrogens, plays a beneficial role in reducing the risk of advanced AMD. The hypothesis that early

TABLE 1 | Role of sex hormones in ocular diseases.

++	+	+/–	/	–
Protective effect	**Modest correlation**	**Non-significant correlation**	**Non-associated factor**	**Inversely correlated factor**

Disease	E	P	T	Studies
AMD	+/–	/	/	Menopausal and reproductive factors and risk of age-related macular degeneration. Feskanich et al.
				The effect of the hormone therapy on the risk for age-related maculopathy in postmenopausal women. Abramov et al.
				Reproductive exposures, incident age-related cataracts, and age-related maculopathy in women: the Beaver Dam Eye Study. Klein et al.
				Female reproductive factors and eye disease in a rural south Indian population: the Aravind comprehensive eye survey. Nirmalan et al.
	/			Clinical risk factors for age-related macular degeneration: a systematic review and meta-analysis. Chakravarthy et al.
				Age-related macular degeneration guidelines for management. The Royal College of Ophthalmologists.
				Sex steroid and AMD in older French women: the POLA study. Defay et al.
AMD (drusenoid or neovascular)	++			Hormone therapy and age-related macular degeneration. The women's health initiative sight exam study. Haan et al.
				Inverse association of female hormone replacement therapy (HRT) with AMD and interaction with ARMS2 polymorphisms. Velez et al.
				HRT, reproductive factors, and age-related macular degeneration: the Salisbury Eye Evaluation Project. Freeman et al.
				Association between reproductive and hormonal factors and age-related maculopathy in postmenopausal women. Snow et al.
	+			Clinical risk factors for age-related macular degeneration: a systematic review and meta-analysis. Chakravarthy et al.
				Risk factors for age-related macular degeneration. Evans
				Five-year incidence of age-related maculopathy lesions: the Blue Mountains Eye Study. Mitchell et al.
				Risk factors associated with age-related macular degeneration: a case-control study in the age-related eye disease study (AREDS): AREDS report number 3. Group, AREDS research
				Smoking, alcohol intake, estrogen use, and AMD in Latinos: the Los Angeles Latino Eye Study. Fraser-Bell et al.
CSCR	/	/	–	The potential role of testosterone in central serous chorioretinopathy. Grieshaber et al.
				Central serous chorioretinis associated with testosterone therapy. Ahad et al.
				Central serous chorioretinopathy in patients receiving exogenous testosterone therapy. Nudleman et al.
				Finasteride for chronic central serous chorioretinopathy. Forooghian et al.
	/	/	/	Serum cortisol and testosterone levels in chronic central serous chorioretinopathy. Tufan et al.
				Serous central chorioretinopathy and endogenous hypercortisolemia. Kapetanios et al.
MACULAR HOLE	++	/	/	Clinical features of idiopathic macular cysts and holes. McDonnell et al.
				Macular holes. James and Feman
				Estrogen antagonist and development of macular hole. Chung et al.
				Estrogen and macular holes: a postal questionnaire. Gray et al.
				Systemic risk factors for idiopathic macular holes: a case-control study. Evans et al.
	–	/	/	Vitreous estrogen levels in patients with an idiopathic macular hole. Inokuchi et al.
Retinitis Pigmentosa	/	++	/	Enhancing survival of photoreceptor cells *in vivo* using the sintetic progestin norgestrel. Doonan et al.
				Norgestrel may be a potential therapy for retinal degenerations. Doonan and Cotter
				Neuroprotective actions of progesterone in an *in vivo* model of retinitis pigmentosa. Sanchez-Vallejo et al.
Diabetic Retinopathy	/	/	+/–	The role of sex hormones in diabetic retinopathy. Grisby et al.
				Dehydroepiandrosterone protects bovine retinal capillary pericytes against glucose toxicity. Briganrdello et al.
	++		/	Gender and estrogen supplementation increases severity of experimental choroidal neovascularization. Espinosa-Heidmann et al.
	If early stage			Effects of tamoxifen versus raloxifene on retinal capillary endothelial cell proliferation. Grigsby et al.
	–			
	If late stage			
	/	/	/	Exogenous estrogen exposures and changes in diabetic retinopathy: the Wisconsin Epidemiologic Study of Diabetic Retinopathy. Klein et al.

(*Continued*)

TABLE 1 | Continued

++	+	+/–	/	–	
Protective effect	**Modest correlation**	**Non-significant correlation**	**Non-associated factor**	**Inversely correlated factor**	
Disease	**E**	**P**	**T**	**Studies**	
Glaucoma	++	/	/	Is estrogen a therapeutic target for glaucoma? Dewundara et al. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Furchgott et al. Estrogen deficiency accelerates aging of the optic nerve. Vajaranant and Pasquae Primary open-angle glaucoma. Weinreb and Khaw The effect of the menstrual cycle on optic nerve had analysis in healthy women. Akar et al. Estrogen pathway polymorphisms in relation to primary open angle glaucoma: an analysis accounting for gender from the United States. Pasquale et al.	

For and against evidences for each disease, with type of correlation.

AMD, age-related macular degeneration; CSRC, central serous chorioretinopathy; E, estrogens; P, progesterone; T, testosterone.

menopause (before age 45 years), because it reduces the duration of exposure to estrogens, could constitute a risk for the development of macular degeneration was evaluated by Vigerling et al. (94) who compared different samples of women, including those that had experienced spontaneous early menopause and others who had experienced iatrogenic early menopause following ovariectomy. The results showed no increased risk for AMD among the women who experienced spontaneous early menopause, whereas the risk of developing macular degeneration was significantly increased in those who had undergone ovariectomy before age 45 years.

Studies Showing No Protective Effect of Estrogens

While some studies have found a neuroprotective effect of estrogens, others have not, leaving the question of their potential neuroprotective effect open. The guidelines for the management of AMD issued by the Royal College of Ophthalmologists (95) state that, on the basis of a meta-analysis (32), female sex is not a factor associated with higher risk of AMD. The guidelines go on to state that the higher prevalence of AMD among women is largely due to their longer life expectancy (87).

Numerous studies cited by the guidelines report that reproductive characteristics, e.g., age at menarche and menopause, and exposure to HRT are inconsistently associated with AMD (50, 96–98). The POLA study, for example, found that advanced AMD and drusenoid AMD or AMD with pigment abnormalities were not associated with oophorectomy or hysterectomy or HRT exposure. The POLA study examined serum levels of diverse hormones and correlated molecules (estradiol, testosterone, DHEAS, sex hormone-binding globulin) and found a correlation, albeit weak, only for high DHEAS levels that appeared to be associated with an increased prevalence of drusenoid AMD. Other studies and a review corroborate the hypothesis for the lack of a correlation between estrogens and macular degeneration. A systematic review (32) of 18 population-based studies and 6 case-control studies on the strength of the relationship among factors, increasing the risk for AMD reported that the risk factors most strongly correlated with degenerative maculopathy were age, smoking (33, 99, 100), history of cataract surgery (101–103), and family history of AMD (104, 105). Moderately correlated factors were body-mass index (99), history of cardiovascular disease (99, 106), hypertension (99, 106, 107), and elevated plasma fibrinogen (108). Factors weakly or inconsistently correlated with AMD were sex (107, 109, 110), ethnic group (107, 111), color of iris (104), history of cardiovascular disease (99, 106) and serum levels of cholesterol (99, 106), and triglycerides (106). Female sex was not considered a risk factor, precluding the role of sex hormones in higher risk for the disease.

Central Serous Chorioretinopathy (CSCR)

Evidence of the Association between CSCR and Sex Hormones: Testosterone and Increased Risk of CSCR

Central serous chorioretinopathy is an acquired eye disease characterized by exudative detachment of the retinal and/or the

RPE. Its pathogenesis is not yet fully understood; however, it is thought that alterations in choroid circulation and RPE function may be implicated in the development of the disease. This notion is corroborated by findings from fluorangiography and OCT that document augmented capillary permeability and pressure of the choroid vessels (107, 108). Risk factors vary widely from psychosocial stress to type A personality, Cushing's syndrome, infections, smoking, alcohol, to pregnancy, and steroid therapy (109–111), all of which are characterized by elevated serum levels of glucocorticoids (112–114).

Given its higher prevalence among men, androgens have been directly implicated in the pathogenesis of CSCR (111, 115). In their study, Nudleman et al. (116) examined patients receiving exogenous testosterone therapy and who had no known risk factors for CSCR. The results showed that testosterone therapy is a probable independent risk factor for CSCR; moreover, disease symptoms and subretinal fluid accumulation resolved after discontinuation of testosterone therapy. Further evidence for the role of testosterone came from a case report of a woman who was not pregnant while receiving testosterone therapy (111), and another case report of a male population receiving testosterone therapy for hypogonadotropic hypogonadism (115), the latter of which demonstrated a temporal correlation between administration of therapy and the development of CSCR, indicating both testosterone and estrogen as the cause. Given the link between testosterone and CSCR, finasteride, an inhibitor of DHT synthesis, was considered as an alternative in the treatment of CSCR. Forooghian et al. (117) investigated the effect of finasteride administered for 6 months and monitored changes in visual acuity, macular thickness, subretinal fluid accumulation, OCT, serum DHT and testosterone, and cortisol levels in urine. Though no changes in visual acuity occurred, macular thickness and subretinal fluid level were lowest at 3 months in therapy before increasing slightly though remaining below basal reference limits. Furthermore, macular thickness and subretinal fluid both increased after therapy was discontinued in four patients and normalized in the patients who continued with therapy. These findings suggest a possible role for finasteride in the treatment of chronic CSCR.

Studies Showing an Absence of Association between CSCR and Testosterone

The study of Kapetanios et al. (114) reported a statistically significant association between elevated cortisol levels and the development of ICSCR, whereas no significant differences in testosterone levels were noted and levels remained within the normal range in both groups. A case-control study (118) of chronic ICSCR reported that cortisol and testosterone levels were similar in both groups. Though there appears to be a correlation between hormones and ICSCR, conflicting evidence leaves many questions about the etiopathogenesis and treatment of CSCR open.

Macular Hole

Evidence of Estrogen Protection in the Macular Hole

Macular hole is a major cause of diminished vision, especially in advanced age. Although in some cases the cause can be identified, e.g., contusive trauma (119), cystoid macular edema (120), or diabetes (121), it is idiopathic in the majority and thought to be due to circumferential vitreoretinal contraction (122). Macular hole affects women far more often than men (123). As in other eye diseases, it is thought that estrogens have a beneficial effect on macular health and protect against the development of macular hole. For example, estrogens stimulate the synthesis of collagen and hyaluronic acid in the skin, suggesting that a similar process might also occur in the eye. With the sudden drop in estrogen production after menopause, this protection is lost, posing the retina to a higher risk than that for men, in whom estrogen levels are generally low throughout life and do not change abruptly. In addition to menopause, early hysterectomy or HRT may influence estrogen activity in relation to the development of macular holes (124, 125). Current evidence indicates strong correlations between macular hole and female sex and postmenopausal age (126), as demonstrated by various studies. A population-based study showed a female-to-male ratio of 3.3:1 for full-thickness macular hole (127). In their case-control study, Evans et al. (128) found that 67% of persons with macular hole were women and that 74% of the women were aged 65 years or older. The study analyzed various risk factors, including ethnic origin, systemic comorbidities, current use and history of medications, alcohol intake, smoking, body weight and height, menstrual and obstetric history, age at menopause and severity of associated symptoms, and exposure to HRT. The study findings indicated few systemic factors associated with idiopathic full-thickness macular hole (IFTMH), and though sex-correlated, no association was found between the principal indicators of exposure to estrogens and the incidence of macular hole. Nonetheless, a role for estrogens was suggested by the fact that women with macular hole generally experienced a more difficult menopause and more bothersome climacteric symptoms such as hot flashes than healthy women. The results also suggested that the development of IFTMH may be more due to the sudden change in hormone levels that chronic exposure, as demonstrated by the higher risk is associated with menopause, hysterectomy, and oophorectomy.

Correlations have been found between tamoxifen and macular hole and its precursor lesions (129). Tamoxifen is an anti-estrogenic nonsteroid drug used in adjuvant therapy for breast cancer (130). In this case report, all three women were receiving tamoxifen therapy and presented with cystic changes of the foveal region and defects of the external retina suggestive of initial macular hole.

A Study Shows That Estrogen Could Have a Negative Effect on Macular Hole

Another study investigated whether there were differences between the estrogen levels in the vitreous of subjects with macular hole and in those with other retinal disorders, who served as controls (131). The estrogen concentration was significantly higher in those with macular hole ($p < 0.05$), implicating it in the pathogenesis of the disorder. Since estrogens activate collagenase, this could be correlated with the development of vitreous collagen disorders; however, owing to the small study sample (10 cases and 9 controls), no definitive conclusions could be drawn.

Retinitis Pigmentosa

Evidence of the Benefical Effect of Progestinic Therapy in Retinitis Pigmentosa

Retinitis pigmentosa refers to a group of inherited retinal degenerative disorders in which genetic mutations lead to the death of retinal photoreceptors (132). Although the mutations in several genes implicated in the development of the diseases are known, the mechanism by which they cause cell death is not fully understood and no effective treatment is currently available (133). Recent studies using mouse models have suggested Norgestrel, a synthetic analog of progesterone, as a potential agent (134). Norgestrel's protective action has been demonstrated on retinal explants *in vitro* and in two different *in vivo* models of retinal degeneration: the one involving mice exposed to photostress-induced damage and the other involving mice with genetic mutations characteristic of retinitis pigmentosa. Both models showed a reduction in cell loss, with improvement in cell survival of about 70% (calculated on the basis of photoreceptor number, structural integrity, and function), indicating that apoptosis was interrupted or slowed. Although the mechanism of action is not yet fully understood, it is thought that norgestrel activates a survival pathway probably based on the increased expression of fibroblast growth factor 2 (FGF-2), a powerful neurotrophic factor whose production is increased by retinal stress. Its action promotes cell survival and inhibits apoptosis by activating the intracellular cascades involving mitogen-activated protein kinase, PI3K, and protein kinase C (PKC). The involvement of FGF-2 was demonstrated by measuring its changes in the control subjects who were administered only the vehicle and in the subjects receiving norgestrel therapy: the finding of higher FDG-2 values after norgestrel administration confirmed the hypothesis. These findings were strengthened by the same research group in a subsequent study (135) in which they investigated the potential of norgestrel as therapy for retinal degeneration.

Other studies investigated the relationship between progesterone and progestrinics and retinitis pigmentosa (136) building on previous studies on progesterone in experimental models of acute brain damage in which the drug had shown a neuroprotective effect (137). The action of progesterone was analyzed to determine the number of surviving cells, as measured by electroretinography, and the potential protective effect of the drug owing to its ability to limit damage by free radicals or to increase antioxidant defenses. The results showed a reduction in cell death and gliosis, with a statistically significant reduction in glutamate and a significant increase in reduced glutathione and oxidized glutathione. The study underlined the beneficial action of progesterone which exerts *via* multiple modes of action in protecting the retina during retinitis pigmentosa, suggesting its use or that of its analogs in the treatment of the disease.

Diabetic Retinopathy

The Uncertain Role of Sex Hormones in Diabetic Retinopathy

Poor glycemic control ultimately results in macrovascular and microvascular complications, affecting the kidney and the eye in persons with diabetes. Diabetic retinopathy is most common complication (138) and is present in 34.6% of diabetics (139). Two forms are distinguished: nonproliferative or early stage diabetic retinopathy, and proliferative or late stage retinopathy in which retinal neovascularization is evident, leading to increased risk of loss of vision due to retinal detachment, neovascular glaucoma, and vitreous hemorrhaging. Another common cause of loss of vision is macular edema, which may arise in any stage of retinopathy (140). Studies investigating the possible links between sex hormones and diabetic retinopathy have analyzed the incidence of the condition by sex and produced conflicting results (49). One possible explanation for the discrepancies is the likely presence of confounding factors. Grisby et al. (141) compared the direct action of sex hormones on retinal cells and their action on vasculature, and found that various hormones are involved in the development and progression of retinopathy in diabetic patients. Androgens and androgen inhibitors appear to play both a causal and a protective role, since they increase blood pressure and the levels of adhesion molecules (ICAM and VCAM-1), with worsening of lipid levels. A low level is associated with the metabolic syndrome and may alter lipid, glycemic, and blood pressure values. Dehydroepiandrosterone has a proven protective effect against the damage of elevated glucose levels in pericytes (142). It is believed that the damage due to elevated glucose levels occurs mainly through oxidative mechanisms. The toxicity of glucose and subsequent vascular damage manifest in four ways: activation of the PKC cascade; activation of aldoso-reductase; protein glycosylation; and activation of the hexosamine pathway. According to the so-called unifying therapy, the formation of free oxygen radicals underlies the mechanisms leading to glucose damage (143, 144).

As oxidative stress is implicated in the development and progression of diabetic retinopathy, studies have investigated the role of estrogens, which possess antioxidant properties. Estrogens stimulate the ERβ receptor and protect retinal cells against oxidative stress through their ability to modulate the transcription of antioxidant genes and protect the mitochondria (145). Estrogens can exert a differential action depending on the stage of retinopathy: during the initial stages, the proliferation of endothelial cells induced by estradiol has a beneficial effect and protects the retina by inducing repair processes, whereas during the proliferative stage, this same effect exacerbates the disease (146, 147). Selective estrogen receptor modulators (SERMs), including tamoxifen and raloxifen, act as antagonists or agonists of estrogen depending on the type of receptor to which they bind. In the retina, both drugs strongly antagonize estrogen-induced angiogenesis (147). From the multitude of data collected so far it is clear that the use of sex hormones or their antagonists in the treatment of retinopathy must be personalized based on sex, age, and stage of disease. Stimulation or hormonal modulation may provide a novel therapeutic option.

Other studies have investigated differences in electroretinographic patterns in relation to neuroretinal function in men and women with type 2 diabetes, but not retinopathy to evaluate risk for neurodegenerative disease (148). The analysis showed that neuroretinal dysfunction leading to diabetic retinopathy was far more common among men than women, suggesting a sex-related protective mechanism. This finding was corroborated in a minireview issued by the Berkeley School of Optometry (49) that noted a more frequent incidence of abnormal neuroretinal function in men with type 2 diabetes. It was also observed that

advanced proliferative retinopathy in type 1 diabetes more often affects men, that retinopathy is more likely present at diagnosis of diabetes in men, and that it is often more severe than in women.

HRT Does Not Influence Diabetic Retinopathy

A possible benefit of HRT for retinopathy in postmenopausal women has been studied (149); however, no connection between changes in retinopathy and the incidence of macular edema and exposure to exogenous estrogen could be established, indicating that, unlike its effects observed in other eye disorders, HRT has no effect on diabetic retinopathy.

Glaucoma

Benefical Effects of Estrogens in Glaucoma

Glaucoma, the second leading cause of blindness in the world (43), is a slow, progressive neurodegenerative disease characterized by gradual loss of RGC (150) and loss of vision (151). A recent review (152) examining possible correlations between glaucoma and estrogens reported that factors influencing the duration of estrogen exposure (e.g., age at menarche, use of oral contraceptives, bilateral ovariectomy, age at menopause) can raise the risk of POAG. A higher risk for POAG was associated with age at menarche over 13 years, as compared to age less than 12 years (153), and a 25% higher risk was noted in women who took oral contraceptives for more than 5 years (154). Bilateral ovariectomy before age 43 increased the risk of developing glaucoma (155), as did spontaneous menopause before age 45 years (156), whereas the risk was significantly lower in women over age 65 who entered menopause after age 54 years (154). Prolonged estrogen exposure reduced the risk of glaucoma or glaucoma-related conditions, as demonstrated by evidence that IOP is lower during pregnancy when estrogen levels are elevated, particularly during the third trimester (157, 158). Evidence for a protective effect of HRT against glaucoma is uneven (79, 159, 160).

Genetic factors may also play a role in increasing the risk for developing glaucoma. SNPs implicated in the estrogen metabolic pathway associated with women, but not men (161), and polymorphisms of the endothelial nitric oxide synthase gene encoding the enzyme regulated by estrogens are correlated with the development of open-angle glaucoma (162). Estrogen exposure may alter the pathogenesis of glaucoma and exert a neuroprotective action. A future area of focus is the use of estrogens in glaucoma treatment or prevention. Numerous studies have reported an association between estrogens and glaucoma; for example, estrogen deficiency was associated with the acceleration of aging of the optic nerve (163). Estrogens have also been implicated in aqueous humor production and drainage via receptors on the ciliary epithelium (10, 159). Changes in estrogen levels appear to influence IOP, which is responsible for optic nerve trophism (164). Epidemiology, clinical, and experimental evidence supports the hypothesis that early reduction of estrogen levels leads to premature aging of the optic nerve and increased susceptibility to glaucoma.

In their study on the role of estrogens in modulating the topography of the optic nerve head, Akar et al. (165) reported that, as observed in diabetic women, hormonal fluctuations during the menstrual cycle affect the central area and the margin of the optic nerve head. Genetic analysis to determine whether there exist associations between certain SNPs of genes involved in estrogen metabolism and the development of POAG (161) showed that SNPs were correlated with global POAG and POAG with elevated, but not with low IOP and no correlation of any type was noted for the men. Among the women, however, the gene that encodes the catechol-O-methyltransferase enzyme was found to be associated with open-angle glaucoma. Summarizing, links between SNPs of the estrogen pathways and the development of glaucoma were noted for certain types of glaucoma and only in women.

FUTURE PROSPECTS AND OPPORTUNITIES

A Place for HRT?

In light of the amount of research conducted so far on sex hormones and neuroretinal diseases, the question arises whether and how it can be applied to the identification and development of new drugs or to expanding indications for drugs already on the market. An emblematic case is that of HRT for the treatment of climacteric symptoms.

Despite the wealth of data, the question remains open. A study evaluated the efficacy of HRT with phytoestrogens on eye function, as measured with short-wavelength automated perimetry in postmenopausal women (166). Phytoestrogens are nonsteroids of plant origin with estrogen-like action in modulating vision sensitivity. Consistent with the theory of timing, which states that the benefits of HRT diminish the later the therapy is initiated, a loss of efficacy was noted in relation to the time between initiation of phytoestrogen therapy and onset of menopausal symptoms, with no benefit gained if the therapy was started in women over 60 years of age. No conclusions could be drawn from this study and no recommendations for the use of HRT for eye diseases could be made.

Implications for Future Study Design

The present review of the literature found evidence for a sex-related difference in the prevalence of certain eye diseases. A plausible explanation for the difference is the differential effect of sex hormones on the development and course of disease, which may be as meaningful as it is complex, though the underlying mechanisms are not yet fully understood.

The link between sex hormones and retinopathies opens new therapeutic horizons. To obtain a better understanding of the interactions between sex hormones and eye diseases, studies should be designed to determine the presence of an association between sex, hormones, and disease, and if such an association exists, the potential therapeutic correlates. To reach these objectives, key areas of focus are: epidemiological studies on the distribution of eye disease in a population, while taking sex and hormonal status into account; experimental studies on the changes in the incidence and/or course of disease in relation to hormone administration or deprivation; and preclinical animal studies comparing differences between the sexes.

AUTHOR CONTRIBUTIONS

All the authors have equally contributed to the search of the literature and cooperated to write the article.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 06 March 2018
doi: 10.3389/fendo.2018.00068

Secretoneurin A Directly Regulates the Proteome of Goldfish Radial Glial Cells *In Vitro*

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *23 November 2017*
Accepted: *16 February 2018*
Published: *06 March 2018*

Citation:
Da Fonte DF, Martyniuk CJ, Xing L and Trudeau VL (2018) Secretoneurin A Directly Regulates the Proteome of Goldfish Radial Glial Cells In Vitro. Front. Endocrinol. 9:68. doi: 10.3389/fendo.2018.00068

Radial glial cells (RGCs) are the main macroglia in the teleost brain and have established roles in neurogenesis and neurosteroidogenesis. They are the only brain cell type expressing aromatase B (*cyp19a1b*), the enzyme that synthesizes estrogens from androgen precursors. There are few studies on the regulation of RGC functions, but our previous investigations demonstrated that dopamine stimulates *cyp19a1b* expression in goldfish RGCs, while secretoneurin A (SNa) inhibits the expression of this enzyme. Here, we determine the range of proteins and cellular processes responsive to SNa treatments in these steroidogenic cells. The focus here is on SNa, because this peptide is derived from selective processing of secretogranin II in magnocellular cells embedded within the RGC-rich preoptic nucleus. Primary cultures of RGCs were treated (24 h) with 10, 100, or 1,000 nM SNa. By using isobaric tagging for relative and absolute quantitation and a Hybrid Quadrupole Obritrap Mass Spectrometry system, a total of 1,363 unique proteins were identified in RGCs, and 609 proteins were significantly regulated by SNa at one or more concentrations. Proteins that showed differential expression with all three concentrations of SNa included H1 histone, glutamyl-prolyl-tRNA synthetase, Rho GDP dissociation inhibitor γ, vimentin A2, and small nuclear ribonucleoprotein-associated protein. At 10, 100, and 1,000 nM SNa, there were 5, 195, and 489 proteins that were downregulated, respectively, whereas the number of upregulated proteins were 72, 44, and 51, respectively. Subnetwork enrichment analysis of differentially regulated proteins revealed that processes such as actin organization, cytoskeleton organization and biogenesis, apoptosis, mRNA processing, RNA splicing, translation, cell growth, and proliferation are regulated by SNa based on the proteomic response. Moreover, we observed that, at the low concentration of SNa, there was an increase in the abundance of proteins involved in cell growth, proliferation, and migration, whereas higher concentration of SNa appeared to downregulate proteins involved in these processes, indicating a dose-dependent proteome response. At the highest concentration of SNa, proteins linked to the etiology of diseases of the central nervous system (brain injuries, Alzheimer disease, Parkinson's disease, cerebral infraction, brain ischemia) were also differentially regulated. These data implicate SNa in the control of cell proliferation and neurogenesis.

Keywords: secretogranin II, secretoneurin, radial glial cells, aromatase, neurogenesis

INTRODUCTION

Radial glial cells (RGCs) are a macroglial subtype present during central nervous system (CNS) development of all vertebrates and are characterized by their bipolar morphology (1) and stem-like progenitor properties (2). The cell bodies of radial glia line the brain ventricles and have an elongated radial fiber terminating on the walls of blood vessels or at the pial surface with end feet (3). Due to this unique morphology, their radial processes are used as scaffolds for the migration of newborn neurons (4, 5). As stem-like cells, RGCs are capable of undergoing neurogenesis and/or gliogenesis to produce neurons or other glial cells (6). RGC populations are transient in mammals as they differentiate into astrocytes at the end of cortical development. In contrast, RGCs are abundant throughout the adult brain in teleost fish, supporting the ability for unsurpassed high levels of neurogenesis and regeneration (7, 8). In addition, teleost RGCs express various steroidogenic enzymes (9, 10) and are the exclusive cell type to express the estrogen-synthesizing enzyme, aromatase B (*cyp19a1b*) (11–13), and thus, they are neuroendocrine cells that produce neuroestrogens and other steroids in the CNS. Although RGCs in fish have established roles in neurogenesis and neurosteroidogenesis, little is known about other functions of these cells and the factors that regulate them. As RGCs are the main macroglia in fish CNS (14), they share close interactions with different neurons and can express neurotransmitters, neuropeptides, and hormone receptors (15). Therefore, these cells may be under functional control through neuronal–glial interactions.

We previously reported on the close neuroanatomical relationship between the soma of secretoneurin A (SNa)-immunoreactive magnocellular preoptic neurons and RGCs along the third ventricle in goldfish (16). The neuropeptide SN is generated from endoproteolytic processing of its precursor protein secretogranin II (SgII) and has well-established functions in endocrine, nervous, and immune systems (17, 18). Currently, there are two known SgII paralogs, SgIIa and SgIIb, that likely arose from the genome duplication events in teleost fish, and these paralogs produce SNa and SNb peptides, respectively (19). Both *in vivo* and *in vitro*, SNa reduces *cyp19a1b* mRNA levels in goldfish RGCs, implicating SNa in the control of neuroestrogen production (16). Furthermore, data from transcriptome sequencing of cultured goldfish RGCs revealed that gene networks related to immune responses and CNS physiology were responsive to 1,000 nM SNa (15). Altogether, these anatomical and transcriptomic data propose that SNa is a regulator of RGC functions. The objectives of this study were to (1) characterize the RGC proteome, (2) determine the differentially regulated proteins over a range of SNa concentrations, and (3) identify protein networks responsive to SNa regulation.

MATERIALS AND METHODS

Experimental Animals

All procedures were approved by the University of Ottawa Protocol Review Committee and followed standard Canadian Council on Animal Care guidelines on the use of animals in research. Common adult female goldfish (*Carassius auratus*) were purchased from a commercial supplier (Mt. Parnell Fisheries Inc., Mercersburg, PA, USA) and allowed to acclimate for at least 3 weeks prior to experimentation. Goldfish were maintained at 18°C under a stimulated natural photoperiod and fed standard flaked goldfish food. Sexually mature female goldfish (18–35 g) were anesthetized using 3-aminobenzoic acid ethyl ester (MS222) for all handling and dissection procedures.

Cell Culture and Exposure

The direct effects of SNa on RGCs were tested using a previously established and validated cell culture method (20). In short, the hypothalamus and telencephalon were dissected from female goldfish and rinsed twice with Hanks Balanced Salt Solution (HBSS; 400 mg KCl, 600 mg KH_2PO_4, 350 mg $NaHCO_3$, 8 g NaCl, 48 mg Na_2HPO_4, and 1 g D-Glucose in 1 L ddH_2O) with Antibiotic-Antimycotic solution (Gibco) and minced into small explants. RGCs were dissociated with trypsin (0.25%; Gibco) and cultured in Leibovitz's L-15 medium (Gibco) with 15% fetal bovine serum (Gibco) and Antibiotic-Antimycotic solution. Cell culture medium was changed 4–7 days after isolation and then once a week thereafter. RGCs were subcultured by trypsinization (0.125%) for three passages and then used for experimentation. Goldfish SNa was synthesized as reported previously (21). Stock solutions of synthetic goldfish SNa peptide were made in water and stored at −20°C until use. Aliquots were thawed on ice then diluted to desired concentrations in serum-free media. Cells were exposed for 24 h to one of the three concentrations of goldfish SNa or the water control.

Protein Extraction, Isobaric Tagging for Relative and Absolute Quantitation (iTRAQ), and LC-MS/MS

Proteins were extracted as previously described (22) prior to labeling and dissolved in denaturant buffer [0.1% SDS (w/v)] and dissolution buffer (0.5 M triethylammonium bicarbonate, pH 8.5) in the iTRAQ Reagents 8-plex kit (AB Sciex Inc., Foster City, CA, USA). For each sample, 100 μg of protein were reduced, alkylated, trypsin-digested, and labeled according to the manufacturer's instructions (AB Sciex Inc.). Two 8-plex iTRAQ labeling reactions were conducted, each with two biological replicates. Peptides from the control group were labeled with either iTRAQ tags 113 or 114. The three treatments were labeled as follows: 10 nM SNa (115, 116), 100 nM SNa (117, 118), and 1,000 nM SNa (119, 121). This labeling scheme was repeated in a second experiment, so the data are derived from a sample size of $n = 4$ biological replicates per group.

Labeled peptides were combined for each iTRAQ experiment, desalted with C18-solid phase extraction, and dissolved in strong cation exchange solvent A [25% (v/v) acetonitrile, 10 mM ammonium formate, and 0.1% (v/v) formic acid, pH 2.8]. The peptides were fractionated using an Agilent HPLC 1260 with a polysulfoethyl A column (2.1 mm × 100 mm, 5 μm, 300 Å; PolyLC, Columbia, MD, USA). Peptides were eluted with a linear

gradient of 0–20% solvent B [2 5% (v/v) acetonitrile and 500 mM ammonium formate, pH 6.8] over 50 min., followed by ramping up to 100% solvent B in 5 min. The absorbance at 280 nm was monitored, and a total of 14 fractions were collected. The fractions were lyophilized and resuspended in LC solvent A [0.1% formic acid in 97% water (v/v), 3% acetonitrile (v/v)]. A hybrid quadrupole Orbitrap (Q Exactive) MS system (Thermo Fisher Scientific) was used with high-energy collision dissociation in each MS and MS/MS cycle. The MS system was interfaced with an automated Easy-nLC 1000 system (Thermo Fisher Scientific). Each sample fraction was loaded onto an Acclaim Pepmap 100 pre-column (20 mm × 75 μm; 3 μm-C18) and separated using a PepMap RSLC analytical column (250 mm × 75 μm; 2 μm-C18) at a flow rate at 350 nL/min during a linear gradient from solvent A [0.1% formic acid (v/v)] to 25% solvent B [0.1% formic acid (v/v) and 99.9% acetonitrile (v/v)] for 80 min and to 100% solvent B for an additional 15 min.

The raw MS/MS data files were processed by a thorough database searching approach considering biological modification and amino acid substitution against the National Center for Biotechnology Information Cyprinidae database (downloaded on March 23, 2016) using the ProteinPilot v4.5 with the Fraglet and Taglet searches under ParagonTM algorithm (23). The following parameters were considered for all the searching: fixed modification of methylmethane thiosulfonate-labeled cysteine, fixed iTRAQ modification of amine groups in the *N*-terminus, lysine, and variable iTRAQ modifications of tyrosine. The MS/MS spectra that (1) were unique to a specific protein and (2) showed a sum of the signal-to-noise ratios for all the peak pairs greater than 9 were used for quantification. A protein was quantified if it was represented with at least three unique spectra in at least two of the biological replicates, along with a Fisher's combined probability of <0.05 and a fold change of <0.5 or >1.5.

Gene Ontology (GO) and Pathway Analysis

Gene ontology terms were assigned using protein annotation through evolutionary relationships to classify all proteins identified in RGC cultures (24). GO categories for biological processes, molecular functions, cellular components, protein classes, and pathways were used to identify the distribution of proteins within each GO category. Pathway Studio 9.0 (Elsevier) and ResNet 10.0 were used to build a protein interaction network for SNa effects in RGCs. Official gene symbols were manually retrieved using Gene Cards (http://www.genecards.org) to map proteins into Pathway Studio. The number of proteins that successfully mapped to Pathway Studios using Name + Alias was as follows: 10 nM SNa = 99, 100 nM SNa = 231, and 1,000 nM SNa = 499. Interaction networks were based on expression, binding, and regulatory interactions in the database and were constructed using direct connections with one neighbor. Subnetwork enrichment analysis (SNEA) for cell processes was performed in Pathway Studio to determine if differentially expressed proteins were related to specific biological functions. *P* value for gene seeds was set at < 0.05 and the criterion of >5 members per cell process or group was required for inclusion as a significantly regulated gene network.

RESULTS

Identification and GO Classification of RGC Proteins

A complete list of proteins ($n = 1{,}363$) that were identified in this study is provided in Table S1 in Supplementary Material. The annotated proteins were assigned GO terms and classified in three main GO categories: biological function, molecular function, and cellular component (**Figure 1**). A variety of biological functions were identified with 1,546 unique biological processes classified into 12 functional groups. The most represented biological functions included the categories of cellular process (438 proteins), metabolic process (414), and cellular component organization or biogenesis (199). Other important biological function allocations included the categories of developmental process (86 proteins), response to stimulus (65), immune system process (30), and biological adhesion (11). A broad array of 893 molecular functions was categorized into 9 functional groups. The molecular functions most represented in the RGC proteome were binding (334 proteins), catalytic (299), structural molecule (177), and transporter (38) activities. When organized by cellular component, RGC proteins were enriched in the categories of cell part (379 proteins), organelle (238), and macromolecular complex (165). Most proteins identified in RGCs were further classified into protein class, with nucleic acid binding (187 proteins), cytoskeletal proteins (147), and enzyme molecular (90) being the most represented classes detected in RGCs (**Figure 2**). Finally, a total of 535 proteins were represented by 102 pathways. The top 25 represented pathway ontologies are shown in **Table 1**. Proteins were most enriched in relation to Parkinson disease (31 proteins), integrin signaling pathway (28), ubiquitin proteasome pathway (26), cytoskeletal regulation by Rho GTPase (26), Huntington disease (25), and inflammation mediate by chemokine and cytokine signaling (24) (**Table 1**). Comparison of the assigned pathway ontologies identified by transcriptomics (15) and proteomics reveals high similarity. For example, of the top 25 pathways from transcriptomic and proteomic datasets, 18 (72%) were identical (**Table 1**). A full list of GO classifications can be found in Table S2 in Supplementary Material.

Quantitative Proteomic Responses in Radial Glia to SNa *In Vitro*

A total of 609 proteins differed in abundance following SNa treatment (**Figure 3**), and there were 17 unique expression patterns recognized within the data set (**Table 2**). At 10, 100, and 1,000 nM SNa, there were 5, 195, and 489 downregulated proteins, respectively, whereas the numbers of upregulated proteins were 72, 44, and 51, respectively. There were more downregulated proteins observed as the concentration of SNa increased in the cultures. The overlap of the differentially regulated proteins between concentrations is shown in **Figure 4**. A total of 10 common proteins were regulated in all three concentrations, and some examples include H1 histone, glutamyl-prolyl-tRNA synthetase, Rho GDP dissociation inhibitor γ, vimentin A2, and small nuclear ribonucleoprotein-associated protein. **Table 3** highlights the top

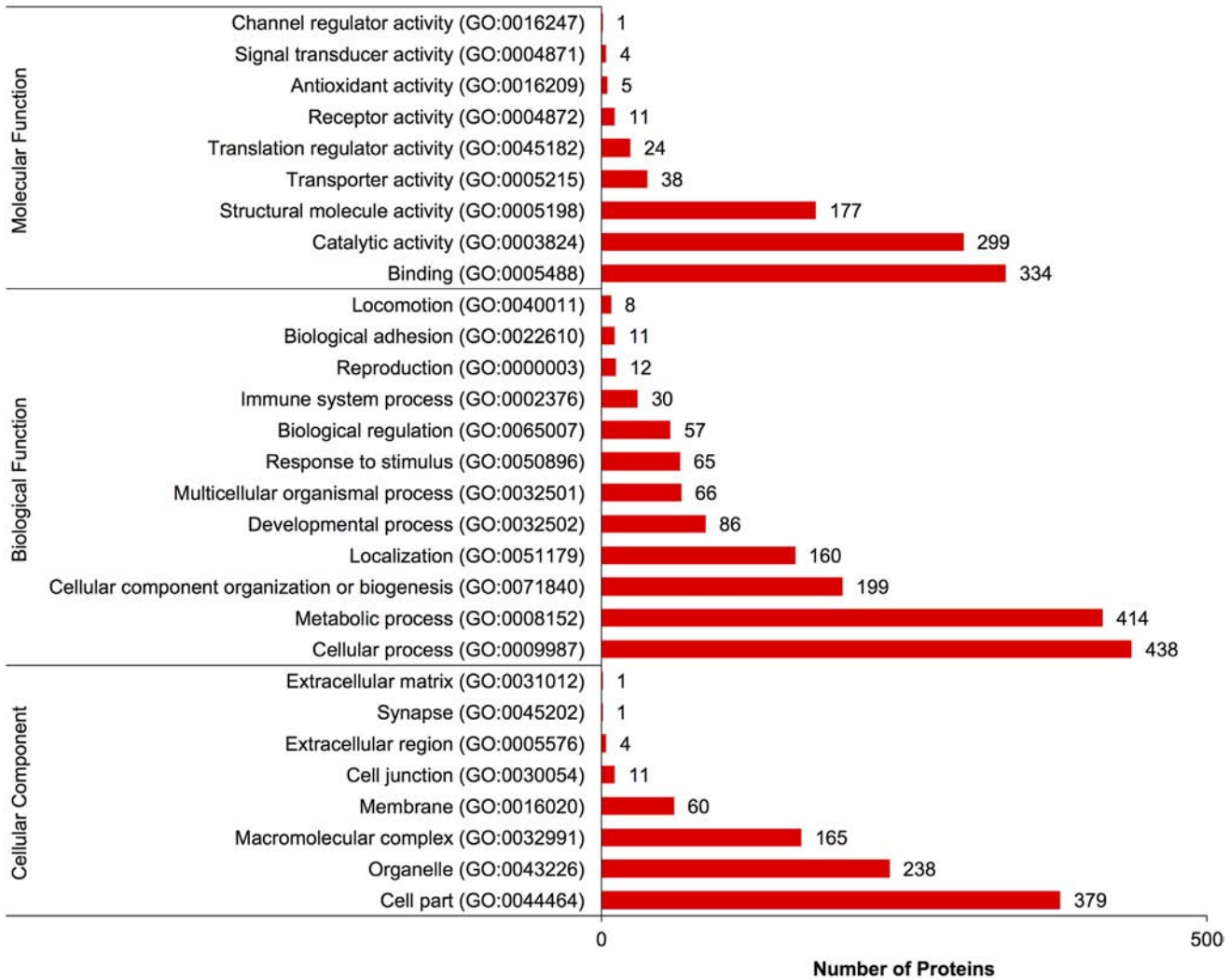

FIGURE 1 | Gene ontology classification of *Carassius auratus* radial glial cell (RGC) proteins into molecular function, biological function, and cellular component categories. The number of proteins ascribed to each classification, along with accession number, is provided.

20 proteins that were most regulated by each concentration of SNa based on the *P* value.

Identifying Cellular Processes Affected by SNa Using SNEA

The major objective of this study is to identify cellular processes that were responsive to SNa through the analysis of significantly regulated RGC proteins. Processes that were regulated by all concentrations of SNa included actin organization, cytoskeleton organization and biogenesis, apoptosis, mRNA processing, RNA splicing, translation, cell growth, and proliferation. At the low 10 nM SNa dose, proteins that were increased are related to processes such as blood vessel development, actin organization, cytoskeleton organization and biogenesis, cell proliferation, growth, and migration (**Figure 5**). The proteins affected by 100 nM SNa control similar processes such as cell proliferation and growth; however, proteins involved in these processes were downregulated compared to 10 nM SNa (**Figure 6.**). Similarly, 1,000 nM SNa treatment resulted in the downregulation of proteins related to cell processes that include actin organization, cytoskeleton organization and biogenesis, cell proliferation, growth, and migration. In addition, protein responses at 1,000 nM SNa suggested enrichment of processes related to neurite outgrowth and nerve fiber regeneration (**Figure 7A**) and tight and gap junction assembly (**Figure 7B**). Both 100 and 1,000 nM SNa caused changes in proteins involved in cell cycle, mitochondrial function (mitochondrial membrane permeability, depolarization, damage), and RNA processing (RNA metabolism, processing, splicing).

In addition, SNEA was used to determine if regulated proteins were known to be associated with human diseases. Major themes in disease networks that were associated with proteins altered by 1,000 nM SNa included diseases of the CNS (brain

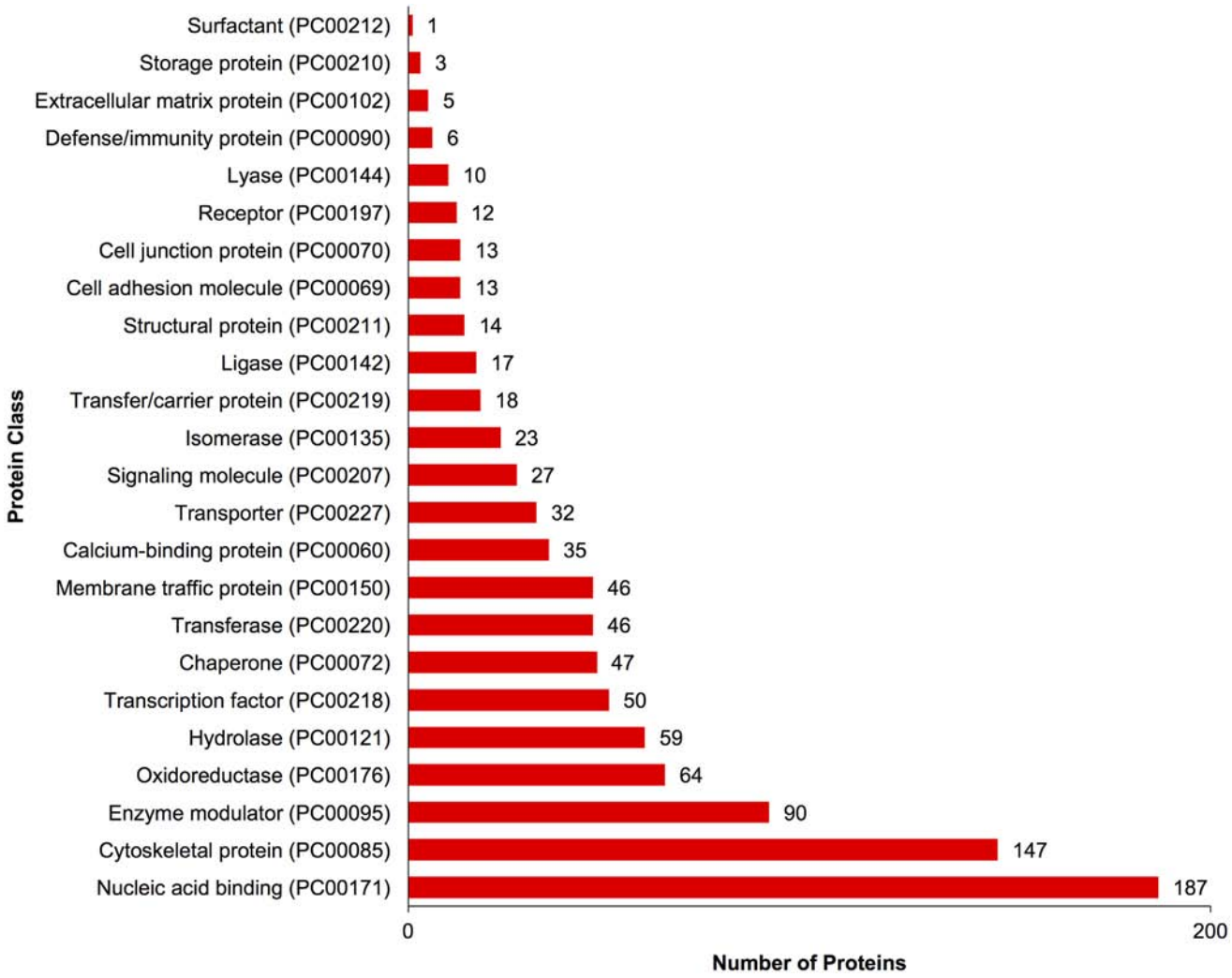

FIGURE 2 | Gene ontology classification of *Carassius auratus* radial glial cell (RGC) proteins by protein class. The number of proteins ascribed to each classification, along with accession number, is provided.

injuries, Alzheimer disease, Parkinson disease, cerebral infraction, brain ischemia), diseases of the cardiovascular system (heart failure, cardiomyopathies, heart injury, cardiac hypertrophy), and neoplasms (lung, ovarian, colorectal, pancreatic, prostatic, breast, endometrial). An example of a network associated with Alzheimer, Parkinson, and neurodegenerative diseases is shown in **Figure 8**. All the identified subnetworks are presented in Table S3 in Supplementary Material.

DISCUSSION

This study used quantitative proteomics to determine the response of RGCs to SNa in an *in vitro* cell culture system. This effort to characterize the goldfish RGC proteome identified 1,363 unique proteins and adds to those previously reported (25). A total of 609 proteins showed changes in relative abundance across three concentrations of SNa, with higher doses eliciting a greater suppressive response in protein abundance. In each concentration, SNa regulated proteins that have a role in cell processes including actin organization, cell growth, proliferation, and migration. Previous research on the effects of SN in the CNS focused on neurons (26), so our work on the effects of SNa on the RGC transcriptome (15) and proteome (current study) is the first to characterize the effects of SNa in any glial cell type.

Assigning pathway ontologies to the identified proteins revealed many relationships to the immune system, including chemokine and cytokine signaling, T cell activation, TGFβ signaling, toll receptor signaling pathway, B cell activation, and interleukin signaling pathway. While the role of RGCs *per se* in neuroinflammation is not well studied, both the transcriptomic and proteomic data support the hypothesis that RGCs contribute to immune-related functions in fish. In the mammalian CNS, this is more typically associated with astrocytes, which possess both pro- and anti-inflammatory functions, depending on the mode of injury (27). In fish brain, RGCs are the main macroglia due to the lack of typical stellate

TABLE 1 | Top 25 pathway ontologies associated with *Carassius auratus* RGC proteins based on number of protein identified in each pathway.

Pathway name	Pathway accession	# of proteins identified
Parkinson disease[a]	P00049	31
Integrin signaling pathway[a]	P00034	28
Ubiquitin proteasome pathway	P00060	26
Cytoskeletal regulation by Rho GTPase[a]	P00016	26
Huntington disease[a]	P00029	25
Inflammation mediated by chemokine and cytokine signaling pathway[a]	P00031	24
FGF signaling pathway[a]	P00021	15
EGF receptor signaling pathway[a]	P00018	15
CCKR signaling map[a]	P06959	14
Wnt signaling pathway[a]	P00057	14
Angiogenesis[a]	P00005	13
Gonadotropin-releasing hormone receptor pathway[a]	P06664	12
Cadherin signaling pathway[a]	P00012	12
Alzheimer disease-presenilin pathway[a]	P00004	10
T cell activation[a]	P00053	10
Nicotinic acetylcholine receptor signaling pathway	P00044	10
Ras pathway[a]	P04393	10
Glycolysis	P00024	10
Cell cycle	P00013	10
Apoptosis signaling pathway[a]	P00006	8
TGF-beta signaling pathway[a]	P00052	7
De novo purine biosynthesis	P02738	7
PDGF signaling pathway[a]	P00047	7
Dopamine receptor-mediated signaling pathway	P05912	7
Axon guidance mediated by Slit/Robo	P00008	6

[a]*These pathways were also identified in the de novo transcriptome assembly data in Ref. (15).*

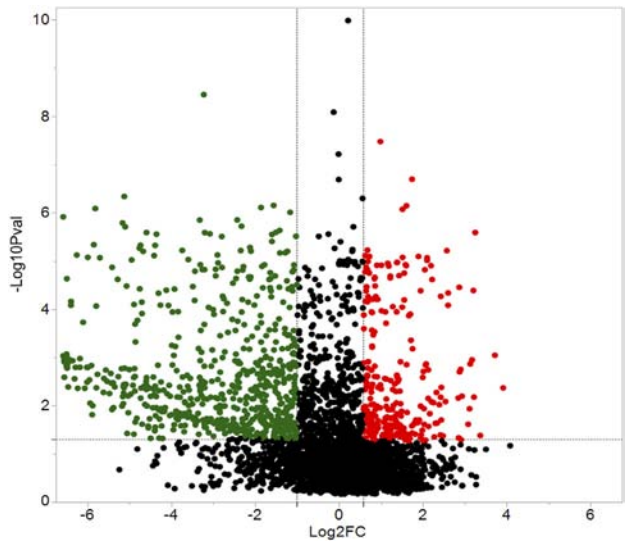

FIGURE 3 | Volcano plots for protein expression in *Carassius auratus* radial glial cells (RGCs) treated with three concentrations of secretoneurin A. Significantly regulated proteins were determined using a cut off of >1.5 or <0.5 and $P < 0.05$. Red and green dots represent proteins that are upregulated and downregulated, respectively.

TABLE 2 | Expression patterns of the differentially regulated proteins in *Carassius auratus* RGCs treated with various concentrations of SNa compared to control.

Expression pattern	10 nM	100 nM	1000 nM	Number of protein IDs
I	↑	↑	↑	5
II	↑	↑	–	9
III	↑	–	↑	3
IV	↑	–	–	31
V	↑	–	↓	20
VI	↑	↓	↓	4
VII	–	↑	↑	11
VIII	–	↑	–	18
IX	–	↑	↓	1
X	–	–	↑	25
XI	–	–	–	757
XII	–	–	↓	288
XIII	–	↓	↑	6
XIV	–	↓	–	7
XV	–	↓	↓	176
XVI	↓	–	–	3
XVII	↓	↓	↑	1
XVIII	↓	↓	–	1

RGC, radial glial cell.

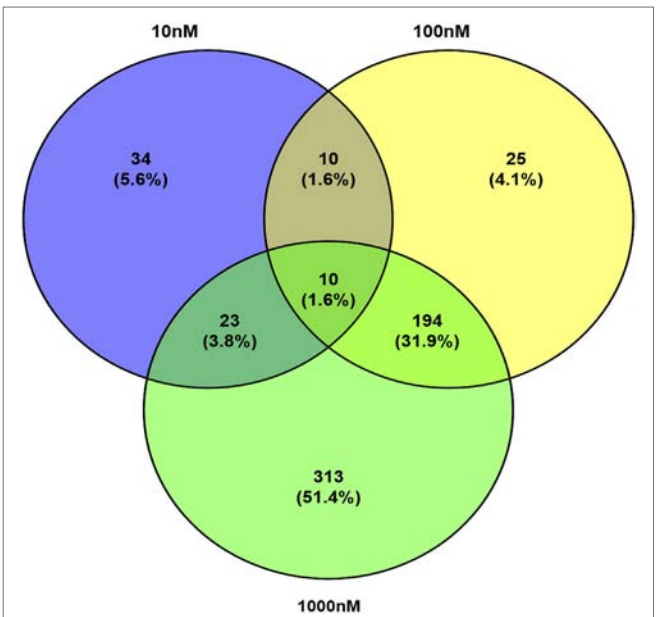

FIGURE 4 | Venn diagram showing the overlap of differentially expressed proteins following secretoneurin A (10–1,000 nM) treatment in *Carassius auratus* radial glial cells.

astrocytes, thus RGCs likely share some functions with mammalian astrocytes in brain homeostasis (14). In addition, many neurotransmitter (5-HT, DA, ACh, glutamate, GABA, histamine) and hormone (thyrotrophin-release hormone, oxytocin, corticotropin-releasing factor) receptor-mediated pathways were identified, providing further evidence that neuronal–glial interactions are important in the control of this cell type. Our proteomic data show the presence of several well-characterized RGC markers including brain lipid binding protein (BLBP),

TABLE 3 | Top 20 proteins identified by iTRAQ as different in abundance among groups in *Carassius auratus* RGCs treated with one of three concentrations of SNa.

Concentration (nM)	Accession	Protein	% coverage protein ID	Fold change	*P* value
10	A7MCL7_DANRE	Cystatin 14a, tandem duplicate 2	47	3.34	2.02E–07
	Q6DN21_CARAU	Calmodulin long form	99.33	5.94	6.11E–06
	A0A0C5Q0E9_MEGAM	Fibroblast growth factor receptor 1b	11.62	1.54	7.76E–06
	E7F1X7_DANRE	Serine and arginine repetitive matrix 1	33.69	1.65	8.06E–06
	VIM2_CARAU	Vimentin A2	44.03	3.72	8.07E–06
	F1QNJ3_DANRE	Testin LIM domain protein	25.51	4.29	8.58E–06
	F1Q5X0_DANRE	Synaptosome associated protein 29 kDa	44.74	4.29	9.73E–06
	B0UXN7_DANRE	C-Abl oncogene 2, non-receptor tyrosine kinase	20.18	3.00	1.19E–05
	C0LYZ3_9TELE	High-mobility group box 1	55.44	2.02	1.23E–05
	A0A0R4IMX7_DANRE	SH3 and PX domains 2B	25.68	1.87	1.23E–05
	X1WGZ7_DANRE	Glutamyl-prolyl-tRNA synthetase	25.03	4.57	1.25E–05
	E7F354_DANRE	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase	57.89	3.03	1.25E–05
	Q802W6_DANRE	Arhgdia protein	39.41	2.70	2.01E–05
	Q6Y3R4_CARAU	H1 histone	34.03	4.66	2.43E–05
	B3DGP9_DANRE	Protein tyrosine kinase 2aa	11.29	1.58	3.44E–05
	Q6NYA1_DANRE	Heterogeneous nuclear ribonucleoprotein A/Bb	36.25	7.30	3.53E–05
	Q802Y1_DANRE	Serine/arginine-rich splicing factor 4	39.34	1.57	4.18E–05
	143BA_DANRE	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein beta	77.46	0.15	6.06E–05
	RBM8A_DANRE	RNA-binding protein 8A	22.99	1.90	6.22E–05
	F1QG80_DANRE	Ribosomal protein L22	34.75	1.60	7.03E–05
100	Q5U7N6_DANRE	Talin 1	18.95	0.45	9.82E–07
	Q4U0S2_DANRE	Myosin, heavy chain 11a, smooth muscle	24.77	0.49	3.08E–06
	E7FEK9_DANRE	Golgin A4	40.17	0.04	4.72E–06
	F1QIN6_DANRE	CAP-GLY domain containing linker protein 2	43.21	0.05	4.76E–06
	Q1LXT2_DANRE	Eukaryotic translation elongation factor 2a, tandem duplicate 1	26.11	1.60	5.96E–06
	F1QTN7_DANRE	Acidic leucine-rich nuclear phosphoprotein 32 family member A	27.38	0.13	7.51E–06
	E7EYW2_DANRE	Epsin 2	16.27	0.02	8.56E–06
	Q7ZW39_DANRE	Phosphoribosyl pyrophosphate synthetase 1A	22.33	0.08	9.34E–06
	F1R1J9_DANRE	AHNAK nucleoprotein	53.03	2.60	1.08E–05
	Q502F6_DANRE	Zgc:112271 protein	75.58	0.29	1.20E–05
	EIF3L_DANRE	Eukaryotic translation initiation factor 3 subunit L	22.57	0.28	1.30E–05
	C0LYZ3_9TELE	High-mobility group box 1	55.44	1.57	1.35E–05
	Q7ZU46_DANRE	Heat shock protein 4a	28.45	0.14	1.36E–05
	Q0PWB8_DANRE	PDZ and LIM domain 3b	32.7	0.11	1.51E–05
	Q9DF20_DANRE	Fragile X mental retardation 1	40.77	0.10	1.51E–05
	A4FUN5_DANRE	ISY1 splicing factor homolog	57.54	0.32	1.75E–05
	Q1LYC9_DANRE	Calpain, small subunit 1 a	47.69	0.35	2.04E–05
	Q6DRC1_DANRE	Small nuclear ribonucleoprotein F	48.84	0.17	2.90E–05
	F1QYM4_DANRE	Eukaryotic translation initiation factor 4 h	55.08	0.50	3.11E–05
	F1Q7S0_DANRE	Vesicle transport through interaction with t-SNAREs 1A	41.94	0.03	3.32E–05
1,000	Q5U7N6_DANRE	Talin 1	18.95	0.11	3.56E–09
	A0JMJ1_DANRE	Scinderin like a	33.06	0.03	4.60E–07
	Q0GC55_CARAU	Heat shock protein 47 kDa	24.81	0.34	7.06E–07
	Q6PBR5_DANRE	ATPase, H+ transporting, V1 subunit G isoform 1	80.51	3.05	7.17E–07
	F1RDG4_DANRE	si:dkey-222f2.1	55.02	0.28	7.79E–07
	Q7ZTZ6_DANRE	STIP1 homology and U-Box containing protein 1	34.51	0.02	8.19E–07
	F1R1J9_DANRE	AHNAK nucleoprotein	53.03	2.86	8.48E–07
	Q804W1_DANRE	Parvalbumin isoform 4b	45.87	0.01	1.22E–06
	F1QFN1_DANRE	ELKS/RAB6-interacting/CAST family member 1b	31.20	0.19	1.41E–06
	EIF3L_DANRE	Eukaryotic translation initiation factor 3 subunit L	22.57	0.10	1.42E–06
	Q7SXA1_DANRE	Ribosomal protein L26	55.17	0.03	1.63E–06
	E7F049_DANRE	Kinectin 1	26.47	0.20	1.90E–06
	Q7ZW39_DANRE	Phosphoribosyl pyrophosphate synthetase 1A	22.33	0.03	1.96E–06
	Q803A9_DANRE	DnaJ (Hsp40) homolog, subfamily B, member 11	11.94	0.38	2.32E–06
	A0A0R4I9C6_DANRE	Ubiquitin-fold modifier 1	73.33	0.35	2.55E–06
	B8JJS6_DANRE	Programmed cell death protein 10-B	32.86	9.51	2.58E–06
	F1QTN7_DANRE	Acidic leucine-rich nuclear phosphoprotein 32 family member A	27.38	0.04	2.58E–06
	A4FUN5_DANRE	ISY1 splicing factor homolog	57.54	0.11	2.59E–06
	Q502F6_DANRE	zgc:112271	75.58	0.12	2.74E–06
	Q9W792_DANRE	T-complex polypeptide 1	26.98	0.05	2.78E–06

All fold changes are relative to control.

iTRAQ, isobaric tagging for relative and absolute quantitation; RGC, Radial glial cell; SNa, secretoneurin A.

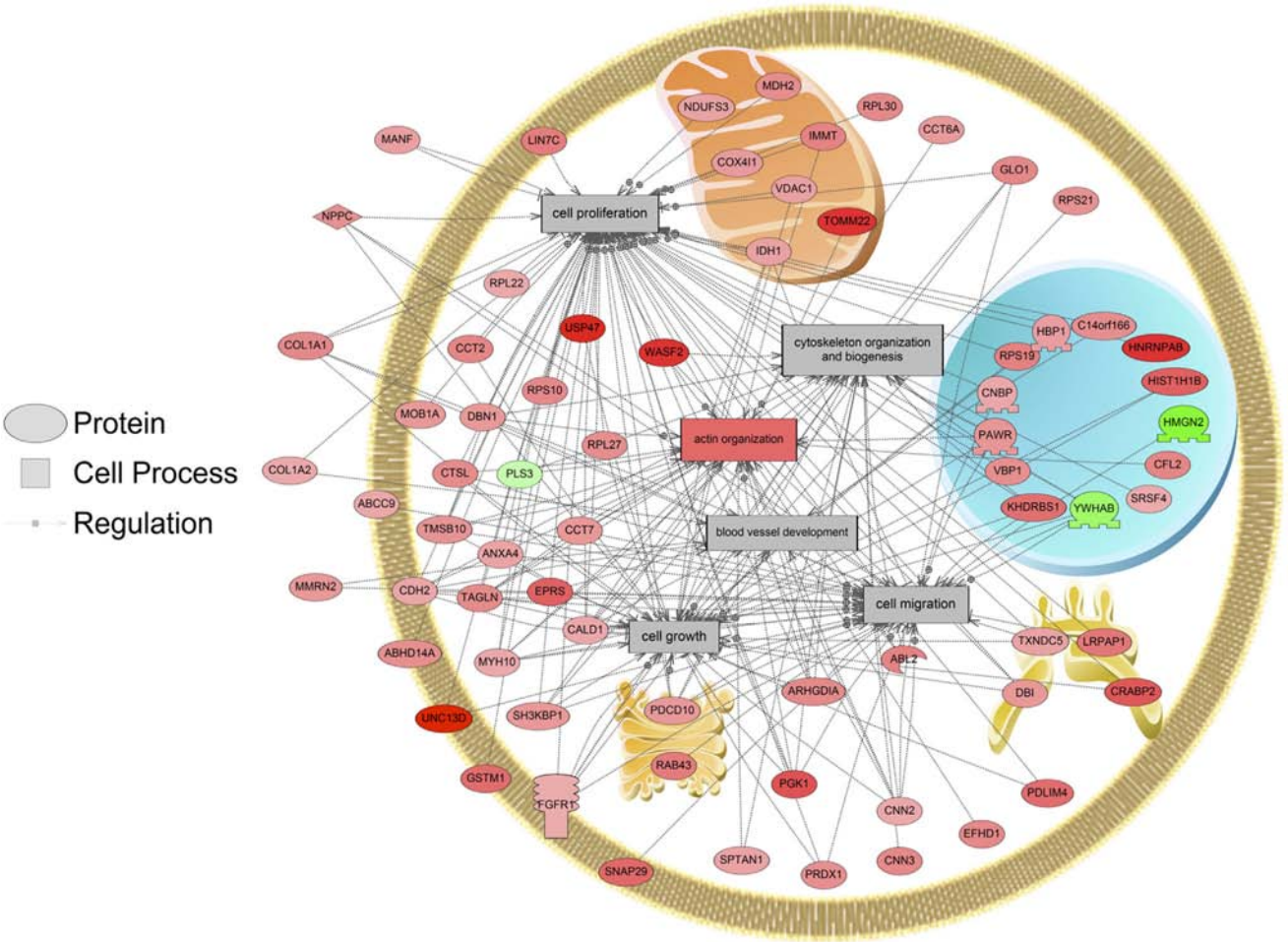

FIGURE 5 | Cellular processes of blood vessel development, actin organization, cytoskeleton organization and biogenesis, cell proliferation, growth, and migration were enriched in primary *Carassius auratus* radial glial cell culture after 10 nM secretoneurin A treatment ($P < 0.05$) based on the protein profiles. Red indicates that the protein is increased, and green indicates that the protein is decreased in abundance relative to the control group. All abbreviations are provided in Table S4 in Supplementary Material.

glial fibrillary acidic protein (GFAP), tight junction protein ZO-1, Sox2, and vimentin (28, 29), confirming that these cultured cells are indeed RGCs. Furthermore, analysis of the differentially regulated proteins shows that SNa can regulate these classical protein markers of RGCs. At each concentration, SNa increased the expression of the intermediate filament protein vimentin. While vimentin is implicated in several glial processes, it has also been shown to be involved in the initial stages of neurite outgrowth in rat hippocampal neurons *in vitro* (30). However, the 1,000 nM SNa treatment decreased BLBP and ZO-1. These proteins are lost when RGCs differentiate into neurons. Moreover, SNa decreases the expression of aromatase B (*cyp19a1b*) in RGCs *in vivo* and *in vitro* in a dose-dependent manner (16). Taken together, these data suggest that SNa downregulates some of the genes related to the progenitor-like characteristics of this cell type.

A low dose of 10 nM SNa upregulated proteins known to be involved in processes of blood vessel development, actin organization, cytoskeleton organization and biogenesis, cell growth, migration, and proliferation. It has been previously established that SN can induce mouse angiogenesis and vasculogenesis (31, 32), which supports our data that 10 nM SNa increases the expression of proteins associated with blood vessel development. Mouse RGCs have been shown to regulate angiogenesis in the brain through their interactions with blood vessels (33). Since SNa can regulate proteins involved in blood vessel formation, this may control the function of RGCs to regulate angiogenesis. As a chemoattractant, SN has the ability to stimulate the migration of various cell types. SN can stimulate the migration of human monocytes both *in vivo* and *in vitro* at nanomolar concentrations (34). Similarly, SN induces eosinophil (35), dendritic cell (36), and fibroblast (37) chemotaxis. These studies indicate that the chemotactic activity of SN is dose dependent with maximal affects between 0.1 and 100 nM, and higher doses elicit an inhibitory affect. Interestingly, here, we report that proteins involved in cell migration are decreased by a high concentration of 1,000 nM

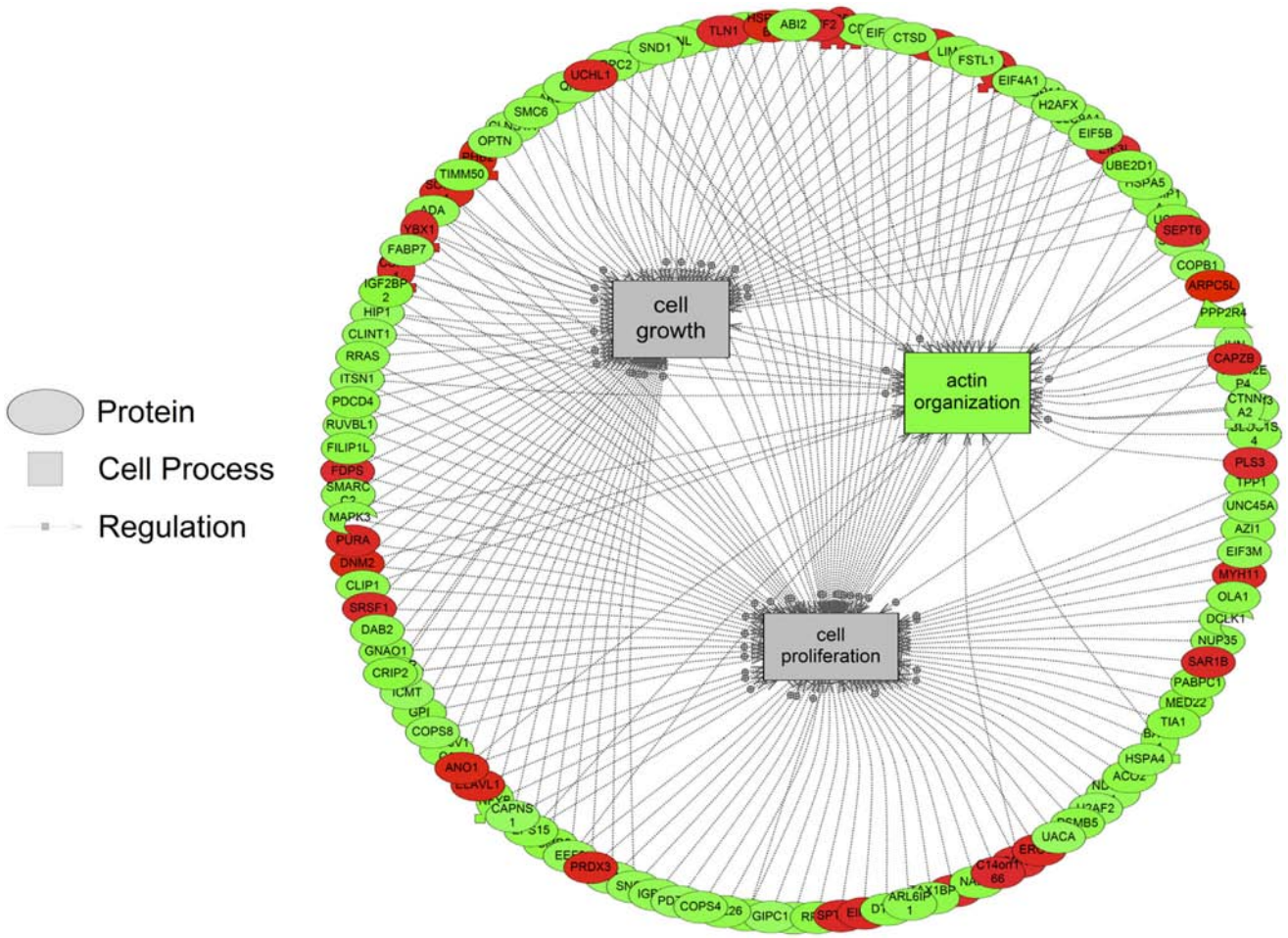

FIGURE 6 | Cellular processes of actin organization, cell proliferation, and growth were significantly enriched in primary *Carassius auratus* radial glial cell culture after 100 nM secretoneurin A treatment ($P < 0.05$) based on the protein profiles. Red indicates that the protein is increased, and green indicates that the protein is decreased in abundance relative to the control group. All abbreviations are provided in Table S4 in Supplementary Material.

SNa, indicating that SNa may regulate proteins involved in cell migration in a dose-dependent manner. In addition, both 100 and 1,000 nM concentrations regulated actin organization, cell growth, and cell proliferation protein networks; however, these higher concentrations decreased expression of proteins involved in these cell processes.

Following treatment with 1,000 nM SNa, subnetworks related to tight junction and gap junction assembly were enriched based on the proteins regulated. These cell processes were also identified by gene set enrichment analysis of transcriptomic data and were increased by SNa at the gene network level (15). Unlike the transcriptomic response, proteins involved in tight junction and gap junction assembly were downregulated, indicating that there is a complex regulation of the transcriptome and proteome that control these processes. Among the adherens junctions that were regulated by SNa, *N*-cadherin (cadherin 2) is a calcium-dependent adhesion molecule (38), and it is expressed to uphold RGC apical–basal polarity while anchoring adjacent RGCs to each other to create stem cell niches (39–42). *N*-cadherin controls mouse RGC function in neuronal migration and directing axon formation (42, 43). Furthermore, *N*-cadherin through its interactions with its effector β-catenin regulates RGC proliferation and differentiation (44–46). Reduction in mouse *N*-cadherin causes RGCs to migrate from the stem cell niche and stimulate neuronal differentiation (46). In our study, *N*-cadherin levels were increased at 10 nM and decreased at 1,000 nM SNa. Therefore, our data on *N*-cadherin expression indicate a role for SNa in the regulation of RGC-mediated neuronal migration, as well as RGC proliferation and differentiation.

Proteins involved in cellular processes such as neurite outgrowth and nerve fiber regeneration were also altered in abundance in RGCs following treatment with 1,000 nM SNa. The high dose of SNa significantly decreased the abundance of proteins known to be involved in neurite outgrowth. Although this cellular process was not enriched at lower doses, these data indicate that SNa has inhibitory effects on proteins that regulate neurite outgrowth. In contrast, SN stimulates neurite outgrowth in mouse cerebellar granule cells with a maximal concentration

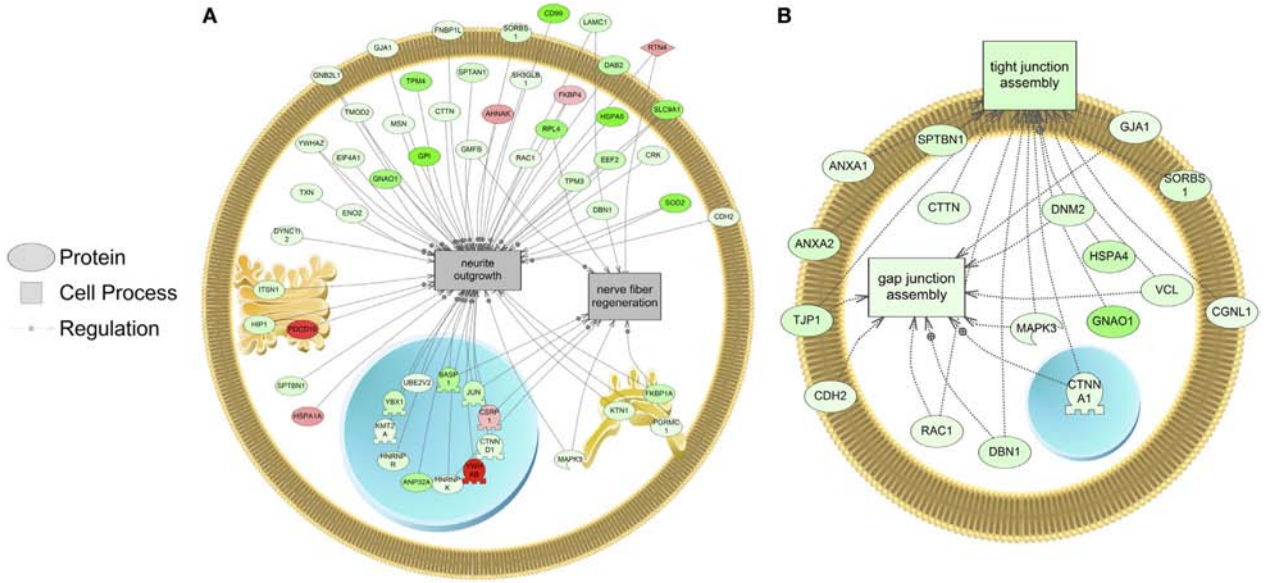

FIGURE 7 | Cellular processes of **(A)** neurite outgrowth and nerve fiber regeneration and **(B)** tight and gap junction assembly were enriched in primary *Carassius auratus* radial glial cell culture after 1,000 nM secretoneurin A treatment ($P < 0.05$) based on the protein profiles. Red indicates that the protein is increased, and green indicates that the protein is decreased in abundance relative to the control group. All abbreviations are provided in Table S4 in Supplementary Material.

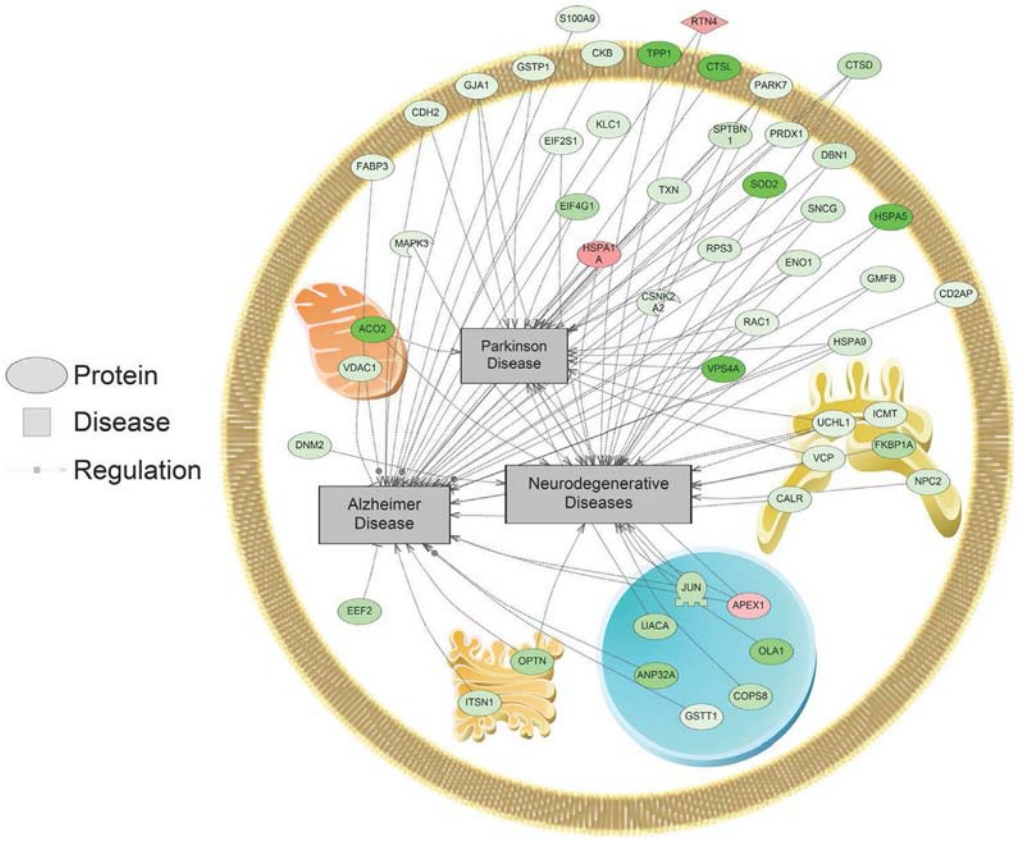

FIGURE 8 | Alzheimer, Parkinson, and neurodegenerative disease pathways were enriched in primary *Carassius auratus* radial glial cell culture after 1,000 nM secretoneurin A treatment ($P < 0.05$) based on the protein profiles. Red indicates that the protein is increased, and green indicates that the protein is decreased in abundance relative to the control group. All abbreviations are provided in Table S4 in Supplementary Material.

of 100 nM (47). Proteins related to nerve fiber regeneration were also decreased in goldfish RGCs exposed to 1,000 nM SNa. Previous findings show that SN treatment induces neural regeneration and neurogenesis in murine models of stroke (48). The data obtained from goldfish reveal protein networks that may underlie the role of SN in tissue repair and specifically in stem cell-like RGCs. SNa increased the expression of reticulon 4 (RTN4) in goldfish RGCs. RTN4 is represented in both the nerve fiber regeneration and the neurite outgrowth protein networks in goldfish RGCs. It is also known as a negative regulator of neurite plasticity in mammalian models by inhibiting neurite outgrowth and axonal regeneration (49–51). However, the major protein region of RTN4 that is involved in neurite growth inhibition is absent in zebrafish RTN4 (52) and is required for successful zebrafish axon regeneration (53). Therefore, given the opposite effects of RTN4 observed in zebrafish compared to mammals, future work should be directed toward elucidating the effects of SNa on neurite outgrowth and axon regeneration in fish through mechanisms involving RTN4.

Many proteome subnetworks were identified that correspond to diseases of the CNS and cardiovascular system along with several neoplasms. Protein networks in RGCs related to Alzheimer disease, Parkinson disease, brain ischemia, and brain injury were all regulated by 1,000 nM SNa. Changes in these networks are consistent with previous studies that have associated SN with these neuropathological conditions. SN is differentially regulated in brain ischemia (48, 54, 55), Alzheimer disease (56–58), and epilepsy (59, 60). It has been reported that circulating SN increases after heart failure and has a protective effect by reducing myocardial ischemia injury, cardiomyocyte apoptosis, and inducing angiogenesis (61, 62). Here, we show that SNa can regulate subnetworks related to cardiovascular diseases such as cardiomyopathies, heart injury, cardiomegaly, and heart failure. In addition, many subnetworks related to neoplasms were enriched after SNa treatment including lung, ovarian, colorectal, pancreatic, prostatic, breast, and endometrial. Importantly, SN has been shown to be expressed in several types of neuroendocrine tumors including prostate, lung, rectal, pancreatic, thyroid, duodenum, and appendix (63–65). Taken together, it is suggested that these networks underlie, in part, the molecular responses to SN that are mechanistically linked to these disease states.

The teleost brain has become a popular model for neurogenesis and neuroendocrinology because of the persistent abundance of progenitor RGCs in the adult brain and the high aromatase activity present in RGCs (29, 66, 67). Our research has implications in these fields as characterizing the RGC proteome can elucidate the molecular mechanisms that underlie these unique functions of fish RGCs. The proximity of SNa-immunoreactive magnocellular preoptic neurons with aromatase B/GFAP expressing RGCs prompted our efforts to characterize the effects of SNa on the transcriptome (15) and now the proteome of these multifunctional cells. For the first time, we show that the RGC proteome is responsive to nanomolar concentrations of SNa. Proteins regulated by SNa are implicated in many cell processes including those associated with blood vessel development, actin organization, cytoskeleton organization and biogenesis, neurite outgrowth, nerve fiber regeneration, cell growth, migration, and proliferation. At lower doses, SNa increased proteins involved in cell growth, migration, and proliferation, whereas higher doses of SNa downregulated proteins involved in these processes. This indicates that SNa has dose-dependent regulatory effects in RGCs. At 1,000 nM of SNa, proteins linked to the etiology of diseases of the CNS (brain injuries, Alzheimer disease, Parkinson disease, cerebral infraction, brain ischemia) were also differentially regulated. These data implicate that SNa in the control of pathways is important in cell proliferation and neurogenesis through direct actions on RGCs.

ETHICS STATEMENT

The study was carried out in accordance with the Canadian Council on Animal Care and approved by the University of Ottawa animal care and veterinary services.

AUTHOR CONTRIBUTIONS

DD performed experiments, analyzed the data, and wrote the paper. CM performed experiments, analyzed the data, and contributed to writing the paper. LX performed experiments, analyzed the data, and contributed to writing the paper. VT helped design experiments and co-wrote the paper.

ACKNOWLEDGMENTS

Funding from the NSERC Discovery program (VLT), NSERC-CGSM (DDF), and Michael Smith Foreign Study Award (DDF), University of Ottawa Research Chair in Neuroendocrinology (VLT), and the Ontario Trillium Scholarship (LX) are acknowledged with appreciation. Some of the results presented are part of the research thesis of DDF at the University of Ottawa (68).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fendo.2018.00068/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 12 March 2018
doi: 10.3389/fendo.2018.00086

11β-HSD Types 1 and 2 in the Songbird Brain

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Glucocorticoid (GC) hormones act on the brain to regulate diverse functions, from behavior and homeostasis to the activity of the hypothalamic–pituitary–adrenal axis. Local regeneration and metabolism of GCs can occur in target tissues through the actions of the 11β-hydroxysteroid dehydrogenases [11 beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and 11 beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2), respectively] to regulate access to GC receptors. Songbirds have become especially important model organisms for studies of stress hormone action; however, there has been little focus on neural GC metabolism. Therefore, we tested the hypothesis that 11β-HSD1 and 11β-HSD2 are expressed in GC-sensitive regions of the songbird brain. Localization of 11β-HSD expression in these regions could provide precise temporal and spatial control over GC actions. We quantified GC sensitivity in zebra finch (*Taeniopygia guttata*) brain by measuring glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) expression across six regions, followed by quantification of 11β-HSD1 and 11β-HSD2 expression. We detected GR, MR, and 11β-HSD2 mRNA expression throughout the adult brain. Whereas 11β-HSD1 expression was undetectable in the adult brain, we detected low levels of expression in the brain of developing finches. Across several adult brain regions, expression of 11β-HSD2 covaried with GR and MR, with the exception of the cerebellum and hippocampus. It is possible that receptors in these latter two regions require direct access to systemic GC levels. Overall, these results suggest that 11β-HSD2 expression protects the adult songbird brain by rapid metabolism of GCs in a context and region-specific manner.

Keywords: glucocorticoid, mineralocorticoid, 11 beta-hydroxysteroid dehydrogenase, stress response, songbird

OPEN ACCESS

Edited by:
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Reviewed by:
Kiran K. Soma, University of British Columbia, Canada
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *15 December 2017*
Accepted: *23 February 2018*
Published: *12 March 2018*

Citation:
Rensel MA, Ding JA, Pradhan DS and Schlinger BA (2018) 11β-HSD Types 1 and 2 in the Songbird Brain. Front. Endocrinol. 9:86. doi: 10.3389/fendo.2018.00086

INTRODUCTION

Glucocorticoid (GC) hormones regulate numerous biological processes, including crucial actions on the central nervous system such as feedback regulation of adrenal function, activation or suppression of cognitive and locomotor activity, and regulation of feeding behaviors (1). GC secretion is dynamic, varying according to time of day (2, 3), developmental and life history stage (4–7), as well as in response to acute and chronic stress (1). Following synthesis and secretion, primarily from the adrenals, GC effects are regulated by various mechanisms, including binding to circulating globulins that regulate access to tissues and local expression of catabolic and anabolic enzymes in target tissues (8). Ultimately, tissue genomic and cellular responses are guided by the degree to which GC receptors

are expressed. Sensitivity to GC effects is mediated through the intracellular mineralocorticoid receptor (MR) and glucocorticoid receptor (GR); acute stress-induced effects are mediated by the lower-affinity GR, while the high-affinity MR mediates baseline and early stress-induced effects (1, 9). Membrane GR and MR, while less studied, are found in both birds and mammals where they likely mediate rapid, non-genomic effects of GCs (10–12).

The 11β hydroxysteroid dehydrogenase enzymes (11β-HSDs) mediate the interconversion of the GC corticosterone (birds, some rodents, and reptiles) or cortisol (other vertebrates; both hereafter referred to as CORT) to an inactive form, 11-dehydrocorticosterone (11-DHC) or cortisone, respectively. The enzyme 11 beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) catalyzes the conversion of 11-DHC or cortisone into CORT *in vivo* but is capable of catalyzing the reverse reaction *in vitro*; 11 beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2) exclusively inactivates CORT by catalyzing its conversion into 11-DHC or cortisone (13). Dysregulation of these enzymes in peripheral tissues is implicated in mammalian models of hypertension, diabetes, obesity, and metabolic disorder (14). 11β-HSD2 plays a critical role in the mammalian kidney, where its expression preserves aldosterone access to MR, which binds both CORT and aldosterone with equal affinity (15).

In rodents, 11β-HSDs are expressed peripherally and in brain, although their localization and functional significance vary across developmental stages. For example, relatively little 11β-HSD1 is expressed in the early developing rodent brain (15, 16), whereas it is widely expressed in the adult brain and periphery. In the adult brain, this enzyme participates in hypothalamic–pituitary–adrenal (HPA) axis regulation (17, 18) and facilitates GC-induced memory impairments in aging animals (19). Conversely, 11β-HSD2 expression in the adult brain has a limited distribution associated with salt-based aldosterone sensitivity, while 11β-HSD2 is expressed abundantly in the fetal brain and placenta and is hypothesized to protect the fetal nervous system from excess GC exposure (15).

Evidence for 11β-HSD2 expression in the adult human brain is contradictory (20, 21), and it is possible that CORT is metabolized in the human brain *via* a unique hydroxysteroid dehydrogenase (22). Given the documented ill effects of stress and elevated GCs on adult neurogenesis and cognition (23), the presence of region-specific GC-inactivating and regenerating enzymatic machinery in the brain should be adaptive and expected.

Unlike the adult rodent brain, metabolism of CORT *via* 11β-HSD2 has been reported in hatchling and adult songbird brain using chicken-specific PCR primers (24) as well as species-specific primers (25). In the latter study, we confirmed expression of 11β-HSD2 in two regions of the adult zebra finch (*Taeniopygia guttata*) brain, the caudal nidopallium (cNp) and hippocampus (HP). In addition, expression covaried with free CORT sampled in these regions using *in vivo* microdialysis. Specifically, we found that CORT levels were higher in the region expressing lower levels of 11β-HSD2 (25) suggesting that 11β-HSD2 limits bioactive GC exposure in specific regions of the brain.

The cNp and HP both lie adjacent to the lateral ventricular zone (VZ), a region of the songbird brain in which adult neurogenesis is conspicuous and critical for seasonal growth of song control nuclei (26) and recruitment of hippocampal neurons (27). As previous work has established differential effects of GCs on neurogenesis (23), we predicted that both 11β-HSD1 and 11β-HSD2 expression in HP and cNp, as well as another adjacent region, the caudomedial nidopallium (NCM), should provide precise control over GCs within these sensitive proliferative zones. We therefore hypothesized that 11β-HSD2 and/or 11β-HSD1 expression regulates exposure of the adult songbird brain in regions with heightened expression of GC receptors.

We tested this hypothesis in adult male and female zebra finches, assessing expression in six brain regions. We included three brain regions, NCM, cNp, and HP because of their proximity to the VZ, the diencephalon (DIEN), because of its role in regulating HPA negative feedback, and the cerebellum and optic tectum as control regions. Previous studies have described expression profiles of GR and MR in songbirds (28–32). To the best of our knowledge, 11β-HSD1 expression has not been reported in songbirds, with little data on 11β-HSD2. To assess how metabolic enzymes might participate in controlling GC access to its receptors, we used quantitative PCR to simultaneously characterize expression of 11β-HSD1 and 11β-HSD2, as well as GR and MR, in adult brain. While we detected 11β-HSD2 in all brain regions, 11β-HSD1 expression was undetectable. To verify this result, which differs significantly from mammalian patterns of expression (15), we confirmed 11β-HSD1 expression in the brains of developing finches as well as two additional target tissues, the liver and kidney (33).

ANIMALS AND METHODS

Animals

This study was conducted at the University of California, Los Angeles. All procedures involving animals were approved by the Chancellor's Animal Research Committee. We utilized adult (>100 days of age) non-breeding zebra finches obtained from our captive colony. For one study, we utilized developing finches of variable ages (see below). Finches in our colony are kept in large open flight aviaries with up to 40 same-sex individuals residing in each enclosure. Breeding cages are comprised of four to five breeding pairs with access to breeding boxes filled with nesting material. Lights are maintained on a 14 h light/10 h dark cycle, and finches are supplied with *ad libitum* seed, water, cuttle bone, and grit at all times. Egg mix and nutritional supplements are provided at least once per week.

Dissections

Groups of four to five adult finches were captured together and transported in a darkened cage to a procedure room where each bird was then processed. Following rapid decapitation, the brain was removed and placed on a Petri dish situated in wet ice (see below). Upon dissection, the six brain regions of interest were immediately frozen on dry ice, transferred to tubes, and kept at −80°C until RNA extraction. The amount of time that passed from initial capture to sacrifice ranged from 1 to 87 min (mean time = 39 ± 6.5 min), and sacrifice time post-capture was included as a covariate in statistical analyses. Sampling times and

ranges were highly similar between males and females (**Figure 1**). A total of 18 birds were sacrificed in this manner, over the course of four sessions in 2 days (one AM and one PM session/day—the order of sexes captured was counterbalanced across days). The same aviary was never entered more than once per day to reduce potential stress effects on gene expression.

Brain regions were dissected as follows: with the brain ventral side down, the whole cerebellum was removed. Next, we made two parasagittal cuts ~1 mm from the midline from the caudal to the rostral end, then removed 5 mm of the rostral portion of the brain. The HP was then carefully separated from the caudal portion and removed bilaterally [see Ref. (25)]. Using watchmaker's forceps, we next collected a roughly 1 mm^2 region of the underlying telencephalon (TEL) containing the caudomedial nidopallium (NCM) [see Ref. (34)]. A 1 mm^2 portion of cNp located lateral to the position of the HP cut was next made bilaterally [see Ref. (25)]. After removal of the remainder of the TEL, the optic tecta were easily separated. Finally, excess optic nerve and myelin was removed and discarded from the ventral DIEN. Liver and kidney were collected next and frozen.

To compare 11β-HSD1 expression across developmental stages, brain tissue was collected from four individuals per age group: 5–7 days post-hatch ("hatchlings"), 25 days post-hatch ("fledglings"), and 75 days post-hatch ("juveniles"). The entire TEL (including underlying DIEN) was collected from hatchlings due to their very small size, while only the caudal TEL was utilized from fledglings and juveniles. The HP was removed from the caudal TEL of these latter ages (for other studies). To enable a direct comparison of expression across ages, the remaining caudal TEL from four of the adults utilized in the main study above was processed in parallel with these developing finch samples.

RNA Extraction and cDNA Synthesis

RNA was extracted from frozen tissue samples using the Trizol method according to the manufacturer's guidelines (Ambion). All centrifugation steps were performed at 4°C. Briefly, tissue was homogenized in 1 ml cold Trizol for a maximum of 45 s, followed by centrifugation at 12,000 *g* for 10 min. The supernatant was decanted and incubated at room temperature for 5 min, and 200 µl of chloroform was then added. Samples were vigorously shaken, incubated at room temp for 3 min and then centrifuged at 12,000 *g* for 15 min. After centrifugation, the aqueous layer was carefully removed and placed in a new tube. Next, 500 µl of isopropanol was added to each tube. For small tissues that produced tiny RNA pellets (HP, NCM, and cNp), we added 1 µl of 15 mg/ml glycoblue (Invitrogen) to improve visibility. Tubes were briefly vortexed, incubated at room temperature for 10 min, centrifuged at 12,000 *g* for 10 min, and the supernatant removed, leaving the RNA pellet in place. Finally, 1 ml of 75% ethanol (4°C) was added to the tube, and the tube was inverted to ensure that the pellet was free-floating. After centrifugation at 7,500 *g* for 5 min, the ethanol was carefully pipetted out of the tube, and residual ethanol was allowed to evaporate. The RNA pellet was then resuspended in 10–120 µl of sterile water based on pellet size, vortexed and then heated in a water bath at 58°C for 10 min. RNA quantity and integrity was then determined *via* nanodrop. Total RNA concentrations ranged from 100 to 600 ng/µl, and $A_{260/280}$ ratios were between 1.8 and 2.15.

To prepare cDNA from RNA samples, 600 ng RNA was reverse transcribed. Briefly, 0.5 µl of DNAse (Promega) and 1.1 µl of DNase buffer were added to each sample, then incubated at 37°C for 30 min and 65°C for 10 min. Next, 1.5 µl of oligoDT (Sigma) and 0.5 µl dNTPs (Bioline) were added, and tubes were incubated for 10 min at 65°C. Finally, a mix of reverse transcriptase (Superscript II, Invitrogen; 1 µl/sample), RT buffer (4 µl/sample), DTT (1 µl/sample), and RNAse inhibitor (RNAsin, Promega; 1 µl/sample) was added, followed by incubation at 42°C for 50 min and 70°C for 15 min. Samples were then frozen at −20°C until qPCR analysis.

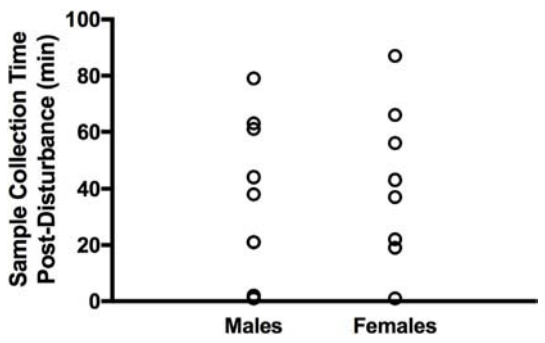

FIGURE 1 | Distribution of tissue collection times post-disturbance in adult male and female zebra finches. Males and females were sampled at similar times.

Quantitative PCR

Mineralocorticoid receptor, 11β-HSD2, and 11β-HSD1 qPCR primers were designed based on the zebra finch genome using Primer3Plus and PrimerBlast (NCBI). GR primers were taken from Banerjee et al. (35). Concentrations were optimized for each primer pair and are listed along with primer sequences and amplicon lengths in **Table 1**. We used an Applied Biosystems 7300 Real-Time PCR system to quantify gene expression relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in samples using the SYBR Green method. Samples were run at a 1:10 dilution and in duplicate wells. Reaction volume was 25 µl, and cycling conditions were as follows: (1) 2 min at 50°C, (2) 10 min at 95°C, (3) 15 s at 95°C, (4) 1 min at 60°C, repeat steps 3 and 4 40 times, (5) 15 s at 95°C, (6) 1 min at 60°C, (7) 15 s at 95°C, and (8) 15 s at 60°C.

Standard curves were prepared for each gene and plate to confirm reaction efficiency (90–110%) and standard curve linearity (≥98%). For GR and MR, each plate contained a standard curve prepared from a mix of 1:1 cDNA representing all samples from the region represented on the plate. We included positive control tissue in the adult 11β-HSD2 standard curves, pooling all brain regions together with a small amount of kidney cDNA. The adult 11β-HSD1 standard curve was constructed from liver cDNA. All standard curves utilized a fourfold dilution with curves extending from 1:1 to 1:256 for GR, MR, and GAPDH, and standard curves ranging from 1:1 to 1:1,024 (11β-HSD1) or 1:4,096 (11β-HSD2).

TABLE 1 | Quantitative PCR primer details.

Gene name (accession #)	Primer sequence (5′–3′)	Amplicon length (bp)	[Primer] per reaction (μM)
Mineralocorticoid receptor (NM_001076690)	F: AAGAGTCGGCCAAACATCCTTGTTCT R: AAGAAACGGGTGGTCCTAAAATCCCAG	150	0.3
Glucocorticoid receptor (XM_002192952.3)	F: TGCAGTACTCCTGGATGTTCC R: GAGCATGTGTTTGCATTGTTC	155	0.3
11 Beta-hydroxysteroid dehydrogenase type 2 (XM_002187455.3)	F: AAAACAGGGACAACATGCGA R: CCCCTCTGTGATGCTGTTCA	189	0.6
11 Beta-hydroxysteroid dehydrogenase type 1 (XM_002196384.1[a])	F: CATCCATAGCGGGTAAAATTG R: CGCTCTCTGTGTTGATGTAGC	162	0.3
Glyceraldehyde-3-phosphate dehydrogenase (NM_001198610.1)	F: TGACCTGCCGTCTGGAAAA R: CCATCAGCAGCAGCCT	70	0.3

[a]*This transcript is "11 beta-dehydrogenase 1-like" in NCBI; Ensembl lists a nearly identical transcript as "HSD11B1." Therefore, we designed qPCR primers to cover a region of the transcript found in both versions.*

All sample values fell within the bounds of the standard curve for each gene and plate (except for 11β-HSD1; see below). Preliminary optimizations for 11β-HSD1 indicated low to no expression in adult brain; therefore, all samples within a given brain region and a given sex were pooled for the assay instead of running individual samples ($n = 12$ pooled samples from males and females in six regions).

In the absence of 11β-HSD1 expression in regions of the adult brain examined, we conducted further tests to verify the specificity and accuracy of our 11β-HSD1 primers. We first created individual pools of caudal and whole TEL (all ages), liver (all ages), and kidney (adults) and optimized the qPCR reaction. After confirming amplification in all three pools, we sequenced products and used NCBI Blast to confirm the specificity of the products. Because we successfully amplified 11β-HSD1 in the TEL pool, we next ran a single qPCR plate with TEL samples from all four ages ($n = 4$ per age). We constructed the standard curve for this plate from a pool of TEL, kidney, liver, and adrenal cDNA.

Specificity of amplification for GR, MR, 11β-HSD2, and 11β-HSD1 was established by (a) confirming the presence of a single peak on dissociation curves and (b) sequencing and subsequent BLAST analysis of qPCR products. Additional sequencing and gel electrophoresis were used to confirm expression of 11β-HSD1 in hatchling brain (see Results). No reverse transcriptase (no RT) controls were run for each gene to assess DNA contamination. We also confirmed that reaction mixes were not contaminated by running no-template controls on each plate.

Expression levels were calculated using the delta cycle threshold (CT) method, where expression = 1,000*power [2, $^{-(\text{CT gene} - \text{CT GAPDH})}$]. GAPDH was utilized as a reference gene for this calculation, as expression is relatively stable in the songbird brain (25, 36).

Statistics

Regional variation in MR, GR, and 11β-HSD2 relative expression was assessed using linear mixed models and general linear models where appropriate. For each gene, brain region, sex, the sex by brain region interaction, and sacrifice time post-disturbance were included as fixed effects, and bird ID ($n = 17$; one female was excluded from analyses due to aberrant GAPDH results) was included as a random factor to control for repeated sampling within individual birds. Initial analyses included the sex by sacrifice time interaction. In the absence of any sex-specific effects, this term was eliminated from the final models. The sample size in some brain regions was less than 17 due to technical error or depletion of sample (see figure legends for sample sizes). For GR and MR, there was no variance due to bird ID in the initial mixed model; therefore, general linear models were utilized (model results were identical with and without bird ID). If the interaction term was non-significant, it was removed and the model was re-run to obtain the final model. Significant effects of brain region or a significant interaction term were analyzed using LSD *post hoc* tests, with a significance value of $P < 0.05$. Liver and kidney expression levels in three to four samples were used as positive controls and were not statistically analyzed in comparison with brain.

We used linear regression to compare mean expression levels of 11β-HSD2 with those of MR and GR across regions. Using the same procedure, we tested whether regional patterns of GR expression predicted patterns of MR expression.

RESULTS

Regional Patterns of Gene Expression in Adult Brain

Mineralocorticoid receptor expression levels differed significantly between brain regions ($F_{5,89} = 26.2$; $P < 0.001$) with highest levels in HP, followed by NCM and cNp, and lowest levels in OT, CER, and DIEN (**Figure 2**). Levels did not differ between males and females ($F_{1,89} = 1.9$; $P = 0.171$) and were unrelated to time of sacrifice post-capture ($F_{1,89} = 0.8$; $P = 0.381$). There was no interaction between sex and region ($F_{5,84} = 0.7$; $P = 0.647$).

Glucocorticoid receptor expression levels also differed between brain regions ($F_{5,82} = 191.5$; $P < 0.001$) with highest levels in CER, followed by NCM and cNp, and lowest levels in DIEN, OT, and HP (**Figure 2**). A significant main effect of sex ($F_{1,82} = 11.4$; $P = 0.001$) was driven by the sex*region interaction term ($F_{5,82} = 6.6$, $P < 0.001$). Specifically, GR expression levels were elevated in the CER of females ($P < 0.001$), while there were no sex differences in any other brain regions (all $P > 0.2$). Time of sacrifice post-capture was unrelated to expression ($F_{1,82} = 0.1$; $P = 0.712$).

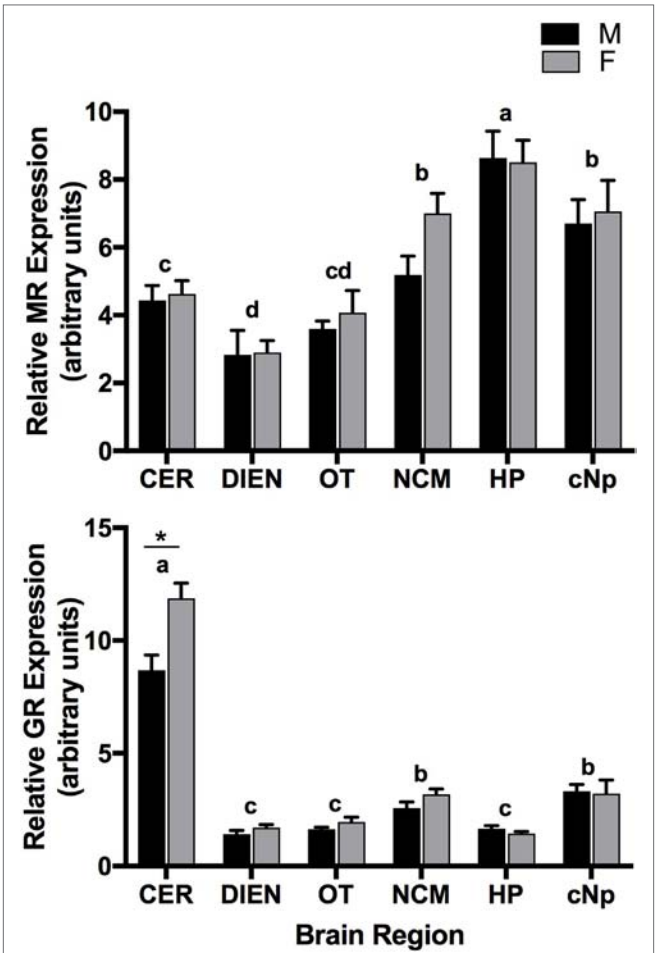

FIGURE 2 | Quantitative PCR results showing mineralocorticoid receptor (MR) (top panel) and glucocorticoid receptor (GR) (bottom panel) expression patterns (relative to glyceraldehyde-3-phosphate dehydrogenase) across six brain regions in adult male (M) and female (F) zebra finches. Letters above bars represent significant differences. *Indicates a significant difference between males and females in CER. Bars are means ± 1 SE. Sample sizes are as follows (*n* = MR/GR): CER, cerebellum (17/16); DIEN, diencephalon (17/16); OT, optic tectum (17/17); NCM, caudomedial nidopallium (16/16); HP, hippocampus (17/17); cNp, caudal nidopallium (13/13).

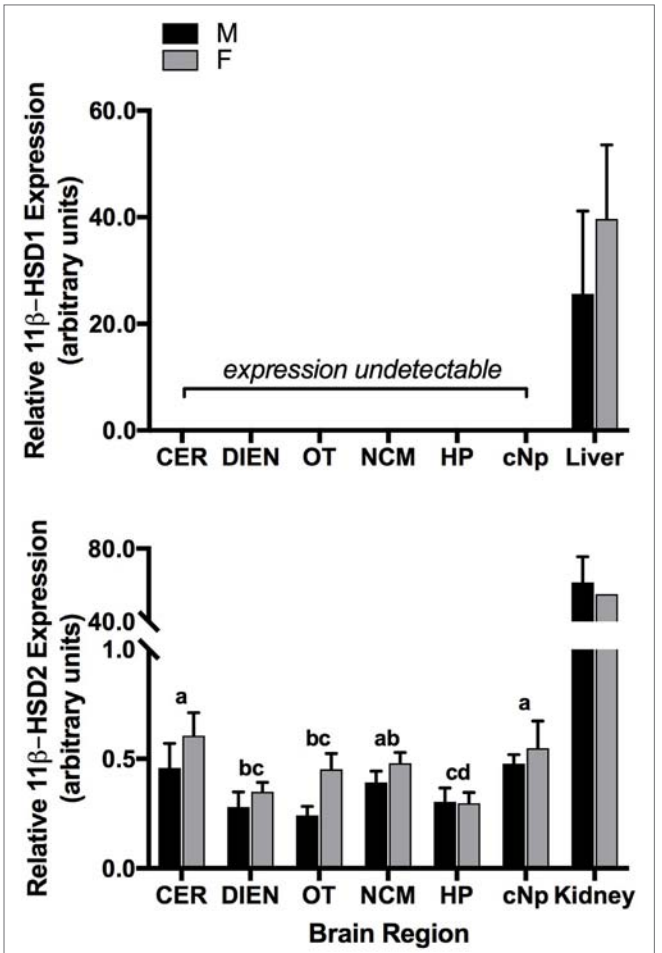

FIGURE 3 | Quantitative PCR results showing 11 beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (top panel) and 11 beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (bottom panel) expression patterns (relative to glyceraldehyde-3-phosphate dehydrogenase) across six brain regions in adult male (M) and female (F) zebra finches. Letters above bars represent significant differences. Positive control tissues are presented for comparison (11β-HSD1: liver; 11β-HSD2: kidney). Bars are means ± 1 SE. Sample sizes are as follows (*n* = 11β-HSD2): CER, cerebellum (17); DIEN, diencephalon (17); OT, optic tectum (17); NCM, caudomedial nidopallium (16); HP, hippocampus (17); cNp, caudal nidopallium (13).

Whereas expression levels were high in liver, we did not detect 11β-HSD1 expression in brain (**Figure 3**). Specifically, CT values were undetermined for all brain samples, indicating a lack of amplification within the 40-cycle qPCR program. The 11β-HSD1 primer concentrations (0.6 μM) were determined based on an optimization in liver, as previous validation attempts in brain with differing concentrations of primers and cDNA indicated low to no expression.

In contrast to 11β-HSD1, 11β-HSD2 was expressed in adult kidney and brain and differed significantly among brain regions ($F_{5,89} = 5.8$; $P < 0.001$). Levels were highest in CER, NCM, and cNp, followed by DIEN, OT, and HP (**Figure 3**). Expression levels did not vary between males and females ($F_{1,89} = 2.3$; $P = 0.136$) or according to time post-capture ($F_{1,89} = 0.2$; $P = 0.687$). There was no interaction between region and sex ($F_{5,84} = 0.8$; $P = 0.525$).

Coexpression Patterns across Brain Regions

Mean regional GR expression levels did not correlate with MR ($F_{1,4} = 0.1$; $P = 0.781$). If the two regions with exceptionally high MR and GR were removed, however (HP and CER), there was a strong and significant correlation between GR and MR in the remaining four regions ($F_{1,2} = 175.7$; $P = 0.006$; $R^2 = 0.99$; **Figure 4**). Similarly, regional 11β-HSD2 levels were not correlated with GR ($F_{1,4} = 5.6$; $P = 0.077$) or MR levels ($F_{1,4} = 0.02$; $P = 0.91$) when all regions were included in the analyses. However, exclusion of CER and HP resulted in a significant positive correlation between 11β-HSD2 and GR ($F_{1,2} = 126.1$; $P = 0.008$; $R^2 = 0.98$) and 11β-HSD2 and MR ($F_{1,2} = 64.5$; $P = 0.015$; $R^2 = 0.97$) among the remaining regions (**Figure 5**).

11β-HSD1 in Developing Zebra Finch Brain and Adult Kidney

After finding that 11β-HSD1 expression was absent in six brain regions of adults, but present in the liver (a major site of expression across taxa), we conducted an additional qPCR optimization to determine whether expression could be detected in a pool of TEL taken from hatchlings, fledglings, juveniles, and adults. We compared the CT value of this pool to those obtained from liver (all ages pooled) and kidney [an additional positive control tissue in rodents (33)]. Liver showed the highest signal, with CT values of ~24, followed by kidney (CTs ~27), followed by TEL (CTs ~30). Lower CT values indicate higher levels of expression in a sample. After sequencing, products from each of these pools correctly BLASTed to the 11β-HSD1 mRNA sequence in NCBI (see **Table 1** for details).

To further assess these findings in brain, we used qPCR to examine 11β-HSD1 expression in individual samples from all four age groups. Expression of 11β-HSD1 was detected in 2 of 3 hatchling whole TEL samples (the fourth hatchling sample was contaminated and omitted), 1 of 4 fledgling caudal TEL samples, 0 of 4 juvenile caudal TEL samples, and 0 of 4 adult caudal TEL samples. CT values for the two hatchlings were between 26 and 32, while all other amplification occurred at CT 35 or higher.

We further investigated the presence of 11β-HSD1 expression in these three brain samples by (1) running qPCR products on a gel and (2) sending samples for sequencing. Gel electrophoresis (5 µl product + 1 µl 6× loading dye; 2% gel) on the three brain samples with positive qPCR amplification revealed a single band at the expected size (~162 bp) for both hatchling samples and a faint band for the fledgling sample. Sequenced qPCR products from these three samples matched the 11β-HSD1 sequence in NCBI (using BLAST).

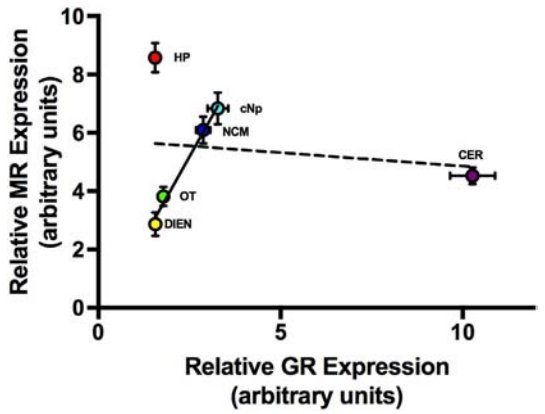

FIGURE 4 | The relationship between average (±1 SE) MR and GR expression levels across six brain regions. Dashed line = best fit line with all six brain regions included. Solid line = best fit line when CER and HP were excluded from the analysis. Abbreviations: CER, cerebellum; DIEN, diencephalon; OT, optic tectum; NCM, caudomedial nidopallium; HP, hippocampus; cNp, caudal nidopallium.

DISCUSSION

This study tested the hypothesis that the enzymes 11β-HSD1 and 11β-HSD2 are expressed in regions of the adult zebra finch brain with elevated sensitivity to GCs. Whereas widespread changes in GC exposure can be initiated by regulation of adrenal synthesis, secretion, and association with binding globulins, local regeneration and metabolism of GCs, respectively, may enable regions requiring precise GC regulation to control access to receptors on a fine scale (8). Our results in the finch vary significantly from expression patterns reported in rodents (15). In particular, we did not detect 11β-HSD1 expression in adult brain, though we confirmed expression of this enzyme in some hatchlings and fledglings. By contrast, 11β-HSD2 was expressed in all adult brain regions examined. In addition, in adult brains we observed distinct regional patterns of GR, MR, and 11β-HSD2 mRNA,

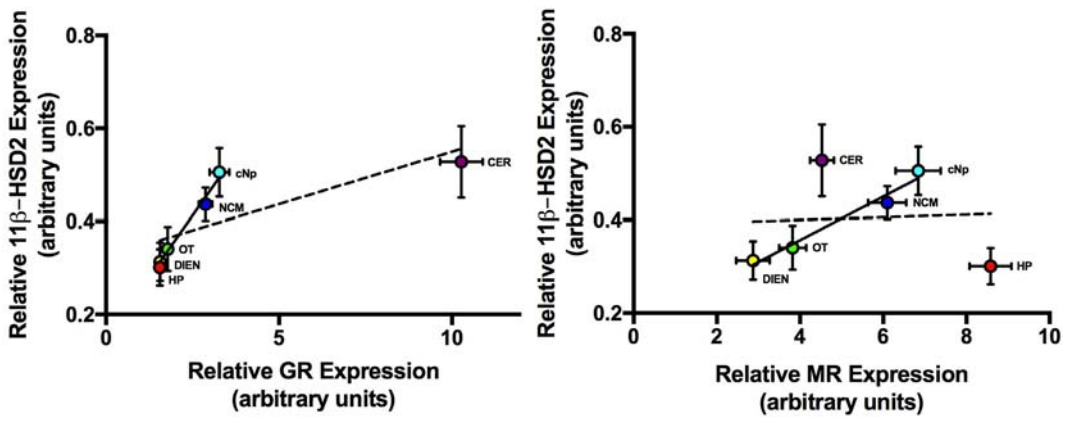

FIGURE 5 | The relationship between average (±1 SE) 11 beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2) and GR (left panel) and average 11β-HSD2 and MR (right panel) expression levels across six brain regions. Dashed line = best fit line with all six brain regions included. Solid line = best fit line when CER and HP were excluded from the analysis. Abbreviations: CER, cerebellum; DIEN, diencephalon; OT, optic tectum; NCM, caudomedial nidopallium; HP, hippocampus; cNp, caudal nidopallium; MR, mineralocorticoid receptor; GR, glucocorticoid receptor.

with highest GR expression in CER and highest MR expression in HP. Regional 11β-HSD2 patterns were similar but not identical to GR and MR patterns. This suggests that 11β-HSD2 plays a role in regulating both baseline and stress-induced CORT access to receptors, but that the nature of this activity varies according to brain region and context.

Regional GR and MR Expression Patterns

Differential expression of GR and MR enables the brain to utilize GCs for a diversity of functions. For example, the paraventricular nucleus of the hypothalamus (PVN), which expresses abundant GR in mammals (37–40), is a key site for stress-induced negative feedback on the HPA axis (41). Similarly, the mammalian HP expresses abundant MR and GR (37, 39, 40) and provides feedback regulation of HPA axis activity (42). The nature of central GR and MR expression and function has been well characterized in mammals (40). In the avian brain, our results on the distribution and abundance of GC receptors align with the handful of other studies, showing elevated GR in CER (30, 31, 35, 43) and MR in the HP [(28, 29, 32, 43); but see Ref. (30, 35)]. We add to these studies the description of relatively low levels of both MR and GR in the songbird DIEN, and relatively high levels in NCM and cNp.

The expression of HP MR and, to a lesser extent, GR suggests that the avian HP plays a similar role in HPA axis regulation in birds and mammals. MR activation in the HP by baseline systemic CORT levels maintains basal activity of the HPA axis through inhibitory projections to the hypothalamus (44). In addition, relatively high GR expression in CER, which we report here, has been observed in the granule and Purkinje cell layers of adult mammals (37, 38) and the external granule cell layer of neonatal mammals (45). We also detected higher CER GR expression in females than males, with no differences elsewhere in brain. The functional significance of this heightened CER sensitivity in females remains unknown. Our results from DIEN differ somewhat from previous observations. For example, several studies show a lack of MR expression in the avian hypothalamus (28, 30, 32) and the mammalian PVN (40), whereas others provide evidence for expression (24, 35). We detected relatively low amounts of both GR and MR in zebra finch DIEN, which includes the hypothalamus. These discrepancies likely result from differential sensitivity of qPCR vs *in situ* hybridization procedures, or because our sampling of the hypothalamus included additional diencephalic regions excluded from other studies.

GR, MR, and 11β-HSD2 Coexpression

Coordinated GC regulation across the brain is necessary for management of the diverse central functions of CORT (44). It is therefore not surprising that we observed a positive correlation between average GR and MR expression levels across several regions, where those with elevated MR also expressed elevated GR. The same pattern was observed when correlating GR or MR expression with 11β-HSD2 expression: regions with elevated GR or MR expression exhibited the highest 11β-HSD2 mRNA levels. This relationship, however, was not identified in all regions, notably the two regions expressing the highest levels of GR (CER) and MR (HP). While 11β-HSD2 expression was relatively elevated in CER, the degree of elevation did not match that of GR expression. In addition, while HP MR was elevated, 11β-HSD2 expression in this region was among the lowest detected. Taken together, these results suggest that the CER and HP may depend on unmodified systemic levels of GCs to initiate appropriate responses on their respective receptors, while other regions may rely on co-regulation by 11β-HSD2, GR, and MR. Future studies should probe the activity and regulation of 11β-HSD2 particularly within the HP and CER, as these remain sites of elevated GC sensitivity.

The HP of adult finches in this study exhibited a greater degree of MR expression relative to GR than other regions examined, while 11β-HSD2 expression remained low. These results may reflect the critical role of HP MR in providing negative feedback on the HPA axis under basal conditions, as MR is virtually absent from the PVN and pituitary, where stress-induced, GR-mediated feedback predominates (46). While 11β-HSD2 preserves MR access for aldosterone in the kidney (47), HP MR is not an aldosterone target, and 11β-HSD2 expression is therefore unnecessary to enable this function. In addition, it is likely that enzymatic metabolism of GCs *via* 11β-HSD2 in HP would be detrimental, as MR binding in this region provides overarching control over the day-to-day activity of the HPA axis. It is therefore not surprising that 11β-HSD2 transcript levels were relatively low in HP.

In contrast to our finding of elevated MR in HP, GR expression was relatively elevated in the CER. Although this relationship has been observed previously in both birds and mammals, its significance is unclear. Very high GR expression relative to other regions has been observed in the neonatal rat cerebellum (48), and exogenous GC treatments can reduce cerebellar volume and initiate long-term cognitive impairments in children (49, 50). The role of GR in the adult CER is less clear, although stress and GC treatments have been shown to impair motor function in adult rats (51). Interestingly, some mood disorders associated with HPA axis dysregulation (52, 53) have been linked to cerebellar atrophy (54). We detected elevated 11β-HSD2 in adult zebra finch CER, although levels were not as high as expected given the correlation between GR and 11β-HSD2 across other brain regions. This suggests that CORT access to GR plays an important role in the avian CER.

High GR expression in the adult CER, which governs motor function, may be partially responsible for rapid, non-genomic changes in activity observed after acute CORT treatment in songbirds. A single dose of GCs administered non-invasively increased perch hopping activity in white-crowned sparrows within 15 min [*Zonotrichia leucophrys gambelii* (55, 56)]. GCs may affect HP-based cognition as well as motor behavior, leading to rapid changes in cache recovery behavior in chickadees within 5 min of administration [*Parus gambeli* (57)]. Rapid, non-genomic GC effects across vertebrate taxa are likely initiated by membrane GC receptor binding (11). A lower-affinity GC membrane receptor has been characterized in house sparrow [*Passer domesticus* (10)] and zebra finch brain (12). In addition, recent work on membrane GC receptors in mammals suggests that a single gene is responsible for both cytosolic and membrane receptors (11, 58). Therefore, it is possible that our characterization

of GR and MR included membrane as well as intracellular GC receptor expression.

Although our study revealed expression patterns for several genes in the GC-signaling pathway, a full understanding of the interaction between GC-metabolizing enzymes and receptors requires assessing whether the molecules in these pathways are co-localized within individual neurons or glia. A higher resolution analysis might also be useful. In mammals, for example, 11β-HSD2 has been localized to the endoplasmic reticulum and the nucleus (59). Thus, cells expressing intracellular 11β-HSD2 might avoid effects of GC metabolism by expressing GR or MR receptors on the cell membrane. An investigation of the subcellular localization of 11β-HSD2 in songbird brain, along with a characterization of membrane GR and MR will provide a more conclusive picture of the nature of 11β-HSD2 expression and function in the songbird brain.

Functional Significance of 11β-HSD2 Expression in Brain

We observed 11β-HSD expression patterns that differ significantly from those in rodents. First, we found that 11β-HSD2 was expressed throughout the adult songbird brain, a finding that differs markedly from the adult rodent brain with its more limited distribution (15). This result builds on previous findings in our lab, in which 11β-HSD2 transcript, as well as dehydrogenase activity, was reported in hatchling and adult zebra finch brain (24, 25). Second, while we found robust expression of 11β-HSD1 in liver, expression was undetected in the adult zebra finch brain, though this enzyme is widely distributed in the brains of adult rodents (15). There are several possible explanations for this result. First, we did not attempt to quantify 11β-HSD1 expression in *all* brain regions of the adult zebra finch. Therefore, it is possible that other regions do express this enzyme. However, we noted an absence of 11β-HSD1 in the HP, a site of prominent expression in the rodent brain, which suggests a fundamental difference between birds and mammals. Another possibility is that differential splicing produces alternate transcripts in the zebra finch brain and liver. Future work will address this possibility by utilizing primers that target different regions of the gene. Finally, it is intriguing that we were able to detect 11β-HSD1 expression in the brains of very young finches. Such a result suggests that this enzyme could be developmentally down regulated. The role for this enzyme in developing brain is currently unknown, but future work should pinpoint the loci of expression in hatchlings and document more precisely its temporal patterns of expression.

Elevated expression of 11β-HSD2 in the developing rodent brain likely protects the growing brain from potential GC damage. This protection lasts through the first few weeks of neonatal life. This is clearly seen in the external granule cell layer of the cerebellum, where 11β-HSD2 reportedly protects neural progenitor cells from CORT-induced apoptosis, cerebellar atrophy, and developmental deficits (45, 60–62). These patterns in the developing mammal brain raise the question of whether 11β-HSD2 plays a similar role in the adult songbird brain, especially in the CER, cNp and NCM, regions where 11β-HSD2 transcript levels were highest. The HP, NCM and cNp are bordered by the VZ, where neurogenesis persists in adult songbirds (63). We found relatively high levels of GR, MR, and 11β-HSD2 expression in NCM and cNp, highlighting the potential importance of appropriate GC signaling in these areas. While adult neurogenesis in mammals is relatively restricted, it is conspicuous and widespread in songbirds and other taxa, including fish, reptiles, and amphibians (64, 65). GCs can impair hippocampal neurogenesis, depending on dose and context (23, 66–68). As 11β-HSD2 protects neurogenesis during mammalian fetal development (60, 69), it is plausible that the enzyme serves a similar function in adult songbirds, and potentially in fish, where 11β-HSD2 is also widely expressed in the adult brain (70).

The results of this study raise an important question: why are neural 11β-HSD1 and 2 expression patterns so different between rodents and songbirds? One possibility is that these taxa-specific patterns arose as a result of differences in HPA axis activity and regulation. For example, adult mice that are similar in body mass to songbirds have higher circulating baseline and stress-induced GCs, as well as higher brain GC levels when compared with zebra finches (71–73) (Rensel, unpublished data). Thus, one could speculate that mice would express higher levels of 11β-HSD2 to limit CORT exposure to the brain and potential damage to CORT-sensitive neural circuits. Obviously, this is not the case; instead, rodents display widespread expression of the GC-regenerating 11β-HSD1. A second possibility is that differences in neural GC metabolism could be accounted for by differences in MR or GR binding affinities in brain between rodents and songbirds. However, these receptors appear to be relatively conserved in their binding affinities in rodents and birds (10, 74). Interestingly, the mammalian and songbird GR respond quite similarly to GC agonists and antagonists, but some traditional MR agonists in mammals do not work well in songbirds (10), suggesting that there may be MR receptor differences which modify GC action on the receptor between species. Finally, this study and others suggest that neural MR is more widely expressed throughout the songbird brain than the rodent brain (10), providing support for the view that differential neural metabolism may exist to regulate GC action on MR instead of or in addition to GR. Thus, more work is needed to determine if GC actions on MR differ across taxa, possibly explaining differential 11β-HSD expression patterns.

Steroid hormone-binding globulins in the circulation also differ between birds and mammals. Mammals possess both corticosterone-binding globulin (CBG) and sex hormone-binding globulin (SHBG), the latter of which binds androgens and estrogens. By contrast, birds appear to lack SHBG, and instead some sex steroids also bind CBG [primarily androgens (75, 76)]. This difference may affect the amount of free CORT accessible to the brain, which in turn could necessitate neural GC regeneration or metabolism. For example, rodent CBG may bind a greater proportion of circulating GCs, necessitating 11β-HSD 1-based regeneration in brain, whereas free CORT may more easily reach the brain of the songbird because a greater portion of circulating GCs is unbound. However, while CBGs are thought to prevent access to target tissues (77), some studies suggest that CBGs are actually necessary for GC delivery to the brain, making any conclusions preliminary at this time (78). In the end, a vital difference between the brains of rodents and

songbirds remains the widespread neuroplasticity that persists into adulthood in the songbird brain and which is present to a much smaller degree in rodents (8). Given the capacity for GCs to influence such plasticity, it seems likely that the expression of CORT metabolic enzymes in the adult avian brain is related to this inherent neuroplasticity.

Overall, our results highlight the complexity of GC signaling in the songbird brain. It is likely that 11β-HSD2 protects sensitive neural circuits from GC access to GR and MR, but the timing and localization of this activity undoubtedly depends on region and dose-specific effects. In addition, it is worthwhile to note that quantification of mRNA expression does not necessarily equate to presence of protein, as Medina et al. (79) documented a disparity between GR and MR mRNA and cytosolic protein in house sparrow brain. Rapid modulation of enzyme activity through post-translational modifications is also likely and may provide an additional layer of control over GC metabolism and action in the songbird brain [e.g., Ref. (80–82)]. Future studies will seek to elucidate the functional importance of 11β-HSD2 in those regions in which it is expressed.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the UCLA Chancellor's Animal Research Committee. The protocol was approved by the Chancellor's Animal Research Committee.

AUTHOR CONTRIBUTIONS

MR and BS conceived and designed the study and wrote the manuscript. MR, JD, and DP conducted the experiments. MR analyzed the data. All the authors provided feedback on the manuscript.

ACKNOWLEDGMENTS

The authors thank Rice Zhang and the UCLA Division of Laboratory Animal Management for their excellent care of the finch colony. This work was supported by NIH R03 grant 5R03MH108921 awarded to MR and BS.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 28 March 2018
doi: 10.3389/fendo.2018.00139

Identification of Transmembrane Protease Serine 2 and Forkhead Box A1 As the Potential Bisphenol A Responsive Genes in the Neonatal Male Rat Brain

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Edited by:
Jae Young Seong, Korea University, South Korea

Reviewed by:
Leo T. O. Lee, University of Macau, China
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *06 December 2017*
Accepted: *15 March 2018*
Published: *28 March 2018*

Citation:
Ubuka T, Moriya S, Soga T and Parhar I (2018) Identification of Transmembrane Protease Serine 2 and Forkhead Box A1 As the Potential Bisphenol A Responsive Genes in the Neonatal Male Rat Brain. Front. Endocrinol. 9:139. doi: 10.3389/fendo.2018.00139

Perinatal exposure of Bisphenol A (BPA) to rodents modifies their behavior in later life. To understand how BPA modifies their neurodevelopmental process, we first searched for BPA responsive genes from androgen and estrogen receptor signaling target genes by polymerase chain reaction array in the neonatal male rat brain. We used a transgenic strain of Wistar rats carrying enhanced green fluorescent protein tagged to gonadotropin-inhibitory hormone (GnIH) promoter to investigate the possible interaction of BPA responsive genes and GnIH neurons. We found upregulation of transmembrane protease serine 2 (*Tmprss2*), an androgen receptor signaling target gene, and downregulation of Forkhead box A1 (*Foxa1*), an ER signaling target gene, in the medial amygdala of male rats that were subcutaneously administered with BPA from day 1 to 3. Tmprss2-immunoreactive (ir) cells were distributed in the olfactory bulb, cerebral cortex, hippocampus, amygdala, and hypothalamus in 3 days old but not in 1-month-old male rats. Density of Tmprss2-ir cells in the medial amygdala was increased by daily administration of BPA from day 1 to 3. Tmprss2 immunoreactivity was observed in 26.5% of GnIH neurons clustered from the ventral region of the ventromedial hypothalamic nucleus to the dorsal region of the arcuate nucleus of 3-day-old male rat hypothalamus. However, *Tmprss2* mRNA expression significantly decreased in the amygdala and hypothalamus of 1-month-old male rats. *Foxa1* mRNA expression was higher in the hypothalamus than the amygdala in 3 days old male rats. Intense Foxa1-ir cells were only found in the peduncular part of lateral hypothalamus of 3-day-old male rats. Density of Foxa1-ir cells in the hypothalamus was decreased by daily administration of BPA from day 1 to 3. *Foxa1* mRNA expression in the hypothalamus also significantly decreased at 1 month. These results suggest that BPA disturbs the neurodevelopmental process and behavior of rats later in their life by modifying *Tmprss2* and *Foxa1* expressions in the brain.

Keywords: transmembrane protease serine 2, forkhead box A1, androgen receptor, estrogen receptor, polymerase chain reaction array, gonadotropin-inhibitory hormone neurons

INTRODUCTION

Bisphenol A (BPA) is an organic synthetic compound widely used to make polycarbonate plastics and epoxy resins utilized in reusable food and drink containers and inner lining of cans and bottles (1). Hydrolysis of the ester-bond linking BPA can occur at high temperature, and BPA can be leached out into food and beverages. Fetuses or young animals during their developmental stages are thought to be susceptible to BPA exposure than adults (2). Therefore, detectable level of BPA in 88% of human cord blood samples (3) generated social concerns about the effect of BPA on the fetuses.

Dodds and Lawson were the first to show the estrogenic property of BPA (4). However, the estrogenic effect of BPA is 10,000 times less potent than estradiol-17β shown by uterine vascular permeability assay in ovariectomized mice (5). Similar level of agonistic activity of BPA to estrogen receptor (ER) was shown in yeast expressing human estrogen or androgen receptor (AR) (6). On the other hand, BPA is a potent ligand for the non-classic membrane bound G protein-coupled receptor for estrogen (GPR30) (7, 8). BPA did not have an agonistic activity but had an antagonistic activity to AR in the same yeast-based assay (6). Stronger antagonistic activity of BPA to human AR was shown in an African monkey kidney cell line (9). It is also known that BPA can disturb steroidogenesis by interfering the activity of steroidogenic enzymes, such as CYP450scc, 3βHSD, and CYP450arom (10). Perera et al. (11) examined the association between prenatal BPA exposure and child behavior. They found that high prenatal BPA exposure was associated with higher emotionally reactive and aggressive behavior syndromes in boys and lower anxious/depressed and aggressive behavior in girls (11). There are numerous studies showing the effect of perinatally administered BPA on social behavior of rodents. BPA exposure during pregnancy increased display of nose-to-nose contacts, play solicitations, and approaches in both sexes (12). Administration of BPA during pregnancy or lactation increased defensive behavior in male and sexual behavior in female rats (13). In the other study, BPA exposed males during gestation and lactation showed persistent deficits in sexual behavior in adulthood (14).

To find BPA responsive genes in the brain mediating its effect on social behavior, we first focused on amygdala, because amygdala is the integrative center for the processing of emotion, which is pivotal for social behavior. It is thought that deficits in the development of amygdala may cause autism, a neurodevelopmental disorder that is characterized by impaired social interaction (the amygdala theory of autism) (15). It is also known that these brain nuclei express ARs and ERs and abnormally enlarge in autism infants (16). We subcutaneously administered a reference dose of BPA (50 μg/kg BW/day) determined by United States Environmental Protection Agency (EPA, www.epa.gov/iris/subst/0356.htm) to neonatal male transgenic rats carrying enhanced green fluorescent protein tagged to gonadotropin-inhibitory hormone [EGFP-GnIH (17)] promoter from day 1 to 3 and differential expressions of AR and ER signaling target genes in the medial amygdala were analyzed by polymerase chain reaction (PCR) array. GnIH is a hypothalamic neuropeptide that decreases gonadotropin secretion directly acting on the pituitary or by decreasing the activity of gonadotropin-releasing hormone (GnRH) neurons (18, 19). Recent studies have shown that GnIH neurons also decrease motivated behavior (20, 21). We found upregulation of transmembrane protease serine 2 (*Tmprss2*) and downregulation of Forkhead box A1 (*Foxa1*) mRNAs in the amygdala by BPA exposure. We further studied the location of Tmprss2 and Foxa1 immunoreactive (ir) cells, effect of BPA on the density of Tmprss2 and Foxa1-ir cells and developmental changes in *Tmprss2* and *Foxa1* mRNA expressions in the male rat brain.

MATERIALS AND METHODS

Animals

EGFP-GnIH Wistar rats (17) were housed under a controlled 12 h light/dark cycle (light on at noon) and the temperature was maintained at 22°C in a specific pathogen free animal facility. All rats had free access to autoclaved food and water. The pups were counted after parturition, weighed, and remained with their biological mother. All procedures were approved by Monash University, Animal Ethics Committee (MARP/2016/037).

PCR Array

Bisphenol A at 50 μg/kg or vehicle (sesame oil) was subcutaneously injected to neonatal male rats after dawn daily from postnatal day 1 (P1) to P3. 2 h after the last injection the rats were deeply anesthetized by Zoletil/Ketamine/Xylazine (Z/K/X) at 13.5 mg/90 μl/kg and brains were collected and stored at −80°C. Medial amygdaloid tissue from both sides of the brain was dissected in a cryostat at −20°C by referring to a rat brain atlas (22). Medial amygdaloid tissues from two male siblings were combined in a tube for homogenization. Total medial amygdaloid tissue samples of six BPA treated rats from three different littermates and six vehicle treated rats from three different littermates were collected. Total RNA was extracted by using an RNA isolation kit (RNeasy mini kit; QIAGEN, Hilden, Germany) and reverse transcribed by using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher SCIENTIFIC, Waltham, MA, USA). Differential gene expression was analyzed by PCR arrays, RT^2 Profiler™ PCR Array Rat AR Signaling Targets, and RT^2 Profiler™ PCR Array Rat ER Signaling Targets (QIAGEN). These PCR array kits analyze the expression levels of 84 AR signaling target genes (Table S1 in Supplementary Material) and 84 ER signaling target genes (Table S2 in Supplementary Material). Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2 (Cited2, NM_053698), insulin-like growth factor binding protein 5 (Igfbp5, NM_012817), kallikrein B plasma 1 (Klkb1, NM_012725), and myelocytomatosis oncogene (Myc, NM_012603) were analyzed in both assays. Real-time PCR was performed using a StepOnePlus™ Real-Time PCR System (ThermoFisher SCIENTIFIC) with conditions of 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min, followed by a dissociation step according to the manufacturer's instruction. Expression levels of AR signaling target genes were normalized by the mean expression levels of housekeeping genes (HKGs), actin beta (Actb, NM_031144), Hypoxanthine

TABLE 1 | Effect of Bisphenol A (BPA) on androgen receptor (AR) and estrogen receptor (ER) signaling target gene expression in the medial amygdala of neonatal male rats.

AR signaling target genes			ER signaling target genes		
Gene symbol	**Fold changes**	***P* value**	**Gene symbol**	**Fold changes**	***P* value**
Abcc4	1.10	0.627	Adora1	1.09	0.735
Abhd2	1.12	0.508	Ahr	1.11	0.837
Acsl3	0.99	0.913	Akap1	1.23	0.424
Adamts1	0.91	0.751	Apbb1	1.11	0.665
Aldh1a3	0.89	0.646	Bcar1	1.13	0.634
Appbp2	0.99	0.944	Bcl2l1	1.08	0.634
Ar	0.95	0.434	Bdnf	1.07	0.810
Atad2	1.13	0.655	Bmp4	0.89	0.673
Camkk2	1.14	0.191	Bmp7	6.46	0.585
Cenpn	0.98	0.948	Brca1	0.62	0.0541
Cited2	1.11	0.696	C3	1.00	0.925
Ackr3	1.01	0.929	Cav1	1.06	0.719
Cyp2u1	1.00	0.897	Ccl12	0.87	0.916
Dhcr24	1.21	0.387	Ccnd1	0.98	0.944
Eaf2	0.56	0.0645	Cited2	1.26	0.526
Elk1	1.14	0.391	Ckb	0.97	0.853
Ell2	1.01	0.935	Ctgf	0.76	0.359
Ern1	1.21	0.392	Ctsd	1.06	0.899
Errfi1	1.16	0.564	Cyp19a1	1.01	0.975
Fam105a	0.99	0.832	Cyp1a1	–	–
Fkbp5	1.08	0.571	Ebag9	15.05	0.266
Fos	0.80	0.252	Efna5	1.27	0.257
Fzd5	0.99	0.795	Egr3	0.88	0.651
Gucy1a3	1.13	0.222	Erbb2	1.19	0.521
Herc3	1.01	0.974	Erbb3	1.52	0.992
Hpgd	1.17	0.482	Esr1	1.21	0.515
Igf1r	1.10	0.564	Esr2	0.86	0.606
Igfbp5	0.96	0.753	Fos	0.79	0.351
Irs2	1.08	0.733	Foxa1	0.32	0.0147*
Jun	1.08	0.639	Fst	0.97	0.814
Klk1c2	2.43	0.135	G6pd	1.11	0.599
Klk4	0.95	0.994	Gper1 G	1.06	0.791
Klkb1	–	–	Hsp90aa1	0.96	0.731
Krt8	1.06	0.931	Igf1	0.86	0.503
Lama1	0.90	0.304	Igfbp4	1.51	0.164
Lifr	0.90	0.545	Igfbp5	0.98	0.867
Lrig1	1.00	0.975	Irs1	1.12	0.629
Lrrfip2	1.03	0.854	Junb	0.99	0.790
Maf	1.09	0.692	Klkb1	–	–
Map7d1	1.01	0.926	L1cam	1.02	0.994
Mme	1.00	0.957	Lgals1	1.01	0.883
Mt2A	1.15	0.563	Lpl	1.02	0.890
Myc	1.23	0.286	Ltbp1	0.89	0.549
Ncapd3	1.00	0.990	Maff	0.71	0.473
Ndrg1	1.27	0.127	Med1	1.07	0.844
Nfkb1	1.05	0.866	Mmp9	2.81	0.357
Nfkb2	0.95	0.816	Mta1	1.06	0.678
Nfkbia	1.01	0.773	Myc	1.33	0.348
Nkx3-1	0.64	0.102	Nab2	1.28	0.685
Orm1	–	–	Ncoa2	1.01	0.979
Pak1ip1	1.11	0.0870	Ncoa3	1.01	0.969
Pgc	–	–	Ncor1	0.81	0.285
Pias1	1.10	0.789	Ncor2	0.98	0.888
Pik3r3	1.07	0.794	Nov	0.89	0.450
Pmepa1	1.14	0.489	Nr0b1	0.84	0.534
Ppap2a	0.92	0.341	Nr0b2	–	–
Rab4a	0.99	0.901	Nr2f6	1.07	0.787
Rel	0.80	0.131	Nr3c1	0.93	0.775
Rela	0.97	0.788	Nr5a2	1.33	0.703
Ripk4	1.01	0.888	Nrip1	1.21	0.467
Sgk1	1.18	0.369	Nrp1	1.39	0.285
Slc26a2	1.04	0.801	Pdzk1	1.02	0.987
Slc45a3	0.75	0.509	Pelp1	0.95	0.650
Sms	0.98	0.685	Pgr	0.71	0.397
Snai2	0.79	0.328	Phb2	1.16	0.178
Sord	0.97	0.896	Ptch1	1.14	0.725
Sp1	1.06	0.692	Ptgs2	1.43	0.504
Spdef	1.15	0.475	Rala	1.06	0.587
Srf	1.12	0.564	Rara	1.27	0.548
Steap4	0.61	0.302	S100a6	0.87	0.539
Stk39	0.93	0.560	Safb	1.05	0.831
Tbc1d8	1.01	0.876	Snai1	1.15	0.725
Tiparp	1.56	0.988	Socs3	1.04	0.885
Tmprss2	2.17	0.0449*	Spp1	0.74	0.650
Tpd52	1.03	0.862	Tff1	–	–
Trib1	1.09	0.715	Tgfa	0.99	0.993
Tsc22d1	1.03	0.370	Tgfb3	1.16	0.466
Tsc22d3	1.12	0.587	Thbs1	1.05	0.861
Vapa	0.99	0.942	Vdr	1.15	0.686
Vipr1	0.96	0.821	Vegfa	1.14	0.719
Wipi1	1.08	0.655	Wisp2	1.01	0.974
Zbtb10	1.10	0.305	Wnt4	0.99	0.997
Zbtb16	0.99	0.889	Wnt5a	1.04	0.804
Zfp189	1.02	0.925	Xbp1	1.07	0.808

Fold changes: ratio of the average of BPA group (n = 3, two siblings in one sample) to control group (n = 3, two siblings in one sample).

–, under detectable.

**P < 0.05 by Student's t-test.*

phosphoribosyltransferase 1 (Hprt1, NM_012583), lactate dehydrogenase A (Ldha, NM_017025), and ribosomal protein large P1 (Rplp1, NM_001007604) using the ΔΔCt method. Expression levels of ER signaling target genes were normalized by the mean expression levels of Hprt1 and Rplp1, which were selected from the HKGs by the software based on their consistent expression levels within all samples. Ct cut-off was set to 35 in both assays and treated as undetectable.

Real-Time PCR

Five P3 male brains and five P35 male brains were collected under deep anesthesia by Z/K/X at 13.5 mg/90 μl/kg and stored at −80°C. Amygdala, hypothalamus, and telencephalon excluding amygdala and hippocampus were collected in a cryostat at −20°C. Tissues were homogenized in TRIzol™ Reagent (ThermoFisher SCIENTIFIC) and total RNA was extracted by chloroform. Total RNA was reverse transcribed by using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher SCIENTIFIC). Expression levels of *Rattus norvegicus* Tmprss2 mRNA (NM_130424.3) and Foxa1 mRNA (NM_012742.1) were measured using Rplp1 mRNA (NM_001007604.2) as a reference HKG. Real-time PCR was performed using SensiFAST SYBR Master Mix (BioLine Reagent, London, United Kingdom) and primers (Tmprss2 forward: 5′-CACCTGCCATCCACATACAG-3′, reverse: 5′-CCAGAACTTCCAAAGCAAGC-3′; Foxa1 forward:

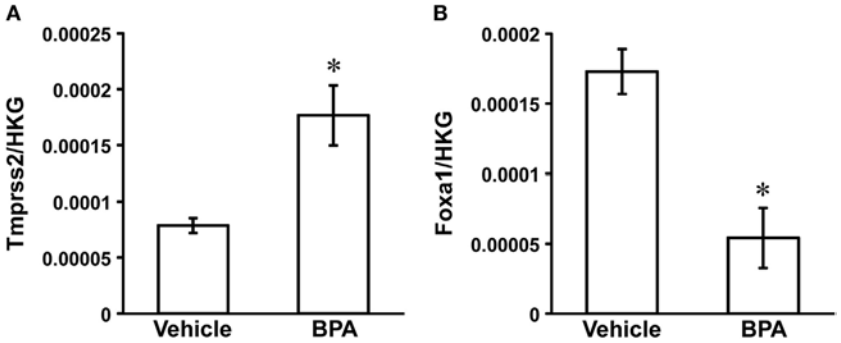

FIGURE 1 | Effect of Bisphenol A (BPA) administration on Tmprss2 and Foxa1 gene expression in the neonatal male rat medial amygdala. **(A)** Effect of BPA administration on the ratio of Tmprss2 gene expression level relative to housekeeping gene (HKG). Each column and the vertical line represent the mean ± SEM ($n = 3$ samples, both sides of medial amygdala of two male siblings in one sample). *$P < 0.05$, vehicle vs. BPA by Student's t-test. **(B)** Effect of BPA administration on the ratio of Foxa1 gene expression level relative to HKG. Each column and the vertical line represent the mean ± SEM ($n = 3$ samples, both sides of medial amygdala of two male siblings in one sample). *$P < 0.05$, vehicle vs. BPA by Student's t-test.

5′-GGAGGCCTACTCCTCTGTCC-3′, reverse: 5′-TTGGCGTA GGACATGTTGAA-3′; Rplp1 forward: 5′-GACGGTCACGGA GGATAAGA-3′,reverse:5′-GCAGATGAGGCTTCCAATGT-3′). Real-time PCR was performed using a StepOnePlus™ Real-Time PCR System (ThermoFisher SCIENTIFIC) with conditions of 95°C for 2 min, 40 cycles of 95°C for 5 s, and 60°C for 30 s, followed by a dissociation step. The levels of each mRNA were normalized to Rplp1 mRNA using the ΔΔCt method.

Immunohistochemistry and Fluorescent Microscopy

Immunohistochemistry was performed to investigate the location of Tmprss2 and Foxa1 protein in P3 male rat brains using rabbit monoclonal anti-TMPRSS2 antibody (EPR3861; abcam, Cambridge, United Kingdom) and anti-FOXA1 antibody (EPR10881: abcam). Briefly, five P3 male rats' brains were collected in 4% paraformaldehyde (PFA) under deep anesthetized by Z/K/X at 13.5 mg/90 µl/kg. After 5 days in 4% PFA at 4°C, brains were soaked in 30% sucrose in 0.1 M phosphate buffer at 4°C until they sank. Brains were kept at −80°C until sectioning at 20 µm thickness on a cryostat at −20°C. Sections were incubated in 0.3% H_2O_2 in 20% methanol in 0.01 M phosphate buffered saline (PBS; pH 7.0) for 20 min to suppress endogenous peroxidase activity. Sections were then washed three times in PBS and incubated overnight at 4°C in the primary antibody at concentrations of 1:300 for anti-TMPRSS2 antibody and 1:200 for anti-FOXA1 antibody in blocking solution (0.5% Triton X, 2% normal goat serum in PBS). The next day, three subsequent washes in PBS were followed by incubation in biotinylated goat anti-rabbit IgG at 1:500 in blocking solution for 40 min. After the sections were washed in PBS three times, they were then incubated for 45 min in avidin–biotin complex (Vectastain Elite Kit, Vector, Burlingame, CA, USA) in blocking solution. The resulting complex was visualized by 3,3-diaminobenzidine after the sections were washed three times in PBS and rinsed in 0.05 M Tris–HCl buffer (pH 7.5). Immunohistochemistry without the primary antibodies served as control. The location of the immunoreactivities was identified by Nissl staining of the adjacent sections.

Number of Tmprss2-ir cells in the medial amygdala was counted in three 100 µm grids and averaged for each P3 male rat that were subcutaneously injected with BPA at 50 µg/kg/day ($n = 5$) or vehicle ($n = 4$) from P1 to P3. Number of Foxa1-ir cells in the hypothalamus was counted in three 100 µm grids and averaged for each P3 male rat that were subcutaneously injected with BPA at 50 µg/kg/day ($n = 4$) or vehicle ($n = 4$) from P1 to P3.

Co-localization of Tmprss2 or Foxa1 immunoreactivity and GnIH-EGFP was studied using a fluorescent microscope with bright field function. The secondary antibody (biotinylated goat anti-rabbit IgG) for immunohistochemistry was used at 1:200 to investigate the co-localization of Tmprss2 or Foxa1 and GnIH-EGFP.

Statistics

Differential expression of AR and ER signaling target genes in the medial amygdala, number of Tmprss2-ir cells in the medial amygdala, and number of Foxa1-ir cells in the hypothalamus between BPA administered male rats and control rats were analyzed by Student's t-test. Differential expression of Tmprss2 and Foxa1 in the amygdala, hypothalamus, and telencephalon excluding amygdala and hippocampus in P3 and P35 male rat brains was analyzed by one-way ANOVA followed by Fisher's protected least significant difference test.

RESULTS

Effect of BPA on AR and ER Signaling Target Gene Expression in the Medial Amygdala of Neonatal Male Rats

Differential expression of AR and ER signaling target genes in the medial amygdala by BPA administration was analyzed in neonatal male rats. Klkb1 (NM_012725), orosomucoid 1 (Orm1, NM_053288), and progastricsin (pepsinogen C) (Pgc, NM_133284) were undetectable within the 84 AR signaling target genes, and cytochrome P450 family 1 subfamily a polypeptide 1 (Cyp1a1, NM_012540), Klkb1 (NM_012725), nuclear receptor subfamily 0 group B member 2 (Nr0b2, NM_057133) were

undetectable within the 84 ER signaling target genes (**Table 1**). Genes analyzed in both assays (Cited2, Igfbp5, Klkb1, Myc) produced similar results in terms of detectability, fold changes by BPA treatment, and *P* values by Student's *t*-test (**Table 1**).

An AR signaling target gene, Tmprss2 (NM_130424) was the only gene that was significantly increased in the medial amygdala of neonatal male rats by BPA treatment (fold change 2.17, $P = 0.0449$; **Table 1**; **Figure 1A**). An ER signaling target gene, Foxa1 (NM_012742) was the only gene that decreased significantly in the medial amygdala of neonatal male rats by BPA treatment (fold change 0.32, $P = 0.0147$; **Table 1**; **Figure 1B**).

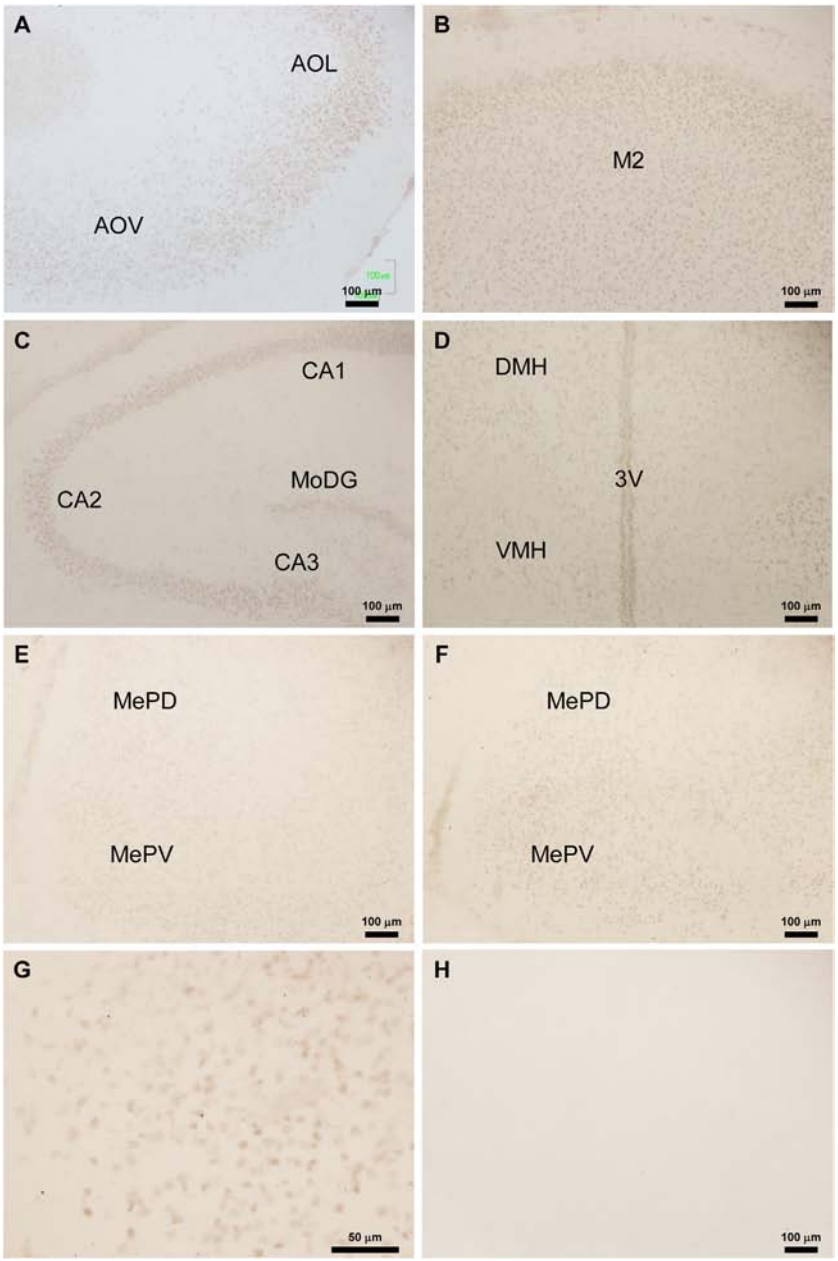

FIGURE 2 | Location of Tmprss2-immunoreactive cells in the neonatal male rat brain. **(A)** Tmprss2-immunoreactive (ir) cells in the olfactory bulb of Bisphenol A (BPA) treated P3 rat. AOL, anterior olfactory nucleus, lateral part. AOV, anterior olfactory nucleus, ventral part. **(B)** Tmprss2-ir cells in the secondary motor cortex (M2) of BPA treated P3 rat. **(C)** Tmprss2-ir cells in the hippocampus of BPA treated P3 rat. CA1, field CA1 of the hippocampus; CA2, field CA2 of the hippocampus; CA3, field CA3 of the hippocampus; MoDG, molecular layer of the dentate gyrus. **(D)** Tmprss2-ir cells in the hypothalamus of BPA treated P3 rat. DMH, dorsomedial hypothalamic area; VMH, ventromedial hypothalamic area; 3V, third ventricle. **(E)** Tmprss2-ir cells in the amygdala of vehicle treated P3 rat. MePD, medial amygdaloid nucleus, posterodorsal part; MePV, medial amygdaloid nucleus, posteroventral part. **(F)** Tmprss2-ir cells in the amygdala of BPA treated P3 rat. **(G)** Higher magnification of MePV of BPA treated P3 rat **(F)**. **(H)** Immunohistochemistry without the first antibody showed no immunoreactive cellular structure.

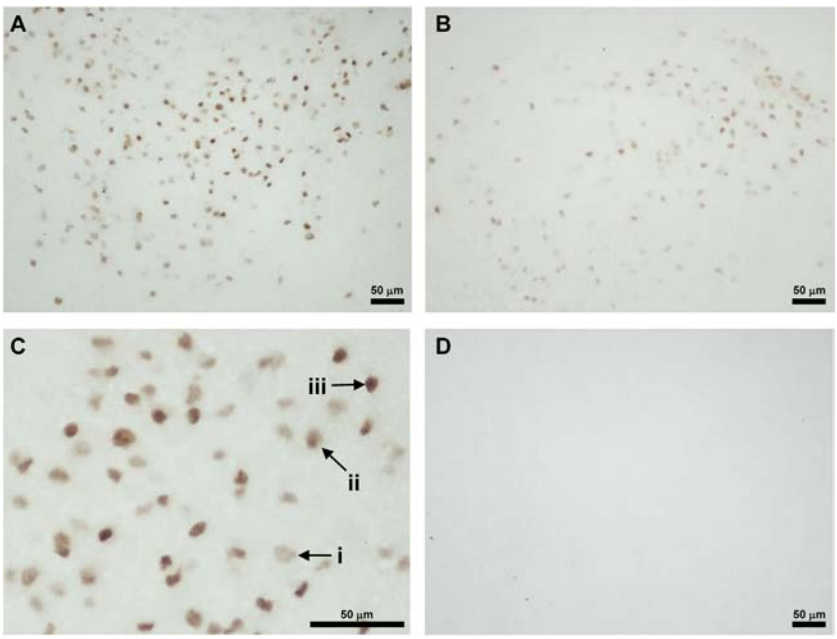

FIGURE 3 | Location of Foxa1-immunoreactive cells in the neonatal male rat brain. **(A)** Foxa1-ir cells in the peduncular part of lateral hypothalamus (PLH) of vehicle treated P3 rat. **(B)** Foxa1-ir cells in the PLH of Bisphenol A treated P3 rat. **(C)** Higher magnification of Foxa1-ir cells in the PLH of vehicle treated P3 rat **(A)**. (i) A cell showing even staining in the cytoplasm and the nucleus. (ii) A cell showing darker staining in the nucleus than the cytoplasm. (iii) A cell showing intense staining only in the nucleus. **(D)** Immunohistochemistry without the first antibody showed no immunoreactive cellular structure.

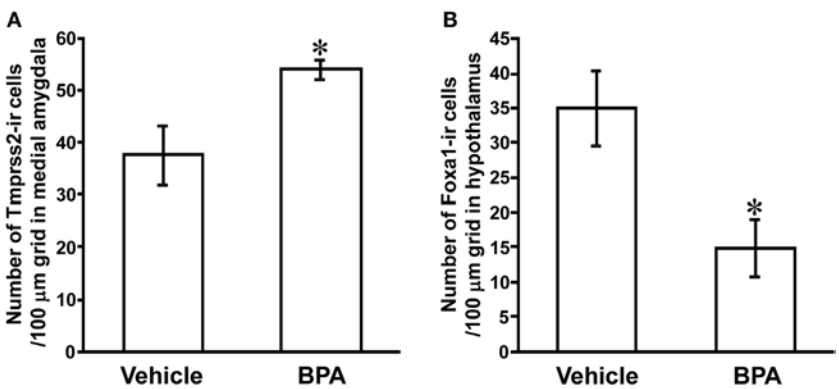

FIGURE 4 | Effect of Bisphenol A (BPA) administration on the number of Tmprss2-ir cells in the medial amygdala and Foxa1-ir cells in the hypothalamus of neonatal male rat. **(A)** Effect of BPA administration on the number of Tmprss2-ir cells in 100 μm grid in the medial amygdala. Each column and the vertical line represent the mean ± SEM. *$P < 0.05$, vehicle ($n = 4$) vs. BPA ($n = 5$) by Student's *t*-test. **(B)** Effect of BPA administration on the number of Foxa1-ir cells in 100 μm grid in the hypothalamus. Each column and the vertical line represent the mean ± SEM. *$P < 0.05$, vehicle ($n = 4$) vs. BPA ($n = 4$) by Student's *t*-test.

Distribution of Tmprss2 and Foxa1 Immunoreactive Cell Bodies in Neonatal Male Rat Brain

Location of Tmprss2 and Foxa1-ir cell bodies in P3 male rat brains was analyzed by immunohistochemistry. Abundant Tmprss2-ir cell bodies were found in the olfactory bulb (**Figure 2A**), cerebral cortex (**Figure 2B**), hippocampus (**Figure 2C**), hypothalamus (**Figure 2D**), and amygdala (**Figures 2E–G**). Spindle-like Tmprss2-ir cell bodies of about 10 μm in diameter (**Figure 2G**) were consistently found in all brain areas. The weak blur staining of Tmprss2-ir cell bodies (**Figure 2G**) suggested that Tmprss2 exists in neuronal cellular membrane. Immunohistochemistry without the first antibody served as control (**Figure 2H**).

Foxa1-ir cell bodies were only found in the peduncular part of lateral hypothalamus (PLH) (**Figures 3A–C**). Three kinds of staining were observed. The first type of staining was even through the cytoplasm and the nucleus, and the cellular diameter was about 10 μm (**Figure 3C**, i). The second type of staining was a weak staining in the cytoplasm and darker staining in the nucleus of about 7 μm in

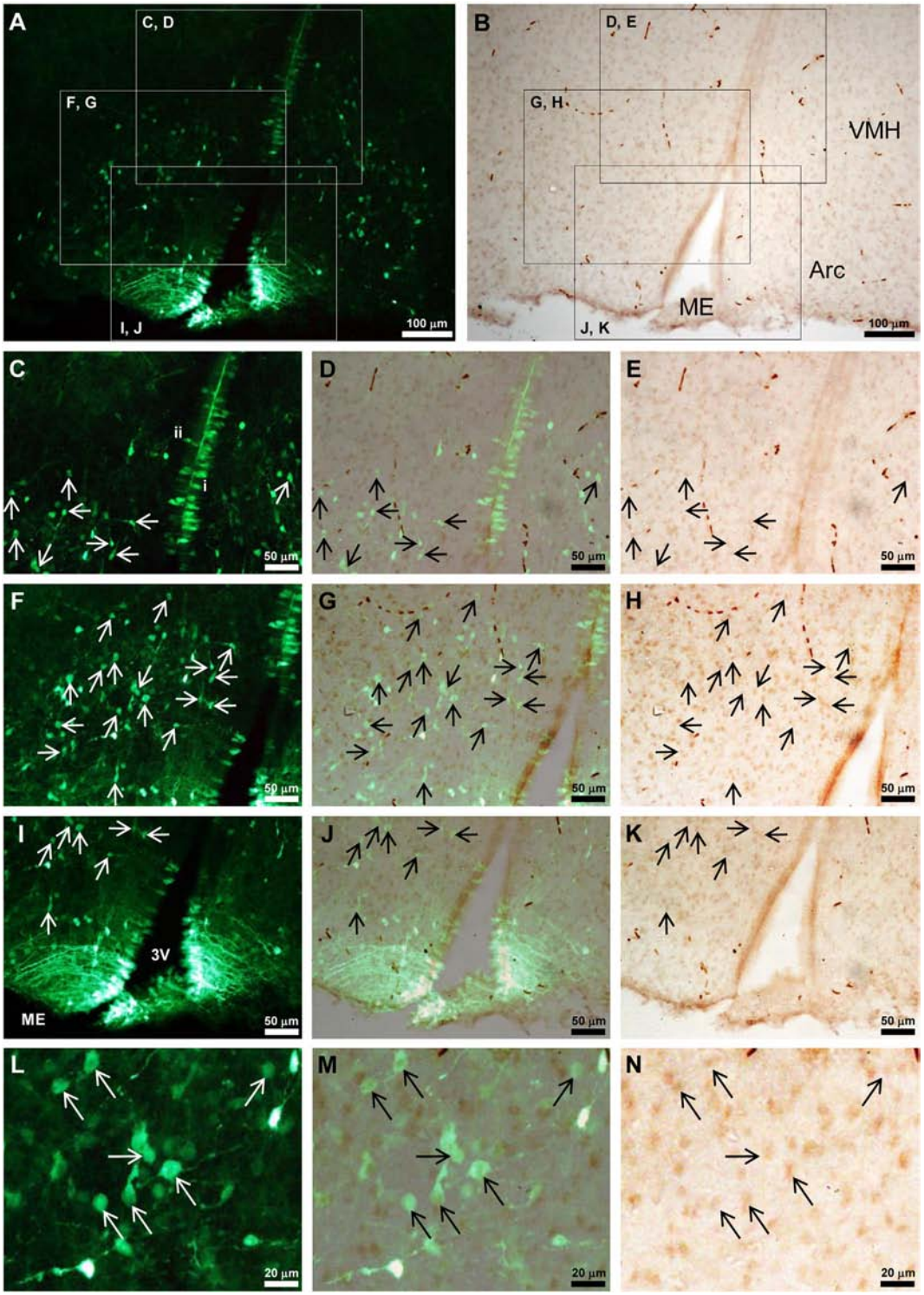

FIGURE 5 | Tmprss2-immunoreactive EGFP-GnIH neurons in the hypothalamus of neonatal male rat. **(A,B)** EGFP-GnIH neurons **(A)** and Tmprss2-immunoreactive (ir) cells **(B)** in the identical field and focus of hypothalamus. VMH, ventromedial hypothalamic area; Arc, arcuate hypothalamic nucleus; ME, median eminence. **(C–E)** Higher magnification of highlighted area in panel **(A,B)** with identical focus. Panel **(D)** is the merged image of **(C,E)**. Arrows show Tmprss2-ir EGFP-GnIH neurons. [**(C)** i] An example of ventricle contacting rod-like EGFP-GnIH cell. [**(C)** ii] An example of EGFP-GnIH cell projecting its process to the ventricle. **(F–H)** Higher magnification of highlighted area in panel **(A,B)** with identical focus. Panel **(G)** is the merged image of **(F,H)**. Arrows show Tmprss2-ir EGFP-GnIH neurons. **(I–K)** Higher magnification of highlighted area in panel **(A,B)** with identical focus. Panel **(J)** is the merged image of **(I,K)**. Arrows show Tmprss2-ir EGFP-GnIH neurons. 3V, third ventricle. **(L–N)** Higher magnification of the central area in panel **(F–H)** with identical focus. Arrows show Tmprss2-ir EGFP-GnIH neurons.

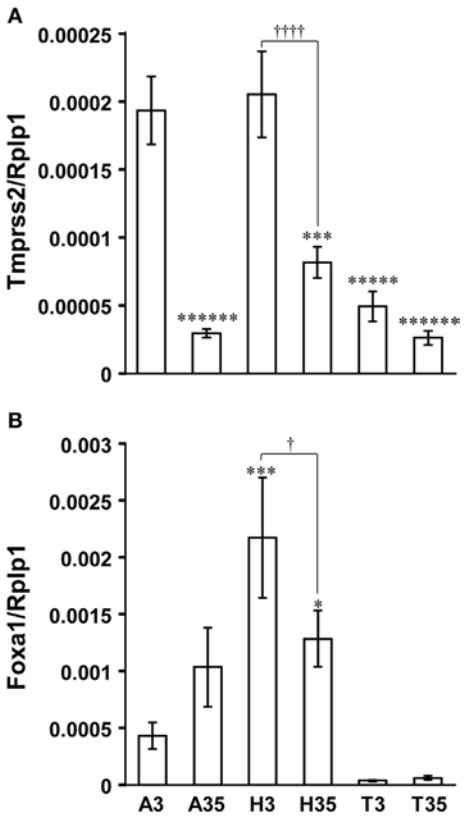

FIGURE 6 | Developmental changes in Tmprss2 and Foxa1 gene expression in the amygdala, hypothalamus, and telencephalon excluding amygdala and hippocampus of male rat. **(A)** The ratio of Tmprss2 gene expression level relative to a housekeeping gene, Rplp1. Each column and the vertical line represent the mean ± SEM ($n = 5$ samples, one sample from one rat). ******$P < 0.000001$; *****$P < 0.00001$, ***$P < 0.001$ vs. amygdala of P3 male rat (A3) by one-way ANOVA followed by Fisher's protected least significant difference (PLSD) test. ††††$P < 0.0001$, hypothalamus of P3 male rat (H3) vs. hypothalamus of P35 male rat (H35) by Student's *t*-test. A35, amygdala of P35 male rat; T3, telencephalon excluding amygdala and hippocampus of P3 male rat; T35, telencephalon excluding amygdala and hippocampus of P35 male rat. **(B)** The ratio of Foxa1 gene expression level relative to Rplp1. Each column and the vertical line represent the mean ± SEM ($n = 5$ samples, one sample from one rat). ***$P < 0.001$; *$P < 0.05$ vs. A3 by one-way ANOVA followed by Fisher's PLSD test. †$P < 0.05$, H3 vs. H35 by Student's *t*-test.

diameter (**Figure 3C**, ii). The third type of staining was an intense staining only in the nucleus (**Figure 3C**, iii). Immunohistochemistry without the first antibody served as control (**Figure 3D**).

Effect of BPA Administration on the Number of Tmprss2-ir Cells in the Medial Amygdala and Foxa1-ir Cells in the Hypothalamus

To investigate the effect of BPA on Tmprss2 and Foxa1 protein expressions, number of Tmprss2-ir cells and Foxa1-ir cells were counted in the medial amygdala and hypothalamus, respectively, of P3 male rats that were daily administered with BPA or vehicle from P1 to P3. The number of Tmprss2-ir cells in the medial amygdala was significantly increased by BPA administration ($P = 0.032$; **Figures 2E,F** and **4A**). On the other hand, the number of Foxa1-ir cells in the hypothalamus was significantly decreased by BPA administration ($P = 0.042$; **Figures 3A,B** and **4B**).

Co-Localization of Tmprss2 and EGFP-GnIH Cells in the Hypothalamus

Because Tmprss2 was highly expressed in the hypothalamus of P3 male rat (**Figure 2D**), where GnIH neuronal cell bodies are located, we investigated if some Tmprss2-ir cells are also EGFP-GnIH positive (17). Abundant EGFP-GnIH cells were observed from the ventral region of ventromedial hypothalamic nucleus (VMH) to the dorsal region of the arcuate nucleus (Arc) as well as along the third ventricle in P3 male rat hypothalamus (**Figures 5A,C,F,I,L**). The diameter of EGFP-GnIH cell bodies in the VMH and Arc was 6–11 μm and had typical GnIH neuronal structure with dendrite or axon-like processes (**Figures 5A,F,L**). 26.5 ± 6.8 (mean ± SD from four different brains) percent of EGFP-GnIH cells in the VMH and Arc were immunoreactive to Tmprss2 antibody (**Figures 5A–N**).

EGFP-GnIH cells along the third ventricle were different between the dorsal population (**Figure 5C**) and the ventral population in the median eminence (ME) (**Figure 5I**). The dorsal population of EGFP-GnIH cells along the third ventricle had two cell types. The first type had a rod-like structure with a diameter of 4–7 μm and they were directly attached to the ventricle (**Figure 5C,i**). The second type was round shape with a diameter of 4–7 μm situated in the sub-ventricular zone and had a protrusion to the ventricle (**Figure 5C,ii**). The ventral population of EGFP-GnIH cells in the ME had an oval shape with a minor axis of 4–7 μm directly contacted the ventricle and sent long processes to the external zone of the ME (**Figure 5I**). No clear cellular co-localization of Tmprss2 immunoreactivity was observed in EGFP-GnIH cells along the third ventricle either in the dorsal population (**Figures 5A–E**) or ventral population in the ME (**Figures 5I–K**).

Developmental Change in Tmprss2 and Foxa1 Gene Expression in the Amygdala, Hypothalamus, and Telencephalon in the Male Rat Brain

Tmprss2 was equally highly expressed in the amygdala and hypothalamus compared with the telencephalon excluding amygdala and hippocampus in P3 male rat brain (**Figure 6A**). However, Tmprss2 expression in the amygdala and hypothalamus decreased significantly in P35 male rat equivalent to Tmprss2 expression levels in the telencephalon (**Figure 6A**).

Foxa1 was expressed significantly higher in the hypothalamus compared with the amygdala of P3 male rats (**Figure 6B**). Although Foxa1 expression in the hypothalamus decreased significantly in P35, it was significantly higher than its expression level in the amygdala of P3 male rats (**Figure 6B**). Foxa1 expression in the telencephalon excluding amygdala and hippocampus was almost under detectable level (**Figure 6B**).

DISCUSSION

To understand how perinatal exposure of BPA can modify the neurodevelopmental process and behavior, we first searched for BPA responsive genes from AR and ER signaling target genes by PCR array in the neonatal male rat medial amygdala that were subcutaneously administered with a reference dose of BPA (50 μg/kg BW/day) determined by United States EPA. We found upregulation of *Tmprss2*, an AR signaling target gene, and downregulation of *Foxa1*, an ER signaling target gene. Number of Tmprss2-ir cells in the medial amygdala was also increased and Foxa1-ir cells in the hypothalamus was also decreased in the hypothalamus by BPA administration to neonatal male rats.

Tmprss2 encodes a multimeric protein that is translated into an N-terminal short cytoplasmic domain, a transmembrane domain, extracellular low-density lipoprotein receptor A domain, a scavenger receptor cysteine-rich domain, and a serine protease domain at the C-terminal (23). In human tissue, *TMPRSS2* is highly expressed in the small intestine, followed by heart, lung, liver, and small amounts in thymus and prostate. *TMPRSS2* is very highly expressed in the fetal brain but its expression level in the adult brain is minimum (23). *TMPRSS2* was found to be induced by androgen exposure to prostate cancer cells by microarray containing 1,500 cDNAs (24). It was shown that TMPRSS2 mediates androgen-induced prostate cancer cell invasion, tumor growth, and metastasis by stimulating a proteolytic cascade (25).

There is no study investigating the function of Tmprss2 in the brain besides only one study showing its possibility to mediate cancer pain by acting on trigeminal neurons (26). Significant decrease of *Tmprss2* expression in the amygdala and hypothalamus at 1 month of age suggests that Tmprss2 is related to neurogenesis or neuronal differentiation. In humans, TMPRSS2 is highly expressed in the fetus brain but its expression decreases to undetectable level in the adult brain (23). Tmprss2-ir cells were consistently distributed in olfactory bulb, cerebral cortex, hippocampus, amygdala, and hypothalamus in 3 days old male rats. We attempted Tmprss2 immunohistochemistry in 1-month-old and adult rat brains but we could not observe positive staining possibly due to its low expression level. The site of Tmprss2 expression corresponds well with that of AR expression in the rat brain (27, 28), suggesting that Tmprss2 expression is regulated by androgen.

In 3-day-old male rat hypothalamus, Tmprss2 was expressed in 26.5% of EGFP-GnIH neurons in the hypothalamus. EGFP-GnIH neurons were clustered from the ventral region of VMH to the dorsal region of Arc at this age, which was similar to fetal rat brain (29), but unlike GnIH neuronal population in the dorsomedial hypothalamic area in the adult (17, 30, 31). Abundant EGFP-GnIH cells were also found along the third ventricle, but clear expression of Tmprss2 was not observed in this region. EGFP-GnIH cells along the third ventricle can be determined as tanicytes from their morphology. Recent lineage-tracing experiments have shown that tanicytes along the third ventricle are hypothalamic stem cells (32) and postnatally differentiate into neurons and astrocytes in the hypothalamus (33). These results suggest that Tmprs2 is involved in the construction of neural and glial cellular architecture as a transmembrane protease in neonatal rat brain. Previous study has found that daily subcutaneous injection of a reference dose of BPA (50 μg/kg BW/day) to female Wister rats whose GnRH neurons express EGFP from P0 to P3 decreases numbers of GnIH-ir cell bodies, fiber density, and contacts on GnRH neurons and advances puberty at 1 month of age (34).

FOXA1 belongs to the FOXA family of transcriptional regulators (FOXA1, FOXA2, and FOXA3) that play pivotal roles in mammalian development (35). FOXA proteins serve as pioneering factors in the early sequence of the transcriptional regulatory program. FOXA induces nucleosomal rearrangement that facilitates binding of other transcriptional regulators such as sex steroid hormone nuclear receptors (36). FOXA1 serves as a pioneering factor that is required for ERα to bind estrogen-response-element after estrogen stimulation (37). FOXA1 also facilitates AR/chromatin interactions at the regulatory loci near androgen responsive genes (38). It was shown that BPA promotes epithelial mesenchymal transition of ER-negative breast cancer cells through downregulation of FOXA1 by PI3K/Akt activation (39). It is possible that BPA also downregulates Foxa1 by activating PI3K/Akt in the brain.

Foxa1 was highly expressed in the hypothalamus of P3 male rat but significantly decreased in the 1-month-old rat brain, suggesting that Foxa1 is also involved in neuronal differentiation. It was shown that Foxa1 is one of the regulators of neuronal differentiation of midbrain dopamine cells (40). A recent study showed that ablation of Foxa1 causes impaired development of subthalamic nucleus in mice (41), a brain region that is next to PLH where intense Foxa1-ir cells were found in P3 male rats in this study. Foxa1 immunoreactivity in the nucleus of the cells in PLH where AR and ER are also expressed (27) suggests that Foxa1 is involved in transcriptional activities of AR and ER in PLH.

Finally, it was shown that BPA upregulates Tmprss2 and downregulates Foxa1 in the neonatal male rat brain. Location of Tmprss2 and Foxa1-ir cells as well as significant decrease in Tmprss2 and Foxa1 expression in 1-month-old male rat brain suggest that BPA disturbs the neurodevelopmental process and behavior by modifying the expression of Tmprss2 and Foxa1 in the brain.

ETHICS STATEMENT

All procedures were approved by Monash University, Animal Ethics Committee (MARP/2016/037).

AUTHOR CONTRIBUTIONS

TU conceived the project. TU, SM, and TS performed the experiments. TU wrote and IP edited the paper.

ACKNOWLEDGMENTS

We thank Dr. Satoshi Ogawa, Marshita Mohd Idris, Ng Kai We, Rachel Shalini Anthonysamy, Indah Maisarah binti Shani, and Vigneswaran Tambirajah for their technical assistances (Brain Research Institute Monash Sunway, Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fendo.2018.00139/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
published: 04 April 2018
doi: 10.3389/fendo.2018.00136

Neural Androgen Synthesis and Aggression: Insights From a Seasonally Breeding Rodent

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Aggression is an essential social behavior that promotes survival and reproductive fitness across animal systems. While research on the neuroendocrine mechanisms underlying this complex behavior has traditionally focused on the classic neuroendocrine model, in which circulating gonadal steroids are transported to the brain and directly mediate neural circuits relevant to aggression, recent studies have suggested that this paradigm is oversimplified. Work on seasonal mammals that exhibit territorial aggression outside of the breeding season, such as Siberian hamsters (*Phodopus sungorus*), has been particularly useful in elucidating alternate mechanisms. These animals display elevated levels of aggression during the non-breeding season, in spite of gonadal regression and reduced levels of circulating androgens. Our laboratory has provided considerable evidence that the adrenal hormone precursor dehydroepiandrosterone (DHEA) is important in maintaining aggression in both male and female Siberian hamsters during the non-breeding season, a mechanism that appears to be evolutionarily-conserved in some seasonal rodent and avian species. This review will discuss research on the neuroendocrine mechanisms of aggression in Siberian hamsters, a species that displays robust neural, physiological, and behavioral changes on a seasonal basis. Furthermore, we will address how these findings support a novel neuroendocrine pathway for territorial aggression in seasonal animals, in which adrenal DHEA likely serves as an essential precursor for neural androgen synthesis during the non-breeding season.

Keywords: aggression, androgens, brain, dehydroepiandrosterone, steroid-converting enzymes, hamster, seasonality

OPEN ACCESS

Edited by:
Takayoshi Ubuka, Monash University Malaysia, Malaysia

Reviewed by:
Brian Trainor, University of California, Davis, United States
Wolfgang Goymann, Max Planck Institute for Ornithology (MPG), Germany

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *29 December 2017*
Accepted: *15 March 2018*
Published: *04 April 2018*

Citation:
Munley KM, Rendon NM and Demas GE (2018) Neural Androgen Synthesis and Aggression: Insights From a Seasonally Breeding Rodent. Front. Endocrinol. 9:136. doi: 10.3389/fendo.2018.00136

INTRODUCTION

Aggression is a well-studied social behavior that is universally exhibited across vertebrates in various social contexts (1, 2). Among conspecifics, aggression is typically displayed when two or more individuals compete for a critical limited resource, such as mates, territories, or food. In seasonally breeding vertebrates, elevated territorial aggression is most often exhibited during the breeding season, when individuals compete for access to resources that will increase their chances of reproductive success. A substantial body of work on male territorial aggression has shown that elevated levels of circulating androgens, including testosterone (T), are correlated with increased aggressive behaviors during the breeding season [reviewed in the studies by Cunningham et al. (3) and Fuxjager et al. (4)]. For example, studies from seasonally breeding birds [(5, 6); but see the study by Apfelbeck et al. (7)]; lizards (8, 9); and rodent species, including mice [(10); but see the study by Trainor

and Marler (11)], gerbils (12, 13), and hamsters (14, 15), have demonstrated that castration decreases intermale aggression in a reproductive context, but that this reduction can be alleviated by exogenous T administration. Moreover, individuals with higher endogenous levels of T oftentimes display greater aggression toward conspecifics and may exhibit dominance over animals with lower circulating T levels (16, 17).

Interestingly, some seasonally breeding species exhibit equivalent or increased levels of territorial aggression outside of the breeding season, despite gonadal regression and reduced circulating levels of androgens [reviewed in the study by Soma et al. (18)]. A particularly remarkable example of a species that displays elevated aggression during the non-breeding season is Siberian hamsters (*Phodopus sungorus*), which exhibit robust changes in morphology, physiology, and behavior on a seasonal basis. Siberian hamsters breed during the summer months and cease breeding and undergo gonadal regression, a reduction in body mass, and changes in thermoregulation during the winter months (19). These natural seasonal adaptations can be elicited in the laboratory by housing animals in light cycles that mimic the photoperiods of the breeding and non-breeding seasons (20, 21). For example, animals exposed to short, winter-like days (i.e., >12.5 h of light/day) in the laboratory, which mimic the photoperiod conditions of the non-breeding season, exhibit gonadal regression, a reduction in body mass, and a change in pelage color from brown to white (22, 23). These characteristic changes in physiology are associated with increased levels of aggression in both male (15, 24) and female hamsters (25, 26). Thus, Siberian hamsters are an excellent model for elucidating how seasonal variations in photoperiod can alter the neuroendocrine mechanisms associated with territorial aggression.

SEASONAL SHIFTS IN NEUROENDOCRINE MECHANISMS UNDERLYING AGGRESSION

Pineal Melatonin Regulates Seasonal Changes in Aggression

Although several biotic and abiotic factors vary on a seasonal basis, photoperiod (day length) is the primary environmental cue that is used by animals to shape seasonal shifts in reproductive physiology and social behavior [reviewed in the studies by Prendergast et al. (27) and Walton et al. (28)]. These differences in physiology and behavior are produced *via* a complex neural circuit, which begins with the perception of environmental light *via* retinal ganglion cells and culminates in the transduction of information about day length into a neuroendocrine signal within the pineal gland. The pineal gland secretes melatonin, an indolamine that plays a prominent role in establishing and maintaining biological rhythms, in response to photoperiodic information. Because melatonin secretion tends to be high at night and low during the day, changes in photoperiod cause associated changes in the pattern and duration of melatonin secretion, which convey information about day length to the central nervous system [reviewed in the studies by Bartness et al. (29) and Goldman (30)].

Previous work has implicated pineal melatonin in the modulation of neuroendocrine mechanisms underlying seasonal aggression *via* direct actions on neural substrates, such as the hypothalamus and pituitary gland, and *via* peripheral actions on the adrenal glands and gonads [reviewed in the studies by Haller et al. (31) and Boringer and Nelson (32)]. In seasonally breeding vertebrates, melatonin has regulatory functions at all levels of the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes and binds to melatonin-type I receptors (MT_1) located on the hypothalamus (33, 34), anterior pituitary gland (35, 36), gonads (37, 38), and adrenals (39, 40). Collectively, the presence of MT_1 at different tiers of the HPA and HPG axes suggests that melatonin alters gonadal and adrenal steroid synthesis on a seasonal basis. More specifically, long days induce a lower duration of melatonin secretion, which upregulates the HPG axis relative to the HPA axis to promote gonadal steroid secretion [reviewed in the study by Tsutsui et al. (41)]. Conversely, short days result in prolonged melatonin secretion, which upregulates the HPA axis relative to the HPG axis and elevates adrenal steroid synthesis (26, 42). The action of melatonin at the level of the adrenals is particularly intriguing, since several species of seasonally breeding animals that display year-round territorial aggression increase serum dehydroepiandrosterone (DHEA), an adrenal androgen, during the non-breeding season.

DHEA Promotes Territorial Aggression During the Non-Breeding Season

In recent years, it has become increasingly evident that DHEA is an important modulator of seasonal aggression in birds and rodents [reviewed in the study by Soma et al. (43)]. DHEA is an androgen that is secreted by the adrenal cortex in certain mammals, including hamsters and squirrels (15, 44). Although DHEA is synthesized peripherally, circulating DHEA is capable of passing through the blood–brain barrier and can be metabolized to active androgens and estrogens, such as T and estradiol (E_2), *via* a multi-step conversion in brain regions that express the appropriate steroidogenic enzymes (45, 46). Alternatively, some seasonally breeding animals are capable of steroid synthesis *de novo* from cholesterol, and these "neurosteroids" can bind directly to androgen receptor (AR) and estrogen receptors (ERα and ERβ) to regulate social behaviors (47, 48). Because DHEA has a low affinity for AR and ER, high endogenous levels of DHEA would be required to activate these receptors and induce changes in behavior (49). Therefore, it is likely that region-specific metabolism of circulating DHEA and/or neurally synthesized DHEA into active androgens and estrogens is primarily responsible for modulating the neural circuits relevant to territorial aggression during the non-breeding season, since these steroids bind with high affinity to AR and ER in neurons and glia.

Results from studies on seasonally breeding animals haveilluminated a few potential neuroendocrine pathways by which androgens can act on relevant neural circuits to modulate seasonal changes in aggression (**Figure 1**). Briefly, the classical neuroendocrine model proposes that circulating gonadal steroids pass through the blood–brain barrier and act on neural circuits relevant to aggression by directly binding to

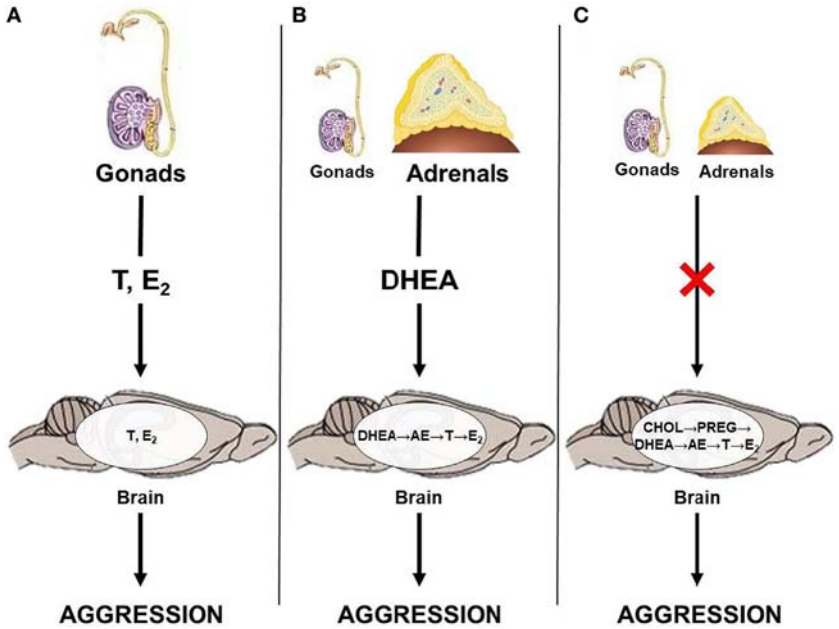

FIGURE 1 | Neuroendocrine pathways by which androgens could affect territorial aggression in seasonally breeding animals. **(A)** Gonadal steroids, such as testosterone (T) and estradiol (E_2), act directly on the brain; **(B)** adrenal dehydroepiandrosterone (DHEA) is locally converted to T and/or E_2; and **(C)** steroids are produced *de novo* in the brain from cholesterol (CHOL) *via* conversion to pregnenolone (PREG) and, subsequently, to DHEA, androstenedione (AE), T, and E_2 in the absence of steroid production from the gonads and adrenal glands.

AR or ER (**Figure 1A**). This mechanism likely predominates during the breeding season, when animals exhibit elevated levels of circulating androgens. However, outside of the breeding season, circulating gonadal steroids are low and adrenal steroids, such as DHEA, may serve as an important source of neural T and E_2. Thus, during the non-breeding season, circulating DHEA may serve as a prohormone and be converted to active androgens and estrogens in the brain after passing through the blood–brain barrier (**Figure 1B**). Alternatively, neurosteroids may be synthesized *de novo* from cholesterol (**Figure 1C**). These different neuroendocrine mechanisms culminate in region-specific binding of active steroids to AR and ER, respectively, which modulates seasonal changes in territorial aggression.

Adrenal DHEA as a Source of Neurally-Derived Androgens in Siberian Hamsters

Both male and female Siberian hamsters exhibit pronounced changes in reproductive physiology and territorial aggression across seasonal phenotypes (19). Several studies have demonstrated that male and female hamsters housed in short-day photoperiods display decreased levels of circulating gonadal steroids (23, 25), but increased levels of serum DHEA (15, 26). Furthermore, short-day males and females display elevated levels of aggression (21, 25), and similar increases in aggressive behaviors are observed in long-day animals administered short day-like levels of melatonin (24, 26). Interestingly, short-day males that receive adrenalectomies exhibit reduced levels of aggressive behaviors, yet adrenal demedullation, in which the catecholamine-secreting adrenal medulla is removed, produces no change in aggression (24). Furthermore, photoperiod has no effect on serum or adrenal cortisol content, and cortisol treatment does not affect levels of aggression in short- or long-day males (50). Collectively, these findings suggest that the neuroendocrine control of territorial aggression is dependent on seasonal changes in photoperiod and melatonin secretion, but is independent of gonadal steroids. Moreover, these data suggest that certain adrenocortical steroids may facilitate increases in aggression during the non-breeding season.

Recent studies from our group have focused on elucidating the neuroendocrine mechanisms responsible for increased territorial aggression during the non-breeding season. Specifically, we have examined the potential role of the HPA axis in modulating aggression. We have shown that male and female hamsters housed in short-day photoperiods decrease serum DHEA levels following an aggressive encounter, suggesting that short days may facilitate the conversion of circulating DHEA to T and E_2 (51, 52). Moreover, short-day female hamsters exhibit changes in adrenal morphology and have significantly higher adrenal DHEA content relative to long-day females. In addition, short- but not long-day females show elevated serum DHEA following adrenocorticotropic hormone (ACTH) challenge, in which the HPA axis is stimulated *via* exogenous administration of ACTH. Exogenous melatonin administration also stimulates adrenal DHEA release and elevates circulating DHEA and aggression in female hamsters (26). Taken together, these findings suggest that melatonin is responsible for coordinating a "seasonal switch" from gonadal to adrenal regulation of aggression by acting directly on the adrenal glands. In addition, these data suggest that DHEA peripherally regulates seasonal aggression.

We have also compared the abundance of gonadal and adrenal steroid receptors in brain regions associated with aggression or reproduction across seasonal phenotypes. We found that photoperiod does not affect glucocorticoid receptor (GR) levels in male hamsters in regions associated with aggressive behavior, including the medial amygdala (MeA) and the medial prefrontal cortex, or in the hippocampus, an area that shows changes in GR levels across photoperiods in other animals (50). In contrast, short-day female hamsters exhibit increases in ERα abundance in brain regions associated with aggression, including the periaqueductal gray (PAG), lateral septum (LS), and bed nucleus of the stria terminalis (BnST), but not in nuclei associated with reproduction, including the preoptic area (POA), arcuate nucleus (ARC), and anteroventral periventricular nucleus of the hypothalamus (53). Likewise, short-day males elevate ERα expression in brain nuclei associated with aggression, including the BnST, MeA, and central amygdala (54). While the presence of steroidogenic enzymes in these brain nuclei has yet to be confirmed, these data suggest that localized variation in neural ERα abundance may be responsible for seasonal changes in territorial aggression.

In summary, our findings provide compelling evidence that adrenal DHEA serves as a critical precursor of neurally-derived androgens in non-breeding Siberian hamsters (**Figure 2**). We have shown that short-day female hamsters display increased levels of aggression, but show no change in other social behaviors, such as investigation [**Figure 2A**; (53)]. In addition, short-day females increase serum DHEA, yet display reduced circulating levels of E_2 compared to long-day females [**Figure 2B**; (25, 53)]. Finally, female hamsters exhibit region-specific changes in ERα abundance, which may be a consequence of increased negative feedback from the HPG axis during the non-breeding season. Specifically, short-day females elevate ERα receptors in regions associated with aggression, such as the PAG, but not in nuclei associated with reproduction, such as the ARC [**Figure 2C**; (53)]. It is likely that these elevations in ERα abundance upregulate the activity of brain nuclei associated with aggressive behaviors and, consequently, increase territorial aggression during the non-breeding season.

NEURALLY-DERIVED STEROID SYNTHESIS IN OTHER SPECIES

Mice

Like Siberian hamsters, some species of deer mice (*Peromyscus sp.*) undergo seasonal changes in reproductive physiology and behavior. For example, male beach mice (*P. polionotus*) and deer mice (*P. maniculatus*) increase territorial aggression during the non-breeding season, in spite of gonadal regression and low circulating levels of androgens (55, 56). Intriguingly, California mice (*P. californicus*) do not undergo gonadal regression in response to short-day photoperiods, yet still exhibit elevated levels of aggression during the non-breeding season (57, 58). Together, these data suggest that increases in aggression during the non-breeding season are independent of circulating gonadal steroid levels and that, instead, localized neural androgen and estrogen synthesis may be responsible.

Recent work on the neuroendocrine mechanisms underlying seasonal aggression in deer mice has primarily examined neural ER abundance in brain nuclei associated with aggressive behaviors. Both short-day male beach mice and male deer mice exhibit increases in ERα abundance and expression in the BnST, yet display decreases in ERβ abundance and expression in the BnST and MeA. These changes in ERα- and ERβ-immunoreactivity are correlated with increased levels of aggression (59). Moreover, selective activation of either ERα or ERβ is associated with increased aggression in short-day male beach mice, and these increases in aggression are not correlated with changes in estrogen-dependent gene expression in the BnST and POA (60). While it is unclear whether adrenal DHEA is a source of neural estrogens in this signaling pathway, these findings suggest that ER abundance increases in a region-specific manner and primarily *via* non-genomic pathways to increase territorial aggression during the non-breeding season in *Peromyscus species* that are reproductively responsive to changes in photoperiod.

In contrast, mice that reproduce year-round or are physiologically non-responsive to changes in photoperiod do not exhibit similar changes in steroid hormone synthesis. For example, house mice (*Mus musculus*) decrease aggression in response to exogenous DHEA administration (61, 62). Furthermore, seasonally breeding male California mice, which do not undergo gonadal regression during non-breeding season, still elevate aggression and circulating E_2 levels in response to short days. However, non-breeding aggression in this species is independent of changes in ERα abundance, as short-day and long-day males show no differences in ERα and ERβ immunostaining in the LS, BnST, MeA, or POA (58, 63). Collectively, these findings indicate that some rodent species may not utilize circulating DHEA to maintain year-round territorial aggression. Additionally, these studies suggest that only seasonally breeding mice that are physiologically responsive to changes in day length alter neural ER abundance to elevate non-breeding aggression.

Birds

Most avian species have distinct breeding and non-breeding seasons, and many of these species display high levels of territorial aggression throughout the year [reviewed in the study by Soma (64)]. Like seasonally breeding rodents, birds undergo gonadal regression and exhibit pronounced reductions in plasma androgen levels during the non-breeding season, yet still exhibit levels of aggression that are often quantitatively and qualitatively similar to that displayed during the breeding season (65, 66). Moreover, castration of non-breeding male song sparrows has no effect on territorial aggression (67). Thus, while breeding season aggression is mediated by gonadal steroids, territorial aggression exhibited outside of the breeding season may be regulated by non-gonadal steroids in some male songbirds (68, 69).

Several studies have provided evidence that DHEA is an indirect source of neural androgens and estrogens in song sparrows (*Melospiza melodia*) during the non-breeding season. Male song sparrows display elevated circulating DHEA levels during the non-breeding season, which typically match seasonal changes in territorial aggression (66, 70). In addition, non-breeding male song sparrows display elevated plasma DHEA in

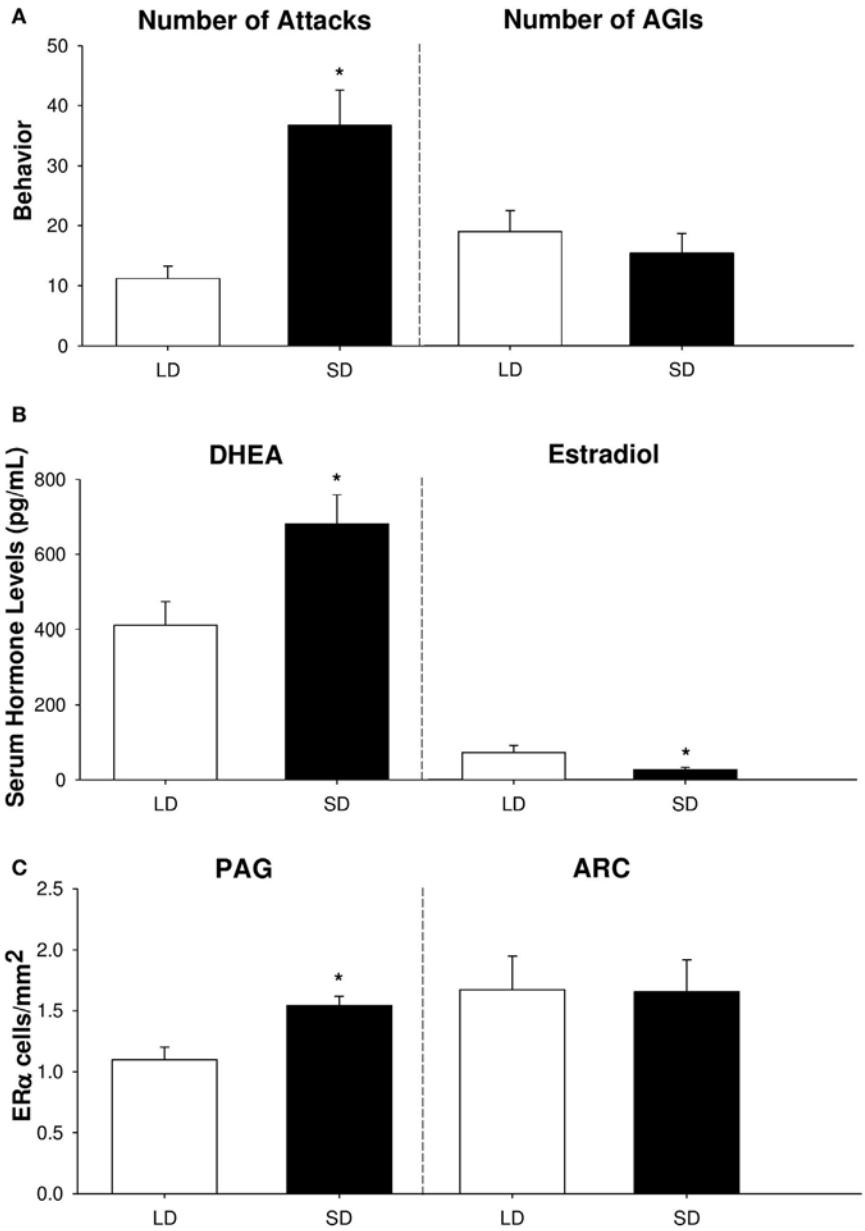

FIGURE 2 | Aggressive behavior, serum hormone profiles, and neural estrogen receptor-α (ERα) abundance differ across reproductive phenotypes of female Siberian hamsters. **(A)** Number of attacks and anogenital investigations (AGIs), **(B)** serum dehydroepiandrosterone (DHEA) and estradiol levels (in pg/mL), and **(C)** ERα cell density (in cells/mm^2) in the periaqueductal gray (PAG) and arcuate nucleus of the hypothalamus (ARC) in short-day and long-day female hamsters following 10 weeks of photoperiodic treatment. Bar heights represent mean ± SEM. "*" indicates a significant difference between groups ($P < 0.05$). Data are modified and reprinted with permission from the authors (25, 53).

the jugular vein exiting the brain, but not in the peripherally-located brachial vein, following a simulated territorial intrusion [STI; (71)]. Non-breeding male song sparrows also have higher 3β-hydroxysteroid dehydrogenase (3β-HSD) activity, an enzyme that converts DHEA to the androgen precursor androstenedione, in brain regions associated with aggression, including the LS, BnST, and the taenial amygdala, the avian equivalent of the MeA (72). Furthermore, sparrows exhibit increased aromatase activity, an enzyme that converts T to E_2, and increased ERα and ERβ mRNA expression in brain nuclei that regulate aggression (73, 74). A more recent study confirmed that neural levels of DHEA, T, and E_2 are higher in brain tissue than in circulation and that a single STI event alone can induce localized changes in neural DHEA levels (75). Taken together, these data suggest that circulating DHEA is an important source of neurally-derived steroids and that DHEA metabolism in the brain stimulates territorial aggression during the non-breeding season of birds.

Interestingly, there is emerging evidence that this mechanism is evolutionarily-conserved in some tropical bird species, but not others. Non-breeding male spotted antbirds (*Hylophylax n. naevioides*) elevate plasma DHEA, reduce circulating T, and display similar aggressive behaviors compared to breeding males (76, 77). In contrast, non-breeding European nuthatches (*Sitta europaea*) do not elevate aggressive behaviors following exogenous DHEA administration (78), and non-breeding male European starlings (*Sturnus vulgaris*) exhibit lower plasma DHEA levels compared to breeding males (79), indicating that these species maintain year-round territorial aggression independently of DHEA. Intriguingly, inhibiting AR and aromatase activity reduces territorial aggression in breeding, but not non-breeding male European stonechats (*Saxicola torquatus rubicola*), suggesting that steroid hormones only regulate territorial aggression in a breeding context (80, 81). Because the neuroendocrine mechanisms underlying seasonal aggression in songbirds vary considerably across species and context, additional studies in this area of research are warranted.

CONCLUSION AND FUTURE DIRECTIONS

Several species of seasonally breeding animals maintain or elevate territorial aggression during the non-breeding season, despite reductions in circulating gonadal steroids. Although our work on Siberian hamsters suggests that non-breeding animals metabolize adrenal DHEA to locally synthesize neural androgens and induce changes in aggressive behaviors, several aspects of these mechanisms have yet to be proven experimentally. Future studies should compare peripheral and central levels of gonadal and adrenal steroids across reproductive phenotypes and investigate whether neurosteroid concentrations and AR and ERβ abundance, in addition to ERα abundance, vary seasonally in brain nuclei associated with aggression. Moreover, little is known about how photoperiod affects the activity of enzymes involved in gonadal steroid synthesis (e.g., 3β-HSD and aromatase), both in the brain and in systemic circulation. It is important that future work addresses this area of research to pinpoint the sex steroid synthesis pathway cascades that are critical in establishing seasonal differences in aggression. Ongoing and future studies in hamsters and other seasonal species will continue to provide important insights into the role of neurally-active steroid hormones in the regulation of aggression.

AUTHOR CONTRIBUTIONS

KM and GD drafted the manuscript, with editorial contributions from NR.

ACKNOWLEDGMENTS

We thank previous and current members of the Demas lab for their assistance in data collection and analysis and for their intellectual contributions to the projects discussed in this review. We also thank Wolfgang Goymann and Brian Trainor for their valuable comments on a previous version of this manuscript and Takayoshi Ubuka, Ishwar Parhar, and Vance Trudeau for the invitation to submit this review.

FUNDING

This work was supported in part by National Science Foundation grant IOB-0543798 (to GD), National Institutes of Health grant MH109942 (to GD), National Science Foundation Doctoral Dissertation Improvement Grant IOS-1406063 (to NR and GD), National Institutes of Health Training Grant T32HD049336 (to NR), and a National Science Foundation Graduate Research Fellowship (to NR).

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Conflict of Interest Statement: The authors declare no competing interests, financial, or otherwise.

frontiers in Endocrinology

MINI REVIEW
published: 09 April 2018
doi: 10.3389/fendo.2018.00156

Sex Steroids and Adult Neurogenesis in the Ventricular-Subventricular Zone

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The forebrain ventricular-subventricular zone (V-SVZ) continuously generates new neurons throughout life. Neural stem cells (type B1 cells) along the lateral ventricle become activated, self-renew, and give rise to proliferating precursors which progress along the neurogenic lineage from intermediate progenitors (type C cells) to neuroblasts (type A cells). Neuroblasts proliferate and migrate into the olfactory bulb and differentiate into different interneuronal types. Multiple factors regulate each step of this process. Newly generated olfactory bulb interneurons are an important relay station in the olfactory circuits, controlling social recognition, reproductive behavior, and parental care. Those behaviors are strongly sexually dimorphic and changes throughout life from puberty through aging and in the reproductive age during estrous cycle and gestation. Despite the key role of sex hormones in regulating those behaviors, their contribution in modulating adult neurogenesis in V-SVZ is underestimated. Here, we compare the literature highlighting the sexual dimorphism and the differences across the physiological phases of the animal for the different cell types and steps through the neurogenic lineage.

Keywords: ventricular-subventricular zone, sexual dimorphism, estrogens, testosterone, neural stem cells, puberty, estrous cycle, pregnancy

OPEN ACCESS

Edited by:
Takayoshi Ubuka, Monash University Malaysia, Malaysia

Reviewed by:
John W. Cave, Weill Cornell Medicine, United States
Emmanuel Moyse, François Rabelais University, France

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *19 November 2017*
Accepted: *22 March 2018*
Published: *09 April 2018*

Citation:
Ponti G, Farinetti A, Marraudino M, Panzica GC and Gotti S (2018) Sex Steroids and Adult Neurogenesis in the Ventricular-Subventricular Zone. Front. Endocrinol. 9:156. doi: 10.3389/fendo.2018.00156

INTRODUCTION

The subventricular zone-olfactory bulb (V-SVZ-OB) system has fascinated scientists for over than 25 years. In fact, this region harbors, in many mammals, a huge neurogenesis persisting until aging (1). In rodents, this process involves multiple steps, each one of them representing a model for different biological and pathological processes with unique features. In fact, this neurogenic process encompasses a germinal layer located in the ventricular-subventricular zone of the forebrain (V-SVZ), along the ventricle in which neural stem cells undergo self-renewal (2) and differentiation to intermediate progenitors (type C cells), then to immature neurons (type A cells) (3–5). Newly generated, type A, cells undergo tangential migration along the rostral migratory stream (RMS) up to the OB (6, 7). There, they migrate radially to the appropriate cell layer and differentiate into interneurons (8). Neurogenesis is thus a complex process consisting in proliferation, migration, apoptosis, and differentiation occurring in each of those levels with specific features (9, 10). The proper turnover enforced by proliferation, migration as well as apoptosis in the OB, is essential for optimizing olfaction [(11); **Figure 1**]. Therefore, the study of V-SVZ is capital for many purposes: understanding unregulated cell growth in tumor formations (12, 13), preventing or replacing cell loss in aging (1, 14, 15), decreasing neurodegenerative disease risks (16–18), and improving stroke treatments (18).

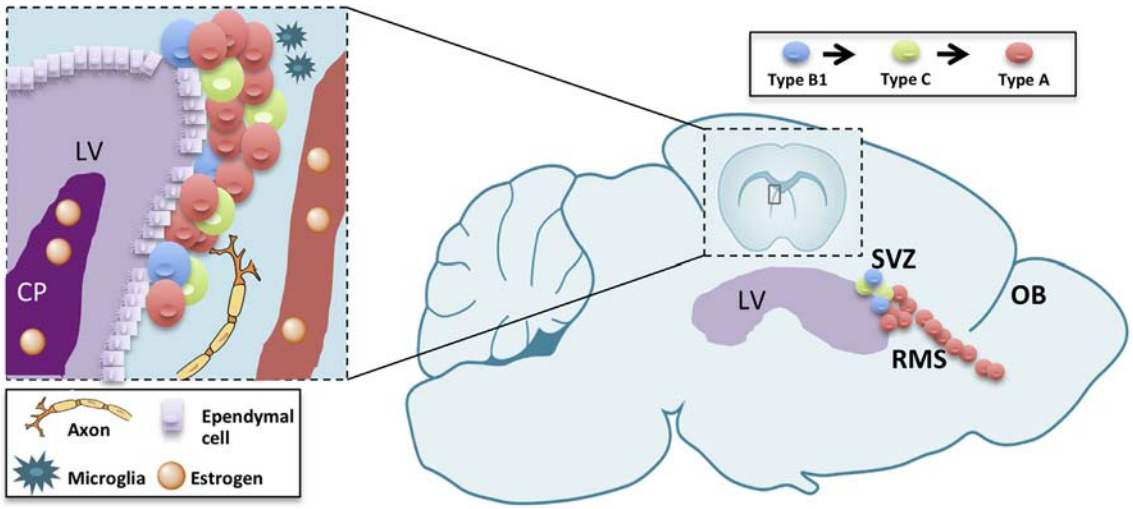

FIGURE 1 | Schematic drawing summarizing adult neurogenesis in the V-SVZ/OB system. Adult neurogenesis is a multiple-step process, occurring in three different subregions: the ventricular-subventricular zone (V-SVZ), the rostral migratory stream (RMS), and the olfactory bulb (OB). Sex hormones reaching the lateral ventricle (LV) through the choroid plexus (CP) or blood vessels (BV) modulate each of those steps either directly on neurogenic lineage or indirectly through other component of the stem-cell niche or the parenchyma.

Despite a huge interest on the endogenous and exogenous factors affecting adult neurogenesis in V-SVZ-OB system (19), few studies have focused on the role of gonadal hormones. This flaw is surprising since steroids have a key role in hippocampal neurogenesis both during development and in adulthood (20–22). Furthermore, V-SVZ-OB system is involved in social and reproductive behaviors, which are strongly regulated by sexual steroids (23, 24) and are targets for xenoestrogens (25–27). Moreover, estrogen receptors (ERs) and enzymes involved in the biosynthesis of steroids such as aromatase, the enzyme converting testosterone (T) into estradiol, are expressed in the V-SVZ (28) and in the OB of adult (29, 30) and developing (31) rats and mice (32). However, while the importance of steroids in the regulation of adult neurogenesis in the hippocampus has been widely studied, its role in the V-SVZ-OB system is more debated. Here, we want to focus on the available data in order to encourage a discussion addressing the open questions in the field.

SEXUAL DIMORPHISM IN V-SVZ-OB SYSTEM

Sexual dimorphism in the V-SVZ-OB system is an open question. Only few studies compared the two sexes and most of them are limited to a few ages. Indeed, the extent of neurogenesis in this region changes along life and it is likely to be affected by changes in the endocrine system.

Neurogenesis is more prominent in adult female mice compared with males. In 3-month-old C57/BL6J mice, females displayed higher proliferating rates in V-SVZ, RMS, and OB, and lower apoptotic cells in V-SVZ in both estrus and pregnancy (33) than males. Similarly, the number of neuronal progenitors (SOX2+) in the V-SVZ of females was higher than males in young adults but not in pups (34).

On the other hand, in other studies, the density of apoptotic cells in accessory (AOB) and main (MOB) OB was similar in the two sexes (35). Some differences affect transiently specific features of the V-SVZ-OB system. For instance, peripubertal males displayed higher rates of apoptosis (33), as well as of proliferation in the V-SVZ compared with females (35), but, in 2-month-old animals, the proliferation rate in the V-SVZ is similar in the two sexes and 1 month later there was a similar supply of newly generated cells in both the MOB and the AOB (35).

Multiple factors may explain the discrepancy among the data. From a technical point of view, the methods used to assess cell proliferation may highlight a different subset of the cycling population. In fact, while the total number of cycling cells identified with PCNA was measured by Diaz (33), the study of Nunez-Parra (35) highlighted only the cells in the S-phase, labeled by BrdU 2 h after the injection of the marker. Thus, it may reflect differences in the cell-cycle length between the two ages, or differences in the composition of the V-SVZ, e.g., a decrease in the number of type C cells, which have a longer S-phase length compared with type A cells (4, 5) or even differential sensitivities of BrdU antibodies (36), although the use of two different anti-BrdU antibodies by Nunez-Parra et al. is likely to have decreased this issue. Moreover, since different subregions in the V-SVZ give rise to different interneurons in the OB (37), it is possible that sexual dimorphism is limited to some of them. In addition to that, the extent of neurogenesis is dissimilar in different mouse strains (38) and it might be differently regulated. In fact, other reports indicate that the higher number of proliferating cells in the V-SVZ of females is limited to mature animals, i.e., 6–8 months old (39). Interestingly, this dimorphism is abolished (SJL/J) or reverted (BALB/C) in different strains (39). Accordingly, in two months old C57BL6 mice, the density of newly generated cells is higher in the AOB of males than females, while no sexual

dimorphism has been reported for MOB (34). Similarly, no sexual dimorphism was observed in the number of newly generated cells in the AOB of young-adult CD1 mice, although the age of those mice was not specified (40).

In Wistar rats, males exhibited a higher number of proliferating cells than females and this sexual dimorphism was already established before puberty (41). The higher proliferation at the level of the ventricle does not lead to a sex difference in the density of newly generated cells in the MOB, but only in the volume of the granular layer in the anterior part of the AOB, larger in males than in females [(42); **Table 1**].

Beside the cells belonging to the neurogenic linage, neural stem-cell niche encompass other structures, namely blood vessels (43, 44), microglia (45, 46), and choroid plexus (47). Both blood vessels (48), microglia (49, 50), and choroid plexus (51) are deeply affected by sex steroids. These structures, thus, may mediate the effect of sex steroids on adult neurogenesis (**Figure 1**).

Moreover, V-SVZ neurogenesis may also be modulated in a trans-synaptic way by other neuronal circuits which may be sensible to sexual steroids, e.g., serotonin or dopamine system (52, 53), and cholinergic neurons (54).

In general, estrogens are neuroprotective and stimulate differentiation and proliferation while progestins and androgens stimulate differentiation and cell survival (21). However, the V-SVZ-OB system has its unique features. In conclusion, a number of factors can affect adult neurogenesis in the V-SVZ-OB system, and it is likely that some of them are sexually dimorphic and change throughout lifetime. In this picture, the endocrine system may play a key role.

HORMONAL REGULATION OF V-SVZ NEUROGENESIS IN ADULT FEMALES

Circulating hormone levels dramatically change during the life of female rodents, during both estrous cycle and pregnancy. These changes may affect neurogenesis. In particular, E_2 levels control the estrous cycle, pregnancy, and sexual behavior (32, 55).

TABLE 1 | Sexually dimorphic features in the subventricular zone-olfactory bulb (V-SVZ-OB) system.

Model	Feature	Higher in:	Where	Reference
Prepubertal Wistar rats	Proliferation rates	Males	V-SVZ	(41)
	Volume of the granule cell layer	Males	Anterior AOB	(42)
	Newly generated cells	Males	Anterior AOB	(42)
Peripubertal mice	Apoptotic cells	Males	V-SVZ	(33)
P60 C57/BL6 mice	Newly generated cells	Males	AOB	(34)
P90 C57/BL6 mice (estrous and pregnancy)	Proliferation rates	Females	V-SVZ, RMS, OB	(33)
	SOX2 + progenitors	Females	V-SVZ, RMS, OB	(34)
P180-P240 C57/BL6 mice	Proliferation rates	Females	V-SVZ, RMS, OB	(39)
P180-P240 BALB/c mice	Proliferation rates	Males	V-SVZ, RMS, OB	(39)

Since OB has a key role in mother's offspring recognition, it is not surprising that the rate of neurogenesis in V-SVZ transiently increase during pregnancy (56). Indeed, two peaks of cell proliferations were observed at gestation day 7 and at postpartum day 7, while at delivery the neurogenic rate is similar to matched aged virgin females (56). The first peak is evident also in females mated with sterile males, so it depends on maternal hormonal levels rather than on the embryo. However, this effect is mediated by prolactin rather than E_2 or progesterone (56, 57). However, E_2 may have an indirect role, since it stimulates prolactin release (58).

In the adult female mouse, E_2 has an inhibitory effect on V-SVZ-OB neurogenesis in both V-SVZ and OB. First, it decreases cell proliferation in the V-SVZ in different models. The number of proliferating cells in the V-SVZ is lower during estrus, than proestrus (39). Moreover, in ovariectomized females, acute E_2 supplementation for one day, with a dose comparable to the estrus, decreases cell proliferation in the V-SVZ (59). On the other hand, this effect was not detected by long-term treatment [3 weeks (60)] or with a lower dose of E_2 (61), comparable with diestrus (62). Differences in the effect of ovariectomy may be due to an interplay of many component of the neural stem-cell niche. In fact, ovariectomized mice express both ERα and ERβ, but E_2 supplementation selectively upregulates ERβ (51). T metabolite 5α-dihydrotestosterone (5αDHT) decreases the expression of AR in the choroid plexus of ovariectomized mice (51).

Male pheromones stimulate the production of ovarian hormones (63) as well as the neurogenesis in adult females (35, 64, 65). However, E_2 does not increase neurogenesis (66), nor cell proliferation in V-SVZ or neuroblasts density in OB, but it decreases cell survival in AOB, but not in MOB (24).

In the OB, E_2 has different effects depending on the region. In the MOB, in adulthood rather than during development, E_2 is able to impair the survival of newly generated cells (59) and MOB functionality (60). Interestingly, as demonstrated in aromatase-KO mice, developmental E_2 has the opposite effect in the AOB: the absence of E_2 during development decreases the survival of adult generated cells in the AOB. This phenotype can be reverted by adult E_2 treatment. On the contrary, the lack of estrogens during development neither alters cell proliferation in the V-SVZ, nor its response to E_2 (60).

In contrast to mice, the proliferation rate in the rat V-SVZ does not change during pregnancy, while it increases at delivery (67). As for mice, E_2 role in female rat is highly debated. Proliferation in the V-SVZ is not affected neither by ovariectomy nor by acute T or E_2 supplementation (41). No studies are available on the long-term effects of ovariectomy despite it deeply alter choroid plexus transcriptome which may indirectly affect the neural stem-cell niche (68). However, E_2 decreases the survival of newly generated cells in the AOB, but not in the MOB [(29, 30); **Figure 2**; **Table 2**].

The different effects of E_2 in mice and rats may be related with the lack of ERα and ERβ in the mouse V-SVZ (76) and with the presence of ERα receptor in the rat (28), although other pathways may be involved (21). For example, no information is available at the moment, concerning the expression of membrane ER (GPER) in rodent V-SVZ.

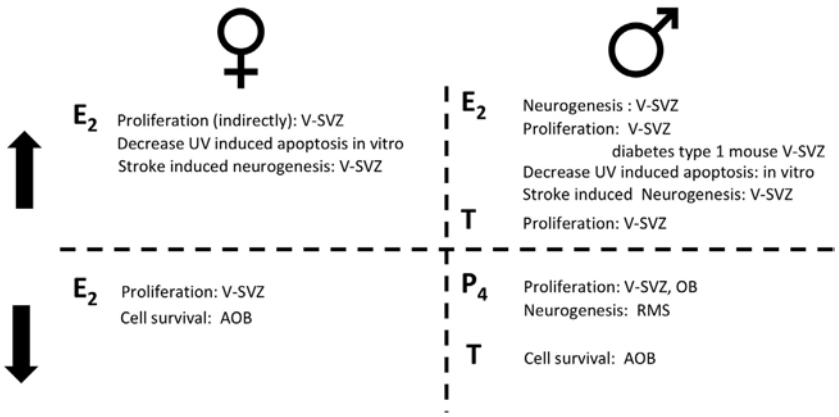

FIGURE 2 | The role of sex hormones in adult neurogenesis for females (left) and for males (right). On the top, steroid hormones induce an increase (↑) in the reported actions; on the bottom steroid hormones induce a decrease (↓) in the reported actions. Estradiol (E_2); progesterone (P_4); testosterone (T).

TABLE 2 | Summary of the hormonal regulations in the subventricular zone-olfactory bulb (V-SVZ-OB) system of adult male and female rodents.

	Hormone	Effect	Where	Reference
Hormonal regulation of V-SVZ neurogenesis in adult females				
Mouse	Estrogen	↓ Proliferation	V-SVZ	(39)
		↑ Proliferation (indirectly)	V-SVZ	(56, 57)
		↓ UV-induced apoptosis	*In vitro*	(34)
		↑ Stroke-induced neurogenesis	V-SVZ	(61, 69, 70)
		↓ Cell survival	MOB	(59)
		↓ Functionality	MOB	(60)
Mouse, rat	5α-dihydrotestosterone	↓ Expression AR	Choroid plexus	(51)
		↓ Cell survival	AOB	(29, 30, 66)
Hormonal regulation of V-SVZ neurogenesis in adult males				
Mouse	Castration	↑ Proliferation	V-SVZ	(39)
		↓ Apoptosis	V-SVZ	(39)
	Estrogen	↓ UV-induced apoptosis	*In vitro*	(34)
		↑ Stroke-induced neurogenesis	V-SVZ	(61, 69, 70)
Type 1 diabetes mouse model		↑ Proliferation	V-SVZ	(71)
		↓ Cell survival	AOB	(72)
Rat	5α-dihydrotestosterone	↓ Expression AR	Choroid plexus	(51)
	castration	↑ Expression ERβ	Choroid plexus	(51)
		↓ Proliferation	V-SVZ	(41)
	Estrogen	↑ Proliferation	V-SVZ	(41)
		↑ Stroke-induced neurogenesis	V-SVZ	(73, 74)
		↑ DCX + cells after stroke	V-SVZ	(73)
		↓ Cell death	V-SVZ	(74)
	Testosterone	↑ Proliferation	V-SVZ	(41)
	Progesterone	↓ Proliferation	V-SVZ/OB	(75)
		↓ Neurogenesis	RMS	(75)

HORMONAL REGULATION OF V-SVZ NEUROGENESIS IN ADULT MALES

The effect of sexual steroids is complex also in males. In fact, castration increased the number of proliferating cells and decreased the number of apoptotic ones in the V-SVZ of C57BL6 and SJL/J adult males, i.e., 6–8 months old (39).

Neurogenesis is influenced by pheromones related to aggressive (35) and paternal behavior (77, 78). In fact, the response to pheromones is sex specific and affected by hormonal levels. Indeed, female pheromones stimulate neurogenesis in adult males (64), although the survival of newly generated cells in the AOB in males does not change after opposite sex pheromones exposure, as in females (65). Interestingly, male pheromones as well as female ones, enhance proliferation in the V-SVZ of males (35), although it did not change the ratio of SOX2 cells among the BrdU labeled ones (64). However, low T levels feminize neurogenic response, increasing newly generated cell survival in the AOB, following male pheromone exposure, without affecting cell proliferation in RMS and V-SVZ, leading to attraction to male cues (72).

E_2 have a neuroprotective effect on V-SVZ precursors. In fact, it is able to restore proliferation in a type 1 diabetes mouse model (71). Only a few choroid plexus genes are altered by castration in rats (68): ERβ expression increased when compared with sham operated rats (51), while ARs expression decreased after 5αDHT treatment (51).

Unlike in females, T or E_2 are required for maintaining physiological neurogenic rate in the V-SVZ of peripubertal rats (41). In fact, the number of proliferating cells and the number of type C progenitors is restored by hormonal treatment in castrated rats, but this effect is restricted to the lateral wall of the V-SVZ (41).

Progesterone and its metabolites, decrease cell proliferation in the V-SVZ-OB of adult rats [2 months old (75)]. The number of newly generated cells in the final part of the RMS is decreased by progesterone metabolites. It is not clear, however, whether this effect is due to a reduction in cell proliferation, of cell survival or, less likely, in the migration rate (**Figure 2**; **Table 2**).

HORMONAL REGULATION OF V-SVZ NEUROGENESIS IN PATHOLOGICAL CONDITIONS

Beside an effect in physiological conditions, sex steroids may have a neuroprotective role after different insults. In fact, while no effect of sex steroid treatment was observed on cell death *in vitro*, E_2 prevented apoptosis after UV insults in both male- and female-derived V-SVZ cells, whereas no T effect was reported [(34); **Table 2**].

Stroke induced an increase in the number of newly generated cells, which was significantly higher in females. As for UV-induced apoptosis, E_2 enhances neurogenesis after ischemic stroke, *in vivo*, in mice of both sexes (61, 69, 79) and rats (73). This increase is present 96 h but not 24 h after stroke (61). The presence of ERα and ERβ, as well as AR is required for the stroke-induced neurogenesis in female mice, since it is abolished in transgenic mice lacking those receptors (69). Interestingly, those receptors are not directly expressed in the V-SVZ (61) suggesting that they may act indirectly through other cells.

Gonadal hormones are supposed to have a key role also in many diseases which display a different incidence and severity in the two sexes (80). V-SVZ neurogenesis may have a prominent role in some of them as: Parkinson disease (17, 81), Multiple sclerosis (82, 83), Alzheimer disease (84), autism (85), schizophrenia (86), and in psychiatric and cognitive disorders (87). However, only limited data are available on the effect of neuroactive steroids on the V-SVZ neurogenesis in those diseases. Moreover, many studies report controversial data on changes in the V-SVZ neurogenesis that may be related on the experimental model as in Parkinson disease (17).

CONCLUDING REMARKS

Despite the huge amount of studies on adult neurogenesis in the V-SVZ-OB system, still few data focus on its regulation by steroids. The role of steroids on V-SVZ-OB neurogenesis is highly complex. Generally, neurogenesis is more affected by T in males, while E_2 has a higher influence on females. However, the same hormone may determine a different effect depending on sex, age, strain, brain region, and neurogenic process. It is also possible that the different extent of V-SVZ-OB neurogenesis may reflects behavioral differences described among many strains of mice (88) as observed in other brain regions (89, 90). Those differences may be genetic (91, 92) or depend on a lack of maternal care during development (93). Profound differences exist between males and females. Some of them are actively determined by steroids levels in adults, while others are established during development. Moreover, sexual hormone's levels changes along life. Important species-specific differences exist between different rodent models. Despite some similarities, adult neurogenesis is regulated by different factors in the V-SVZ-OB system compared with the SGZ of the hippocampus. Furthermore, different cell populations, or different steps of the neurogenic lineage may be sensible to a specific hormone.

The extent of adult neurogenesis in the V-SVZ-OB changes along with each of the above mentioned parameters. However, it is not clear which features are directly or indirectly involved. It is, thus, important to consider all those parameters altogether.

AUTHOR CONTRIBUTIONS

All the authors equally contributed to the search for the sources and to the writing of the manuscript.

ACKNOWLEDGMENTS

We are grateful to the Fondazione Cavalieri Ottolenghi for hosting our laboratories in its premises.

FUNDING

We acknowledge the support from Fondazione Cavalieri Ottolenghi, the University of Torino (Ricerca locale to SG, GP, and GCP), and Fondazione CRT—RF 2015.2564 (to SG). This study was supported by Ministero dell'Istruzione, dell'Università e della Ricerca—MIUR projects "Dipartimenti di Eccellenza 2018–2022" to the Dept. of Neurosciences "Rita Levi Montalcini" and to the Dept. of Veterinary Sciences.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 18 April 2018
doi: 10.3389/fendo.2018.00173

Sex Differences in Medium Spiny Neuron Excitability and Glutamatergic Synaptic Input: Heterogeneity Across Striatal Regions and Evidence for Estradiol-Dependent Sexual Differentiation

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OPEN ACCESS

Edited by:
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Reviewed by:
Richard Anthony DeFazio, University of Michigan, United States
Wudu E. Lado, University of Toronto, Canada

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *19 December 2017*
Accepted: *03 April 2018*
Published: *18 April 2018*

Citation:
Cao J, Willett JA, Dorris DM and Meitzen J (2018) Sex Differences in Medium Spiny Neuron Excitability and Glutamatergic Synaptic Input: Heterogeneity Across Striatal Regions and Evidence for Estradiol-Dependent Sexual Differentiation. Front. Endocrinol. 9:173. doi: 10.3389/fendo.2018.00173

Steroid sex hormones and biological sex influence how the brain regulates motivated behavior, reward, and sensorimotor function in both normal and pathological contexts. Investigations into the underlying neural mechanisms have targeted the striatal brain regions, including the caudate–putamen, nucleus accumbens core (AcbC), and shell. These brain regions are of particular interest to neuroendocrinologists given that they express membrane-associated but not nuclear estrogen receptors, and also the well-established role of the sex steroid hormone 17β-estradiol (estradiol) in modulating striatal dopamine systems. Indeed, output neurons of the striatum, the medium spiny neurons (MSNs), exhibit estradiol sensitivity and sex differences in electrophysiological properties. Here, we review sex differences in rat MSN glutamatergic synaptic input and intrinsic excitability across striatal regions, including evidence for estradiol-mediated sexual differentiation in the nucleus AcbC. In prepubertal animals, female MSNs in the caudate–putamen exhibit a greater intrinsic excitability relative to male MSNs, but no sex differences are detected in excitatory synaptic input. Alternatively, female MSNs in the nucleus AcbC exhibit increased excitatory synaptic input relative to male MSNs, but no sex differences in intrinsic excitability were detected. Increased excitatory synaptic input onto female MSNs in the nucleus AcbC is abolished after masculinizing estradiol or testosterone exposure during the neonatal critical period. No sex differences are detected in MSNs in prepubertal nucleus accumbens shell. Thus, despite possessing the same neuron type, striatal regions exhibit heterogeneity in sex differences in MSN electrophysiological properties, which likely contribute to the sex differences observed in striatal function.

Keywords: sex, estradiol, hormones, striatum, medium spiny neuron, nucleus accumbens, caudate–putamen, electrophysiology

INTRODUCTION

Steroid sex hormones and biological sex are important factors influencing neuron function (1–4). Historically, research into the roles of biological sex and hormones targeted brain regions directly involved in sex-specific reproduction-related behaviors in adult animals (5, 6). These regions display large sex differences in volume, neuron cellular anatomy, and/or electrophysiological properties. Examples of these regions include the spinal nucleus of the bulbocavernosus (7), the sexually dimorphic nucleus of the preoptic area (8), and the song control nuclei in sexually dimorphic songbirds (9). Years of work have built upon these early studies to make significant advances. Notable among these is the discovery that biological sex and steroid sex hormones can modulate brain regions not directly involved with sex-specific reproductive behaviors. Although the extent of the influence on biological sex in the nervous system remains vastly underexplored, especially since few studies documented experimental animal sex (10–12), contemporary research has detected sex differences in neural substrates across the brain (13–18). The newly appreciated role of sex in modulating neural substrate also includes the striatal brain regions, comprising the caudate–putamen (also called dorsal striatum), the nucleus accumbens core (AcbC) and the nucleus accumbens shell (AcbS).

Biological sex, exogenous 17β-estradiol (estradiol), and endogenously circulating hormones *via* the estrous or menstrual cycle can modulate striatal-mediated cognitive, locomotor, and sensorimotor behaviors, including those related to motivation and reward (19–30). Many striatal pathologies are sensitive to estradiol and/or show sex differences in incidence and/or phenotype. These include depression, Parkinson's disease, drug addiction, schizophrenia, tardive dyskinesia, Huntington's disease, ADHD, and Tourette's syndrome, among others (31–41). The majority of published research in this field probes the link between sex and estradiol-induced influences on striatal-mediated normal and pathological behaviors (20, 42–46), especially regarding critical neurotransmitter/modulator systems such as dopamine and acetylcholine (43, 47–64). This research has established that sex and estradiol can influence striatal function *via* action on neurotransmitter and modulator systems, especially since the striatal regions show no robust sex differences in regional volume, neuron density, or soma size (65, 66). One area that has historically received less attention is how sex and estradiol modulate the electrophysiological properties of striatal neurons, including both the output neuron of the striatum, the medium spiny neuron (MSN) (67), and striatal interneurons (68). The term MSN is synonymous with striatal projection neuron. This is unfortunate, given that to change striatal circuit output and ultimately function, estradiol and sex must in some respect influence the electrophysiology of the MSN. This mini-review will focus upon the current state of knowledge regarding sex differences in rat MSN electrophysiological properties across striatal regions, with a focus on glutamatergic inputs and intrinsic excitability.

MSNs IN ADULT CAUDATE–PUTAMEN SHOW SEX-SPECIFIC AND ESTRADIOL-INDUCED DIFFERENCES IN EXCITABILITY *IN VIVO*

This research began in the 1980s, when Vincent and colleagues discovered that estradiol exposure increased *in vivo* spontaneous action potential generation and dopamine sensitivity in striatal neurons in ovariectomized adult female rat caudate–putamen (69). Tansey and colleagues then identified that the striatal neurons showing increased *in vivo* spontaneous action potential generation in response to high estradiol levels included nigrostriatal MSNs (other MSN subtypes and striatal interneurons were not examined). This increase in spontaneous action potential generation was induced either *via* exogenous estradiol exposure in ovariectomized animals or endogenously during specific phases of the estrous cycle (70). MSN spontaneous action potential firing rates were elevated in females compared with males only during the phases of the estrous cycle associated with the effects of increased estradiol and progesterone (proestrus and estrus). MSNs outside of the caudate–putamen were not examined in either study. The next breakthrough in targeting MSN electrophysiology came in the mid-1990s, when Mermelstein and colleagues established that estradiol rapidly decreases L-type calcium channel currents in both prepubertal and adult female rat caudate–putamen MSNs (71). In this case, estradiol acted within seconds in a steroid- and dose-dependent method on a membrane-associated estrogen receptor. The receptor was eventually identified as membrane-associated estrogen receptor β (72). Later research encompassing both the caudate–putamen and the nucleus accumbens established the presence of membrane-associated estrogen receptors α, β, and GPER-1 in MSNs, striatal interneurons, presynaptic terminals, and glia (72–78). The presence of aromatase, the enzyme that converts testosterone into estradiol, was also confirmed (79–82). There is little to no evidence of nuclear estrogen receptors in the striatal regions in adult rodents (83–85), although an exhaustive search across development, estrus cycle stages, and relevant species has not been performed.

These studies established several foundational themes for more recent research on the influences of estradiol and sex on MSN electrophysiology. First, estradiol can act *directly* on MSNs to modulate electrophysiological properties in addition to *indirectly* acting on MSNs by manipulating neuromodulatory influences such as those encompassed by the dopaminergic and cholinergic systems. Second, MSN sensitivity to estradiol can occur in a sex-specific fashion. Third, estradiol can manipulate MSN excitability, and in particular can increase MSN excitability in adult female animals. This sex-specific increase in MSN excitability could be potentially induced by multiple cellular mechanisms, broadly grouped into two types: mechanisms inducing alterations in synaptic input onto MSNs and mechanisms inducing alterations in the intrinsic electrophysiological properties of MSN. These mechanisms are not necessarily mutually exclusive and are not necessarily active in every striatal region. Recent research has

uncovered that both mechanism types can influence MSN excitability, with heterogeneity across striatal regions (**Table 1**).

EXCITATORY SYNAPSE NUMBER IS INCREASED ONTO FEMALE COMPARED WITH MALE AcbC MSNs, AND THESE SYNAPSES ARE MODULATED BY ESTRADIOL IN ADULTHOOD

Regarding the AcbC, in the early part of this decade, Woolley and colleagues formulated the hypothesis that excitatory synapses onto MSNs in this region are increased in females compared with males (86). Anatomical studies employing electron microscopy, immunocytochemistry, and other techniques established that increased excitatory synapses are present on MSNs in the AcbC of adult rat females in proestrus compared with gonad-intact males (86–88). Alterations in excitatory synapse activity instruct AcbC function in many contexts (89–93), including the responsiveness to drugs of abuse (94). Even minor sex differences in these excitatory synaptic inputs are potentially influential on AcbC function.

One neuroanatomical correlate of increased excitatory synapse number onto female compared with male rat AcbC MSNs was concomitant increased dendritic spine density (87, 88). This sex difference in dendritic spine density was also identified in adult human AcbC, with increased dendritic spine density on female compared with male MSNs (95). In other brain regions, dendritic spine density has long been documented to be sensitive to estradiol exposure, either *via* endogenous exposure *via* the estrous or menstrual cycles, or exogenous (96–98). Regarding the AcbC, Staffend et al. elucidated that a 2-day exposure to exogenous estradiol altered dendritic spine density on MSNs in gonadectomized female hamsters (99). This exogenous estradiol-induced change in spine density is also detected in gonadectomized rat females and is dependent upon estradiol activating mGluR5 and endocannabinoid signaling *via* CB1 receptors (100, 101). This estrogen receptor/mGluR5 signaling pathway in the AcbC is speculated to induce an increased drive for sex (23), although this has not been tested in the context of the estrous cycle. Regarding neurological disorders, this pathway is implicated as one mechanism underlying estradiol-induced potentiation of cocaine-induced locomotor sensitization and cocaine self-administration (102, 103). This estradiol-induced change in dendritic spine density seems specific to the AcbC, at least in the absence of other interacting variables. No estradiol-induced changes in dendritic spine density were measured in the caudate–putamen, and only one of three experiments showed an estradiol-induced change in spine density in the AcbS (99, 101). There is select evidence that postsynaptic excitatory synapse markers are also increased in female AcbS (86), but this is not a robust finding (88, 104). There is evidence that sex differences in excitatory synapse in the AcbS are induced by the effects of stress and potentially other environmental factors (105). In AcbS MSNs, a stress paradigm induced sex-specific alterations in presynaptic but not postsynaptic excitatory synapse markers (106). Investigating the interactions between sex, hormones, and environmental inputs such as stress or environmental chemical exposure is an essential future line of research. Regarding male MSNs, another possibility is that testosterone regulates excitatory synaptic input onto nucleus accumbens neurons, as suggested by experiments analyzing dendritic spine density in response to week-long exogenous testosterone exposure in gonad-intact male rats (107), and the role of androgens in reward-related behaviors (108).

INCREASED EXCITATORY SYNAPSE ACTIVITY IN FEMALE AcbC MSNs IS PRESENT BEFORE PUBERTY AND IS BLOCKED BY NEONATAL EXPOSURE TO ESTRADIOL

This body of data indicates that excitatory synapse number is increased onto adult female compared with male MSNs in the AcbC. Whether these differences in excitatory synapse number are functional has been assessed by analyzing miniature excitatory postsynaptic current (mEPSC) properties (**Table 1**). Wissman and colleagues discovered increased mEPSC frequency in adult gonad-intact female compare to male rat AcbC MSN, with estrous cycle not formally assessed (88). No differences were detected in mEPSC amplitude, decay, or in paired pulse properties, consistent with a model of increased excitatory synapse number in female compared with male AcbC, although sex differences in synaptic release probability

TABLE 1 | Development of sex differences in MSN electrophysiological properties varies by striatal region.

Electrophysiological property	Developmental stage	Caudate–putamen	Nucleus Accumbens Core	Nucleus Accumbens Shell
Intrinsic neuronal excitability	Prepubertal	Female > male	Female = male	Female = male
	Adult	?[a]	?	?
Excitatory synaptic Input	Prepubertal	Female = male	Female > male	Female = male
	Adult	?[a]	Female > male[b]	Female = male?[b,c]

[a]*Adult caudate–putamen medium spiny neurons (MSNs) show increased excitability in vivo in females compared with males, but the mechanisms underlying this phenomenon remains unknown and are not included in this table.*

[b]*Animals were gonad intact, but female estrous cycle stage has not been comprehensively examined.*

[c]*Most but not all accumbens shell (AcbS) literature shows no evidence of sex differences or estrogen sensitivity in excitatory synaptic input in adult animals unexposed to adverse environmental stimuli.*

?—Data not available or complex.

Color signifies the presence of a sex difference.

remain possible. This increase in excitatory synaptic input could potentially be generated either in puberty or during the early natal sensitive period, and then modulated in adult females by the estrous cycle. Cao and coauthors addressed this question in three respects (109). First, increased mEPSC frequency onto female compared with male MSN in the rat AcbC was present prepuberty, determining that sex differences in excitatory synapse are generated before puberty and adulthood. Second, MSNs from females exposed to masculinizing levels of estradiol or testosterone during the neonatal sensitive period to organizational hormone action lacked increased mEPSC frequency compared with control females or males. This finding shows that neonatal masculinization/defeminization *via* estrogen exposure is sufficient to permanently downregulate excitatory synaptic input onto MSNs, an endocrine process which would normally occur only in male animals. This suggests an overall model that sex differences in AcbC excitatory synapse number are organized by steroid sex hormone action during early development, with estrogen exposure in males permanently decreasing AcbC synapse number and possibly adult estrogen sensitivity. Finally, Cao and colleagues found no evidence that MSN intrinsic membrane or action potential properties differed by sex, including those mediating intrinsic excitability. These findings tentatively focus the working model explaining sex differences in AcbC function toward the neuroanatomical inputs onto MSNs, most prominently glutamatergic synapse, but also dopamine inputs. Intriguing possibilities include interactions between sex, estradiol, glutamate, and dopamine. Consistent with this speculation, female compared with male AcbC MSNs demonstrate an increased proportion of large-head dendritic spines adjacent to dopaminergic terminals (87). Estradiol may also differentially modulate dopamine signaling across striatal regions, as anatomical studies revealed membrane-associated estrogen receptor α and GPER-1 expression in dopaminergic terminals in the nucleus accumbens, but not the caudate–putamen (73, 74). Dysregulation of sex differences in glutamatergic and dopaminergic signaling may potentially induce sex differences in the phenotype and incidence of AcbC-related disorders.

The anatomical source of increased excitatory input into the AcbC is unknown. One possibility of many is that the AcbC receives a sex-specific excitatory input distinct from other striatal regions. Consistent with this possibility, the AcbC receives a different set of glutamatergic inputs than other striatal regions (110, 111). These differential inputs are consistent with the AcbC mediating separate aspects of behavior than other striatal regions, including locomotor behaviors (112, 113), but also maternal, social, reward, learning, sensorimotor, and sex-related behaviors (112–121). Relatively new transgenic techniques could potentially be applied to address gaps in anatomical knowledge (122). However, inbred laboratory mice may not be effective tools to address the role of sex in modulating AcbC properties, as domestication induced female mice to lose sex- and AcbC-relevant behaviors compared with non-domesticated mice (123). In addition, many drug-abuse and other reward-mediated behaviors regulated by the nucleus accumbens are difficult or impossible to observe in mice (124). Regarding other avenues of investigation, it is unknown whether excitatory synaptic input, intrinsic excitability, and other components such as estrogen receptor α and β gene expression are modulated across estrous cycle stage. This should be a future avenue of research given the differences in striatal-mediated behaviors across the estrous and menstrual cycles. Another unknown is how GABA receptor activation and sex interact in MSNs. This question is interesting given that in select cases GABA receptor activation can facilitate MSN action potential generation (125), and that estradiol exposure can decrease extracellular GABA concentration in adult female rat caudate–putamen (126).

NO EVIDENCE FOR SEX DIFFERENCES IN EXCITATORY SYNAPTIC INPUT ONTO MSNs IN THE PREPUBERTAL AcbS AND CAUDATE–PUTAMEN

Prepubertal sex differences in excitatory synaptic input seem specific to the AcbC. MSNs in prepubertal male and female AcbS and caudate–putamen show no evidence of sex differences in mEPSC frequency, amplitude or decay (104, 127). Although there is no evidence of sex differences in excitatory synaptic properties so far, it is always a possibility that sex or estradiol modulates unexamined aspects of glutamatergic or other neurotransmission, including but not limited to NMDA/AMPA ratios/function, synaptic plasticity, and/or silent synapse formation. At this point it is unknown whether MSNs in adult AcbS or caudate–putamen exhibit sex differences in excitatory synaptic input, although one study used anterograde but not retrograde tracing methods to provide evidence of increased projections from the orbital frontal cortex to the caudate–putamen in adult gonad-intact female compared with male rats (128). Regarding synaptic plasticity, a recent study established that pharmacological inhibition of aromatase blocked the induction of long-term potentiation of glutamatergic excitatory inputs onto MSNs in adult gonad-intact male rat caudate–putamen neurons (81). This study did not include females, but increased estradiol concentrations in adult female caudate–putamen compared with estradiol concentrations in circulating plasma levels (129), along with the presence of aromatase in caudate–putamen and nucleus accumbens (79–82) hint that aromatase may be locally active across the striatum in both sexes.

MSNs SHOW INCREASED INTRINSIC EXCITABILITY IN FEMALE COMPARED WITH MALE PREPUBERTAL CAUDATE–PUTAMEN BUT NOT THE AcbC OR AcbS

Up to this point, this review has focused on sex differences in excitatory synaptic input. Alternatively, sex differences could occur in the intrinsic electrophysiological properties of MSNs, including alterations in action potential properties, the frequency of evoked action potentials to injected current properties, and passive (membrane) properties. Intrinsic electrophysiological properties regulate action potential generation in response to

synaptic input (130), making these values integral in producing the functional output of MSNs. For example, augmented MSN intrinsic excitability would increase the number of action potentials that an MSN generates in response to a unit current. Alterations in MSN intrinsic excitability have been implicated in a number of striatal functions and disorders, including the responsivity to abused drugs, homeostatic plasticity, and striatal neuron subtypes (94, 131–136).

Female MSNs in prepubertal caudate–putamen show increased intrinsic excitability compared with male MSNs (127), unlike MSNs in the AcbC and AcbS (104, 109). This excitability is manifested in several respects. Female MSNs on average produce more action potentials than male MSNs for similar moderate amounts of injected positive current. This difference in action potential to current ratio is generated by an increased initial action potential firing rate in female compared with male MSNs, along with a decreased action potential after hyperpolarization peak and a mildly hyperpolarized action potential threshold. No sex differences were detected in passive membrane properties such as input resistance or in mEPSC properties. This lack of difference in mEPSC properties is essential for interpreting sex differences in MSN intrinsic excitability, as intrinsic excitability can be altered either independently or in concert with other attributes such as changes in excitatory synaptic input (94, 137–140). These findings support a model where increased excitability in prepubertal caudate–putamen MSNs is driven by internal changes to MSN electrical properties, rather than changes in excitatory synaptic input. Elucidating the ionic and receptor basis underlying this increased intrinsic excitation in female MSNs will be critical future experiments, along with assessments of adult MSNs, possible regional differences between dorsolateral and dorsomedial caudate–putamen, and the rostral–caudal axis. Changes in intrinsic excitability may be responsible for the increased excitability of female compared with male MSNs *in vivo* recorded in adult caudate–putamen (50, 69, 70). However, it is possible that intrinsic excitability and/or excitatory synaptic input in the caudate–putamen may be reorganized during puberty, such as glutamatergic synaptic input in the medial amygdala (141, 142), or striatal dopamine receptors (31, 143, 144). Either or both of these properties may be modulated by estrous cycle stage (145, 146), as suggested by *in vivo* recordings of caudate–putamen MSNs (69, 70). Overall, data from caudate–putamen MSNs support the theme of increased excitability in female compared with male MSNs, but indicate that the mechanism underlying these differences varies by striatal region.

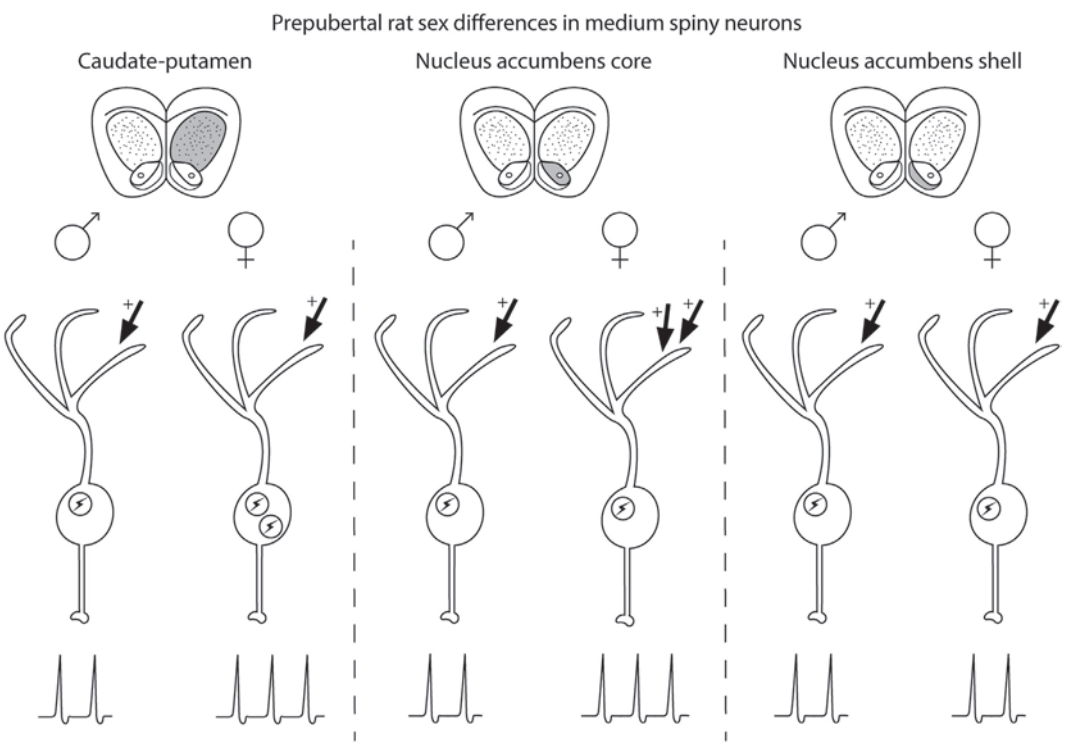

FIGURE 1 | Schematic of sex differences in medium spiny neurons (MSNs) in prepubertal rat caudate–putamen, nucleus accumbens core (AcbC), and nucleus accumbens shell (AcbS). In general, MSNs recorded from prepubertal female rats exhibit increased excitation compared with male MSNs in the caudate–putamen and nucleus AcbC. However, the exact nature of the increased excitability in female MSNs differs by striatal region, encompassing changes in either intrinsic excitability (indicated in the schematic with an encircled lightning bolt) or excitatory synaptic input (indicated with arrows with plus signs). Differences in intrinsic excitability and excitatory synaptic input are indicated with more or less lightning bolts and arrows, respectively. Caudate–putamen MSNs show increased intrinsic excitability in prepubertal females compared with males. Nucleus AcbC MSNs receive augmented excitatory synaptic input in prepubertal females compared with males. Nucleus AcbS MSNs exhibit little evidence for sex differences in either intrinsic excitability or excitatory synaptic input in prepubertal animals.

CONCLUSION

Medium spiny neuron electrophysiological properties are sensitive to sex and steroid sex hormone action in a striatal region-specific manner (**Figure 1**). In prepubertal caudate–putamen, MSNs show on average increased intrinsic excitation in females compared with males. In adult caudate–putamen, MSNs likewise show increased excitability in females compared with males, but the mechanism underlying this difference remains unknown. In both prepubertal and adult AcbC, MSNs receive augmented excitatory synaptic input in females compared with males, and early-life exposure to estradiol is instrumental in the sexual differentiation of this property. In the AcbS, there is little evidence for sex differences in intrinsic excitability or excitatory synaptic input in prepubertal animals unexposed to adverse environmental stimuli. These data argue for heterogeneity across striatal regions in the sensitivity to MSNs to sex and steroid sex hormones and the relative amount and nature of masculinization and feminization in MSN electrophysiological properties differs by striatal region. These findings highlight the importance of differentiating between striatal regions, developmental stage, sex, and estrous cycle. These data extend earlier mosaic models of brain sexuality, in that not only individual brain regions but also individual neuron types, in this instance MSNs, show differential degrees of masculinization, feminization, and homogeny (147, 148). These sex differences and similarities in MSN electrophysiological properties, along with developmental, individual, subtype, and the other documented variables in MSN properties, induce a dizzying variety *within the same neuron type* both between and within males and females.

AUTHOR CONTRIBUTIONS

JM wrote the manuscript; DD prepared the figure; and JC, JW, DD, and JM edited and approved the manuscript and figure.

FUNDING

We acknowledge NIH MH109471 to JM and P30ES025128 (Center for Human Health and the Environment).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 23 April 2018
doi: 10.3389/fnins.2018.00256

Childhood Emotional Abuse Moderates Associations Among Corticomotor White Matter Structure and Stress Neuromodulators in Women With and Without Depression

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Edited by:
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience

Received: *15 January 2018*
Accepted: *03 April 2018*
Published: *23 April 2018*

Citation:
Frost CP, Meyerand ME, Birn RM, Hoks RM, Walsh EC and Abercrombie HC (2018) Childhood Emotional Abuse Moderates Associations Among Corticomotor White Matter Structure and Stress Neuromodulators in Women With and Without Depression. Front. Neurosci. 12:256. doi: 10.3389/fnins.2018.00256

Adverse caregiving during development can produce long-lasting changes to neural, endocrine, and behavioral responses to stress, and is strongly related to elevated risk of adult psychopathology. While prior experience of adversity is associated with altered sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal (HPA) axis activity, the underlying neural pathways are not completely understood. In a double-blind crossover study, we used diffusion tensor imaging (DTI) to examine whether variation in white matter structure predicts differences in HPA-SNS interactions as a function of early adversity. Participants included 74 women who exhibited a wide range of depression severity and/or childhood emotional abuse (EA). Participants attended two experimental sessions during which they were administered 20 mg cortisol (CORT) or placebo and after 90 min, viewed emotionally laden pictures while undergoing MRI scanning. Immediately after emotional picture-viewing, we collected salivary alpha-amylase (sAA) to index SNS activation. We tested whether EA moderated the relation between fractional anisotropy (FA), a measure of white matter fiber structure, and sAA. In the placebo condition, for participants with minimal history of EA, higher FA in corticomotor projections was negatively correlated with sAA, whereas in participants with severe EA, the correlation was trending in the opposite direction. Following CORT administration, FA and sAA were not related, suggesting that SNS tone during acute cortisol elevation may depend on neural pathways other than corticomotor projections. The results suggest that at baseline—though not during cortisol elevation—increased FA in these tracts is associated with lower levels of SNS activity in women with minimal EA, but not in women with severe EA. These findings provide evidence that corticomotor projections may be a key component of altered neural circuitry in adults with history of maltreatment, and may be related to alterations in stress neuromodulators in psychopathology.

Keywords: cortisol, sympathetic nervous system, hypothalamic pituitary adrenal axis, corticomotor system, emotional abuse, depression, diffusion tensor imaging, tract-based spatial statistics

INTRODUCTION

Experience of adverse caregiving in childhood is a risk factor for a variety of psychopathologies in adulthood, including major depressive disorder (MDD) and posttraumatic stress disorder (PTSD). Studies in both humans and animals show that responses to acute stress are shaped by the history of the organism (Pavlides et al., 2002; Alfarez et al., 2003; Ellis et al., 2005; Joëls and Krugers, 2007; Meewisse et al., 2007; Ehring et al., 2008). Prior adversity is at times associated with high sympathetic nervous system (SNS) response (featuring the systemic release of the catecholamine epinephrine in the medulla of the adrenal gland; Otte et al., 2005), and low hypothalamic-pituitary-adrenal (HPA) response (featuring systemic release of glucocorticoids [GCs], primarily cortisol in primates or corticosterone in rodents) to acute stress (Resnick et al., 1995; McFarlane et al., 1997; Walsh et al., 2013; Drury et al., 2016). Furthermore, it has been hypothesized that the balance between SNS and HPA responses to acute stressors is altered in individuals previously exposed to chronic and/or severe stress, so that cortisol signaling does not sufficiently contain the SNS response (Yehuda et al., 1998). The neural pathways supporting such alterations in peripheral stress physiology in individuals previously exposed to adversity are unknown.

Functional activity in a variety of brain regions and circuits, such as hippocampus, amygdala, and fronto-limbic circuitry, regulates and is modulated by stress neuromodulators such as catecholamines and GCs (Roozendaal et al., 2009; Ulrich-Lai and Herman, 2009; Hermans et al., 2014; Vogel et al., 2016). More recent research (Dum et al., 2016; Abercrombie et al., 2018) in humans and nonhuman primates has indicated a possible role for cortical premotor areas including supplementary motor area (SMA), cingulate motor areas (CMAs), and premotor cortex (PMC) in neural response to and control of stress neuromodulators. For instance, anatomical tracing in Cebus monkeys shows these areas project densely to spinal circuits innervating sympathetic preganglionic cells, which terminate in adrenal medulla, likely modulating peripheral SNS activation (Dum et al., 2016).

In addition, dorsomedial frontal cortical areas, which share substantial overlap with these premotor regions, are known substrates for GC modulation of neural activation and HPA tone (McEwen et al., 1986; Diorio et al., 1993; Radley et al., 2006). Such a pathway could constitute a potential interface by which trauma and adversity may give rise to altered HPA/SNS relationships. Indeed, numerous studies have found that patterns of dorsomedial prefrontal activation and connectivity during emotion regulation differ in individuals with history of maltreatment (McLaughlin et al., 2015; McCrory et al., 2017) and may also distinguish between individuals resilient to or at risk of psychopathology related to early adversity (Herringa, 2017).

To further elucidate the neural pathways by which adversity relates to alterations in stress response systems, we tested relations between measures of structural connectivity and SNS activation in a sample of women with varying history of childhood emotional abuse (EA) and psychopathology. We further tested how exogenous cortisol altered these relationships. The use of exogenous cortisol permits inferences about the effects of cortisol *per se*, rather than the effects of elevations in endogenous cortisol elicited by a potent, social-evaluative stressor (Dickerson and Kemeny, 2004). We pharmacologically manipulated cortisol (CORT) vs. placebo before participants viewed emotionally evocative pictures, and measured salivary alpha-amylase (sAA) following the task to index SNS activation. Alpha-amylase is an enzyme whose salivary concentration can be used as an index of SNS activity, such as when detecting autonomic dysregulation or treatment response (Nater and Rohleder, 2009). Brain structure was assessed using T1- and diffusion-weighted magnetic resonance imaging. Using functional magnetic resonance imaging (fMRI) in this sample, we previously reported that childhood EA moderated the effects of CORT on activation in SMA and adjacent premotor cortical areas, and that this activation was related to sAA, an index of sympathetic adrenal-medullary output. Because descending tracts such as corticospinal tract (CST) likely carry corticomotor axons en route to sympathetic preganglionic cells (Dum et al., 2016), we therefore hypothesized that CORT's effects on sAA would be related to their white matter structure, and that this relationship would be moderated by history of childhood EA.

MATERIALS AND METHODS

Participants

Participants were women aged 18–45 with varying depression severity and history of childhood EA. We did not specifically recruit women with anxiety disorders or PTSD, but these were not exclusionary (**Table 1**; full eligibility criteria in Supplementary Materials). Of 85 eligible participants, 80 completed the study and full data were available for 75. Data was lost to experimenter error (1 participant), scanner malfunction (1 participant), poor image quality (2 participants), and a medical condition (1 participant), and 1 participant was excluded as an extreme outlier during data analysis (see next section), bringing the final N to 74. The study protocol was approved by the University of Wisconsin Health Sciences IRB. Participants provided written informed consent and were paid for participation.

Measuring Childhood EA and Depression Severity

Childhood EA was retrospectively assessed using the Emotional Abuse subscale of the Childhood Trauma Questionnaire (CTQ), a well-validated instrument that can be used to measure aversive caregiving continuously or by categorizing participants into groups using standard cut scores (Bernstein et al., 2003). In the final sample, 14 women experienced moderate-to-extreme ("severe"), 14 experienced low-to-moderate ("moderate"), and 46 experienced none-to-minimal ("minimal") childhood EA. Timing of EA was assessed using a life history calendar (Caspi et al., 1996), which confirmed that all women endorsing EA experienced abuse prior to menarche, and many experienced ongoing EA from early childhood through adolescence.

Consistent with the NIMH Research Doman Criteria (RDoC) framework (Insel, 2014), which emphasizes the continuous nature of psychiatric disorders, we recruited women with a range of depression, anxiety, and PTSD severity. Psychopathology was assessed using the SCID-I/P for DSM-IV-TR with additional questions to assess DSM-5 criteria (First et al., 2002). Some participants experienced clinically significant anxiety (36%) and PTSD (12%); these disorders were comorbid with depression, for which we specifically recruited and which was more prevalent in our sample (61% lifetime, 43% current; **Table 1**). Depression severity was indexed using the average of Beck Depression Inventory-II (BDI-II) scores across two experimental sessions (Beck et al., 1996). A square-root transformation was applied to reduce negative skew and undue influence of extreme BDI-II scores as in prior research (van Minnen et al., 2005; Roelofs et al., 2013). In scatter plots, scores are back-transformed to preserve initial range.

Although we sought to recruit a sample in which EA and depressive symptoms were not entirely overlapping (**Table 1**), EA was nevertheless strongly associated with adult depression: the correlation in this sample is $r_{(73)} = 0.45$, $p < 0.01$. One participant's scores on the BDI-II and EA subscale of the CTQ were 0 and 25, which are respectively the lowest and highest possible scores. These values exerted extreme and anomalous influence on results: standardized residuals > 2, Cook's distance > 4 times mean, leverage >> $2p/n$, thus meeting criteria for outlier identification proposed by Rawlings et al. (1998). This was the only participant in the sample who concurrently reported extreme EA and no depressive symptomatology. Thus, this participant skewed statistical distributions and was ultimately classified as an outlier and excluded from analysis. For discussion, see Limitations Section.

TABLE 1 | Demographic and clinical characteristics.

Characteristics	CTQ emotional abuse groups		
	Minimal ($n = 46$)	Moderate ($n = 14$)	Severe ($n = 14$)
Age, years[a]	26.1 ± 6.4	31.4 ± 7.1	28.1 ± 8.0
Lifetime depressive disorder	23 (50.0)	9 (64.3)	13 (92.9)
Current depressive disorder	12 (26.1)	7 (50.0)	13 (92.9)
Current anxiety disorder	12 (26.1)	6 (42.9)	9 (64.3)
Current PTSD	0	3 (21.4)	6 (42.9)
Race[b,c]			
White	34 (73.9)	9 (64.3)	12 (85.7)
Asian	8 (17.4)	3 (21.4)	2 (14.2)
African American	3 (6.5)	1 (7.1)	0
Unknown	1 (2.2)	1 (7.1)	0
Ethnicity[b]			
Hispanic/Latina	4 (8.7)	2 (14.3)	0
Not Hispanic/Latina	42 (91.3)	11 (78.6)	14 (100)
Unknown	0	1 (7.1)	0
Education level[d]	4.4 ± 1.4	5.2 ± 1.1	4.9 ± 1.3
Childhood caregivers' education level[d]	4.5 ± 1.7	4.9 ± 1.2	5.1 ± 1.7

Values are mean ± SD, or n (groupwise %). CTQ, Childhood Trauma Questionnaire; PTSD, posttraumatic stress disorder.

[a] There was a small but significant group difference in age, $F_{(2,73)} = 3.25$, $p = 0.04$.

[b] Chi-squared tests confirmed the CTQ Emotional Abuse groups did not significantly differ by racial or ethnic composition, p's > 0.34.

[c] Because of rounding, percentages may not total 100.

[d] Education categories: 1, Less than high school; 2, High school diploma or equivalent (i.e., GED); 3, Some college, no degree; 4, Associate's degree; 5, Bachelor's degree; 6, Master's degree; 7, Doctoral degree.

Procedure

Cortisol levels were manipulated by administering 20 mg oral hydrocortisone (i.e., exogenous cortisol; CORT). In a separate experimental session, participants were given an identically appearing placebo capsule. The CORT and placebo sessions were conducted in the late afternoon into the evening (beginning at 4:15 PM) and were typically separated by 1 week (minimum of 5 days). The order of drug administration was randomized and double-blinded. Neuroimaging (diffusion tensor imaging [DTI] and fMRI) was conducted during both visits. fMRI data are described elsewhere (Abercrombie et al., 2018).

SNS Activation During CORT vs. Placebo

To index SNS activation in the context of an emotionally evocative experience, sAA was measured immediately after participants completed an emotional picture viewing task, which occurred 90 min after drug administration during fMRI (**Figure 1**). Participants viewed one of two sets of psychometrically matched pictures from the International Affective Picture System (IAPS; Lang et al., 2008) during each session.

For measurement of sAA and salivary cortisol levels, saliva samples were collected from participants throughout each session with Salivettes (Sarstedt, Nümbrecht, Germany), according to previously-described guidelines (Rohleder and Nater, 2009).

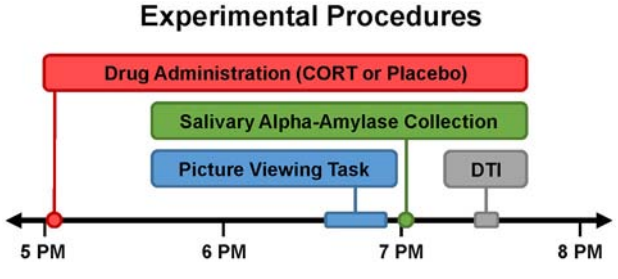

FIGURE 1 | Timing of experimental procedures. Study participation entailed two experimental sessions, which were typically separated by 1 week (minimum of 5 days). All experimental sessions were conducted late in the day (beginning at 4:15 PM) when endogenous cortisol levels are relatively low. Drug (20 mg oral hydrocortisone [CORT] or placebo) was administered slightly after 5PM. Order of drug administration across the two sessions was randomized and double-blinded. Approximately 90 min after drug administration, participants viewed emotional pictures during fMRI (described elsewhere; Abercrombie et al., 2018). Salivary alpha-amylase (sAA) was collected immediately after the emotional picture viewing task to index sympathetic nervous system (SNS) activity during CORT vs. placebo. Diffusion tensor imaging (DTI) was conducted to assess white matter structure.

sAA concentrations were assayed with an enzyme kinetic method (e.g., Bosch et al., 2003). Intra- and inter-assay coefficients of variation were below 11%. Cortisol concentrations were measured with a high sensitivity chemiluminescence immunoassay (IBL International, Hamburg, Germany; intra- and inter-assay coefficients of variation below 8%) to confirm that post-drug administration cortisol levels did not vary by group (see **Table 2**). Log-transformation was used to normalize distributions for sAA and salivary cortisol (Rohleder and Nater, 2009).

Image Collection and Preprocessing

Brain images were collected using a 3T General Electric MRI scanner (Discovery MR750; GE Medical Systems, Waukesha, WI) equipped with an 8 channel RF coil (GE Healthcare, Waukesha, WI). Structural data were acquired using a T1-weighted BRAVO pulse sequence (TI:450 ms, TR/TE/flip:8.16 ms/3.2 ms/12°, matrix:256 × 256 × 160, FOV:215.6 mm, slice thickness:1 mm). Diffusion-weighted data were acquired using a 2D echo planar imaging (EPI) sequence with ASSET parallel imaging at a geometric reduction factor of 2 in order to correct magnetic field inhomogeneities (TR/TE/flip:8,000 ms/66.2 ms/90°, matrix:128 × 128 × 76, FOV:256 mm, slice thickness:2 mm). Each axial slice was encoded with 48 directions, b = 1,000 s/mm^2, and eight b0 images. Field maps to correct for magnetic field distortions were also collected. Images were corrected for eddy current and fieldmap distortions, and skull-stripped (FMRIB Software Library, FSL; Smith et al., 2004). Images from the first scan session were used for analysis. Tensors were fitted with nonlinear optimization, constrained to be positive semi-definite using Camino (camino.cs.ucl.ac.uk; Cook et al., 2006). Tensor images were normalized, and scalar maps for fractional anisotropy (FA) and axial, radial, and mean diffusivity were calculated, using Diffusion Tensor Imaging ToolKit (DTI-TK, dti-tk.sourceforge.net).

To create seed regions with which to virtually dissect tracts of interest, T1 images were processed using FreeSurfer's automatic recon-all pipeline including preprocessing steps (motion correction, intensity normalization, affine registration to Talairach atlas, brain extraction), linear registration to a Gaussian classifier array for automatic subcortical segmentation (v5.3, https://surfer.nmr.mgh.harvard.edu; Fischl et al., 2002), and estimation and parcellation of cortical surfaces into functional and anatomical compartments (Desikan et al., 2006; Destrieux et al., 2010).

TABLE 2 | Salivary analytes by childhood emotional abuse (EA) severity and drug.

Measure	CTQ emotional abuse groups		
	Minimal (n = 46)	Moderate (n = 14)	Severe (n = 14)
sAA LEVELS, U/mL			
Placebo	191.8 ± 155.9	172.9 ± 155.6	236.6 ± 193.8
CORT	166.0 ± 115.1	221.7 ± 225.9	187.4 ± 134.1
SALIVARY CORTISOL LEVELS, nmol/L			
Placebo	1.3 ± 1.6	1.7 ± 2.1	1.3 ± 0.7
CORT	55.4 ± 33.6	54.2 ± 32.7	50.6 ± 41.9

Values are mean ± SD. CORT, cortisol administration; CTQ, Childhood Trauma Questionnaire; sAA, salivary alpha-amylase.

Raw values for sAA and cortisol are shown in the table. Log-transformed values were used for analyses to normalize distributions. Neither sAA nor cortisol showed main effects or interaction for Drug (Placebo vs. CORT) or CTQ Emotional Abuse (EA), p's > 0.33.

Data Analysis

CORT's Effects on SNS Activation in Relation to EA

Proc GLM in SAS (SAS version 9.4, Cary, NC) was used to test (1) whether there was a main effect of Drug (CORT vs. Placebo) on sAA, and (2) whether effects of Drug on sAA differed with respect to EA. Analyses were conducted on log-transformed (i.e., normalized) values of sAA.

DTI

Tract-based spatial statistics (TBSS).

Individual subject maps were co-registered to a bootstrapped group template, and warps to that template were computed, by implementing DTI-TK (Zhang et al., 2007) using a custom tool (Diffusion Image Processing and Analysis, DIPA; github.com/pegasus-isi/dipa-workflow) implemented through Condor, developed by the Center for High Throughput Computing at the University of Wisconsin-Madison & Wisconsin Institutes for Discovery (chtc.cs.wisc.edu). Whole-brain, voxelwise statistical analysis of white matter structure was carried out using the TBSS method in FSL, which entails the creation of a white matter skeleton along which each subject's projected data are then analyzed (Smith et al., 2006).

To elucidate the relationship of childhood EA and adult white matter structure, general linear models were constructed accounting for age as a nuisance regressor and for depression severity, and were entered into FSL's Randomize program. Randomize performs nonparametric permutation to allow inference on data whose null distribution is not known (Winkler et al., 2014). Family-wise error rates were corrected using threshold-free cluster enhancement, which assigns voxel-wise values based on cluster-like local spatial agreement (Smith and Nichols, 2009). Next, we tested whether EA moderated the relationship between white matter structure (FA) and effects of CORT (vs. Placebo) on sAA. The difference in sAA between the CORT and Placebo days ($sAA_{CORT-Placebo}$) therefore represented our experimental variable, and an interaction term specified whether EA moderated these effects. To confirm that depression severity did not also moderate the relationship between $sAA_{CORT-Placebo}$ and FA, a model correcting for age and including terms for BDI, $sAA_{CORT-Placebo}$, and their interaction was also entered into Randomize.

Once clusters of interest were identified, they were warped from template space to participant space and mean FA values over the cluster were extracted from corresponding regions in each participant. For *post hoc* analyses, these FA values were entered into ANCOVA using SAS to characterize the relations between FA and sAA, and differences in these relations across EA groups.

Deterministic tractography

TBSS-derived clusters often include fiber bundles from multiple tracts of interest, which may be disambiguated to aid in interpreting their functional significance. To anatomically validate the affected tracts, deterministic tractography was performed in subjects' native space using Camino and visualized in TrackVis (trackvis.org; Wang et al., 2007). Significant voxels were used as seeds for streamline tractography using the fiber assignment by continuous tracking (FACT) algorithm. Streamlines were terminated at voxels with FA values less than 0.1 or when local curvature exceeded 60°. Among streamlines passing through the significant white matter clusters, tract membership was determined using anatomical landmarks and subject-specific segmentation-derived seed regions from FreeSurfer, as described in the Results. In general, when referring to "clusters," we mean voxels found significant in the TBSS model; by "tracts" we mean those a priori, anatomically-identifiable tracts whose streamlines were found to pass through the clusters of interest.

RESULTS

Participants Could Not Distinguish Between Drug Conditions

Consistent with prior research (Abercrombie et al., 2005, 2011; Wirth et al., 2011), participants did not perform above chance in distinguishing between CORT and Placebo conditions: when asked on each of the first and second visits what they thought had been administered, 60 and 67% of participants said they did not know, respectively, and of those that guessed, accuracies were 52 and 38%, p's > 0.05.

Effects of CORT on SNS Activation

No main effect of Drug (CORT vs. Placebo) on sAA was observed, $F_{(1,74)} = 0.09$, $p = 0.76$ (**Table 2**). In addition, sAA level was not related to EA in either the Placebo or CORT condition or its interaction with Drug, p's > 0.18. sAA was also not related to depression or its interaction with Drug, p's > 0.59.

SNS Activation and White Matter Structure

TBSS-derived clusters of interest shown in **Figures 2** and **3**, and **Table 3** represent regions in which FA was significantly associated with EA, $sAA_{CORT-Placebo}$, or their interaction. Clusters associated with the interaction were centered in bilateral white matter tracts ventral to motor and premotor cortices at the level of the centrum semiovale (**Figure 3A**), at the intersection of the CST, corpus callosum (CC), thalamic radiations/corona radiata (CR), and inferior fronto-occipital fasciculus (IFOF). Corticomotor projections were identified as intersecting at least one of precentral, paracentral, caudal middle frontal, superior frontal, posterior cingulate or caudal anterior cingulate gyri, and reaching spinal cord. In addition, we adapted landmarks from prior studies (e.g., Bleyenheuft et al., 2007) to identify fibers belonging to CST proper (Figure S1). IFOF fibers terminated anteriorly in precentral, inferior or middle frontal gyri and posteriorly in parietal or superior temporal areas. CR terminated in thalamus.

The relation between tractographic "streamline" count and anatomical fiber count is highly conditional, and should be understood as a qualitative rather than quantitative tool (Jones et al., 2013b), but may offer a very coarse measure of comparative connectivity. In all participants examined, a large quantity of streamlines belonging to CST and other motor cortical projections to spinal cord were readily identified, of which the overwhelming majority traversed the cluster of interest. Approximately half or fewer of those streamlines belonging to IFOF did so, and those that did were generally limited to the tract's superior extent (i.e., terminating in superior parietal areas). CR terminating in thalamus varied in cluster membership.

Posthoc analyses were conducted to disentangle the significant TBSS interaction. Using mean cluster FA, we found that the correlation between $sAA_{CORT-Placebo}$ and FA differed across EA groups, $F_{(6,74)} = 3.90$, $p < 0.01$, $R^2 = 0.26$. This effect was driven by Placebo-day differences in slope, $F_{(6,74)} = 3.02$, $p = 0.01$, $R^2 = 0.21$ (**Figure 3C**), such that for women with minimal childhood EA, post-task sAA levels were negatively associated with higher cluster FA ($r = -0.30$, $p = 0.02$), but for women with history of severe EA the association was positive though did not reach significance ($r = 0.37$, $p = 0.20$). Cluster FA was unrelated to CORT-day sAA and its interaction with EA, p's $>$ 0.19 (**Table 3**). Further investigation showed that effects were mainly driven by opposing group-wise relations between sAA and radial diffusivity; axial and mean diffusivity were unrelated to sAA and EA.

FA was associated with depression severity in a cluster localized to the anterior CC (Table S1, Figure S2), but was not associated with sAA or the interaction between depression severity and sAA.

DISCUSSION

Consistent with our hypothesis, we found that the structural properties of bilateral corticomotor projections were related to SNS activation following exposure to emotional stimuli. Specifically, we found that the relation of corticomotor FA to SNS activation differed among women with history of severe childhood emotional abuse vs. those without, and CORT administration eliminated these differences. Depression severity did not moderate these effects. These findings provide evidence that these corticomotor projections may constitute an important pathway underlying alterations in stress neuromodulators in individuals with a history of abuse.

Differences in FA Are Likely Driven by Corticomotor Projections

Streamlines belonging to a variety of tracts traversed the clusters extracted from the TBSS analysis. Although tractography cannot definitively confirm or exclude the contribution of individual tracts to detectable FA effects, it can aid in interpreting likely drivers. In all participants examined, the most reliable corticofugal projections linked brainstem and spinal cord to

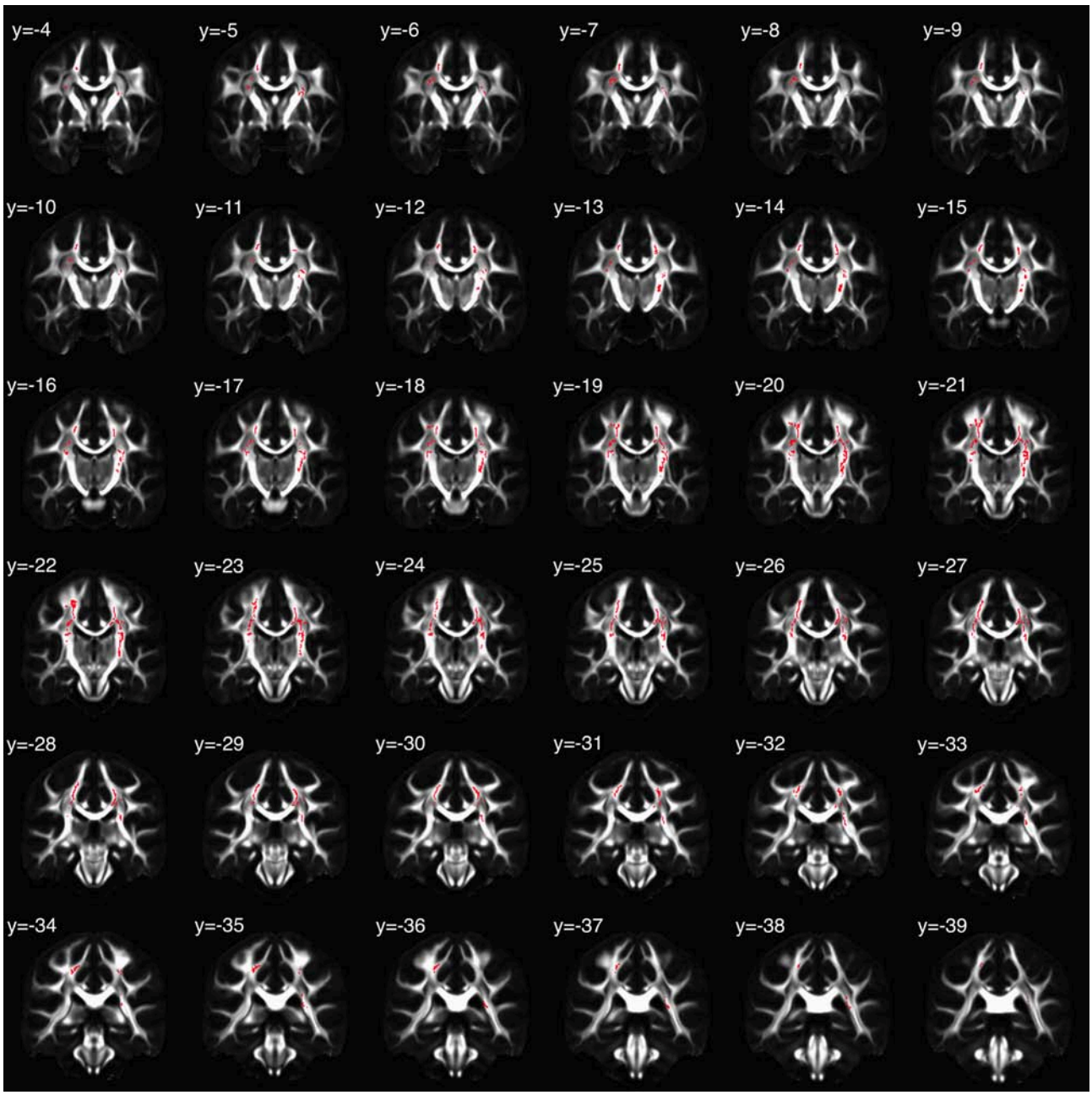

FIGURE 2 | Clusters in which the relation between fractional anisotropy (FA) and salivary alpha-amylase (sAA) was moderated by childhood emotional abuse (EA). Coronal slices are presented in montage, beginning with $y = -4$ in MNI space, radiological orientation (i.e., participants' right is on viewer's left).

sensorimotor areas (Figure S3). Using anatomical landmarks to positively identify its descending course, CST streamlines were identified as traversing the clusters of interest more reliably and in greater proportion than other tracts: of streamlines belonging to CST, over 90% were identified in multiple participants. Most such streamlines appeared to terminate on the dorsal surface of precentral and superior frontal gyri, although precise departures of fibers from a larger tract can be difficult to resolve (e.g., Jones et al., 2013a). Other premotor and sensorimotor projections reaching spinal cord showed similarly high rates of capture by our clusters.

Although a majority of streamlines of the mid-to-posterior CC traversed the clusters, the structure's interhemispheric extent—one of the most robust and undiluted white matter tracts in the brain—did not reach significance, and bilaterally the significant clusters extend well inferior to the main CC projections. This suggests that interhemispheric connectivity, while possibly involved, likely is not the principal driver our effects.

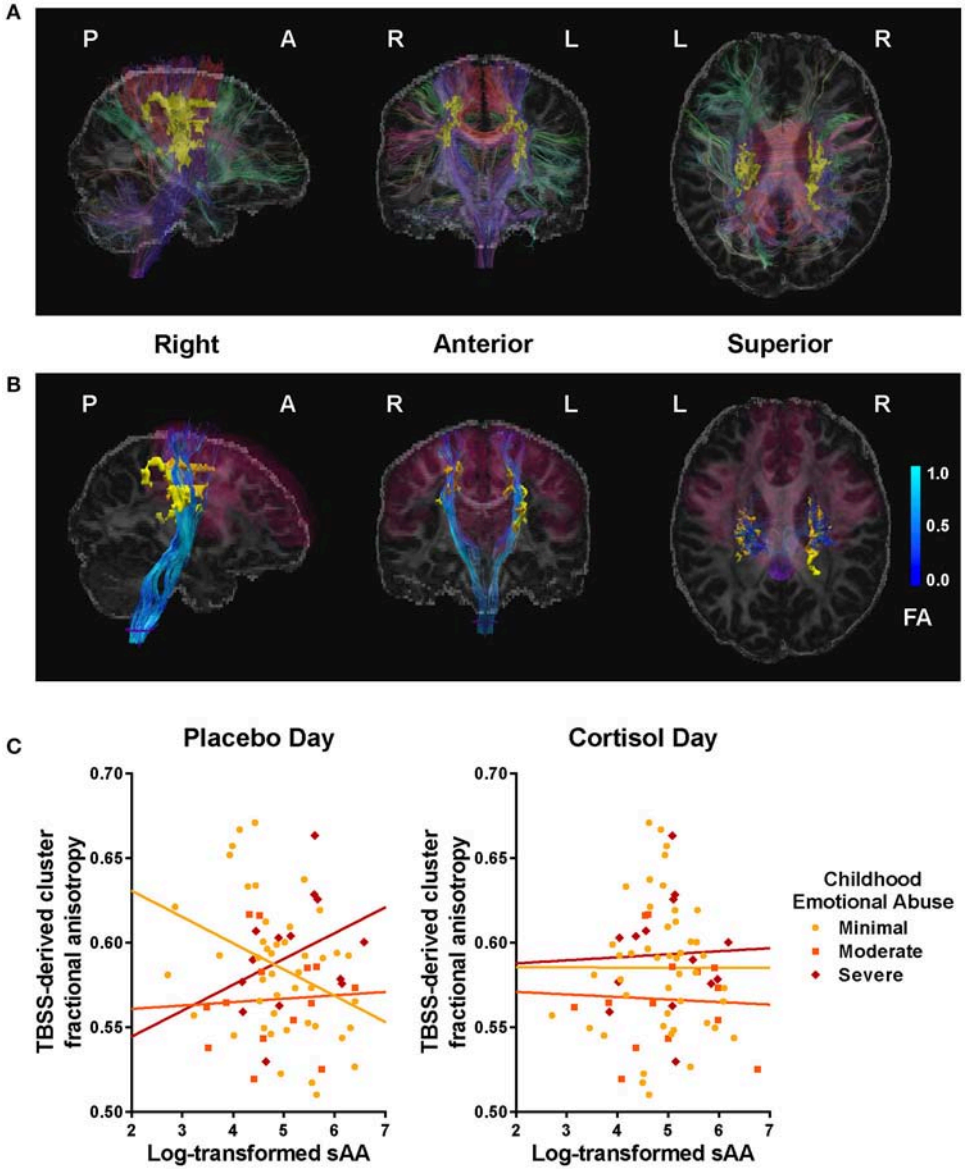

FIGURE 3 | Clusters and tracts in which the relation between fractional anisotropy (FA) and salivary alpha-amylase (sAA) was moderated by childhood emotional abuse (EA). **(A)** Tract-based spatial statistics (TBSS)-derived clusters (yellow) significantly associated with EA × $sAA_{CORT-Placebo}$ interaction, illustrated with a representative participant, and fiber tracts intersecting clusters (color indicates direction at fiber midpoint: red fibers run primarily left-right, green anterior-posterior, blue superior-inferior). See **Table 3** for listing of significant clusters**. (B)** Projection fibers (blue) originating from corticomotor regions (magenta, derived from FreeSurfer segmentation: precentral gyrus, paracentral gyrus, posterior & caudal anterior cingulate gyrus & sulcus, superior frontal gyrus, and caudal middle frontal gyrus), passing through TBSS-derived cluster (yellow), and reaching spinal cord (purple) after virtual dissection using FreeSurfer segmentation. Brighter blue indicates higher FA. **(C)** Scatter plots for sAA and cluster FA, plotted by EA. At left, Placebo-day correlations; at right, CORT-day correlations. EA moderated the association between cluster FA and sAA levels during placebo administration but not during CORT, $F_{(6,74)} = 3.90$, $p < 0.01$.

A variable proportion, typically half or fewer, of IFOF streamlines intersected the clusters, and those generally in dorsal parietal areas, in contrast to more ventral tracts such as those identified in Rodrigo et al. (2016). However, future research can aid in disentangling the possibility of crossing-fiber effects; see following section.

From a theoretical standpoint, corticomotor projections were a hypothesized structure of interest. As described in Abercrombie et al. (2018), CORT increased activation in SMA/PMC and rescued depression-related deficits in memory formation for positive stimuli in severely maltreated women. On CORT day both sAA and rescue effects were significantly related to activation in these areas. No cortical regions innervated by IFOF fibers showed this group x drug interaction. And insofar as corticomotor and adrenal activity are related, projections via CST and other descending circuits would represent a parsimonious route.

TABLE 3 | Childhood emotional abuse (EA), salivary alpha-amylase (sAA), and fractional anisotropy (FA).

Cluster	Volume (voxels)	Coordinate (peak)	Coordinate (CM)	T-Stat (peak)	*P*-value (corrected)
EA					
None	–	–	–	–	–
$sAA_{CORT-Placebo}$					
Left corticofugal tracts	2,373	(−28,−3,4)	(−23,−4,13)	3.74	0.01
Right corticofugal tracts	2,136	(20,−3,34)	(22,−4,15)	4.14	0.01
Left mid superior CC	114	(−18,20,27)	(−17,22,16)	3.33	0.05
Left centrum semiovale	17	(−27,4,27)	(−27,4,27)	3.71	0.05
Left superior CR	13	(−24,21,16)	(−24,21,16)	3.34	0.05
EA * $sAA_{CORT-Placebo}$					
Left corticomotor tracts	1,341	(−28,−3,4)	(−25,−4,17)	3.56	0.02
Right corticomotor tracts	1,307	(18,−3,34)	(21,−5,24)	4.22	0.03

CM, center of mass; CC, corpus callosum; CR, corona radiata; EA, Emotional Abuse subscale of the Childhood Trauma Questionnaire; $sAA_{CORT-Placebo}$, the difference in salivary alpha-amylase between the CORT and Placebo days.
Significant clusters associated with model factors.

Possible Sources of Biological Variation in Corticomotor Projection Tract Structure

Although the precise changes in axonal microstructure or fiber architecture cannot be resolved by FA (Jones et al., 2013b), interpretation depends at least in part on whether effects represent disrupted signaling or some other change in circuit function. Recently-published research (Mortazavi et al., 2018) suggests a possible, if highly speculative, mechanism to explain these effects. Histology of macaque CST found that, contrary to following smooth contours, many axons at the level of the centrum semiovale executed "microscopically sharp axonal turns and/or branches (radii $\leq$ 15 mm) into 2 sharply defined orientations, mediolateral and dorsoventral." Further, high-angle diffusion MRI, which is capable of resolving crossing fiber orientation, was unable to distinguish between crossing fibers and sharp axonal turns.

The influence of intra-voxel discrepancy in fiber orientation on FA is thought to be large, relative to that of axonal physiology (Jones et al., 2013b). It is therefore conceivable that small variations in the proportion of descending fibers deflecting to mediolateral targets in this span of CST could yield statistically detectable changes in FA, even if restricted to a small population of axons bound for spinal circuits innervating sympathetic preganglionic cells. This would also be consistent with the fact that our FA effects were driven by changes in radial, rather than axial, diffusivity. Given the novelty of the above findings, further studies in animal models—which offer the additional advantage of well-characterized adverse caregiving paradigms—may serve to test the generalizability of our results, and fully elucidate any biological source of our structural findings.

EA Moderated Associations Between Cluster FA and sAA

Under placebo, i.e., during basal levels of cortisol, the relation between SNS activation and bilateral corticomotor tract FA depended on severity of EA. Among participants with minimal history of childhood EA, FA was negatively associated with sAA, indicating that women with greater corticomotor white matter FA showed relatively lower SNS activation. For individuals with severe EA, however, results trended in the opposite direction such that higher white matter FA was weakly associated with relatively higher SNS activation.

These findings at baseline are interesting in light of recent nonhuman primate tracing studies indicating that spinal circuits influencing adrenal medulla are likely innervated by fibers from cortical motor areas (Dum et al., 2016). SMA was already known to be robustly associated with SNS tone and autonomic function (Medford and Critchley, 2010), consistent with its classical role preparing for strenuous action involving core musculature (Graziano, 2006); spinal projections therefore constitute a previously unknown, but logical, pathway with respect to modulation of peripheral stress-related activation. Previous analyses from this sample (Abercrombie et al., 2018), as well as other studies, have found functional alterations to SMA and adjacent premotor areas in individuals with history of maltreatment, especially in paradigms related to emotion regulation and executive control (for review, cf. McCrory et al., 2017). These alterations may also distinguish between individuals resilient to or at risk of psychopathology related to early adversity (Herringa, 2017). Taken together, one speculative interpretation might hold that history of exposure to EA alters the function of these areas and their projections to the SNS.

SMA and adjacent premotor areas, including dorsal cingulate, are known to integrate cognitive and affective inputs in motor planning (Picard and Strick, 2001; Shackman et al., 2011) along a continuous functional and physiological gradient: from anterior to posterior, cognitive processing converges on motor planning, and density of spinal cord-bound projections to adrenal medulla increase (Nakamura et al., 1998; Dum and Strick, 2002; Morecraft and Tanji, 2009). These systems appear to coordinate response selection and inhibition under complex, uncertain, and/or emotionally salient conditions (Nachev et al., 2008; McRae et al., 2010). However, despite their documentation

in research concerning motor and some cognitive function, nomenclature for these areas varies in the emotion research discipline—perhaps due in part to limitations on spatial fidelity and resolution in functional imaging, or to challenges specific to more commonly-studied prefrontal association cortices. Study coordinates or clusters that overlap with preSMA, SMA, anterior PMC and other areas may be described using only directional or topographic terms: dorsomedial prefrontal cortex, superior frontal gyrus, dorsal anterior- or mid-cingulate, etc., whose boundaries may be defined differently across studies (see reviews: Ray and Zald, 2012; McCrory et al., 2017). It is possible that they may therefore play an under-recognized role linking emotion to behavior, particularly in the context of stress.

Furthermore, SMA in particular appears to facilitate voluntary emotion regulation (Buhle et al., 2014), alterations to which are considered a link between EA and risk of psychopathology (O'Mahen et al., 2015). Animal evidence for the role of these premotor circuits in inhibiting automatic and enabling intentional responses includes microstimulation of preSMA neurons (Isoda and Hikosaka, 2007), and macrostimulation in humans produces similar effects (Swann et al., 2012). A rodent paradigm found altered connectivity patterns in prefrontal and motor areas in female rats subjected to chronic social stress as pups (Nephew et al., 2017, 2018). Silvers et al. (2017) found that as typically-developing children reach adulthood, reactivity to aversive relative to neutral stimuli shifted from ventromedial (vmPFC) to dorsomedial prefrontal cortex (dmPFC), including preSMA, whereas Herringa and colleagues (Keding and Herringa, 2016; Wolf and Herringa, 2016) found a reverse profile of activation and limbic connectivity in children suffering from PTSD. In addition to SMA function, there is some independent evidence that CST structure is itself related to strategic aspects of emotional intelligence (Pisner et al., 2017).

That these systems should vary by exposure to early life adversity may represent divergent calibration of the neural circuitry supporting emotion regulation for vastly different environmental demands. Indeed, in a review of studies in EA populations, McCrory et al. (2017) found maltreatment-related effects on emotion regulation may persist in the absence of overt psychopathology, and suggest neural responses that are adaptive in an adverse caregiving environment confer "latent vulnerability" in adulthood. Other authors have converged on similar frameworks (e.g., Del Giudice et al., 2011; Blair and Raver, 2012; Teicher and Samson, 2013).

CORT Eliminated Effects of EA on Relations Between Cluster FA and sAA

While EA moderated the relation of corticomotor tract FA and SNS activation under baseline conditions, CORT administration eliminated these associations. Following acute cortisol elevation SNS tone may depend on neural pathways other than corticomotor projections. There is a relative dearth of studies directly examining effects of GCs on neural control of SNS; however, GCs affect a variety of structures that regulate SNS output, including the hypothalamus, amygdala, and bed nucleus of the stria terminalis (Ulrich-Lai and Herman, 2009). Research in rodents implicates amydalofugal pathways during GC elevation: whereas GCs exert negative feedback on the HPA axis in hypothalamus and cortical areas, they increase CRH in the amygdala. This affects fear-related behaviors associated with SNS activity, such as startle (Erickson et al., 2003). Indeed, a study (Song et al., 2014) specifically examining FA in tracts connecting to brainstem nuclei found depression-related alterations not in CST, but in the solitary tract, which is known to reciprocally connect to amygdala; however, they did not report on alterations due to EA. Future research is needed to determine the neural circuitry through which GCs interact with SNS function, and how circuits are affected by EA. This is relevant as evidence suggests that in some disorders, such as PTSD, there are alterations in the relation between GCs and SNS (Yehuda et al., 1998).

Implications for Etiology and Treatment of Stress-Related Psychopathology

Our findings lend further evidence to a large body of literature showing that history of maltreatment is an important etiological factor in depression and other forms of psychopathology, which has bearing on treatment selection (Nemeroff et al., 2003; Williams et al., 2016). Furthermore, early adversity alters stress response systems, and alterations in cortisol signaling and HPA regulation are more often observed in depressed adults with vs. without history of childhood adversity (Heim et al., 2008; Abercrombie et al., 2018). The current findings extend upon these literatures by implicating corticomotor projections as part of a stress responsive neural circuit whose functioning is associated with variation in early caregiving.

These premotor areas and their influence on SNS function may represent an important target for clinical intervention. In addition to their involvement in emotion regulation—an important buffer to psychopathology, as discussed in the section above, "EA moderated associations between cluster FA and sAA"—recruitment in medial premotor areas may play a compensatory role in individuals with maltreatment and/or psychiatric disorders according to several lines of evidence, including a meta-analysis (Herringa et al., 2016; McTeague et al., 2017). Corticomotor projections influencing SNS could comprise a neural circuit important for psychotherapeutic benefit in individuals with history of adverse caregiving.

This circuit may therefore be an important target for behavioral, pharmacological, or neuromodulatory interventions. It could play a role in effective treatments such as behavioral activation (Dimidjian et al., 2011; Ekers et al., 2014), aerobic exercise (Rebar et al., 2015; Schuch et al., 2016), or yoga (Pascoe and Bauer, 2015; Cramer et al., 2017). Our prior research may also suggest that interventions targeting cortisol signaling could operate in part through activation of SMA and PMC (Abercrombie et al., 2018). Repetitive Transcranial Magnetic Stimulation (rTMS) to dmPFC reduced depression severity (Downar et al., 2014), although this was applied to an area anterior to preSMA, was effective specifically in patients with lower anhedonia symptomatology, and initial replication efforts have not been successful (Bakker et al., 2015). Gutman et al. (2009) performed probabilistic structural connectivity for

a target of deep brain stimulation (DBS) previously found effective in treating depression, located in the anterior limb of the internal capsule (IC), and its connectivity patterns bear some superficial resemblance to those of our TBSS-derived cluster, although of course anterior to our cluster intersecting the posterior limb of IC. Though evidence is mixed, cortical motor circuits may represent targets for psychiatric treatment with neuromodulation, possibly in combination with context-specific cognitive therapy related to emotion regulation. Based on findings suggesting that functioning within these circuits is related to early experience, effectiveness of such treatments may vary based on history of childhood adversity.

As mentioned previously, a "mismatch" may arise between phenotype—which developed in response to a stressful early environment—and post-developmental context, that increases risk of depression or other psychopathology. Progress in understanding how to re-align phenotype and environmental demands may improve the specificity of treatments beyond that achieved by accounting for early experience alone.

Limitations

As this is a cross-sectional study, we are unable to draw firm conclusions as to how childhood EA altered developmental trajectories leading to adult differences in brain structure and stress neuromodulator function. In both the current analyses and in fMRI analyses in the same sample (Abercrombie et al., 2018), we found that EA but not depression severity was associated with neural alterations. However, due to the relatively low rate of PTSD in this sample, we are unable to examine neural correlates related to PTSD.

Another limitation of our study is that we were not able to recruit a sample of adults who experienced extreme EA but who do not exhibit psychopathology, and for statistical reasons we needed to exclude a single participant who presented with extreme EA but no depressive symptoms. Future research should endeavor to include such participants with targeted recruiting to allow investigation of the role of EA in stress-related physiology along a full range of severity of psychopathology.

In addition, emerging methods for disambiguating crossing fibers may aid in establishing the exact contributions of each tract captured by our clusters of interest. Further study comparing these methods against histological studies are vital for interpretation. Finally, the present study investigates the effects of exogenous cortisol; future research must also elucidate the impact of naturalistic environmental stressors on relations among white matter structure, cortisol, and SNS activation.

CONCLUSION

We found that history of emotional abuse—but not severity of depression—moderated relations among corticomotor white matter structure and SNS activation in a sample of women with levels of depression along a broad continuum of severity. The findings may suggest that such pathways supporting neural influence on SNS activity vary based on prior experience of adverse caregiving. Furthermore, cortisol administration abolished these associations, consistent with evidence that cortisol acutely alters neural systems supporting the regulation of peripheral stress physiology.

Functional systems-based approaches to the etiology of mental disorders (Insel, 2014) offer insights into biological mechanisms that may be affected by life history, and which can be targeted for clinical applications. Disruptions to both SNS and HPA responses to stress are candidate systems in stress-related disorders such as depression and PTSD. Cortical motor systems and their efferent fibers, whose outputs appear to ultimately modulate adrenal medulla (Dum et al., 2016), may represent a neural circuit affecting alterations in stress response systems. Future studies should further characterize the action of this pathway under different neural, endocrine, and cognitive conditions, and test whether such action differs according to exposure to adverse caregiving and other forms of early life adversity.

AUTHOR CONTRIBUTIONS

HA designed the study and obtained funding. HA, EW, RH, and RB refined the design and implemented the study. EW and RH programmed the picture viewing task. EW and RB programmed scan parameters. EW, RH, CF, and HA collected data. EW and RH organized and cleaned data. EW, CF, and HA analyzed data. CF processed and analyzed neuroimaging data in consultation with RB, MM, and HA. CF wrote the first draft of the manuscript in consultation with MM and HA. HA wrote sections of the manuscript. CF, RH, and HA composed figures and tables. All authors read and approved the manuscript.

FUNDING

This research was funded by grants to HA from the National Institute of Mental Health (R01MH094478) and the University of Wisconsin-Madison Office of the Vice Chancellor for Research and Graduate Education with funding from the Wisconsin Alumni Research Foundation; a Training Program in Emotion Research grant (5T32MH018931-25, PI: Richard J. Davidson) and a Dissertation Completion Fellowship to CF; and a NCCIH grant (T32AT003378) to EW. Data were collected with the assistance of the ICTR Mobile Research Team, which is supported by the Clinical and Translational Science Award (CTSA) program through the NIH National Center for Advancing Translational Sciences (NCATS), grant UL1TR000427. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. The NIH had no further role in study design; in the collection, analysis, and interpretation of the data; in the writing of the report; or in the decision to submit the article for publication.

ACKNOWLEDGMENTS

We thank all of the volunteers who participated in this study, as well as A. Blumenfeld, C. Siwik, M. Dennison, A. Ehlers, C. Ernstoff, S. Goldberg, M. Kalambokidis, A. Lang, J. Nelson,

E. Osterbauer, R. Svoboda, R. Vohnoutka, A. Winter, Lane Neuroimaging Laboratory staff, and ICTR Mobile Research Team for assistance with data collection. We thank Stacey M. Schaefer, Christine Heim, Marilyn J. Essex, Ned H. Kalin, and Richard J. Davidson for consultation and advice. We thank Clemens Kirschbaum's laboratory for conducting salivary cortisol and alpha-amylase assays. We thank Do Tromp, Nagesh Adluru, Andrew Pohl, and Lea Ziskind-Conhaim for consultation on data analysis. Diffusion Image Processing and Analysis (DIPA) software package was conceived and developed primarily by Andrew J. Schoen (schoen.andrewj@gmail.com) and Nagesh Adluru (nagesh.adluru@gmail.com). Please visit https://github.com/pegasus-isi/dipa-workflow for more details. This research was supported by the computing resources and assistance of the UW-Madison Center for High Throughput Computing (CHTC) in the Department of Computer Sciences.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00256/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 23 April 2018
doi: 10.3389/fendo.2018.00183

Neurosteroids in Adult Hippocampus of Male and Female Rodents: Biosynthesis and Actions of Sex Steroids

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The brain is not only the target of steroid hormones but also is able to locally synthesize steroids *de novo*. Evidence of the local production of steroids in the brain has been accumulating in various vertebrates, including teleost fish, amphibia, birds, rodents, non-human primates, and humans. In this review, we mainly focus on the local production of sex steroids in the hippocampal neurons of adult rodents (rats and mice), a center for learning and memory. From the data of the hippocampus of adult male rats, hippocampal principal neurons [pyramidal cells in CA1–CA3 and granule cells in dentate gyrus (DG)] have a complete system for biosynthesis of sex steroids. Liquid chromatography with tandem-mass-spectrometry (LC-MS/MS) enabled us to accurately determine the levels of hippocampal sex steroids including 17β-estradiol (17β-E2), testosterone (T), and dihydrotestosterone (DHT), which are much higher than those in blood. Next, we review the steroid synthesis in the hippocampus of female rats, since previous knowledge had been biased toward the data from males. Recently, we clarified that the levels of hippocampal steroids fluctuate in adult female rats across the estrous cycle. Accurate determination of hippocampal steroids at each stage of the estrous cycle is of importance for providing the account for the fluctuation of female hippocampal functions, including spine density, long-term potentiation (LTP) and long-term depression (LTD), and learning and memory. These functional fluctuations in female had been attributed to the level of circulation-derived steroids. LC-MS/MS analysis revealed that the dendritic spine density in CA1 of adult female hippocampus correlates with the levels of hippocampal progesterone and 17β-E2. Finally, we introduce the direct evidence of the role of hippocampus-synthesized steroids in hippocampal function including neurogenesis, LTP, and memory consolidation. Mild exercise (2 week of treadmill running) elevated synthesis of DHT in the hippocampus, but not in the testis, of male rats, resulting in enhancement of neurogenesis in DG. Concerning synaptic plasticity, hippocampus-synthesized E2 is required for LTP induction, whereas hippocampus-synthesized DHT is required for LTD induction. Furthermore, hippocampus-synthesized E2 is involved in memory consolidation tested by object recognition and object placement tasks, both of which are hippocampus-dependent.

Keywords: hippocampus, neurosteroids, estradiol, testosterone, dihydrotestosterone, estrous cycle, synaptic plasticity, neurogenesis

OPEN ACCESS

Edited by:
Takayoshi Ubuka, Monash University Malaysia, Malaysia

Reviewed by:
Giuseppe Talani, Consiglio Nazionale Delle Ricerche (CNR), Italy
Giuseppe Biagini, University of Modena and Reggio Emilia, Italy

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *02 February 2018*
Accepted: *04 April 2018*
Published: *23 April 2018*

Citation:
Hojo Y and Kawato S (2018) Neurosteroids in Adult Hippocampus of Male and Female Rodents: Biosynthesis and Actions of Sex Steroids. Front. Endocrinol. 9:183. doi: 10.3389/fendo.2018.00183

INTRODUCTION

Extensive evidence has been accumulated that the systems of local steroid synthesis exist in the organs other than gonads and adrenal since 1980s (1, 2). Local production of steroids in the brain has been investigated in various vertebrates, including teleost fish (3, 4), amphibia (5–7), birds (8, 9), rodents (10–14), non-human primates, and humans (15–17).

For clinical purposes, the importance of neurosteroids is increasing. Because of the limitation to invade human brain tissues, quantitative determination of steroids in cerebrospinal fluid (CSF) has been applied to detect the alteration of the allopregnanolone (Allo) level under physiological/pathological conditions, including epilepsy (18, 19), and reproductive mood disorders (20, 21).

Sex steroids including 17β-estradiol (17β-E2), testosterone (T), and dihydrotestosterone (DHT) are also synthesized in the brain. In this review, we mainly focus on the local production of sex steroids, particularly, E2, T, and DHT in the hippocampal neurons of adult rodents (rats and mice).

In addition to the genomic effects, sex steroids modulate neural functions in a rapid/non-genomic manner [reviewed in Ref. (22)]. Using the hippocampal slices of rodents, rapid effects of sex steroids have been extensively investigated. E2 modulates long-term potentiation (LTP) (23, 24) and long-term depression (LTD) (25, 26) in CA1 synapses. E2 induces LTP in CA1 under the weak theta burst stimulation (weak-TBS), which is not strong enough to induce LTP alone (27). Exogenous application of E2, T, and DHT to rat hippocampal slices, rapidly increases dendritic spines in CA1 pyramidal cells (27, 28).

Using exogenous application of steroids, these investigations demonstrated that rapid effects of sex steroids are mediated through estrogen receptors (ERα and ERβ) or androgen receptors (AR), located at the pre/post synapses (25, 28–30), followed by the activation of kinases which phosphorylate the molecules essential for synaptic plasticity. Upon LTP-induction, E2 drives src tyrosine kinase and the extracellular signal-related protein kinase/MAPK (Erk MAPK), resulting in phosphorylation of NMDA receptor (23). In case of E2-induced LTP by weak-TBS, Erk MAPK, PKA, PKC, PI3K, and CaMkII phosphorylate NR2B subunit (27). In addition to postsynaptic modulation, E2 also activates ERs in presynapses, resulting in potentiation of glutamate release (31) or disinhibition of GABAergic axon terminal (32). Concerning spinogenesis, E2, T, or DHT drives Erk MAPK, p38 MAPK, PKA, PKC, PI3K, and LIMK (27, 28), which may phosphorylate cortactin (33, 34) and cofilin (35, 36), leading to actin polymerization and spinogenesis.

Do the effects of hippocampus-synthesized E2, T and DHT share the common mechanism described above? It is difficult, however, to directly demonstrate the roles of hippocampus-synthesized E2, T, and DHT, because of the supply of E2, T, and DHT from testis or ovary. It is necessary to perform the experiment under the depletion of circulation-derived E2, T, and DHT, although the possibility is not excluded that peripherally produced precursors (e.g., pregnenolone and progesterone) convert into E2, T, or DHT in the hippocampus (**Figure 1A**). Several investigations are introduced in the Section "Physiological Roles of Hippocampus-Synthesized Steroids."

STEROID BIOSYNTHESIS IN THE HIPPOCAMPUS

Steroid Biosynthesis in the Hippocampus of Adult Male Rodents

Until about 15 years ago, it had not been elucidated whether adult hippocampal neurons have a complete system for synthesis of sex hormones (from cholesterol to androgens or estrogens) since P450(17α), which is required for synthesis of dehydroepiandrosterone (DHEA) from pregnenolone (PREG), had been thought to be absent in the brain of mammals. Any effort to demonstrate the existence of P450(17α) or its enzymatic activity had been unsuccessful (38–41) despite the presence of DHEA in the rodent brain even after castration (1, 2).

By using the hippocampus of adult male rats, the localization of P450(17α) in the principal neurons [pyramidal cells in CA1–CA3 and granule cells in dentate gyrus (DG)] was demonstrated (42, 43). Immunohistochemistry and *in situ* hybridization studies revealed that StAR and other enzymes, including P450scc, 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-HSD, 5α-reductase (types 1 and 2), and P450arom, are also localized in the hippocampal principal neurons of adult male rats and mice (37, 42–48). Studies with radioactive steroids directly demonstrated synthesis of PREG, DHEA, T, DHT, and E2 in slices or cultured hippocampal neurons from adult male rats in early 2000s (42, 43, 47, 49). These results suggest that complete systems for steroidogenesis exist in the hippocampal neurons of adult male rodents.

Interestingly, an electron microscopic (EM) analysis revealed synaptic localization of steroidogenic enzymes including P450 (17α), P450arom, and 3β-HSD in the hippocampus of adult male rats (42, 50), implying the synaptic synthesis of sex steroids. EM and Western immunoblot analysis revealed localization of steroid receptors including ERα, ERβ, AR, and progesterone receptor (PR) in the hippocampal synapses of rodents (25, 28–30, 51, 52).

Although these results give information about the potential to synthesize steroids in the hippocampus, it remains unclear whether hippocampus-synthesized steroids are effective enough to modulate hippocampal functions. To answer this question, it is necessary to quantitatively determine the concentration of steroids in the hippocampus. From early 2000s, quantitative determination of steroids, such as PREG, DHEA, and T in brain with mass-spectrometry (MS) began to emerge (53–58). The presence of pregnenolone sulfate (PREGS) in the brain of mammals had been a matter of debate (59).

To detect small amounts of steroids in the brain, purification of samples and selection of appropriate derivatization reagents are indispensable. Extracts from brain tissue contain various kinds of impurities (lipids and other steroids) which mask derivatization and ionization of the steroid of interest, resulting in decrease of detection efficiency of MS. Purification of the extracts by hybrid-SPE cartridges before LC–ESI-MS/MS enabled the detection of PREGS in the rat hippocampus (60). Concerning the detection of sex steroids, we removed impurities from hippocampal extracts and separated into fractions containing an individual steroid, with C18 column and normal phase HPLC before derivatization.

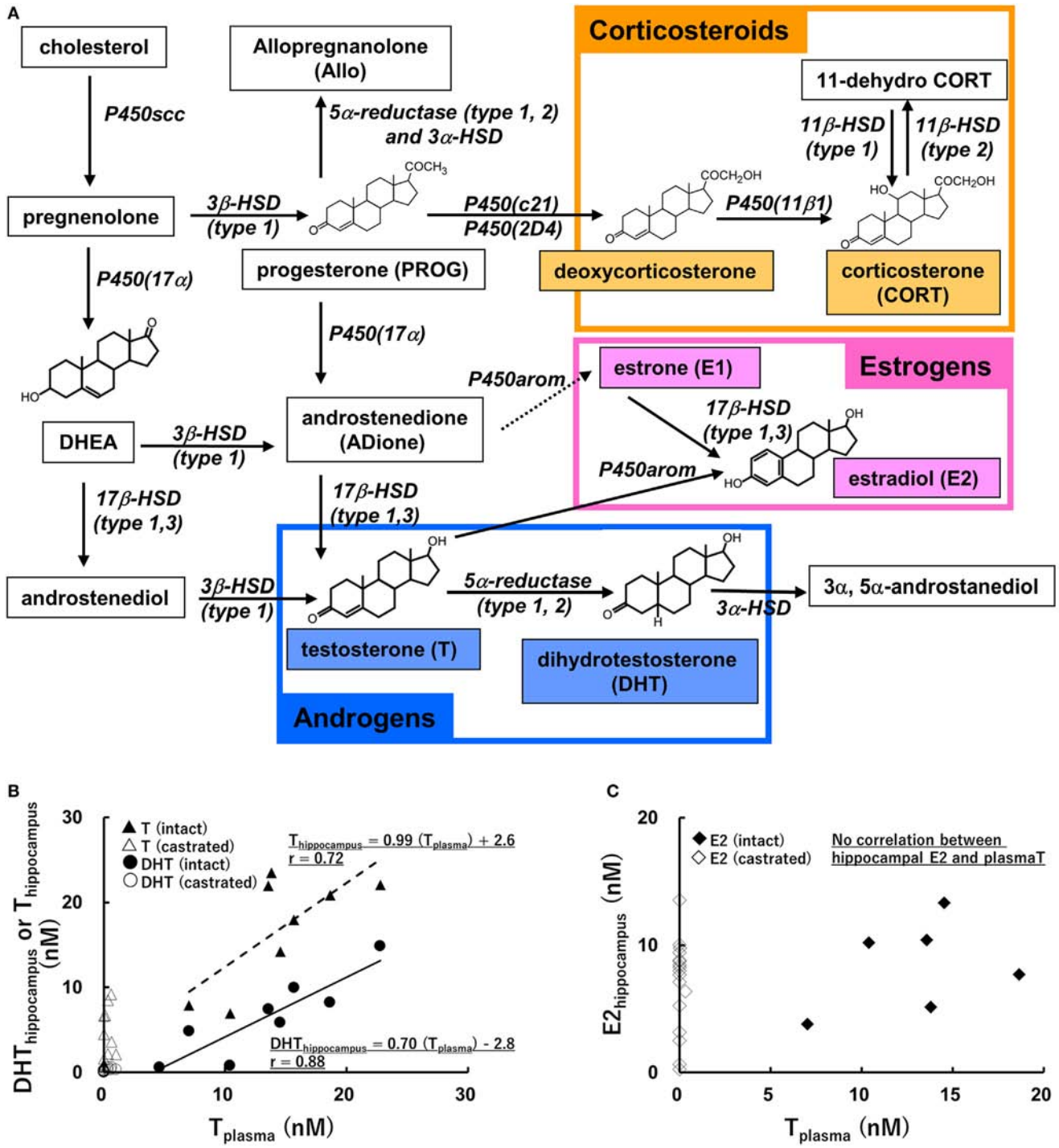

FIGURE 1 | **(A)** Biosynthetic pathway of steroids in rat hippocampus [modified from Ref. (11)]. Estrogens, androgens, and corticosteroids are represented with pink, blue, and orange boxes, respectively. **(B,C)** Relationship between the level of plasma T and those of hippocampal androgens [T and dihydrotestosteron (DHT)] **(A)**, or that of hippocampal E2 **(B)**. Vertical axis represents hippocampal steroids and horizontal axis represents plasma T, a precursor of DHT and E2. The regression lines and Pearson's "*r*" are indicated [modified from Ref. (37)].

Next, picolinoyl-derivatization was selected for steroids of interest (E2, T, DHT, and E1) to increase ionization efficiency (61, 62). Concerning E2, further derivatization with pentafluorobenzyl was performed to elevate volatility. In combination with these improvements, LC-MS/MS enabled us to quantitatively determine the concentration of E2, T, DHT, and E1 in the hippocampus, with high accuracy and reproducibility (37). Caruso and collaborators also determined the levels of steroids including E2, T, and DHT in

rat hippocampus, with LC-MS/MS methods (63). These results support that the significant amount of sex steroids exists in the hippocampus.

Correlation between the level of hippocampal androgen (T and DHT) and that of plasma T (**Figure 1**) was observed (37, 64). Hippocampal estrogen (E2), however, did not correlate with plasma T (**Figure 1**) (37, 64). Using male and female rats, Melcangi's laboratory extensively analyzed the correlation of steroid levels among plasma, CSF and various brain regions (63). According to this work, the levels of E2, T, and DHT in the hippocampus or CSF positively correlated with those in plasma, but no significant correlation of E2 level was observed between in the hippocampus and CSF (63).

Synthesis and Fluctuation of Steroids in the Hippocampus of Adult Female Rodents

Equally important is to clarify whether local steroid production occurs in female hippocampus, because sex hormones have a great impact on functions of female hippocampus [reviewed in Ref. (65)]. The knowledge of the hippocampus-synthesized steroids, however, had been biased toward the data from males (11–13) because of the estrous cycle in female animals. In case of rats and mice, estrous cycle comprises of four stages [proestrus: Pro, estrus: Est, diestrus1 (also called metestrus): D1, and diestrus2 (also called diestrus): D2], and each stage switches in 1 day in this order (66). Hippocampal functions such as spatial memory (67–71), LTP (72, 73), and spine/synapse density (74–78), fluctuate across the estrous cycle. To investigate hippocampal steroid synthesis in female rodents, therefore, fourfold as much data as those of male must be acquired.

LC-MS/MS analysis revealed the accurate concentrations of progesterone (PROG), androstenedione (ADione), T, E1, and E2 in the hippocampus of adult female rats at each stage of estrous cycle (**Table 1**) (79). The levels of plasma steroids exhibit typical estrous cycle dependent changes, in agreement with the previous study (80). The level of hippocampal E2 highly correlates with that of plasma E2, in agreement with other study (63). Concerning the correlation of PROG level between in hippocampus and in plasma, our data exhibit highly positive correlation, whereas others have no correlation (63). It may be due to the difference of samples used for calculation of correlations, rats of both sexes (male and diestrus female) in Caruso et al. (63) and only female (all four stages of the estrous cycle) in our study (79).

Surprisingly, mRNA levels of steroidogenic enzymes, including StAR, P450(17α), 17β-HSD (types 1 and 3), 5α-reductase (types 1 and 2), and P450arom, did not fluctuate in the hippocampus across the estrous cycle (79, 81). Steroid receptors, including ERα, ERβ, AR, and PR, also kept their expression level constant. Moreover, no sex difference was observed concerning these enzymes in the hippocampus whose expression levels are approximately 1/300 ~ 1/1000 of those in gonads or adrenals(79, 81, 82).

Penetration of plasma E2 into the hippocampus, however, is not able to account for the level of hippocampal E2 because hippocampal E2 is much higher than that in plasma. There are two possibilities for explanation of hippocampal E2 fluctuation. The first is the fluctuation of blood PROG which is well known to fluctuate across the estrous cycle (80). This peripherally produced PROG may penetrate hippocampus and be converted into E2, resulting in E2 fluctuation. The other is the fluctuation of activity of kinases including MAPK, Akt, and LIMK, across the estrous cycle (72, 83, 84). The activity of P450arom

TABLE 1 | Mass spectrometric analysis of the concentration of steroids in the hippocampus and plasma of adult rats.

	Male	Female[b]				
		Proestrus	Estrus	Diestrus 1	Diestrus 2	OVX
(A) Hippocampus[a]						
17β-E2	8.4[c] (n^d = 6)	4.3 (n = 6)	1.0 (n = 4)	0.51 (n = 3)	0.67 (n = 4)	0.70 (n = 4)
T	16.9 (n = 8)	1.1 (n = 12)	2.3 (n = 4)	1.3 (n = 3)	1.2 (n = 4)	0.17 (n = 4)
Dihydrotestosteron (DHT)	6.6 (n = 8)	0.62 (n = 7)				
Progesterone (PROG)	14.6 (n = 4)	55.7 (n = 4)	40.7 (n = 4)	87.0 (n = 3)	48.0 (n = 4)	24.5 (n = 5)
Androstenedione	1.5 (n = 4)	1.6 (n = 4)	0.7 (n = 4)	1.1 (n = 4)	0.83 (n = 4)	
E1	0.015 (n = 4)	0.36 (n = 4)	0.045 (n = 4)	0.05 (n = 4)	0.10 (n = 4)	0.025 (n = 3)
Allopregnanolone	1.0 (n = 3)	16.4 (n = 3)				
(B) Plasma						
17β-E2	0.014 (n = 5)	0.111 (n = 6)	0.017 (n = 6)	0.009 (n = 5)	0.029 (n = 6)	0.005 (n = 5)
T	14.6 (n = 8)	0.10 (n = 4)	0.013 (n = 4)	0.020 (n = 3)	0.06 (n = 4)	0.005 (n = 5)
DHT	0.63 (n = 8)					
PROG	6.8 (n = 4)	20.5 (n = 4)	16.7 (n = 4)	51.6 (n = 3)	24.1 (n = 4)	10.1 (n = 5)
Androstenedione	0.61 (n = 4)	1.0 (n = 4)	0.06 (n = 4)	0.119 (n = 4)	0.33 (n = 4)	
E1	0.007 (n = 4)	0.082 (n = 4)	0.004 (n = 4)	0.009 (n = 4)	0.031 (n = 4)	0.002 (n = 4)

[a]Hippocampus was homogenized immediately after dissection from a decapitated head. This condition reflects the basal concentration of steroids in hippocampus.

[b]Female samples were prepared from rats at each stage of estrous cycle (Proestrus, Estrus, Diestrus1, and Diestrus 2) and ovariectomized (OVX) rats.

[c]Data are expressed as mean and are represented as nanomolar. Concentration in nanomolar is calculated using the average volume of 0.14 mL for one whole hippocampus that has 0.14 ± 0.02 g wet weight (n = 86). We assume that tissue having 1 g of wet weight has an approximate volume of 1 mL, since the major part of tissue consists of water whose 1 mL weight is 1 g (47).

[d]Number of animals.

Modified from Ref. (37, 79, 81).

(E2 synthase) changes upon phosphorylation (85). If the activity of kinases fluctuates, then following the fluctuation of P450arom activity may generate hippocampal E2 fluctuation, even if the mRNA levels of steroidogenic enzymes do not change across the estrous cycle.

Female hippocampus is equipped with systems for androgen synthesis from PROG [P450(17α), 17β-HSD (types 1 and 3), 5α-reductase (types 1 and 2)] and synthesizes DHT (**Table 1**) (81). In female hippocampus, a large amount of Allo is also synthesized from peripherally produced PROG because 5α-reductase is responsible for Allo synthesis (53, 63, 81).

Regulation of Local Production of Steroids in Hippocampus

A stimulation with NMDA for 30 min increases the levels of PREG and E2 in the hippocampal slices of adult male rats (42, 43, 47), suggesting that neural activity-dependent Ca^{2+} influx drives local production of PREG and E2.

Reduction of P450arom activity by phosphorylation *via* kinases (PKA and PKC) is an important mechanism which regulates E2 synthesis. Balthazart et al. demonstrated that this phosphorylation occurred in the quail brain within 15 min (85–87). In the cultured hippocampal neurons of female rats, E2 application facilitated the phosphorylation of P450arom, suggesting negative feedback mechanism (88).

As slow/genomic modulators, *cis*-retinoic acid (89) and gonadotropin-releasing hormone (GnRH) (75) were examined using hippocampal slice culture from neonatal rats. Forty-eight-hour treatment with 1 μM of 9-*cis*-retinoic acid increased the expression levels of P450(17α) and P450arom in the cultured hippocampal slices from male rats, *via* retinoid X receptor signaling (89). On the other hand, 8 days of treatment with GnRH enhanced local E2 production (75, 90). Hippocampal E2 synthesis was also increased by a stereotaxic injection of GnRH into the hippocampus of adult female rats (91).

Interestingly, behaviors, including social interaction (92, 93) and exercise (94), alter local production of steroids in the hippocampus. Social isolation (housing individually for 8 weeks) upregulated the mRNA levels of P450arom and StAR in the hippocampus of adult male rats, compared with pair housed rats (92), whereas environmental enrichment (housing in a group of nine in a large cage for 8 weeks) increased the mRNA levels of 5α-reductase type 1 and 3α-HSD (93).

PHYSIOLOGICAL ROLES OF HIPPOCAMPUS-SYNTHESIZED STEROIDS

Hippocampus-Synthesized DHT Enhances Neurogenesis in DG

Adult hippocampal neurogenesis occurs in DG throughout life in mammals (95). Sex steroids (96–99) and exercise (100, 101) enhance adult hippocampal neurogenesis of rodents, but the involvement of sex steroids in the exercise-induced neurogenesis, had been poorly understood.

Recently, Okamoto et al. revealed that mild exercise (30 min/day for 2 weeks) increased synthesis of hippocampal DHT, resulting in the neurogenesis enhancement (94). Injection of flutamide, an AR antagonist, suppressed the exercise-induced increase in neurogenesis, suggesting the involvement of androgens. However, surprisingly, castration (depletion of androgen from blood circulation) did not suppress this effect, suggesting the involvement of hippocampus-synthesized androgens. Indeed, the increase in DHT and 5α-reductase (DHT synthase) mRNA, were observed in the hippocampus of castrated rats after exercise (94). This study provides the direct evidence of the role of hippocampus-synthesized steroids in hippocampal functions.

Modulation of LTP/LTD Induced by Hippocampus-Synthesized Steroids

The physiological roles of hippocampus-synthesized sex steroids (E2 and DHT) in LTP/LTD were demonstrated *in vitro* studies using acute hippocampal slices and selective inhibitors of steroidogenic enzymes. A perfusion with letrozole, a selective inhibitor of P450arom, suppressed the magnitude of LTP in CA1–CA3 synapses of adult male rats (102), and in DG synapses of young (3- to 4-week old) male rats (103–104), within 10–20 min. ICI182,780, a selective antagonist of ERα/β, mimicked this suppressive effect (102), suggesting that hippocampus-synthesized E2 is required for full induction of LTP *via* synaptic ER. Conversely, hippocampus-synthesized DHT is required for the induction of LTD, from the data showing that low frequency stimulation (1 Hz, 15 min)-induced LTD was suppressed in the presence of finasteride, an inhibitor of 5α-reductase (105). In addition to sex steroids, the effect of hippocampus-synthesized PREG is reported, in which the application of aminoglutethimide, an inhibitor of P450scc, decreased the field excitatory postsynaptic potentials in granule cells in 20 min (103). Although the molecular mechanism underlying these effects remains unclear, a possible explanation may be provided by analogy from the data, showing that exogenous application of E2 rapidly (within 30 min) enhanced LTP by driving kinase network (Erk MAPK, PKA, PKC, PI3K, and CaMKII) in a non-genomic manner (27, 106).

Role of Hippocampus-Synthesized E2 in Hippocampus-Dependent Memory

Recently, the role of hippocampus-synthesized E2 in hippocampus-dependent memory consolidation was provided using OVX mice (107). Immediate after the training, bilateral infusion of letrozole into the dorsal hippocampus blocked the transient elevation of hippocampal E2 (within 30 min), and impaired object recognition and object placement memory consolidation (107). Under the same condition except for infusion of E2, this group previously demonstrated that E2 enhanced hippocampal memory consolidation *via* rapid activation of Erk MAPK and PI3K/Akt (108, 109), suggesting that learning experience-induced E2 elevation in the hippocampus rapidly activates kinase cascades.

CONCLUSION

Hippocampus-synthesized steroids as well as circulation-derived ones, are of importance for hippocampal functions.

A possible molecular mechanism for rapid effect of hippocampus-synthesized steroids may be kinase networks which modulate hippocampal functions, including spinogenesis (106, 110), LTP (27), learning, and memory (108, 109).

AUTHOR CONTRIBUTIONS

YH wrote the manuscript. SK brushed up the initial draft of the manuscript written by YH.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 30 April 2018
doi: 10.3389/fendo.2018.00205

Sex Steroids, Adult Neurogenesis, and Inflammation in CNS Homeostasis, Degeneration, and Repair

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Sex steroidal hormones coordinate the development and maintenance of tissue architecture in many organs, including the central nervous systems (CNS). Within the CNS, sex steroids regulate the morphology, physiology, and behavior of a wide variety of neural cells including, but not limited to, neurons, glia, endothelial cells, and immune cells. Sex steroids spatially and temporally control distinct molecular networks, that, in turn modulate neural activity, synaptic plasticity, growth factor expression and function, nutrient exchange, cellular proliferation, and apoptosis. Over the last several decades, it has become increasingly evident that sex steroids, often in conjunction with neuroinflammation, have profound impact on the occurrence and severity of neuropsychiatric and neurodegenerative disorders. Here, I review the foundational discoveries that established the regulatory role of sex steroids in the CNS and highlight recent advances toward elucidating the complex interaction between sex steroids, neuroinflammation, and CNS regeneration through adult neurogenesis. The majority of recent work has focused on neuroinflammatory responses following acute physical damage, chronic degeneration, or pharmacological insult. Few studies directly assess the role of immune cells in regulating adult neurogenesis under healthy, homeostatic conditions. As such, I also introduce tractable, non-traditional models for examining the role of neuroimmune cells in natural neuronal turnover, seasonal plasticity of neural circuits, and extreme CNS regeneration.

Keywords: apoptosis, astrocyte, microglia, neural homeostasis, neural plasticity, neurodegenerative, neurogenesis, steroidal hormone

OPEN ACCESS

Edited by:
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Reviewed by:
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Ayanabha Chakraborti, University of Alabama at Birmingham, United States

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *15 January 2018*
Accepted: *12 April 2018*
Published: *30 April 2018*

Citation:
Larson TA (2018) Sex Steroids, Adult Neurogenesis, and Inflammation in CNS Homeostasis, Degeneration, and Repair. Front. Endocrinol. 9:205. doi: 10.3389/fendo.2018.00205

INTRODUCTION

Sex steroids coordinate the development and maintenance of male and female reproductive systems, and a multitude of other organ systems, including the digestive, metabolic, skeletal, immune, and nervous systems. Sex steroids are robust regulators of many diseases that, unsurprisingly, occur in organ systems in which steroidal hormones regulate development and homeostasis. Steroidal hormones influence morphology, physiology, and ultimately behavior from the cellular level to the organismal level through a variety of effectors, or classes of sex steroids, their specific receptor types, and several intracellular signaling pathways. In this manner, sex steroids spatially and temporally coordinate vast molecular networks. These networks regulate processes including, but not limited to, cellular proliferation, differentiation, motility, survival, and apoptosis. Specifically within the central nervous system (CNS) sex steroids directly regulate neural activity, nitric oxide signaling,

and growth factor expression, among others (1). Through these and other direct processes, sex steroids also secondarily affect the birth and survival of neuronal, glial, and endothelial cells.

Within the context of immune function, sex steroids exert effects on and are one of many effectors of inflammatory cells and immune responses within the CNS. It has become increasingly evident over the last 30 years that the prevalence and severity of neuropsychiatric and neurodegenerative diseases with a neuroinflammatory component are linked to genetic sex [i.e., male versus female (2, 3)]. For example, women are more frequently diagnosed with atypical depression, depression with anxiety, and more severe progression of Alzheimer's Disease than men [reviewed in Ref. (3)]. As such, recent efforts have focused on understanding the implications of estrogen interactions with the neuroimmune system, primarily in the context of neurodegenerative disease and the possible use of estrogens and inhibitors of estrogen signaling as therapeutics. However, there is mounting evidence that the two other classes of sex steroids, androgens and progestogens, also modulate immune responses. Moreover, all of the sex steroids along with the classically described inflammatory cells have emerged as critical players in maintaining cell and tissue integrity under homeostatic and non-pathological conditions. Thus, understanding the role of all sex steroids, in all sexes, and under non-pathological conditions is paramount for unraveling the complex interactions between the hormonal, specifically sex steroidal, and immune systems, and for ultimately developing therapeutics for maintaining and restoring proper cognitive function.

In this review, I aim to present a comprehensive and integrative view of the independent, antagonistic, and synergistic impacts of sex steroids, the neuroinflammatory system, and neural stem cells (NSCs) for neural homeostasis, neuroprotection, and repair following neural insult. After providing a brief overview on sex steroid biosynthesis and intracellular signaling mechanisms, I discuss the steroidal hormone regulation of neural activity and synaptic plasticity, growth factor expression and responses, and apoptosis. With the intent to bridge three vast and recently converging areas of focus in neuroscience, I first lay a framework by describing the impacts of sex steroids on various neural and inflammatory cell types, including NSCs, neural endothelial cells, microglia, astrocytes, and leukocytes, among others. I then discuss the most recent findings at the intersections of sex steroids, neuroinflammation, and postnatal neurogenesis. I finally present several new models that have great potential to provide novel insights into inflammatory regulation of homeostasis, natural plasticity of neurogenesis, and extreme functional repair and recovery following neural damage and degeneration.

SEX STEROID BIOSYNTHESIS AND SIGNALING MECHANISMS

Two major classes of sex steroids, androgens and estrogens, were classically delineated as male- and female-specific hormones as a result of their respective pronounced synthesis in the testes and ovaries and their generalized roles in promoting secondary masculinizing and feminizing effects. Yet, the discovery of extra-gonadal biosynthesis of estrogens from androgens in adipose tissue in 1947 (4) and the birth of the "organizational hypothesis" in 1959 from work that demonstrated testosterone feminizes the CNS of the guinea pig [(5) and reviewed in Ref. (6)] revolutionized the thought and study of sex steroid function. Subsequently, estrogens and androgens have been discovered to be synthesized and exert regulatory effects in multiple organ systems in both males and females.

The three major types of endogenous estrogens are estrone (E_1), 17β-estradiol (E_2), and estriol (E_3), with E_2 being the most prevalent and potent form of circulating estrogen. The primary mode of E_2 biosynthesis occurs *via* the precursor, estrone, which itself is synthesized from androstenedione produced in the theca internal cells of the ovaries in females (**Figure 1**). The enzyme 5α-reductase, also called aromatase, converts androstenedione to E_1, which itself can be converted into E_2 by 17β-hydroxysteroid dehydrogenase (17β-HSD). In both males and females, 17β-HSD can also convert androstenedione into testosterone, which can be converted into E_2 by aromatase [reviewed in Ref. (7)]. After the discovery of extra-gonadal biosynthesis of E_2 in adipocytes, evidence of E_2 biosynthesis has been observed in astrocytes, neurons, and ependymal cells of the brain, osteoblasts, fibroblasts, adrenocortical cells, parietal cells of the intestines, smooth arterial muscle cells, and splenic T cells [reviewed in Ref. (8)].

The androgens testosterone (T) and androstenedione are synthesized primarily by the Leydig cells of the testes (in males) and the zona reticular and zona fasciculate of the adrenal cortex in males and females (**Figure 1**). Circulating plasma T and androstenedione can be converted to dihydrotestosterone (DHT), a potent androgen receptor ligand, by the enzyme 5α-reductase in target tissues. Likewise, dehydroepiandrosterone (DHEA), also known as androstenolone, is produced in both males and females in the adrenals [reviewed in Ref. (7)]. Circulating DHEA can be converted into both DHT and estrogens in peripheral tissues and itself can bind the different estrogen receptors with varying affinities [for more see Ref. (9)]. As mentioned, T and androstenedione can also be converted to E_2 by aromatase in both males and females.

A third class of sex steroids, progestins, includes progesterone, which is synthesized from pregnenolone in the corpus luteum of the ovaries and in the adrenal cortex (**Figure 1**). Progesterone can be converted by 7α-hydroxyprogesterone into androstenedione, which, again, is a precursor of T and E_1 [reviewed in Ref. (7)]. Extra-gonadal and extra-adrenal production of progesterones has also been observed in the pineal, thalamus, cerebellum, and tectum among other areas of the CNS (10).

At peripheral target sites, all steroid hormones bind nuclear and transmembrane receptors to drive complex, yet precisely tuned cell-type specific signaling responses. Briefly, the estrogens E_1, E_2, and E_3 diffuse across the lipid membrane and bind cytoplasmic estrogen receptors alpha (ERα) and beta [ERβ; for more on affinities, see Ref. (11)]. Ligand-bound ERα and ERβ dimerize, rapidly translocate to the nucleus, and directly regulate transcription of over 3,600 mammalian genes (12). Estrogens can also bind the G-protein-coupled receptors GPER (primarily expressed in adipocytes and intestinal cells), GPER1 (neural and adrenocortical cells), and GPER30 [endothelial cells; reviewed in Ref.

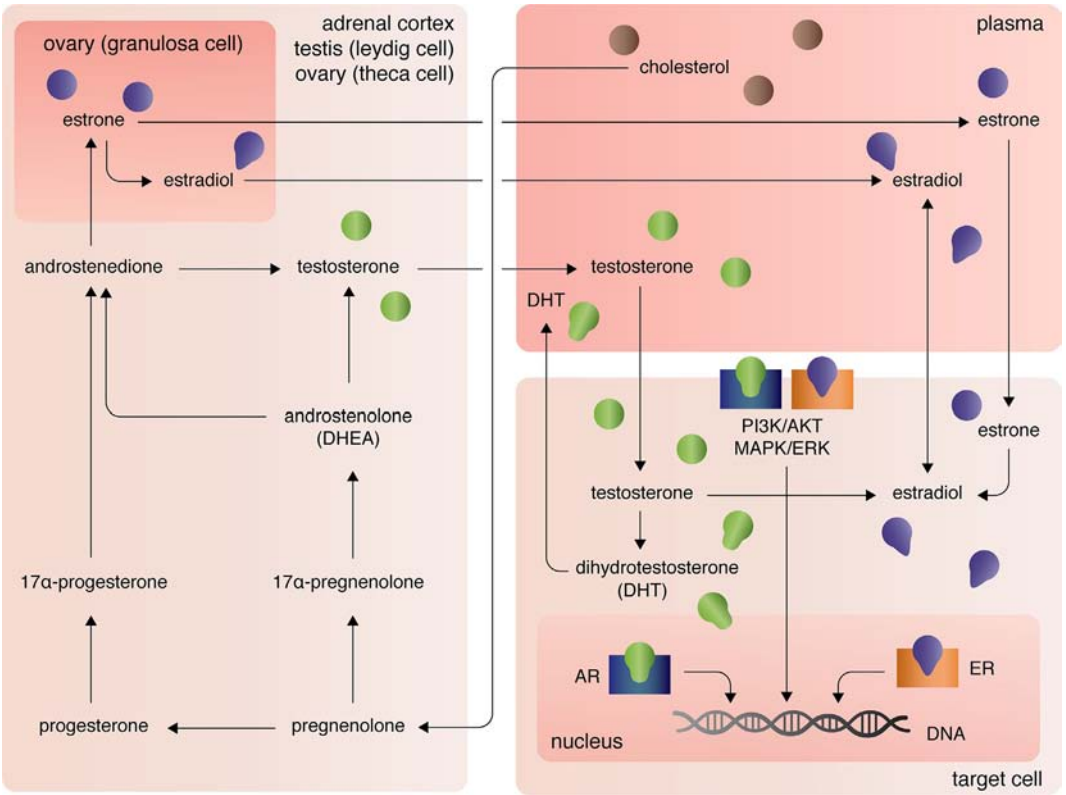

FIGURE 1 | Biosynthesis and localization of sex steroids, with a focus on estrogens and androgens.

(13)]. Estrogen-activation of the GPERs (and likely membrane-associated ERs, as well) initiates PI3K/AKT and MAPK/ERK signaling pathways (13), that in turn coordinate the transcription and translation of whole gene families that regulate cellular processes including, but not limited, to cellular differentiation, proliferation, survival, and apoptosis (14). Similarly, androgens and progesterones exert their functional effects on transcription and translation through intracellular canonical genomic signaling and the non-genomic signaling cascades PI3K/AKT and MAPK/ERK (15, 16). Differential androgen signaling depends on cytoplasmic- versus membrane-targeted isoforms of the androgen receptor gene (16). By contrast progesterone receptors, like estrogen receptors, are encoded by distinct genes and include the intracellular receptors PR-A and PR-B and the membrane progesterone receptor isoforms alpha (mPRα), beta (mPRβ), and gamma (mPRγ) (17). Further, contributing to the complexity of sex steroid receptor signaling mechanisms, unbound estrogen and androgen receptors can bypass activation by their cognate ligands and functionally bind to transcription response elements, albeit with much lower affinity (16).

OVERVIEW OF SEX STEROID FUNCTIONS WITHIN THE CNS

Through their various metabolites, receptors, receptor isoforms, and intracellular signaling mechanisms, sex steroids regulate vast molecular networks to spatially and temporally coordinate a multitude of processes related to cellular proliferation, motility, differentiation, and survival. Within the CNS, sex steroid signaling directly regulates neural activity, growth factor expression, endothelial cell and NSC proliferation, survival, and apoptosis (15, 18).

Neural Activity and Synaptic Plasticity

During development sex steroids, especially estrogens, play a pivotal role in arborization, synaptogenesis, and circuit formation (19). For example, the structural, connective, and physiological differences between the male and female brain are a result of gonadal steroid action during development. Regardless of genetically determined sex, morphological, and physiological masculinization of the brain occurs with exposure to E_2 but not DHT during a restricted developmental window [reviewed in Ref. (6)]. Feminization of the brain and behavior results from a lack of early steroidal hormone exposure (6). One mechanism through which E_2 affects the development of neural connections is through the modulation of glutamatergic synapse formation. For example, in the developing hippocampus, E_2 bound ERα stimulates expression of vesicular glutamate transporter 1 and the post-synaptic NMDA receptor, which in turn increases glutamatergic synapse formation of hippocampal neurons (20). For more information on these and other mechanisms driving sexual differentiation of the brain see Ref. (6).

Sex steroids also critically regulate neural activity and synaptic plasticity to influence circuit structure and activity in the adult CNS (**Figure 2**). In hippocampal CA1 pyramidal neurons of adult female rats, E_2 increases NMDA and AMPA receptor activity, which in turn increase neuronal sensitivity to NMDA (21). This E_2-mediated enhancement in NMDA sensitivity drives increased dendritic spine density of the pyramidal neurons (21). The influence of progesterone in modulation of dendritic spine formation and neural activity is, however, less clear and complicated by age and prior steroidal hormone exposure. For example, Edwards et al. found that progesterone enhances evoked responses from CA1 field recordings of adult female hippocampal slices (22), whereas Ito et al. observed no effect of progesterone on long-term potentiation in CA1 slices from 4-week-old rats (23). Moreover, progesterone and E_2 treatment together diminishes the enhanced glutamate-mediated release of intracellular calcium that each hormone has when administered independently (24).

Aside from canonical genomic signaling at the nucleus, sex steroids can rapidly modulate synaptic transmission through changes in spine density and neuron sensitivity within the synaptic process itself. For instance, T and DHT positively regulate spine formation through extranuclear ARs localized within dendritic spines and activation of local MAPK/ERK signaling (25). Local activation of MAPK/ERK drives rapid cytoskeletal and proteomic changes necessary for spinogenesis and plasticity (discussed further below). Similarly, ERα localizes not only within post-synaptic CA1 spines (26) but also pre-synaptic CA3 terminals, where it rapidly promotes long-term depression and spine formation through MAPK signaling (27).

Growth Factor Regulation

Over 40 years ago, sex steroids, specifically estrogens, were hypothesized to act in conjunction with neurotrophins to induce classic growth factor responses including increased cellular proliferation, differentiation, and survival (28). Subsequently, interactions between sex steroids and neurotrophins have been identified as critical regulators of sex-associated differences in body mass, cardiac function, bone mineral density; hepatic regeneration; tumor formation, and growth; and neural cell function and plasticity [reviewed in Ref. (29)]. Specifically within the CNS, sex steroid receptors co-localize with growth factor receptors including the insulin growth factor (IGF1) receptor IGFR, the low-affinity nerve growth factor receptor p75NTR, and the tropomyosin receptor kinase (trk) family—trkA (nerve growth factor; NGF), trkB (brain-derived neurotrophic factor and neurotrophin 4; BDNF and NT-4), trkC (NT-3). Co-localization occurs within, but is not limited to, the developing mammalian forebrain (30), cerebellum (31), and cortex [*in vitro* (32)], and the telencephalic nucleus HVC [proper name (33)] of the avian song production circuit (**Figure 3**) among other regions functionally related to the song system (34, 35). Such co-localization of steroidal hormone receptors and neurotrophic receptors drives neurotrophic-dependent processes through one or more of several mechanisms including: (1) convergence on the PI3K/AKT and MAPK/ERK signaling pathways to increase transcription and translation of gene families related to well-characterized neurotrophic processes, (2) autonomous enhancement in expression of neurotrophins and their cognate receptors, and (3) a cascade effect driving non-autonomous expression of neurotrophins.

Sex steroids and growth factors synergistically interact through convergence on the PI3K/AKT and MAPK/ERK signaling pathways to directly and indirectly modulate activity of downstream signaling cascades, transcription factors, and translation machinery. For example, PI3K activation by ligand-bound sex steroid receptors and/or growth factor receptors results in phosphorylation of AKT, which ultimately increases CREB-mediated transcription and ribosomal S6 kinase translation *via* mTOR activation (36). Likewise, both PI3K and MAPK activation increase

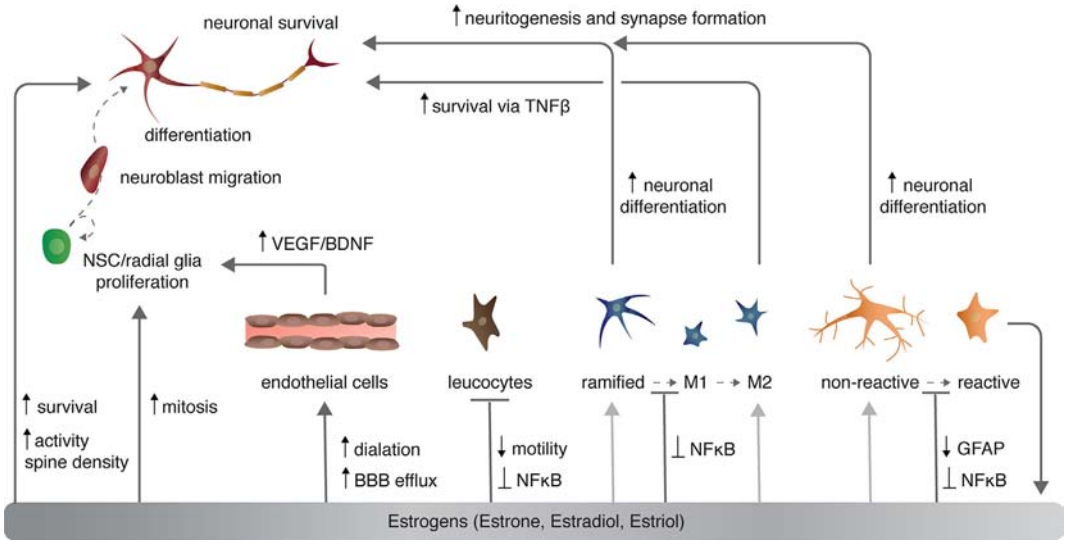

FIGURE 2 | The effects of estrogen on various cell types in the central nervous system. Light gray arrows indicate that estrogens have an effect on neurons via the given cell type to which the arrow points.

phosphorylation of GS3Kβ, inhibiting GS3Kβ's pro-apoptotic effects (37). As eluded to above, convergence on the PI3K and MAPK cascades can occur not only within the neuronal soma but also in pre- and post-synapses to drive rapid local responses to sex steroids and growth factors. Within the nucleus, E_2 directly regulates transcription through ER activity at estrogen response elements located in the promotor region of several growth factors—VEGF, transforming growth factor alpha (TGF-α), BDNF, NT-4, and NGF [reviewed in Ref. (2)]. Alternatively, within the dendritic spines, sex steroids can bind extranuclear ERs and ARs to enhance local BDNF translation *via* activation of PI3K/AKT and MAPK/ERK (25, 38). This in turn supports cytoskeletal rearrangement of dendritic spines and ultimately long-term potentiation by upregulating: the structural proteins growth-associated protein 43 and microtubule-associated protein 2 [reviewed in Ref. (19)]; the phosphorylation of the actin cleaving protein coffilin (39); and synthesis of essential synaptic proteins such as PSD-95 (40). In this manner, sex steroids and neurotrophins, specifically BDNF, rapidly modulate dendritic spine formation and stabilization to facilitate synaptic and circuit plasticity. At the pre-synaptic terminal, E_2 also increases retrograde transport of BDNF through a trkB-dependent mechanism in olfactory bulb neurons (41), possibly supporting antagonistic neurotrophic signaling, neuronal competition, and circuit plasticity (42).

Sex steroids can also drive autonomous and non-autonomous up regulation of both self and different neurotrophins and their cognate receptors. To highlight one example of many, in songbirds, testosterone, and specifically its E_2 metabolite, upregulates the expression of VEGF and the VEGF receptor-R2 in endothelial cells within the highly neurogenic nucleus HVC (**Figure 3**). VEGF in turn induces expansion of the local microvasculature through a sex steroid-independent increase in endothelial cell mitosis (43). Within 1 week of testosterone treatment not only new and mature endothelial cells, but also neurons and astroglia within HVC, upregulate synthesis of BDNF (43). BDNF is both required for and promotes the addition of adult-born projection neurons into HVC (43, 44), thus facilitating growth of the song control circuit and increased quality of singing behavior [reviewed in Ref. (45)]. This example not only demonstrates the neurotrophic cascade effect of sex steroids acting in concert with growth factors, but also introduces effects on vasculature and NSCs that sex steroids have often in conjunction with activation of growth factor signaling.

Vasculature

Sex steroids modulate cerebrovasculature function through several mechanisms, of which I will briefly discuss three [for more, see Ref. (46, 47)]: angiogenesis and endothelial cell survival, vascular contractility, and blood–brain barrier (BBB) permeability (**Figure 2**). Angiogenesis requires proliferation, migration, alignment, and differentiation of endothelial cells. Sex steroids, specifically estrogens, regulate all of these processes. Estradiol directly and indirectly increases the expression of growth factors including VEGF (discussed above) and fibroblast growth factor 2 (FGF2) that in turn increase the proliferation of endothelial cells (48). Estrogens also stimulate endothelial cell adhesion and migration through FGF-mediated increases in expression of surface integrins (49) and the formation of capillary-like structures with an internal lumen *in vitro* (50). Conversely, progesterone suppresses endothelial cell proliferation through nuclear progesterone receptor-mediated arrest of endothelial cells in the G1 phase (51).

Sex steroids also modulate cerebrovascular contractility. Chronic exposure to estrogens decreases vasculature tone and increases nitric oxide-dependent dilation in both males and females (52, 53). Estrogens promote vasodilation through both transcriptional increases in vasodilators including prostacyclin, endothelial nitric oxide synthase (eNOS), among others [reviewed in Ref. (46)], and posttranscriptional mechanisms. For example, E_2 not only enhances eNOS protein and mRNA expression through nuclear ERα (54, 55), but also eNOS activation through PI3K/AKT-mediated phosphorylation of eNOS at serine 1177 (56, 57). Together, increased production and activation of eNOS promotes nitric oxide production and, consequently, endothelium relaxation.

The BBB is a specialized cerebrovascular structure composed of endothelial cells encased in a basement membrane. The BBB is supported by astrocytic feet, pericytes, and local neurons that collectively form the neurovascular unit [NVU; reviewed in Ref. (47)]. The NVU regulates the influx and efflux of ions, molecules, and cells across the BBB to maintain homeostasis (47). Unique from other endothelial cells, the endothelial cells of the BBB are connected with tight junctions and adherin junctions that restrict the paracellular permeability of the BBB (47). By controlling the flow of ions, the BBB creates a high trans-endothelial electrical resistance, which decreases the ability of polar molecules to penetrate the BBB (58). Transport of substances across the BBB is limited to the passive diffusion of low molecular weight lipophilic molecules, polar nutrients including glucose and amino acids *via* solute carriers, and receptor- and adsorptive-mediated transcytosis of specific high molecular weight molecules such as insulin (59).

Sex steroids modulate the permeability of the BBB by enhancing nutrient and efflux transporter expression. Specifically, estrogens increase endothelial expression of glucose transporter 1, which facilitates 2-deoxy-D-glucose uptake into the BBB endothelial cells and transcytosis (60). Estrogens also modulate efflux transporter expression. Efflux transporters, predominately the adenosine triphosphate (ATP) binding cassette (ABC) transporters, prevent many lipophilic molecules from entering the brain and thus limit exposure to potentially harmful molecules (47). The activity of the ABC transporter sub family G member 2 (ABCG2)—alternatively known as the breast cancer related protein—is controlled by estrogens *via* ERβ (61). In endothelial cells, ligand-bound ERβ reduces phosphorylation of AKT, which subsequently reduces the expression and activity of ABCG2 through ubiquitination and proteosomal degradation (61). The decreased expression of ABCG2 results in not only decreased efflux of harmful molecules out of the brain, including amyloid-β protein (62), but also increased transcytosis of molecules such as tyrosine kinase inhibitors and xenobiotics (e.g., pharmaceuticals).

Although untested to my knowledge, the ability of sex steroids to alter BBB permeability through changes in growth factor expression and non-genomic PI3K/AKT signaling seems likely.

The growth factor VEGF increases tight junction permeability *via* VEGFR2-mediated phosphorylation and decreases in the expression of a critical regulatory protein of paracellular permeability, occludin (63). Given the previously discussed E_2-mediated increases in VEGF expression in endothelial cells, E_2 likely increases permeability of the BBB *via* VEGF-mediated decreases in occludin expression. Likewise, protein kinase C, an upstream regulator of PI3K/AKT signaling also reduces the expression of another tight junction protein claudin-5 (64). Thus, activation of PKC and PI3K/AKT signaling *via* ligand-bound sex steroid receptors may also decrease claudin-5 expression to increase permeability across the BBB.

Estrogens also repress inflammatory-mediated increases in leukocyte diapedesis, or transmigration, across the BBB. Secreted inflammatory factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and interleukin-1 beta (IL1β) increase transcriptional activation of cellular adhesion molecules (CAMs). CAMs mediate leukocyte–endothelial cell interactions are necessary for diapedesis and infiltration of leukocytes into the CNS. E_2 attenuates the release IL1β by astrocytes, activation of NFκB, and expression of ICAM1 (64), thus decreasing the permeability of the BBB to leukocytes. In this manner, E_2 is thought to mitigate inflammation following stroke or traumatic brain injury.

Oligodendrocytes

Oligodendrocytes are type of neuroglia that support and maintain the integrity of axons in the CNS. During early development oligodendrocyte precursors arise from neuroepithelial cells in the ventral ventricular zone (VZ) of the embryonic spinal cord and then migrate to and populate the VZs of the brain (65). As the last cell type to populate the CNS, oligodendrocyte precursor cells (OPCs) migrate into the gray and white matter of the entire embryonic CNS. The migration of OPCs is guided by growth factors including platelet-derived growth factor and FGF, netrins, semaphorins, and the chemokine CXCL1 [reviewed in Ref. (66)]. After migration, OPCs differentiate into other two types of oligodendrocytes: (1) myelin forming oligodendrocytes, which ensheath axons with an approximately 1 μm think myelin sheet and (2) non myelin forming oligodendrocytes, also known as perineuronal or satellite oligodendrocytes, which might play a role in the demyelination of nude neurons [reviewed in Ref. (67)]. Oligodendrocytes not only provide trophic support with production and secretion of glial cell line-derived neurotrophic factor, BDNF, and IGF1, but also electrically insulate axons, promote clustering of sodium channels at nodes of Ranvier, and promote microtubule stability in the axon (66).

Interestingly, myelination of axons by oligodendrocytes is tightly controlled by the neuron rather than a preprogrammed developmental clock within the oligodendrocytes themselves. The primary cue for oligodendrocyte ensheathment of axons is electrical activity of neurons. Electrically active neurons downregulate the polysialylated neural cell adhesion molecule (PSA-NCAM), allowing NCAM–NCAM adhesion required for oligodendrocyte–neuron interactions. Neuron firing also causes release of adenosine and ATP during the action potential. Adenosine and ATP promotes OPC differentiation and stimulation of astrocytes, which in turn secrete leukemia inhibitory factor to induce oligodendrocyte myelination (66). After associating with and receiving signals to ensheath by neurons, oligodendrocytes upregulate transport of myelin proteins transcripts, including proteolipid protein (PLP) and myelin basic protein (MBP), to the wrapping processes. PLP and MBP facilitate ensheathment by assisting with membrane trafficking and vertically coupling the multiple layers of membrane of the sheath, respectively (66).

Oligodendrocytes express both nuclear and non-nuclear ERs and PRs, and as such are directly modulated by sex steroids. During development estradiol promotes myelination in the neonatal rat brain, giving rise to higher numbers of oligodendrocytes in female-derived oligodendrocyte cultures than male-derived cultures (68) and possibly contributing to the well-documented higher incidence of neurological autoimmune diseases in females. More specifically, E_2, but not progesterone or testosterone, delays the exit of OPCs from the cell cycle, thereby increasing OPC proliferation (68). E_2 also enhances sheath formation through genomic ER signaling at an estrogen response element for MBP (68, 69) and resulting increases of MBP expression (70). Likewise, progesterone also enhances the formation of MBP and PLP positive membrane sheets through the progesterone receptor, although the precise signaling mechanism is still unknown (68, 71). Progesterone, as well as E_2, also facilitate remyelination following pathological demyelination in the CNS (72, 73). Given the high incidence of autoimmune disorders like multiple sclerosis (MS) in women, demyelination and subsequent remyelination unsurprisingly involves complex interactions between the immune system and steroidal hormones, and as such, will be discussed further below.

Postnatal Neurogenesis

Contrary to popular belief, new neurons are generated in the CNS from NSCs and neural progenitor cells (NPCs) throughout the entirety of vertebrates' lives. Pioneering studies in mammals and songbirds in the 1960s and 1980s provided the first evidence that new neurons arise in the adult brain (74) and that adult-born neurons functionally integrate into pre-existing neural circuits and acquire mature neuronal phenotypes (75). Since the discovery of adult neurogenesis several hypotheses regarding the function of adult-born neurons and neuronal turnover have been proposed. The replacement of older neurons by adult-born neurons has been proposed to enable the learning of new memories (76–78), the maintenance older memories (79, 80), the replacement of over-excited neurons (80), or the replacement of neurons weakened by DNA damage or loss of trophic support (81). Many studies have tested these and other hypotheses, however, no studies have conclusively supported or eliminated any of these possible functions of neuronal turnover (78, 81). Here, I will limit discussion to the complex, yet fine-tuned processes of adult neurogenesis, as broadly defined to include proliferation of NSCs, migration of neuroblasts, and the incorporation and survival of adult-born neurons in functional neural circuits. I will briefly introduce studies that highlight the modulation of adult neurogenesis by sex steroids, as the topic has been extensively reviewed in the past. Although many extrinsic and intrinsic factors such as non-steroidal hormones (e.g., glucocorticoids), morphogens (e.g., Shh, Wnt, BMP), neurotrophins, and neural activity also

coordinate the processes of adult neurogenesis, in this review I will primarily focus on the role of the neuroinflammatory cells as a major regulator (below). For exhaustive discussion of these and a broad range of other adult neurogenesis related topics including functional importance, consider consulting Ref. (82).

Niches and Proliferation

Adult neurogenesis occurs across all investigated vertebrates (and some invertebrates) with varying spatial localization: addition of adult-born neurons is limited primarily to the olfactory bulb and the hippocampus of mammals, but occurs broadly across the telencephalon of birds, and the telencephalon, diencephalon, optic tectum, cerebellum, and hindbrain of fish [**Figure 3** (83)]. True stem cells are defined by two critical characteristics: (1) the ability to self-renew through proliferation and (2) the ability to give rise to multiple cell types through differentiation of progeny. The NSCs of the adult vertebrate brain are self renewing and thought to be multipotent, giving rise to neurons, astrocytes, and oligodendrocytes. Two major models have been proposed to describe the identity of NSC. Briefly, one model posits that non radial glial cells characterized by their expression of sex determining region Y-box 2 (Sox2) are the putative multipotent NSCs, whereas the other model suggests that radial glial cells expressing glial fibrillary acidic protein (GFAP) give rise to adult-born neurons and glia [reviewed in Ref. (84, 85)]. Given that both models are supported by experimental evidence (84, 85) and are not necessarily mutually exclusive, diversity in putative NSCs might represent the spatiotemporal diversity of adult neurogenesis not only across species, but also between neurogenic brain regions within a given species. Alternatively, true stem cells in the adult brain might not exist as a single cell, but rather might represent a whole population of unipotent NPCs, which independently give rise to either neurons, astroglia, or oligodendrocytes.

The dynamic lineages of progeny derived from NSCs, including both lineage-non-restricted NPCs and lineage-restricted NPCs like OPCs, further complicate the identity of putative NSCs in the adult vertebrate brain. For example, in the mammalian brain there are two major neurogenic regions: the ventricular–subventricular zone (V–SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG). The NSCs of the V–SVZ are GFAP positive radial glia-like B cells. B cells are generally quiescent but can be activated by various signaling factors from other cells in the neurogenic niche and transform into GFAP negative transit-amplifying C cells [for mechanisms see Ref. (85)]. Type C NPCs actively proliferate giving rise to doublecortin (DCX) expressing type A neuroblasts that ultimately populate the olfactory bulb [reviewed in Ref. (84, 86)]. Likewise, the NSCs of the SGZ include type I GFAP expressing radial glial-like cells, which give rise to GFAP negative type II NPCs. The type II NPCs are further divided into two subpopulations: (1) type IIa, which express Mash1 and Sox2 and are highly prolific and (2) type IIb, which express Prox1, NeuroD1, and DCX for early commitment to the neuroblast fate [reviewed in Ref. (84, 86)]. Type IIb neuroblasts ultimately incorporate into the local network in the granular cell layer of the DG.

The conservation of NSCs/NPCs lineage relationships across neurogenic niches within mammals and more generally across vertebrates, suggests a common evolutionary origin for the development of NSC/NPC pools in the adult vertebrate brain. For example, the adult avian brain contains proliferative type B cells in the VZ of the telencephalon that give rise to type A migrating neuroblasts (87). NSCs of the adult zebrafish telencephalon exists as type I GFAP positive cells that are quiescent until transforming into type II GFAP positive radial glial cells. Interestingly, the slowly proliferative type II cells can give rise to both new type I cells through Notch signaling and highly proliferative type III GFAP negative neuroblasts [reviewed in Ref. (88)]. These transit-amplifying type III neuroblasts can continue to proliferate, but can also begin fate specification by turning on PSA-NCAM and the pro-neural gene ascl1 (88). The NSCs/NPCs of the neurogenic niches in the zebrafish cerebellum and optic tectum express the classic NPC marker Sox2 and PCNA, but not GFAP (88).

Although it is generally accepted that adult neurogenesis continuously occurs across all non-human vertebrate lifespans [albeit to a lesser extent with age (89, 90)], the degree to which NSC/NPC proliferation occurs across the human lifespan has become a matter of recent intense debate. In 1998 NPCs in the human brain were first identified in postmortem hippocampal tissue of cancer patients who previously received an injection of the thymidine analog bromodeoxyuridine (91)—a commonly used reagent for labeling mitotic cells. BrdU-labeled cells were identified in the granule cell layer, the SGZ, and the hilus of the DG (91). Given that the average age of the patients examined in this study was 64.4 ± 2.9 years, the authors concluded that adult neurogenesis occurs throughout life in the human DG (91). In 2013 Spalding et al. birth dated hippocampal neurons in humans exposed to nuclear-bomb-test-derived 14C and calculated that around 700 new neurons are generated in the hippocampus per day with a turnover rate of 1.75% annually in nearly one-third of the population of hippocampal neurons (92). Again, the authors concluded that although the majority of hippocampal neurons do not turnover (thus the average age of hippocampal neurons increases with the age of the individual), turnover does indeed continue throughout the entire human lifespan (92). Moreover, in April of 2018 neurogenesis was found to persist in postmortem adult hippocampi of otherwise healthy humans ranging in age from 14 to 79 years (93). The degree to which hippocampal neurogenesis occurs subsequent to the first year of life, however, has recently been challenged. Using postmortem and postoperative samples of human hippocampi, Sorrells et al. reported in March of 2018 that proliferation in the DG peaks at 14 gestational weeks and decreases rapidly by 22 gestational weeks (94). By 7 years of age, nestin, vimentin, or GFAP positive cells with NSC/NPC morphology were not detectable in the SGZ or hilus of the DG, suggesting that the proliferative progenitor pool is near completely, if not entirely, depleted by 7 years of age (94). The apparent contradictions across studies regarding the degree to which adult neurogenesis occurs throughout the human lifespan likely arises from the large diversity in the manner through which the human samples were obtained. Given the difficulty in obtaining human samples in general and the near impossibility of controlling for the plethora of factors that alter adult neurogenesis in these human studies participants, the issue of degree to which NSC/

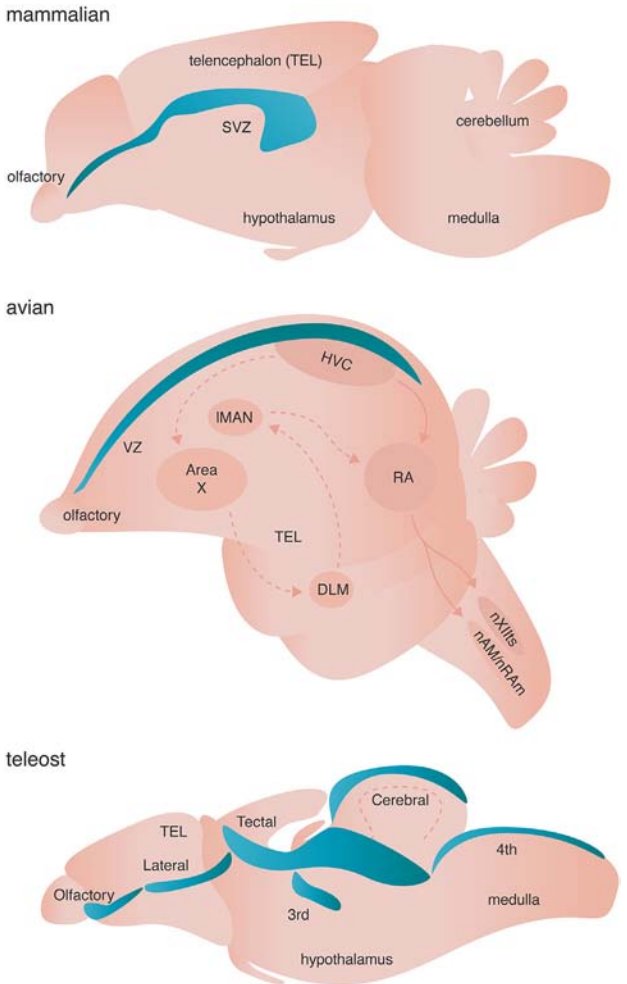

FIGURE 3 | Neurogenic niches in mammals, songbirds, and teleost fishes. Niches are colored blue. Dashed arrows in the avian brain represent the anterior forebrain pathway responsible for song learning, whereas solid arrows in the avian schematic represent the song motor pathway of songbirds. Both pathways are simplified for clarity.

NPC proliferation occurs in humans will likely continue to be debated for some time.

Migration

Generally, migration of neuroblasts occurs between one and fourteen days following birth by means of one of three modes—radial, tangential, or undirected wandering migration. In the mammalian CNS, neuroblasts originating from precursors in the SGZ translocate by means of radial migration into layer CA1 of the hippocampus, although typically after tangentially migrating independent of radial glia cells along the local vasculature (95). Alternatively, neuroblasts from the V-SVZ tangentially migrate to the olfactory bulb along chains of PSA-NCAM positive cells forming the rostral migratory stream (96). These PSA-NCAM positive chains consist of a network of differentiated neurons, astrocytes, and tanycytes (97). Migration of neuroblasts along the rostral migratory stream into the olfactory bulb occurs at a rate of nearly 120 μm/h [*in vitro* (98)] and as such generally occurs over 2 days and 2 weeks (99). In the mammalian brain (and other vertebrate brains too), radial glia, microvasculature, and the cells of the PSA-NCAM chains provide trophic support for the migrating neuroblasts through a multitude of factors including, but not limited to, IGF1 and BDNF (95, 100).

In songbirds, a small fraction of the neuroblasts originating from the lateral ventricles migrate mediolaterally along the fibers of radial glia projecting through the telencephalon (101, 102). However, not all neurogenic regions of the avian brain contain radial fibers (102). Rather, neuroblasts translocating to the olfactory bulb migrate tangentially on chains of PSA-NCAM in the avian telencephalon (74). Neuroblasts destined for HVC wander from the VZ without apparent directionality to and within HVC (103).

Integration and Survival

Newly generated neuroblasts undergo fairly dramatic attrition before successful incorporation into their respective neural circuits: generally around 50% of neuroblast die via programmed cell death across all neurogenic regions of all vertebrate brains investigated. The "selection process" on neuroblasts is tightly regulated by a plethora of factors, including sex steroids, neurotrophins, and inflammatory cytokines, among many others [for mechanisms see Ref. (82)]. In the DG nearly half of adult-born neurons die within 2 weeks of birth. The neuroblasts that survive the initial culling begin neurite outgrowth with dendrites extending into the molecular layer of the DG and axon extension into the hilus of the DG. By 2 weeks, nascent neurons in the DG begin spinogenesis and the axons begin forming functional connections on CA3 pyramidal neurons in the hilus. By 28 days, adult-born neurons of the DG become fully mature and indistinguishable in morphology, physiology, and behavior from pre-existing mature CA1 neurons [reviewed in Ref. (104)].

After 2–14 days, neuroblast arising from the V–SVZ exit the rostral migratory stream and migrate radially into the olfactory bulb, but not before nearly half of neuroblasts undergo apoptosis (99). Between 15 and 30 days, the persisting olfactory bulb neuroblasts differentiate into one of two types of local interneurons. The vast majority, around 95%, differentiate into GABAergic granule cells, whereas the remaining 5% differentiate into periglomerular neurons expressing either GABA, or dopamine, or both (99).

As is true with the other populations of new neurons in the mammalian brain, nearly 50% of all cells born from NSCs/NPCs undergo apoptosis during migration to their final destination in the avian brain as well (105). The majority of investigations characterizing the fate of neuroblasts in the avian brain originate from studies in the song control circuit, although recently studies have also examined adult neurogenesis in the avain hippocampus (106). Within the song control circuit nascent HVC projection neurons begin to express neuronal markers and form synapses on targets up to 4 mm away in the robust nucleus of archopallium (RA) as early as 2 weeks of age (105). By 28 days to 8 months, most new HVC neurons that survive the initial stages of culling form synapses onto RA neurons (105, 107). These new HVC to RA projection neurons persist for months (108) to years (109, 110),

depending on their time of birth and the presence of sex steroids [reviewed in Ref. (81)].

Sex Steroidal Modulation

Sex steroids have complex roles in regulating adult neurogenesis, as broadly defined. Both E_2 and progesterone treatment independently increase mitosis of NSCs/NPCs [**Figure 2** (111, 112)]. However, as is the case with modulation of dendritic spine density (above), progesterone and E_2 reduce the effect of each steroidal hormone when administered together (112). Several studies have shown that natural seasonal plasticity of androgens and castration with supplementation of exogenous androgens enhance the survival of new neurons in both mammals and birds [reviewed in Ref. (3, 81)]. More specifically, the androgen metabolite DHT consistently promotes new neuron survival, whereas the metabolite E_2 has mixed effects, depending on the mammalian species examined, the sex of the animal, and timing and duration of E_2 exposure [reviewed in Ref. (3)]. Alternatively, in HVC of both male and female birds, both DHT and E_2 act independently and synergistically to enhance addition and survival of new neurons (113, 114). Sex steroids exert these pro-neurogenic effects by promoting increased DNA synthesis, transcription, and growth factor signaling and processes [for more see Ref. (3, 15, 81)].

Apoptosis

As discussed throughout, sex steroids generally promote the survival of new and mature neurons. Estrogens, however, have also been proposed to potentiate excitotoxic neuronal death, especially following pathological damage (2). This working hypothesis is supported by the increased sensitivity and excitability of neurons exposed to E_2 *via* estrogen-mediated increases in NMDA and AMPA receptor activity (discussed above). Increased firing of neurons, specifically through NMDA signaling in turn rapidly increases E_2 synthesis (115), and thus can drive a feed-forward cycle that could result in excitotoxic cell death. In this context, estrogens facilitate seizure activity in both animal models and humans (116, 117) through the initiation of an NMDA–E_2 positive-feedback loop (118). Clearly, there exists a fine balance between the beneficial and harmful effects of sex steroids in the maintenance of homeostasis and on neuron survival. And so, careful analysis of the spatiotemporal expression of sex steroids across the sexes and the effects of interactions between the classes of sex steroids on cell proliferation, behavior, and survival is critical for garnering a more thorough understanding of the basic biology and potential restorative value of sex steroids.

SEX STEROID ACTION ON NEUROINFLAMMATORY CELLS

Sex steroids play a pivotal role in the development and function of the immune system through both rapid and long-term mechanisms. Besides regulating the proliferation, migration, and differentiation of immune cells, sex steroids are generally neuroprotective under inflammatory conditions. The anti-inflammatory potential of sex steroids, specifically estrogens, originates from observations that the drop in steroidal hormones associated with menopause increases the incidence of neuroinflammatory associated neuropathologies [reviewed in Ref. (119)]. Subsequently, estrogens, and progesterones to a more limited degree, have been demonstrated to exert their generally protective regulatory action through direct and indirect regulation of immune cell morphology and behavior.

Microglia

Microglia are macrophage-like cells that originate from myeloid precursors during early development and migrate to and take up permanent residence in the developing CNS (120). The primary role of microglia is to continuously survey the microenvironment, remove cell debris and pathogens, and activate reactive inflammation. Microglia have both protective and destructive impacts in the CNS that largely depend on their state of activation (**Figure 4**). In the un-challenged, healthy brain "resting" or ramified microglia are highly motile (121) using their branches for surveilling their territorial domains for excess neural cells and synapses to phagocytose (122). Microglia remain in the resting or non-activated state through inhibitory signals from the neurons on which the microglial processes directly reside (123). The loss of connection between microglia and neurons, for example during neuronal death, accelerates the activation and response of microglia (123). With neural damage or loss of homeostasis, microglia become polarized to one of two states: M1 and M2 [mechanisms reviewed in Ref. (124)]. Inflammatory M1 microglia induce classical, and often harmful, cytotoxic responses through enhanced antigen presentation and the release of cytotoxic mediators, including NO, tumor necrosis factor alpha (TNFα), IL-1β, prostaglandin, and reactive oxygen species following activation by cytokines, interferons, and endotoxins [reviewed in Ref. (125)]. M1 microglia can molecularly switch to M2 microglia [reviewed in Ref. (124)], which phagocytose dying neurons and promote neurite outgrowth, oligodendrocyte fate specification, and angiogenesis following neuronal death (126–128). The switching between cell types and the balance between protective and restrictive outcomes depends on regulatory molecules in the local environment, including sex steroids, among many others [reviewed in Ref. (124, 125, 129)].

Administration of sex steroids before or after insult generally reduces infarct volume following middle cerebral artery occlusion [MCAO; reviewed in Ref. (129)], with evidence that this neuroprotective effect of sex steroids is mediated in part through microglia. For example, administration of E_2 and progesterone reduces the attraction of microglia (and leukocytes) to the MCAO-induced penumbra in both male and female rats (130). Moreover, the typically observed pro-inflammatory cytokines CCL2, CCL5, and IL-6 are lower in the penumbra following E_2 and progesterone treatment, suggesting that activation of local microglia is also suppressed by E_2 and progesterone (130).

Likewise, E_2 disrupts microglia activation following stimulation with lipopolysaccharide (LPS)—a lipoglycan endotoxin isolated from the outer membrane of Gram-negative bacteria and commonly used to mimic CNS infection and induce microglia activation (131). Typically, LPS activates the innate immune receptor toll-like 4 [TLR4 (132)] to induce microglia activation *via* MAPK phosphorylation and NFκB nuclear translocation (133,

134). In turn, microglia stimulated by LPS increase expression of pro-inflammatory cytokines (e.g., TNFα), cyclooxygenase-2), and NOS (135). E_2 activation of microglial ERα, but not ERβ, inhibits the LPS-induced translocation of NFκB family proteins to the nucleus and, consequently, reduces NFκB-dependent transcriptional activity and the resultant inflammatory response [**Figure 2** and **4** (136)]. Further supporting a role for E_2–ERα in microglia reactivity, exogenous systemic supplementation of E_2 prevents LPS-induced microglial activation and expression of neuroinflammatory mediators, specifically, metalloproteinase 9, and complement C3 receptor in mice (137). However, this impact of exogenous E_2 on microglia reactivity is lost in ERα-null mice (137).

The effects of steroidal hormones, however, might not be universal across the sexes: in cultured microglia isolated from neonatal hippocampus of male and female rats, E_2 administration following LPS stimulation of the *ex vivo* microglia attenuates IL-1β mRNA upregulation in male but not female microglia (138). The anti- and pro-inflammatory impact of E_2 on male and female neonatal microglia, respectively, indicates that E_2 drives differential microglia responses in males and females during development likely contributes to sexual differentiation of the CNS (139) and that the differential impact of E_2 across the sexes have effects beyond development. Given the above evidence that E_2 plays pro-neuroprotective and anti-inflammatory roles in the adult female CNS, additional studies directly testing the role of E_2 and the other sex steroids to determine when the switch between E_2-dependent pro- and anti-inflammatory effects occurs in females are necessary.

Astroglia

Astroglia, a sub-type of macroglial cells, comprise nine biochemically and developmentally distinct forms including radial, fibrous, protoplasmic, among others (140). As discussed above, radial astroglia (more commonly referred to simply as radial glia) are organized in planes perpendicular to the ventricle with one of their two projections extended toward the ventricle and the other deep into the gray matter. As such, radial glia structurally and trophically support migrating neuroblasts and, in doing so, play an extensive role in neural development and adult neurogenesis (**Figure 2** and **4**). Fibrous astroglia reside along white matter tracts and exhibit long unbranched fiber-like process, whereas protoplasmic astroglia are found throughout gray matter and are characterized by many fine branching processes uniformly distributed around the soma in the classic stellate pattern. Both fibrous and protoplasmic astrocytes establish highly organized territorial domains with their processes, one of which, the "end foot" contacts an intraparenchymal blood vessel whereas the other processes extend into and survey the surrounding microenvironment. In this manner, all astrocytes bi-directionally connect nutrient sources (e.g., cerebrospinal fluid and blood) to nearby glia, neuropil, and synapses, allowing for dynamic responses to microenvironmental changes [reviewed in Ref. (141)].

Under healthy physiological conditions, astrocytes maintain homeostasis and play key specialized roles in the CNS [reviewed in Ref. (142)]. For example, astrocytes not only facilitate high frequency neuronal firing through buffering of local pH (143),

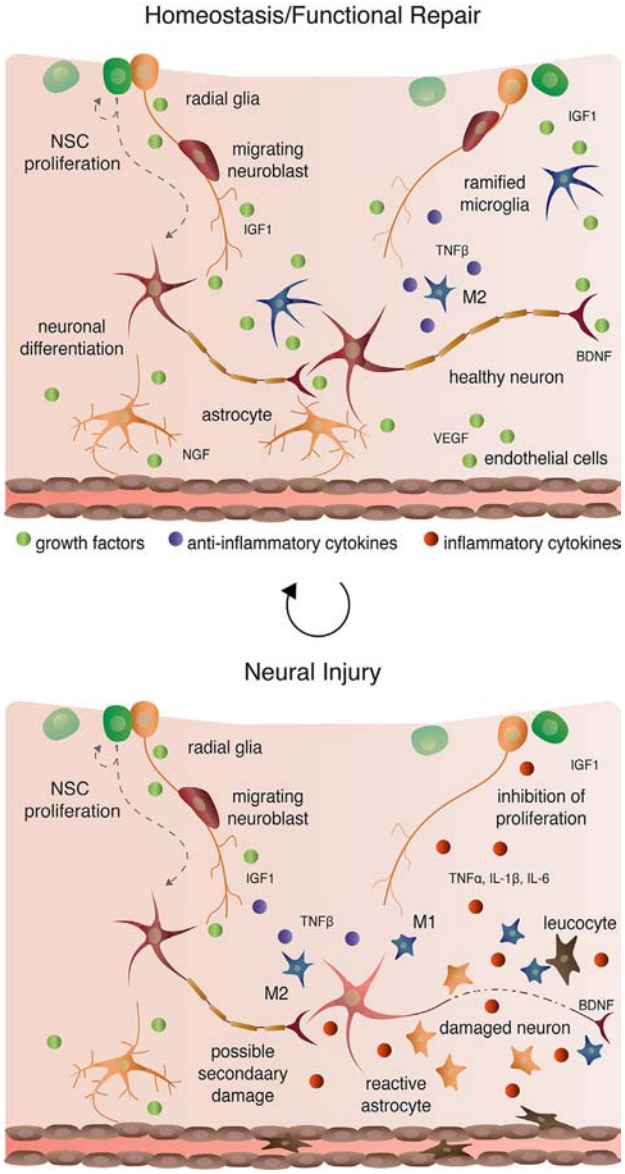

FIGURE 4 | Cellular interactions in homeostatic, injury-induced, and reparative conditions.

uptake of interstitial K+ (144) and glutamate recycling (145), but also modulate neural activity and the propagation of action potentials through the release of glutamate, GABA and calcium, among other "gliotransmitters" (141, 146). Astrocytes also supply energy and nutrients to cells of the CNS through several mechanisms, including, but not limited to: (1) rapid vasodilation during neural activity to increase the availability of oxygen and glucose (147), (2) storage and release of glycogen [reviewed in Ref. (148)], and (3) production, secretion, and distribution of cholesterols that cannot cross the BBB (149). And finally, under normal physiological conditions, non-reactive astrocytes, like ramified microglia, regulate the formation and pruning of synapses (150, 151).

Aside from maintaining stable physiological conditions, astroglia also perform immunological functions similar to CNS-resident microglia and macrophages (**Figure 2** and **4**). In response to neural damage, astroglia become reactive and undergo morphological and behavioral changes characterized by the upregulation of intermediate filament proteins including vimentin and GFAP, which facilitate reactive retraction of the astrocytic perisynaptic processes (152). As with microglia, the morphological and behavioral changes associated with reactive astrogliosis vary along a continuous spectrum dependent on the severity and the spatiotemporal extent of neural damage. For example, with "mild to moderate" reactive astrogliosis, GFAP upregulation and hypertrophy of perisynaptic processes occurs, but reactive astrocytes maintain their territorial domains and do not proliferate (141). "Severe" reactive astrogliosis occurs as either "diffuse" or "with compact glial scar formation" in response to severe focal lesions, infection, or other chronic neurodegeneration (141). Both severe forms are characterized by upregulation of GFAP, processes hypertrophy, extensive intermingling of territorial domains, cytokine release and leukocyte, microglia, and T-cell recruitment, astrocytic proliferation, and reorganization of tissue architecture [reviewed in Ref. (141, 153)]. Moreover, the increased GFAP expression by astrocytes during reactive astrogliosis suppresses neurite outgrowth *in vitro* (154) and axonal regeneration *in vivo* (155). Severe diffuse astrogliosis progresses to compact glial scar formation as a means to cordon off severe necrosis and leukocyte infiltration and to prevent further neural damage (156). This continuum of reactivity highlights both the beneficial roles of astroglia in the acute stages after neural damage and their detrimental effects on regeneration in the context of chronic neural damage.

Not surprisingly, sex steroids modulate the behavior of astroglia under both normal physiological and pathogenic conditions. In the developing and healthy brain, E_2 regulates astrocytic expression and secretion of various molecules involved in neurotransmitter transport, mitochondria respiration, steroidal hormone synthesis, and neuritogenesis [for more see Ref. (157)]. Under pathological conditions, E_2 decreases astrocyte proliferation both *in vitro* and *in vivo* in addition to reducing cytokine secretion and glial scar formation (158–160). The modulation of the astrogliotic response by E_2 begins at the morphological activation of astrocytes: E_2 decreases the expression of GFAP and vimentin after injury-induced neuronal loss, ultimately preventing reactive astrogliosis [**Figure 2** (159, 161)]. Interestingly, E_2 stimulates GFAP expression in olfactory bulb astrocytes (162) and increases astrocytic proliferation in white matter (163), suggesting regional and astrocyte-type differences in response to sex steroids and possibly even impacts on neuroprotection.

Generally, sex steroids suppress astrocytic secretion of pro-inflammatory molecules and the subsequent recruitment of additional inflammatory cells. For instance, as with microglial activation, E_2 prevents LPS stimulated translocation of NFκB to the nucleus and production of TNFα, NO, IL-1β, and IL-6 in astrocytes (164–166). As mentioned above, E_2 suppression of NFκB in astrocytes also results in decreased astrocytic expression of ICAM1 (64), a CAMs that mediates leukocyte–endothelial cell interactions necessary for transmigration of leukocytes across the BBB into the CNS. In this manner, E_2 synthesis and secretion by astrocytes may decrease the permeability of the BBB to leukocytes and contribute to the mitigation of inflammation and glial scaring following neural damage.

Neuronal injury within the mammalian and avian CNS routinely enhances the expression and activity of aromatase (167, 168), which, consequently, permits increases in local estrogen synthesis and confers neuroprotection [**Figure 2** (169)]. Important to understanding mechanisms of injury-induced aromatase activity, upregulation of aromatase occurs in and around the lesion site regardless of the whether or not the affected brain region produces aromatase under normal physiological conditions (170, 171). In fact, this upregulation of aromatase occurs specifically within the locally activated astrocytes. These astrocytes in turn synthesize and secrete estradiol to decrease local neuronal apoptosis (169), as well as likely inducing nearby proliferation of NSCs and migration of neuroblasts to the region of damage [(discussed above and below (172)].

Macrophages, Neutrophils, Monocytes, Dendritic Cells (DCs), and Lymphocytes

In addition to microglia and macroglia, sex steroids modulate inflammation through other inflammatory cells as well. CNS-resident macrophages, neutrophils, non-proliferative monocytes, antigen presenting myeloid cells (i.e., DCs), and lymphocytes (i.e., T-, B-, and natural killer cells) continuously surveil the CNS, rapidly infiltrate sites of injury to clear dead cells and pathogens, recruit additional inflammatory cells for tissue repair, and in some contexts, provide protection from similar future insults. Generally, macrophages, monocytes, and neutrophils minimize inflammation through the phagocytosis of dead cells and debris and the release of cytokines and chemokines to recruit additional leukocytes (e.g., monocytes, neutrophils, etc.) to the CNS and site of injury [reviewed in Ref. (173)]. Another phagocyte, the DC, serves as a sentinel by sensing the local environment through continuous and regular endocytosis. Depending on the environmental signals perceived, DCs dictate the type of inflammatory response initiated through differential secretion of cytokines, some of which promote the differentiation of activated T cells, reviewed in Ref. (174). Differentiated T cells can in turn secrete cytokines, stimulate the activation other cell types (e.g., DCs, macrophages, B cells, etc.), and initiate cytotoxic "killer" functions (174). Together, the degree of collaboration of different inflammatory cell types promotes a unique step-wise program and cytokine profile dictating the impact of the inflammatory response. Although initially beneficial, extensive or overreactive activation and recruitment of leukocytes, DCs, and lymphocytes can push the inflammatory response beyond beneficial toward detrimental in terms of neuroprotection and repair (175).

The majority of evidence supporting a role for sex steroids in regulating these other inflammatory cells originates with male-female differences in insult-responsive cell numbers, with a few exceptions [reviewed in Ref. (173)]. For instance, resident macrophages have higher TLR expression and phagocytic activity in females than males, which possibly contributes to the greater response efficiency and survival of females during sepsis (176).

Likewise, plasmic neutrophil counts correlate with systemic estrogen levels (177). Although vastly understudied, the mechanistic role of estrogens in regulating leukocytes, lymphocytes, and DCs is becoming more evident. In addition to decreasing NκFB nuclear translocation and TNFα, IL-1β, and IL-6 expression in microglia and astrocytes, E_2 regulates macrophages and monocytes stimulated with LPS or other neural trauma through these same mechanisms [**Figure 2** (178, 179)]. Neutrophils decrease IL-1β and IL-6 secretion following injury to the carotid arteries when exposed to E_2 (180). Estrogens also regulate the motility of monocytes and neutrophils: E_2 not only decreases transcytosis of leukocytes across the BBB through changes in BBB permeability (discussed above), but also modulates leukocyte migration through direct action on the leukocytes themselves. Specifically, E_2 decreases CXCR2, a chemokine receptor that mediates monocyte and neutrophil adhesion and chemotaxis, following cytokine stimulation or injury (180–182). As such, E_2 suppression of leukocyte migration across the BBB and within the CNS may restrict recruitment of inflammatory cells and, in turn severe inflammatory responses to promote sex steroid dependent neuroprotection.

Estrogens also regulate myeloid cell and lymphocyte differentiation and function, and thus DCs and T cells are involved in mediating the neuroprotective effects of estrogens. For instance, estrogen signaling *via* ERα is necessary for DC differentiation and modulates the acquisition of DC effector functions (183). The estrogen estriol (E_3) promotes immune self-tolerance functions of DCs through increased expression of anti-inflammatory cytokines IL-10 and TGF-β and decreased expression of pro-inflammatory cytokines IL-12 and IL-6 (183). Likewise, E_2 promotes the development and function of regulatory T cells, which participate in the maintenance of self-tolerance and, when aberrant, autoimmune disorders through upregulation of the transcriptional factor FoxP3 within T cells (184). Together these data provide the beginnings of a mechanistic understanding for the well-known female sex bias in the development of neurological autoimmune disorders and psychiatric disease [for more, see Ref. (185)]. Future efforts examining the influence of steroidal hormones on myeloid cell and lymphocyte physiology and impacts are undoubtably justified.

INTEGRATION OF SEX STEROIDS, NEUROGENESIS, AND NEUROINFLAMMATION

The first conclusive evidence that inflammation impacts adult neurogenesis came in 2003 from a study showing that a 4-week infusion of LPS into the hippocampus decreased NSC proliferation (186). The same year another study demonstrated that systemic inflammation induced through intraperitoneal injection of LPS similarly decreased NSC proliferation (187). Moreover, the LPS-mediated impairment of adult neurogenesis was ameliorated by the administration of inhibitors of microglia activation, minocycline and indomethacin, suggesting microglia were likely regulating NSC behavior (186, 187). Given the 4-week time course of LPS infusion, the observed decrease in NSC proliferation could have resulted from increased NSC apoptosis (122) and reduction of neuroblast incorporation into circuits (188). Yet, subsequent studies have confirmed that LPS promotes a neuroinflammatory response, including depression of NSC proliferation, through direct stimulation of astrocytes and microglia in addition to indirect recruitment of leukocytes, specifically monocytes and neutrophils (189, 190).

Recent studies highlight the dynamic role of inflammatory cells, their interactions, and their responses in modulating adult neurogenesis and the regenerative capabilities of the CNS. Most of such efforts have focused on neuroinflammatory responses to physical damage, such as ischemia and traumatic brain injury, or pharmacological insult, including treatment with LPS or pro-inflammatory cytokines and chemokines. Currently, few studies have directly assessed the role of immune cells in regulating adult neurogenesis, as broadly defined here to include NSC proliferation, neuroblast migration, and nascent neuronal differentiation and survival, under healthy, homeostatic conditions. Below, I briefly discuss advances in understanding the damage-induced inflammatory response on NSC and their nascent progeny after first highlighting the few studies that have addressed the impact of non-reactive and alternatively activated (i.e., M2) microglia on neurogenesis.

Regulation of Adult Neurogenesis by Microglia

Ramified, unchallenged microglia play a pivotal role in homeostasis not only in the parenchyma (above) but also in the neurogenic niches of the CNS. Ramified microglia instruct neuronal differentiation of neuroblasts through the secretion of a still unknown factor [**Figures 2** and **4**; (191)] and facilitates clearance by phagocytosis of the ~50% of neuroblasts that do not differentiate and functionally incorporate into circuits (122). Likewise, alternatively activated M2 microglia promote pro-neurogenic effects through the release of anti-inflammatory cytokines including IL-4, IL-10, and TGFβ; tpysinogen, a precursor of the enzyme trypsin; and growth factor such as IGF1 and BDNF [**Figures 2** and **4**; reviewed in Ref. (124)]. For instance, chronic elevation of microglial TGFβ enhances the survival of proliferative NSC and the differentiation of progeny into the neuronal (as opposed to glial) lineage (192). Likewise, microglia stimulated with IL-4 or IL-10 enhance NPC proliferation, perhaps through the upregulation of IGF1 production following anti-inflammatory cytokine stimulation (193, 194).

Reactive M1 microglia also have immediate beneficial impacts on recovery from neural insult, as they facilitate the initial clearance of debris. Typically, however, in the context of neurogenic repair following clearance of injury- or pathogen-induced neuronal death, M1 microglia have detrimental effects on neurogenesis (**Figure 4**). M1 microglia secretion of pro-inflammatory cytokines, including TNFα, IL-1β, IL-6, and INF-γ (195), directly impair NSC proliferation, suggesting that the NSCs themselves are capable of detecting and responding to certain cytokines. Besides modulating NSC proliferation, these cytokines impair other processes of adult neurogenesis (as broadly defined) from

the migration and differentiation of neuroblasts to final incorporation and survival of new neurons. For instance, IL-1β not only decreases NSC proliferation but also impairs the survival of neuroblasts and hinders neuronal differentiation (196). Although not tested directly as an output of microglia, administration of TNFα, a secreted factor of M1 microglia, promotes the apoptosis of NSC (197) and increases the proportion of daughter cells that differentiate into the astrocytic as opposed to neuronal lineage (198). Likewise, IL-6 reduces NPC proliferation through promotion of progenitor differentiation, albeit into neurons (199).

The differential beneficial and detrimental impacts of microglia underscore the need to further characterize microglial behaviors in healthy and neurodegenerative conditions, and to elucidate mechanisms driving molecular and behavioral switches between states of microglial activation. Additional, carefully controlled studies will also be important for deciphering upstream and downstream regulatory mechanisms of cytokines, growth factors, and other signaling factors: research efforts should be made to disentangle the beneficial versus detrimental impacts of microglia on themselves, other inflammatory cells (astrocytes, leukocytes, DCs, T cells), and CNS-resident cells (endothelial cells of the parenchymal vasculature and BBB, neurons).

Astroglia, Aromatase, and Adult Neurogenesis

As mentioned above, astroglia can synthesize and secrete E_2. In songbirds, the number of newly generated cells positively correlates with the number of precursor radial glial cell processes expressing aromatase (200). Likewise, the degree of neurogenesis also correlates positively with the number of aromatase expressing non-precursor astrocytes, and this correlation is abolished with ovariectomy and administration of aromatase inhibitors (172, 201). Non-precursor astrocytes also actively regulate the differentiation and survival of newly generated neurons (**Figures 2** and **4**). For instance, astrocytic expression of ephrin-B2 induces neuronal differentiation *via* EphB4 receptors on NSCs and subsequent activation of Wnt signaling (202). Moreover, astrocytes facilitate dendritic spine maturation and synapse formation of adult-born neurons through vesicular release of D-serine, a co-agonist (along with glycine) of the NMDA receptor expressed by new neurons (203). However, very few studies have directly tested roles for activated, immunogenic astroglia in regulating NSC proliferation, neuroblast migration and differentiation, or neuronal incorporation and survival. Given the similarities in activation responses between microglia and astroglia, one could easily deduce that if astroglia directly regulate NSCs, then the initial astrogliotic response following injury would likely promote neurogenesis, whereas chronic astrogliosis likely hinders NSC proliferation and new neuron differentiation and survival.

Regulation of Myelination Through Sex Steroid Impacts on Immune Cells

Sex steroids also enhance the myelination of new neurons and the remyelination of mature neurons following pathological demyelination. As discussed above, under non-pathological conditions sex steroids directly promote the proliferation of OPCs, differentiation of new oligodendrocytes and stimulate oligodendrocyte activities including sheath synthesis and wrapping of axons. The impact of sex steroids on oligodendrocyte behavior also occurs indirectly through immune cell-mediated interactions. For example, the CNS autoimmune disorder MS is more prevalent in women than men. Moreover, the characteristic relapsing and progressive sclerotic plaques and demyelination of MS can be alleviated by treatment with sex steroidal hormones (204). In a murine model of MS administration of the copper chelator cuprizone induces toxic demyelination, which is lessened in severity by treatment with progesterone (73). Likewise, treatment with E_2 following cuprizone administration also ameliorates demyelination and promotes myelin repair (163). Progesterone and E_2 both independently induce IGF1 expression in astrocytes, which in turn promotes the proliferation of OPCs and the differentiation of progeny into myelinating oligodendrocytes (163, 205). The beneficial impact of estrogens is, at least in part, mediated through ERα-dependent signaling in reactive astroglia. Treatment of experimental autoimmune encephalomyelitis (EAE) mice, another model for MS, with the selective ERα agonist PLP peptide 139–151 (PPT), decreases the production of TNFα, interferon-γ, and IL-6 in addition to decreasing the recruitment of macrophages and T cells and EAE-associated demyelination (206–208). Conditional gene deletions of ERα in astroglia, but not neurons, reversed the effect of PPT treatment on macrophage and T cell recruitment and prevented estrogen-mediated attenuation of gliosis and axonal degeneration in EAE mice (208). The ERβ agonist WAY-202041 has minimal reported effects on EAE-induced cytokine production (206). Interestingly, however, ERβ, but not ERα, is expressed in microglia. Treatment of EAE mice with the selective ERβ agonist LY3201 promotes microglia to retain their ramified morphology and, as such, reduces microglia NFκB activation and iNOS expression (209).

Microglia and astroglia, in addition to other immune cell types, facilitate not only debris removal following demyelination, axonal damage, and neuronal death, but also neurogenesis, gliogenesis, nascent cell fate specification, arbor and synapse maturation, and nude axonal ensheathment. Although sex steroids clearly modulate the behavior and impact of immune cells, gaps in our understanding of the complex interactions between immune cells, neural cells, and sex hormones still exist. Thus, directly testing the relative impact of reactive astrogliosis and activated microglia on adult neurogenesis and gliogenesis will be essential for understanding not only the unique roles of micro- and macroglia during regeneration but also the manner through which astrocytic and microglial responses integrate into the larger orchestra of neuroinflammation.

FUTURE DIRECTIONS: EMERGING MODELS OF NEUROINFLAMMATION AND REGENERATION

The role of inflammation in neurocognitive disorders and employment of sex steroidal and anti-inflammatory therapeutics has been discussed extensively in past reviews. So instead, here, I limit the discussion to emerging models of

neuroinflammation and regeneration. For more information on the role of inflammation in relatively well-characterized psychiatric and neurodegenerative disorders and classes of therapeutics see **Table 1**.

Neurological Diseases With Recently Discovered Immune Component

The reemergence of fetal microcephaly as a result of maternal Zika viral infection has recently leapt onto the main stage as a neurodevelopmental disorder with an underlying neuroimmune component. This is not to suggest that Zika virus (ZIKV) is the only neurological disease with developing evidence for an inflammatory component, but it certainly of recent piqued interest, and thus, I will briefly discuss ZIKV-induced microcephaly here. Following the detection of ZIKV in the fetal brain (220) and establishment of a causal relationship between ZIKV infection and microcephaly (221) in 2016, ZIKV was discovered to infect NPCs and attenuate their growth (222). Specifically within radial glia progenitor cells, ZIKV reduces proliferation and promotes premature differentiation through ZIKV protein NS2A destabilization of the adherens junction complex, and as a result, mis-scaffolding of the radial processes (223). Two other ZIKV proteins, NS4A and NS4B, cooperatively inhibit AKT and mTOR signaling in NSCs, leading to decreased proliferation and increased autophogy of the NSC (224). ZIKV also infects microglia of human fetal brain, rendering the microglia reactive. ZIKV-infected microglia secrete high levels of pro-inflammatory factors TNF-α, IL-1β, IL-6, among several others (225), which likely further decreases embryonic neurogenesis through mechanisms discussed above. Cleary, additional studies determining the mechanisms of ZIKV's negative impact on neurogenesis and neuroinflammation are warranted and these mechanisms will certainly be taken into consideration during the development of therapeutics and vaccines. Given that ZIKV infects both stem cells and microglia consequently decreasing neurogenesis, it will be important to evaluate potential long-term consequences of infection in the adult brain. Postfetal infection with ZIKV will most certainly negatively impact the maintenance of neural homeostasis through aberrations in NSC and neuroinflammatory cell behavior. Thus, it seems likely that postfetal infection with ZIKV could result in the manifestation of neuropsychiatric and neurodegenerative disorders associated with disruptions in neurogenesis and chronic neural inflammation (see **Table 1**).

Non-Traditional Model With High Natural Neuronal Turnover and Plasticity

Songbirds provide a powerful model for understanding the mechanisms, including those of the neuroimmune system, that regulate natural neuronal turnover during periods of homeostatic stability and robust plasticity. Avian song learning and production are regulated by discrete, well-characterized and intertwined circuits—the anterior forebrain pathway and song production pathway, respectively (**Figure 3**). The song production pathway includes the highly neurogenic nucleus HVC and its target nucleus, the robust nucleus of the archopallium (RA). In temporal resident and migratory (226) birds (most often the males), both HVC and RA undergo dramatic sex steroid dependent physiological and morphological changes between environmental seasons [reviewed in Ref. (227)]. Early each breeding season (spring) HVC and RA nearly double in volume. The increase in HVC volume results largely from the addition of over 50,000 new neurons to a pre-breeding season neuronal population of around 100,000 [numbers from Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) (228, 229) and vary across species (226)]. Alternatively, the growth of RA volume results from increases in neuron size and spacing, but not number (228, 229). Both HVC and RA demonstrate evidence of increased neural activity during the breeding season (230, 231), which has been proposed to be one function of the seasonal uptick in addition of new HVC neurons (81). The seasonal growth of the song production pathway nuclei is concomitantly linked to increased rate and quality of singing behavior (232), which is used most typically by male birds for territory maintenance and mate attraction. As seasonally plastic songbirds transition into the non breeding season (fall), plasmic levels of sex steroids decrease and the song production pathway regresses (89, 233, 234). Regression of HVC occurs very rapidly, with the caspase-mediated apoptosis of 50,000 neurons (a mixture of new and old neurons) occurring between one and four days following transition into non-breeding conditions (89, 235). As soon as one day following transition, song rate and quality also decreases dramatically (89).

Importantly, yet unsurprisingly given the evidence above, systemic inflammation negatively impacts hormonal responses and behavior in songbirds. Subcutaneous injection of LPS in the songbird Gambel's white-crowned sparrow prompts a rapid increase in plasma corticosterone levels, suppresses luteinizing hormone (a trigger of gonad growth and production of sex steroids), and impairs total activity encompassing decreases in food and water intake, singing, and territorial aggression (236). These observations in conjunction with the known upregulation of aromatase expression by astroglia in the zebra finch telencephalon following

TABLE 1 | Neurological disorders with known inflammatory component and therapeutics.

Psychiatric disorders	
Depression	(119, 210)
Schizophrenia	(119)
Autism	(211, 212)
Neurodegernative disorders	
Multiple sclerosis	(119, 213)
Amyotrophic lateral sclerosis	(119)
Parkinson's disease	(119, 214, 215)
Alzheimer's disease	(15, 119)
Traumatic injury	
Cerebral ischemia	(119, 216)
Aging	(3, 210)
Therapeutics	
Selective estrogen receptor modulators	(15, 217)
Blood–brain barrier	(1, 46, 47)
Macrophage delivery	(218)
Mesenchymal stem cells	(216, 219)
Neural stem cells	(216, 219)

injury (171, 201) firmly establish that inflammation impacts the avian CNS and likely plays a role in neural homeostasis and seasonal plasticity of the song circuits.

Interestingly, the seasonal regression of HVC through caspase-mediated apoptosis is necessary for reactive proliferation of neural stem/progenitor cells in the nearby VZ and subsequent HVC neuronal addition (89, 235, 237). Given the likely role of phagocytic cells in the clearance of dying HVC neurons, microglia, and astrocytes may confer the signal of neuronal death in HVC to the VZ neural stem/progenitor cells. If so, the potential for understanding the functions and mechanisms of microglia and astrocytes during natural death or turnover of neurons and the functional incorporation of new long-range projection neurons [as opposed to interneurons or locally projecting adult-born neurons in mammals (81, 104)] is unparalleled. However, to date, any functional mechanistic connections between sex steroid dependent neural plasticity, natural reactive neurogenesis, and neuroinflammatory involvement remains to be directly tested.

Songbirds also provide a tractable model for investigating the interactions between neurogenesis, neuroinflammation, and sex steroid levels, both elevated and basal in both males and females. Although all passerine songbirds have the neural circuits controlling song learning and production, not all songbirds are seasonally plastic. For example, zebra finches are highly social opportunistic breeders, and as such, do not have photoperiod-dependent seasonality of either systemic testosterone levels or HVC neuronal addition. Yet, even in non-seasonally plastic species of songbirds, new neurons do continuously enter and incorporate within HVC, replacing older HVC to RA projection neurons (110). Thus, comparative studies across species or manipulative studies in both seasonal and opportunistic species could provide mechanistic insight into the neuroimmune modulatory effects of sex steroids across the sexes and under varying reproductive states.

Non-Traditional Model With High Regenerative Capacity

Teleost fishes have the most widespread and pronounced adult neurogenesis of any vertebrate examined, with 12–16 proliferative zone distributed across telencephalon, diencephalon, optic tectum, cerebellum, and hindbrain of fish [**Figure 3**; (83)]. Teleosts have indeterminate growth (i.e., continue to grow throughout life), and adult neurogenesis has been proposed to function not only in corresponding growth of the CNS but also in replacing naturally dying neurons [reviewed in Ref. (81, 238)]. The mechanisms of adult neurogenesis in teleost fish are characteristic of those in mammals and birds, with a few exceptions—for more on localization of proliferative zones and mechanisms, see Ref. (238).

Across all vertebrates traumatic brain injury is characterized by apoptosis of neurons, glia, and endothelial cells, inflammation, proliferation of micro- and macroglial cells, and increased neural stem/progenitor cell proliferation. Depending on the severity of the lesion, the secondary effects can include a penumbra of degeneration, inflammation, and breakdown of the BBB, all of which further complicate regenerative abilities and functional recovery of behavior. After traumatic brain injury, bony fishes have the remarkable ability to not only upregulate the proliferation of NSCs and migration of neuroblasts, but to functionally incorporate new neurons that survive long term and to near completely restore tissue architecture at the site of the lesion [reviewed in Ref. (239)]. The first evidence of such brain regeneration in fish came from optic tectum lesion experiments in juvenile carp (*Carassium carassium*), in which the size of the remaining progenitor zone correlated with the degree of tissue restoration (240). Reactive proliferation also occurs in the cerebellum of weakly electric fish (*Apteronotus leptorhynchus*) as soon as one day following apoptosis-mediated clearance of cells at the lesion site (241, 242). Apoptosis rather than necrosis at the lesion promotes a "clean" type of cell death, which has been proposed to contribute to the remarkable regenerative ability of fishes (241). Likewise, zebrafish exhibit reactive progenitor cell proliferation, neuroblast migration, nascent neuronal incorporation, and restoration of tissue architecture following stab lesions through the olfactory bulb and into the telencephalon (243).

Many factors regulate teleost reactive neurogenesis [reviewed in Ref. (239)], including inflammation. Traumatic injury to the zebrafish telencephalon stimulates the activation of microglia, infiltration of leukocytes, and the secretion of pro-inflammatory cytokines TNFα, IL-1β, and IL-8 (244). Upregulation of cysteinyl leukotriene receptor 1 expression in the NPC niche is also induced by traumatic brain injury as wells as cerebroventricular microinjection of the immunogenic zymosan A BioParticles (244). Moreover, administration of leukotriene C4, a ligand for CysLT1, enhances NPC proliferation without physical injury, suggesting cysteinyl leukotriene signaling is an essential regulator of the inflammatory-mediated neurogenic response in zebrafish (244). The chemokine receptor CXCR5 is expressed within and is a critical regulatory signal for the proliferation of the radial neural progenitors of the zebrafish telencephalon following stab lesion (245).

The profound regenerative ability of the fish CNS begs the question, "why is regeneration relatively limited in the mammalian brain?" Comparative studies uncovering the conserved and evolutionarily distinct molecular mechanisms that govern regenerative neurogenesis will inform not only neuroimmune and regenerative biology generally but also neurological disease etiology, and treatments that enhance NSC activity and successful neuroblast incorporation and survival.

CONCLUDING REMARKS

Although still not extensively examined, there clearly exist important links between sex steroids, neuroinflammatory responses, and adult neurogenesis. In this review, I have provided convincing evidence that sex steroids independently influence the processes and mechanisms of adult neurogenesis and neuroimmune responses. Moreover, sex steroids are secreted as an effector of the inflammatory response, and so likely influence immune cell-mediated homeostatic neurogenesis and neural repair. Integration of the sub-fields of sex steroids, neuroinflammation, and neurogenesis will embolden a better

understanding of basic biology of NSCs and neuroimmune function, as well as the etiology of neural disorders—those with established and emerging evidence of a neuroimmune component. Ultimately, future studies exploiting the intersection of sex steroids, inflammation, and neurogenesis will spur the development of novel therapeutic strategies for the treatment of neuropsychiatric and neurodegenerative disorders. The future of neuroinflammation and adult neurogenesis research will no doubt be complicated, but will also certainly be exciting and rewarding!

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

ACKNOWLEDGMENTS

Supported by Pratt and UVa startup funds to TL. The author thanks Sandy R. Larson for extensive discussions, guidance, and editorial comments and David M. Parichy for editorial comments.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 01 May 2018
doi: 10.3389/fnins.2018.00282

Src Kinase Dependent Rapid Non-genomic Modulation of Hippocampal Spinogenesis Induced by Androgen and Estrogen

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Dendritic spine is a small membranous protrusion from a neuron's dendrite that typically receives input from an axon terminal at the synapse. Memories are stored in synapses which consist of spines and presynapses. Rapid modulations of dendritic spines induced by hippocampal sex steroids, including dihydrotestosterone (DHT), testosterone (T), and estradiol (E2), are essential for synaptic plasticity. Molecular mechanisms underlying the rapid non-genomic modulation through synaptic receptors of androgen (AR) and estrogen (ER) as well as its downstream kinase signaling, however, have not been well understood. We investigated the possible involvement of Src tyrosine kinase in rapid changes of dendritic spines in response to androgen and estrogen, including DHT, T, and E2, using hippocampal slices from adult male rats. We found that the treatments with DHT (10 nM), T (10 nM), and E2 (1 nM) increased the total density of spines by ∼1.22 to 1.26-fold within 2 h using super resolution confocal imaging of Lucifer Yellow-injected CA1 pyramidal neurons. We examined also morphological changes of spines in order to clarify differences between three sex steroids. From spine head diameter analysis, DHT increased middle- and large-head spines, whereas T increased small- and middle-head spines, and E2 increased small-head spines. Upon application of Src tyrosine kinase inhibitor, the spine increases induced through DHT, T, and E2 treatments were completely blocked. These results imply that Src kinase is essentially involved in sex steroid-induced non-genomic modulation of the spine density and morphology. These results also suggest that rapid effects of exogenously applied androgen and estrogen can occur in steroid-depleted conditions, including "acute" hippocampal slices and the hippocampus of gonadectomized animals.

Keywords: androgen, estrogen, Src kinase, spine, hippocampus, non-genomic, synapse

OPEN ACCESS

Edited by:
Takayoshi Ubuka,
Monash University Malaysia, Malaysia

Reviewed by:
Barney A. Schlinger,
University of California, Los Angeles, United States
Arturo Ortega,
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience

Received: *29 December 2017*
Accepted: *10 April 2018*
Published: *01 May 2018*

Citation:
Soma M, Kim J, Kato A and Kawato S (2018) Src Kinase Dependent Rapid Non-genomic Modulation of Hippocampal Spinogenesis Induced by Androgen and Estrogen. Front. Neurosci. 12:282. doi: 10.3389/fnins.2018.00282

INTRODUCTION

Accumulating evidence over the past two decades supports the conclusion that some sex steroid responses in the brain involve rapid non-genomic mechanisms (Mukai et al., 2010) in addition to slow/genomic actions (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992; MacLusky et al., 2005). In rat and mouse hippocampus, androgen, and estrogen, including

testosterone (T), dihydrotestosterone (DHT), and estradiol (E2), induced rapid modulation of dendritic spines, which occurred between 30 and 120 min after the application (MacLusky et al., 2005; Murakami et al., 2006; Mukai et al., 2007). Sex steroid-induced rapid effects were also observed in electrophysiological investigations, such as the long-term potentiation (LTP) (Foy et al., 1999; Bi et al., 2000; Grassi et al., 2011; Ooishi et al., 2012b; Hasegawa et al., 2015). E2-induced rapid synaptic modulation occurred not only *in vitro* but also *in vivo* conditions in the hippocampus (MacLusky et al., 2005) (Luine and Frankfurt, 2012; Luine, 2014). The rapid signaling of E2 may depend on multiple kinases, including mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) in the hippocampus (Bi et al., 2000, 2001; Znamensky et al., 2003; Mannella and Brinton, 2006; Mukai et al., 2010; Luine, 2014; Tuscher et al., 2016). The contribution of many other essential serine/threonine kinases have also been studied. These important kinases are LIM domain kinase (LIMK), protein kinase A (PKA), and protein kinase C (PKC) all of which are essential for regulation of the synaptic plasticity (Frick, 2015; Hasegawa et al., 2015; Tuscher et al., 2016).

On the other hand, androgen (T and DHT)-induced rapid effects on synaptic modulation in the hippocampus or other brain regions have not been extensively investigated (Foradori et al., 2008; Hajszan et al., 2008), while rapid effects of androgen were extensively studied in prostate cancer cells and gonadal cells (Migliaccio et al., 2000; Cheng et al., 2007). We tried to examine molecular mechanisms of rapid effects in the hippocampus, and showed that DHT and T induced rapid increase of CA1 dendritic spines via non-genomic signaling, including activation of several serine/threonine kinases (including MAPK, LIMK, PKA, PKC; Hatanaka et al., 2015). Also in CA3 region, T, and DHT rapidly increased thorns in stratum lucidum via MAPK and PKC, but not via PKA (Hatanaka et al., 2009).

Rapid synaptic action may require new arrangement of sex steroid receptor systems. In the hippocampus, androgen receptor (AR), and estrogen receptor (ER), classic nuclear steroid receptors, appear to be primarily located in the glutamatergic neurons (Simerly et al., 1990; Clancy et al., 1992; Brown et al., 1995; Kerr et al., 1995; Mukai et al., 2007). AR and ER are located not only in the cytoplasm and the nuclei but also within dendritic spines (Tabori et al., 2005; Mukai et al., 2007; Hatanaka et al., 2015). Therefore, classic receptors AR and ER, localized in the synaptic membrane, could act as membrane receptors, triggering rapid effects of sex steroids as indicated from many recent investigations (Milner et al., 2005; Mukai et al., 2007, 2010; Pedram et al., 2007; Hojo et al., 2008; Hasegawa et al., 2015; Levin and Hammes, 2016). Synaptic membrane localization of these receptors might be accomplished via palmitoylation of receptors (Pedram et al., 2007; Levin and Hammes, 2016)

Since adult hippocampus locally synthesizes androgen and estrogen (Hojo et al., 2004), their levels in the hippocampus are key factors for action through synaptic AR and ER. Mass-spectrometric analysis revealed that the levels of androgen and estrogen in freshly isolated male hippocampus are higher than those in plasma (Hojo et al., 2009). The levels of male hippocampal sex steroids were ∼17 nM for T, ∼7 nM for DHT, and ∼8 nM for E2 (Hojo et al., 2009), which levels are much higher than that of circulating T (∼15 nM), DHT (∼0.6 nM), and E2 (∼0.01 nM). Importantly, after preparation of "acute" hippocampal slices, sex steroid levels were considerably decreased to below 0.5 nM due to recovery incubation in artificial cerebrospinal fluid (ACSF) (Hojo et al., 2009, 2011; Ooishi et al., 2012a,b; Hatanaka et al., 2015). Therefore, exogenous application of sex steroids may help to elevate steroid levels back to the *in vivo* situation.

We here investigated the possible involvement of Src tyrosine kinase in rapid spine modulation of DHT, T, and E2, with considering similarity and difference in signal pathways between DHT, T, and E2 in male rats. Although Src kinase was known to be activated by androgen and E2 in prostate and breast cancer cells (Migliaccio et al., 1996, 2000), its role in hippocampal synaptic plasticity has not been well-documented.

MATERIALS AND METHODS

Animals

Young adult male Wistar rats (12 week old, 320–360 g) were purchased from Tokyo Experimental Animals Supply (Japan). All animals were maintained under a 12 h light/12 h dark cycle and free access to food and water. The experimental procedure of this research was approved by the Committee for Animal Research of Teikyo University.

Chemicals

DHT, T and PP2 were purchased from Sigma-Aldrich (USA). Estradiol was from Wako Pure Chemicals (Japan). Lucifer Yellow was obtained from Molecular Probes (USA).

Slice Preparation

Adult male rats were deeply anesthetized by isoflurane and decapitated. Immediately after decapitation, the brain was removed from the skull and placed in ice-cold oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 22 $NaHCO_3$, and 10 D-glucose (all from Wako); pH was set at 7.4. The hippocampus was then dissected and 400 μm thick transverse slices to the long axis, from the middle third of the hippocampus, were prepared with a vibratome (Dosaka, Japan). These slices were "fresh" slices without ACSF incubation. Slices were then incubated in oxygenated ACSF for 2 h (slice recovery processes) in order to obtain widely used "acute slices."

Imaging and Analysis of Dendritic Spine Density and Morphology

Drug Treatments and Current Injection of Lucifer Yellow

The "acute" slices (used worldwide) were incubated for 2 h with 10 nM DHT, 10 nM T, or 1 nM E2, together with 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine (PP2), a Src kinase inhibitor. Slices were then fixed with 4% paraformaldehyde in PBS at 4°C overnight. Neurons within slices were visualized by an injection of Lucifer Yellow (Molecular Probes, USA) under Nikon E600FN microscope (Japan) equipped with a C2400-79H infrared camera (Hamamatsu

Photonics, Japan) and with a 40× water immersion lens (Nikon, Japan).

Current injection was performed with glass electrode filled with 4% Lucifer Yellow for 2 min, using Axopatch 200B (Axon Instruments, USA). Approximately two neurons within a depth of 100–200 μm from the surface of a slice were injected with Lucifer Yellow (Duan et al., 2002).

Confocal Laser Microscopic Imaging and Analysis

The imaging was performed from sequential z-series scans with super-resolution confocal microscope (Zeiss LSM880; Carl Zeiss, Germany) using Airy Scan Mode, at high zoom (× 3.0) with a 63 × oil immersion lens, NA 1.4. For Lucifer Yellow, the excitation and emission wavelengths were 458 and 515 nm, respectively. For analysis of spines, three-dimensional image was reconstructed from ∼30 sequential z-series sections of every 0.45 μm. The applied zoom factor (×3.0) yielded 23 pixels per 1 μm. The confocal lateral resolution was ∼0.14 μm. The z-axis resolution was ∼0.40 μm. Our resolution limits were regarded to be sufficient to allow the determination of the head diameter of spines in addition to the density of spines. Confocal images were deconvoluted with the measured point spread function using Processing Mode of LSM880.

The density of spines as well as the head diameter were analyzed with Spiso-3D (automated software calculating mathematically geometrical parameters of spines) developed by Bioinformatics Project of Kawato's group (Mukai et al., 2011). Spiso-3D has an equivalent capacity with Neurolucida (MicroBrightField, USA), furthermore, Spiso-3D considerably reduces human errors and experimenter labor. The single apical dendrite was analyzed separately. The spine density was calculated from the number of spines along secondary dendrites having a total length of 40–60 μm. These dendrites were present within the stratum radiatum, between 100 and 200 μm from the soma. Spine shapes were classified into three categories as follows. (1) A small-head spine, whose head diameter is smaller than 0.4 μm. (2) A middle-head spine, which has 0.4–0.5 μm spine head. (3) A large-head spine, whose head diameter is larger than 0.5 μm. These three categories were useful to distinguish different responses upon kinase inhibitor application. Small-, middle-, and large-head spines probably have different number of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and therefore these three types of spines might have different efficiency in memory storage. The number of AMPA receptors (including GluR1 subunits) in the spine increases as the size of postsynapse increases, whereas the number of N-methyl-D-aspartate (NMDA) receptors (including NR2B subunits) might be relatively constant (Shinohara et al., 2008). Because the majority of spines (>93–95%) had a distinct head, and stubby spines and filopodia did not contribute much to overall changes, we analyzed spines having a distinct head.

Statistical Analysis

Drug-treated dendrite images were used for spine analysis, and typical images were shown in **Figures 1–4**. Each dendrite has ∼50 μm in length including ∼50 spines. For statistical analysis, we employed two-way ANOVA, followed by Tukey-Kramer multiple comparison's test. For each steroid application analysis, we used ∼50 dendrites with 2,300–2,700 spines obtained from 3 rats, 12 slices, 30 neurons. For control dendrite analysis without steroid application, we used 80 dendrites with ∼4,000 spines from 6 rats, 24 slices, 50 neurons.

RESULTS

We investigated the involvement of Src protein kinase in the modulation effects of DHT, T, and E2 on spinogenesis. Dendritic spine imaging was performed for Lucifer Yellow-injected glutamatergic neurons in acute hippocampal slices of male rats. We analyzed secondary branches of the apical dendrites located 100–200 μm distant from the pyramidal cell body around the middle of the stratum radiatum of CA1 region.

Analysis of Spine Head Diameter as Well as the Total Spine Density

The morphological changes in spine head diameter induced after 2 h treatments of drugs were analyzed. Since observing the total spine density cannot describe well the complicated different kinase effects, the changes in spine head diameter distribution were also analyzed. Because the majority of spines (>93–95%) had distinct heads and necks, and stubby spines and filopodia did not contribute much to overall changes (<5–7%), we analyzed spines having distinct heads. We classified these spines with clear heads into three categories based on their head diameter, e.g., 0.2–0.4 μm as small-head spines, 0.4–0.5 μm as middle-head spines, and larger than 0.5 μm as large-head spines.

Statistical analyses based on classification of the spines into three categories were performed. In control slices (without sex steroids supplementation), the spine density was 0.52 spines/μm for small-head spines, 0.30 spines/μm for middle-head spines, and 0.20 spines/μm for large-head spines (**Figure 1**). In order to investigate intracellular signaling pathways of kinases involved in the sex steroid-induced spinogenesis, here, we analyzed the contribution of Src protein kinase by blocking Src kinase with a selective inhibitor, PP2.

DHT Effects and Src Kinase Blocking

The treatments with DHT and PP2 induced significant changes in the total spine density ($F = 20.46$, $p < 0.0001$, two-way ANOVA). After 2 h treatment with 10 nM DHT, the total spine density was significantly increased to 1.26 spines/μm from the control density of 1.04 spines/μm ($p < 0.0001$ for control vs. DHT, Tukey–Kramer multiple comparison's test) (see **Figure 1**). This increase in the total spine density was suppressed by blocking of Src kinase through co-incubation of 10 nM DHT and 10 μM PP2 ($p < 0.0001$ for DHT vs. PP2+ DHT, Tukey–Kramer multiple comparison's test).

From spine head diameter analysis, after 2 h treatments with DHT, the density of middle- and large-head spines significantly increased ($p = 0.0304$ for middle-head and $p < 0.0001$ for large-head, for control vs. DHT, Tukey–Kramer), while the density of small-head spines was not significantly altered. Blocking Src kinase by PP2 abolished the DHT effects on the dendritic spine densities, by decreasing the density of the middle-head spines

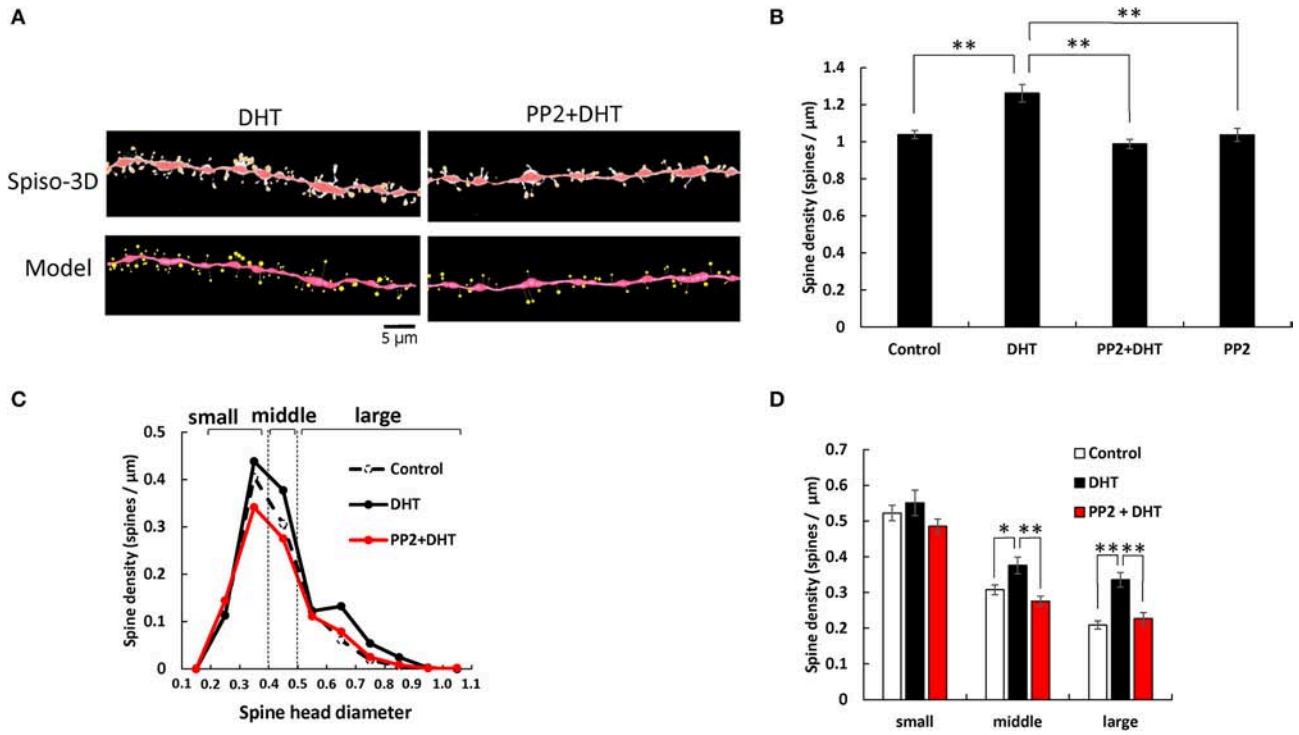

FIGURE 1 | Effects of Src kinase blocker (PP2) on DHT-induced spine increase and change in morphology in hippocampal slices. **(A)** Spines were analyzed along the secondary dendrites of pyramidal neurons in the stratum radiatum of CA1 neurons. Dendrite after DHT-treatment for 2 h (DHT) and dendrite after DHT plus PP2 treatment for 2 h (PP2+DHT). (Spiso) shows the image of dendrite and spines analyzed with Spiso-3D software. Maximal intensity projections onto XY plane is shown. Traced dendrite is shown in red color and spines are indicated in yellow color. (Model) shows 3 dimensional model illustration of (Spiso) image. Bar, 5 μm. **(B)** Effect of treatments by DHT or PP2 on the total spine density in CA1 neurons. Vertical axis is the average number of spines per 1 μm of dendrite. A 2 h treatment in ACSF without drugs (Control), with 10 nM DHT (DHT), with 10 nM DHT and 10 μM PP2 (PP2 + DHT), and with PP2 only (PP2). **(C)** Histogram of spine head diameters after a 2 h treatment in ACSF without drugs (Control, black dashed line), with 10 nM DHT (black line), with 10 nM DHT and 10 μM PP2 (red line). Spines were classified into three categories depending on their head diameter, e.g., 0.2–0.4 μm as small-head spines, 0.4–0.5 μm as middle-head spines, and larger than 0.5 μm as large-head spines. Vertical axis is the number of spines per 1 μm of dendrite. **(D)** Density of three subtypes of spines. Abbreviations are same as in **(B)**. Vertical axis is the number of spines per 1 μm of dendrite. From left to right, small-head spines (small), middle-head spines (middle), and large-head spines (large) type. ACSF without drugs (Control, open column), DHT (black column), PP2 + DHT (red column). Vertical axis is the number of spines per 1 μm of dendrite. Results are represented as mean ± SEM. Statistical significance yielded $^{*}P < 0.05$, $^{**}P < 0.01$ vs. DHT sample. For DHT, PP2+DHT, and PP2 only treatments, we investigated, 50 dendrites with 2,300–2,700 spines from 3 rats, 12 slices, and 30 neurons. For control, we used 80 dendrites with ∼4,000 spines from 6 rats, 24 slices and 50 neurons.

and large-head spines ($p = 0.0016$ for middle and $p = 0.0004$ for large-head, for DHT vs. PP2+ DHT, Tukey–Kramer), while significant changes in the small-head spines did not occur (**Figures 1B–D**).

Note that, only PP2 did not significantly affect the total spine density, implying that the observed inhibitory effects are not due to simple non-specific effects by blockers (**Figure 1B**).

T Effects and Src Kinase Blocking

T and PP2 treatments induced significant changes in the total spine density ($F = 25.68$, $p < 0.0001$, two-way ANOVA). The total spine density was significantly increased to 1.31 spines/μm through 2 h incubation with 10 nM T ($p < 0.0001$ for control vs. T, Tukey–Kramer) (see **Figure 2**). This increase in the total spine density was suppressed by blocking Src kinase through co-incubation of 10 nM T and 10 μM PP2 ($p < 0.0001$ for T vs. PP2+T, Tukey–Kramer) (**Figure 2B**).

From spine head diameter analysis, upon treatments with T, the density of small- and middle-head spines significantly increased ($p = 0.0004$ for small-head and $p = 0.0005$ for middle-head, Tukey–Kramer), while the density of large-head spines was not significantly altered. Inhibition of Src kinase abolished the effect of T on the spine density, by decreasing the density of small-and middle-head spines ($p = 0.0248$ for small-head and $p < 0.0001$ for middle-head, Tukey–Kramer), while there were no significant changes in large-head spines (**Figures 2B–D**).

E2 Effects and Src Kinase Blocking

E2 and PP2 treatments induced significant changes in the total spine density ($F = 19.71$, $p < 0.0001$, two-way ANOVA). After 2 h treatments with 1 nM E2, the total spine density was significantly increased to 1.29 spines/μm ($p < 0.0001$ for control vs. E2, Tukey–Kramer) (**Figure 3**). This increase in the total spine density was suppressed by blocking of Src kinase through

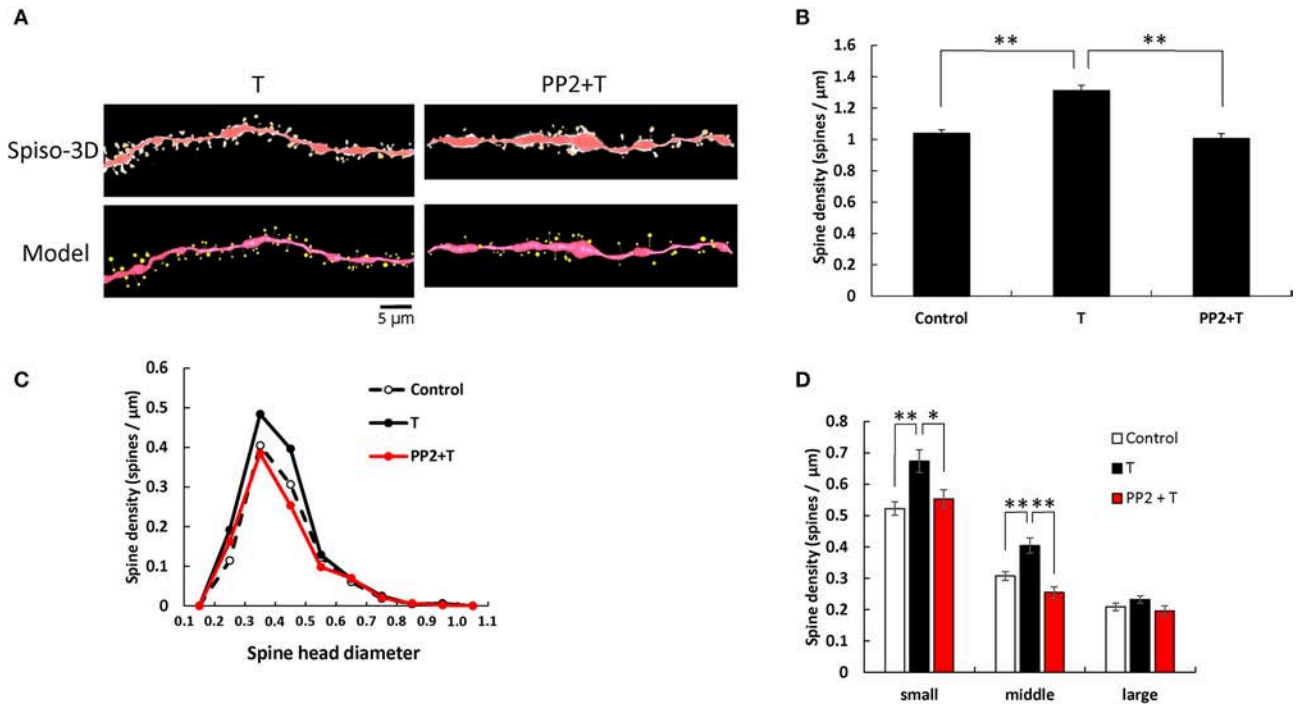

FIGURE 2 | Effects of Src kinase blocker on T-induced spine increase and change in morphology in hippocampal slices. **(A)** Spines were analyzed along the secondary dendrites of pyramidal neurons in the stratum radiatum of CA1 neurons as in **Figure 1**. Dendrite after T-treatment for 2 h (T) and dendrite after T plus PP2 treatment for 2 h (PP2+T). (Spiso) shows the image of dendrite and spines analyzed with Spiso-3D software. Maximal intensity projections onto XY plane is shown. Traced dendrite is shown in red color and spines are indicated in yellow color. (Model) shows 3 dimensional model illustration of (Spiso) image. Bar, 5 μm. **(B)** Effect of treatments by T or PP2 on the total spine density in CA1 neurons. Vertical axis is the average number of spines per 1 μm of dendrite. A 2 h treatment in ACSF without drugs (Control), with 10 nM T (T), with 10 nM T and 10 μM PP2 (PP2 + T). **(C)** Histogram of spine head diameters after a 2 h treatment in ACSF without drugs (Control, black dashed line), with 10 nM T (black line), with 10 nM T and 10 μM PP2 (PP2 + T, red line). **(D)** Density of three subtypes of spines. Abbreviations are same as in **(B)**. From left to right, small-head spines (small), middle-head spines (middle), and large-head spines (large) type. ACSF without drugs (Control, open column), T (black column), PP2 + T (red column). Results are represented as mean ± SEM. Statistical significance yielded $^{*}P < 0.05$, $^{**}P < 0.01$ vs. T sample. For T and PP2 + T treatments, we investigated 50 dendrites with 2300–2700 spines from 3 rats, 12 slices, 30 neurons. For control, we used 80 dendrites with ∼4,000 spines from 6 rats, 24 slices, and 50 neurons.

co-incubation of 1 nM E2 and 10 μM PP2 ($p < 0.0001$ for E2 vs. PP2+ E2, Tukey–Kramer) (**Figure 3A**).

From spine head diameter analysis, treatments with E2 significantly increased the density of small-head spines ($p = 0.0079$, Tukey–Kramer), while the density of middle- and large-head spines was not significantly altered. Blocking Src kinase by PP2 suppressed the effects of E2, by decreasing the density of small-head spines ($p = 0.0075$, Tukey–Kramer), while significant change was not observed in middle- and large-head spines (**Figures 3B–D**).

Improvement of Image Analysis With Super Resolution Confocal Microscopy

Airy scan mode of LSM880 super resolution confocal microscopy is equipped with 32 channel area detectors with honeycomb arrangement in the back of confocal pinhole. With these detectors we can directly measure the main part of the point spread function with which we perform deconvolution of confocal images. Therefore, the resultant final spine images were much clearer particularly for neck images with super resolution confocal microscopic imaging (**Figure 4A**) than those with conventional confocal microscopic imaging (**Figure 4B**) which uses blind deconvolution method with AutoDeblur software (AutoQuant, USA). Particularly necks of spines were clearly observed in Airy Scan mode, while sometimes spine heads spatially isolated from dendrites, without visible necks which connect spine heads to the dendritic shafts, were observed with conventional confocal microscopic imaging (**Figure 4B**). Identification of necks is critical in classification of spine types between stubby type (without neck) and spines with necks (thin and mushroom types).

We quantitatively compared control dendritic spines (without steroid supplementation) obtained from super resolution confocal microscopic analysis (LSM880) with those obtained from conventional confocal microscopic analysis (with Zeiss PASCAL confocal microscopy) (Hasegawa et al., 2015). We observed no significant difference in spine densities within experimental error concerning not only the total spine density but also the head diameter distribution, between these two confocal microscopic analyses. (**Figures 4C–E**). However, the ratio of large-head spine population and middle-head

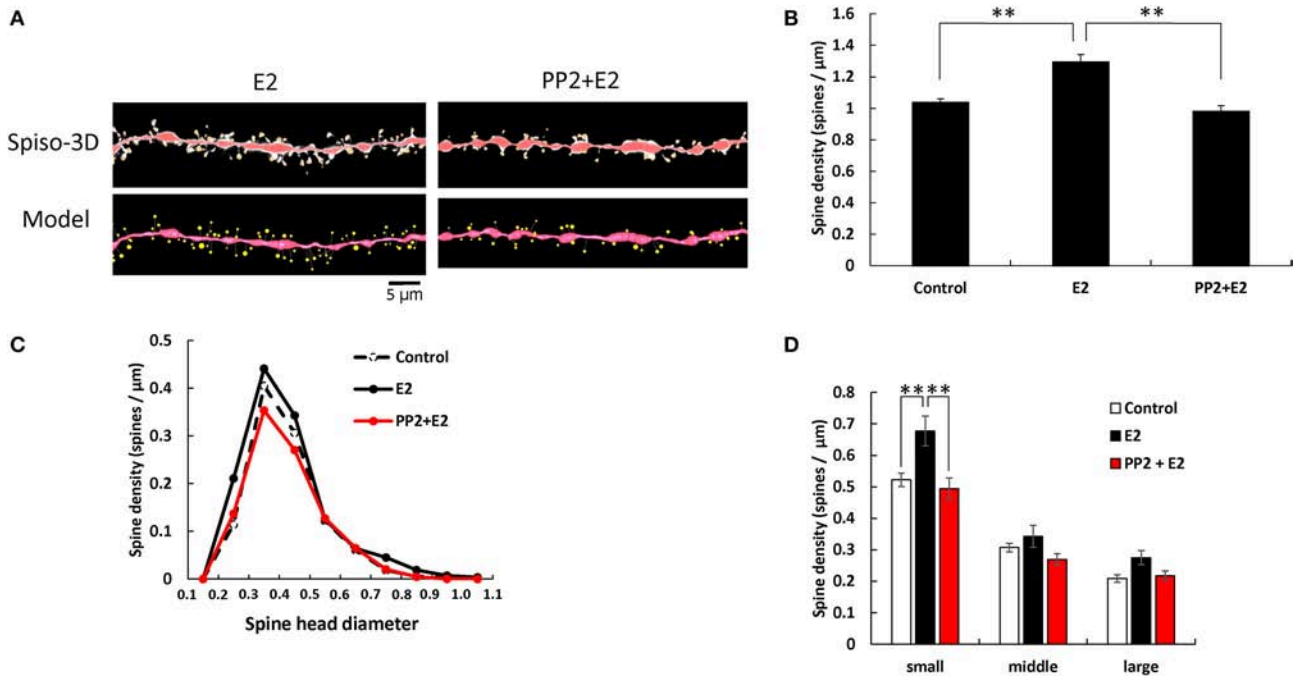

FIGURE 3 | Effects of Src kinase blocker on E2-induced spine increase and change in morphology. **(A)** Spines were analyzed along the secondary dendrites of pyramidal neurons in the stratum radiatum of CA1 neurons as in **Figure 1**. Dendrite after E2-treatment for 2 h (E2) and dendrite after E2 plus PP2 treatment for 2 h (PP2+E2). (Spiso) shows the image of dendrite and spines analyzed with Spiso-3D software. Maximal intensity projections onto XY plane is shown. Traced dendrite is shown in red color and spines are indicated in yellow color. (Model) shows 3 dimensional model illustration of (Spiso) image. Bar, 5 μm. **(B)** Effect of treatments by E2 and PP2 on the total spine density. Vertical axis is the average number of spines per 1 μm of dendrite. A 2 h treatment in ACSF without drugs (Control), with 1 nM E2 (E2), and with 1 nM E2 and 10 μM PP2 (PP2 + E2). **(C)** Histogram of spine head diameters. A 2 h treatment in ACSF without drugs (Control, dashed line), with E2 (black line), with E2 + PP2 (red line). **(D)** Density of three subtypes of spines. From left to right, small-head spines (small), middle-head spines (middle), and large-head spines (large) type. In each group, control (open column), E2 (black column), and PP2+E2 (red column). Results are represented as mean ± SEM. Statistical significance was defined as $^{*}p < 0.05$, $^{**}p < 0.01$ vs. E2 sample. For E2 and PP2+E2 treatments, we investigated 50 dendrites with 2300–2700 spines from 3 rats, 12 slices, and 30 neurons. For control, we used 80 dendrites with ∼4,000 spines from 6 rats, 24 slices, and 50 neurons.

spine population was little bit higher in super resolution than conventional confocal analysis.

DISCUSSION

We consider and discuss about Src kinase dependent signaling mechanisms in non-genomic modulation of sex steroid-induced dendritic spinogenesis.

Src Kinase-Mediated Signaling With Sex Steroids Treatments in Peripheral Tissues

Involvement of Src kinase (nonreceptor tyrosine kinase) in non-genomic rapid signaling upon androgen and estrogen stimulation has been extensively investigated in non-neuronal cells, including prostate cancer cells (e.g., LNCaP cells) (Migliaccio et al., 2000), breast cancer cells (e.g., MCF-7 cells) (Migliaccio et al., 1996), epithelial cells (Castoria et al., 2004), and Sertoli cells (Cheng et al., 2007).

Both T and E2 induced complex formation of AR, ERβ and Src kinase in LNCaP (Migliaccio et al., 2000). E2-induced complex formation of ERα with Src kinase was observed in MCF-7 cells (Migliaccio et al., 2000). Src kinase phosphorylated Erk MAPK in MCF-7 cells (Migliaccio et al., 1996). Upon T stimulation in Sertoli cells, association of AR with Src kinase occurred, leading to activation of Erk MAPK (Cheng et al., 2007).

Taken together, upon stimulation of T or E2, AR or ER may form complex with Src kinase, leading to activation of MAPK in prostate cancer cells, breast cancer cells, or other gonadal tissues (Foradori et al., 2008). Androgen-induced Ca influx may be a trigger of these events in these cells (Rusanescu et al., 1995; Foradori et al., 2008). Src kinase activation is induced by dephosphorylation of tyrosine residue, and this might occur within complexes of AR, ER, and Src kinase, via AR binding to SH3 domain or via ER binding to SH2 domain (Migliaccio et al., 2000). Note that Src kinase is anchored to the membrane via myristoylation (**Figure 5**; Kim et al., 2017).

Src Kinase-Mediated Signaling With Sex Steroid Treatments in Neurons

We observed association of Src kinase, Erk MAPK, and ERα with post synaptic density (PSD), using Western blotting of PSD fractions prepared from male rat hippocampi, implying the spine membrane binding of Src kinase, MAPK, and ER (Mukai et al., 2007). Therefore, from the current results in combination

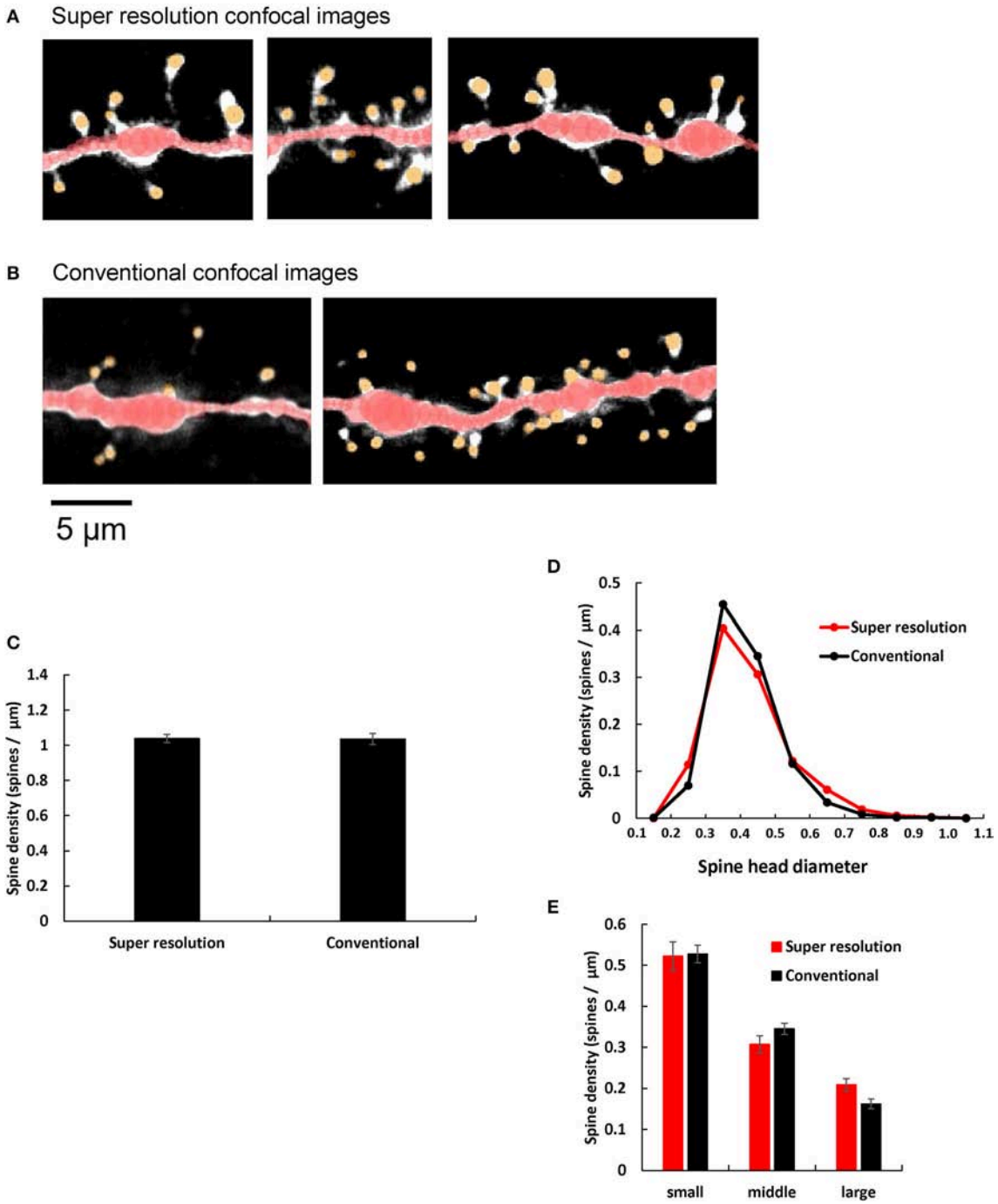

FIGURE 4 | Comparison of spine images obtained by super resolution confocal microscopy and conventional confocal microscopy. **(A,B)** Typical dendrites with spines of CA1 neurons are shown after analysis with Spiso-3D software. Maximal intensity projections onto XY plane are shown. Traced dendrites are shown in red color and spines are indicated in yellow color. Images are obtained with **(A)** Super resolution confocal microscopy and **(B)** Conventional confocal microscopy. Bar, 5 μm. **(C)** Comparison of the total spine density of control dendrites (without steroid supplementation) obtained with super resolution confocal microscopy (Super resolution) and Conventional confocal microscopy (Conventional). Vertical axis is the average number of spines per 1 μm of dendrite. **(D)** Histogram of spine head diameters of control. Super resolution (red line), and Conventional (black line). **(E)** Density of three subtypes of spines of control dendrites. From left to right, small-head spines (small), middle-head spines (middle), and large-head spines (large). In each group, Super resolution (red column), and Conventional (black column). Abbreviations are same as in **(C)**. Results are represented as mean ± SEM. No statistical significance was observed. For Super resolution, we investigated 80 dendrites with ∼4,000 spines, 6 rats, 24 slices, and 50 neurons. For Conventional, we used 40 dendrites with ∼2,000 spines from 4 rats, 10 slices, and 21 neurons.

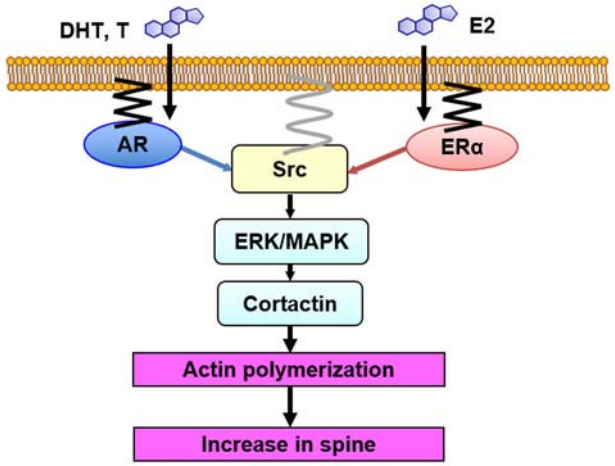

FIGURE 5 | Model illustration of Src kinase-mediated signaling in non-genomic effects of sex steroids on spinogenesis. DHT, T and E2 bind to synaptic receptors (AR and ER) within spines. Then, Src kinase may form complex with AR and ER, resulting in activation of Src kinase. Erk MAPK is then activated, leading to phosphorylation of cortactin, resulting in actin polymerization and new spine formation. AR and ER are anchored to the membrane via palmitoylation. Src kinase is localized to the membrane via myristoylation.

with the results from endocrine tissues (see section Src Kinase-Mediated Signaling With Sex Steroids Treatments in Peripheral Tissues), we can speculate that Src kinase-mediated signaling, triggered by sex steroids in hippocampal spines, may occur as follows: upon T, DHT, and E2 binding to their synaptic receptors (AR and ERα), these receptors activate Src kinase via conformational change of Src, leading to phosphorylation of Erk MAPK (**Figure 5**).

Until recently, however, inadequate amounts of investigations had been accumulated for Src kinase signaling upon androgen and estrogen stimulation in central nervous systems. In one of a few reports, Src tyrosine kinase involvement was suggested from PP2-induced suppression of E2-enhanced LTP in acute hippocampal slices (Bi et al., 2000). Chronic DHT-treatment for 24 h elevated Ca concentrations in the endoplasmic reticulum of primary hippocampal neurons, but this might not directly contribute to non-genomic actions (Foradori et al., 2007).

On the other hand, downstream signaling of Src kinase, from MAPK to spine increase, has been extensively studied in neurons (see section MAPK-Mediated Signaling, Downstream of Src Kinase in Spinogenesis).

MAPK-Mediated Signaling, Downstream of Src Kinase in Spinogenesis

Erk MAPK (serine/threonine kinase) was involved in the rapid non-genomic effects on CA1 hippocampal spinogenesis through DHT, T, and E2 treatments (Hasegawa et al., 2015; Hatanaka et al., 2015). These results were obtained by using MAPK inhibitors, U0126 and PD98059. E2-induced rapid CA1 spinogenesis via Erk MAPK was found in the male and female hippocampus (Mukai et al., 2007; Phan et al., 2011; Luine and Frankfurt, 2012). *In vivo* E2 infusion induced CA1 spinogenesis as well as phosphorylation of Erk MAPK in female ovariectomized (OVX) mouse hippocampus (Frick et al., 2015; Tuscher et al., 2016). T and DHT individually phosphorylated Erk MAPK within 1 h, leading to neuroprotection in primary cultured hippocampal neurons (Nguyen et al., 2005).

Since both steroid receptors (AR and ER), Src kinase and MAPK are present in dendritic spines, an efficient coupling between these proteins could occur in spines, resulting in activation of Src kinase, followed by activation of Erk MAPK (**Figure 5**) (Milner et al., 2005; Tabori et al., 2005; Mukai et al., 2007; Hojo et al., 2008; Hatanaka et al., 2015).

In spinogenesis, the target of Erk MAPK may be cortactin. Erk MAPK phosphorylates cortactin which is associated with actin (MacQueen et al., 2003). Cortactin interacts with both F-actin and actin-related protein (Arp) 2/3 complex as well as scaffold protein Shank (Weed et al., 1998; Campbell et al., 1999), leading to promotion of actin fiber remodeling within spines. Therefore, it is possible that DHT, T, and E2 might modulate spines via cortactin-actin pathway. Cortactin has multiple phosphorylation sites, such as Ser^{113}, Ser^{405}, and Ser^{418}, which are putative phosphorylation sites of MAPK (Campbell et al., 1999). Phosphorylation of cortactin triggered by DHT, T, and E2 may promote assembly of actin cytoskeleton, which either leads to increasing spines or modulating the spine morphology (Hering and Sheng, 2003). The involvement of cortactin in androgen-induced modulation of spines is suggested from the results that AR inhibitor induced actin depolymerization via tyrosine phosphorylation of cortactin (Anahara et al., 2006).

Localization of AR and ER in Spines

The involvement of classic AR in androgen-induced rapid action was indicated from suppressing effects of hydroxyflutamide, a specific inhibitor of AR, on androgen-induced spinogenesis in acute slices of male rat hippocampus (Hatanaka et al., 2015). The AR immunostaining was localized in CA1 neurons with optical microscopic analysis, and synaptic localization of AR was indicated with immuno-electron microscopic analysis (Tabori et al., 2005). Western blot analysis showed the AR expression in PSD fractions as well as in nuclear and cytoplasmic fractions, which implies that AR localized in the PSD can participate in the T- and DHT-induced spine increase (Hatanaka et al., 2015). These results suggest that AR localized in spines could mediate androgen-induced rapid spine increase through activation of Src kinase and MAPK.

The involvement of classic ER in rapid E2 action was indicated from suppressing effects of ICI182,780 (ICI), a specific antagonist of both ER, on E2-induced spinogenesis in male hippocampal slices (Mukai et al., 2007; Hasegawa et al., 2015). Involvement of ERα or ERβ in rapid spinogenesis was also examined by using estrogen receptor agonists. ERα agonist, (propyl-pyrazole-trinyl) tris-phenol (PPT), unlike ERβ agonist such as (4-hydroxyphenyl)-propionitrile (DPN), acutely increased the density of spines in hippocampal CA1 neurons in male rat slices (Hasegawa et al., 2015) and OVX female mice (Phan et al., 2011). These results support the exclusive involvement of ERα in rapid signaling. ERαKO mice and ERβKO mice studies further

confirmed the involvement of ERα, but not ERβ, in the rapid E2 signaling (Murakami et al., 2015).

Expression of ERα in neurons of CA1 in rat and mouse hippocampus was demonstrated by immunostaining with purified antibody RC-19 (Mukai et al., 2007). ERα was located in spines in addition to nuclei/cytoplasm, as revealed by the immunogold electron-microscopic analyses (Mukai et al., 2007). Spine membrane localization of AR and ER may be accomplished via palmitoylation of receptors (**Figure 5**), as judged from recent studies, including the finding that some populations of ERα and ERβ were plasma membrane-bound in cultured breast cancer cells MCF-7 (Pedram et al., 2006), and they were anchored via palmitoylation (Pedram et al., 2007).

Difference Between DHT, T, and E2 in Rapid Effects on Spine Density and Morphology in CA1

Although treatments with DHT, T, and E2 increased the total spine density to almost the same level, three subclass analysis showed clear differences between DHT, T, and E2 in modulation of the spine morphology of hippocampal CA1 neurons (**Figures 1**–**3**). DHT treatments considerably increased the density of large-head spines. On the other hand, all the small- and middle-head spines were increased with T treatments, whereas E2 increased only small-head spines. Since large-head spines have significantly more AMPA receptors than small-head spines (Shinohara et al., 2008), DHT might increase synapses with higher memory storage capacity than T and E2.

The T-effect is not dependent on the conversion from T to E2, and neither from T to DHT. This is concluded from the fact that T-effect was not blocked by inhibition of P450arom (E2 synthetase) and 5α-reductase (DHT synthetase) (Hatanaka et al., 2015). Moreover, the effect of T was blocked by AR antagonist. T-effect must be therefore directly mediated by AR, and not mediated by E2.

Difference in Sex Steroid Levels Between Acute Hippocampal Slices and *in Vivo* Hippocampus

Sex steroid levels in the hippocampus play a key role for rapid action through synaptic AR and ER as modulators of synaptic plasticity. The average concentrations of male rat hippocampal DHT, T, and E2 *in vivo* were determined to be ∼7, 17, and 8 nM, respectively, in freshly isolated hippocampi, with mass-spectrometric analysis (Hojo et al., 2008, 2009) (Okamoto et al., 2012). These hippocampal sex steroid levels are significantly higher than plasma sex steroid levels, due to hippocampal synthesis of sex steroids, in addition to penetration of T (∼15 nM in plasma) to the hippocampus via blood circulation (Hojo et al., 2009). Interestingly, E2 level in the male hippocampus (∼8 nM) is higher than that in female (0.6–4.3 nM), due to conversion of high level T into E2 within the hippocampus by P450arom (Kato et al., 2013). Importantly in "acute" slices (used for current analysis of synaptic plasticity), the levels of DHT, T, and E2 decreased to below 0.5 nM due to the slice recovery incubation for 2 h with ACSF after fresh slice preparations (Hojo et al., 2009, 2011; Ooishi et al., 2012a,b). In the current study, the exogenous application of 10 nM DHT, 10 nM T, and 1 nM E2 individually was used to rapidly elevate the hippocampal sex steroid levels from the steroid-depleted levels in "acute" slice (<0.5 nM), to nearly the endogenous levels of male rat (Ooishi et al., 2012a), resulting in rapid action of sex steroids.

Toward *in Vivo* Analysis of Sex Steroid Effects on Spine Modulation

The currently obtained knowledge encourages analysis of *in vivo* mechanisms of rapid sex steroid actions on spines. Recent *in vivo* investigations implied that E2 treatments with s.c. injection rapidly (within 30–40 min) increased the spine density in CA1 hippocampal neurons of OVX female mice (Phan et al., 2011, 2012; Jacome et al., 2016; Tuscher et al., 2016). These *in vivo* investigations can be interpreted that E2 supplementation recovered the spine density, because OVX surgery once decreased spine density as well as declined hippocampal E2 level due to depletion of circulating E2 (Kato et al., 2013). Such approaches in combination with kinase inhibitors may be useful to analyze *in vivo* molecular mechanisms of rapid synaptic modulation by E2 in female rodents. Concerning male rodents, approaches with castration followed by rapid androgen supplementation could be promising to analyze mechanisms of rapid modulation effects of androgen on spines, because castration decreases hippocampal T and DHT as well as the spine density.

AUTHOR CONTRIBUTIONS

SK: conceived and designed the study; MS, JK, and AK: conducted the experiments and analysis of the data; SK: wrote the manuscript. All authors provided feedback on the manuscript.

ACKNOWLEDGMENTS

We thank Mr. J. Murakami, Mr. W. Tokuyama for spine data analysis. Profs. K. Inoue, J. Kurihara, and A. Kittaka (Teikyo University) are acknowledged for helpful discussions.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 02 May 2018
doi: 10.3389/fendo.2018.00213

Temporal Expression Patterns of Genes Related to Sex Steroid Action in Sexually Dimorphic Nuclei During Puberty

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Sex steroids play a major role in sexually dimorphic brain development during not only the perinatal period but also the pubertal period. We previously showed that, in male mice, the estrogen receptor-α (*Esr1*) and aromatase (*Cyp19a1*) genes are essential to the sexually dimorphic formation of the anteroventral periventricular nucleus (AVPV) and the principal nucleus of the bed nucleus of the stria terminalis (BNSTp), but the estrogen receptor-β (*Esr2*) gene is not necessary. We also showed that the androgen receptor (*Ar*) gene is essential to the sexually dimorphic formation of the BNSTp. These genes are expressed in the AVPV and BNSTp of perinatal mice. However, it remains unknown whether these genes are expressed in the AVPV and BNSTp during puberty, and whether the expression, if any, differs by sex, age, and brain region. Here, we dissected the AVPV and BNSTp from Nissl-stained brain sections of male and female mice on postnatal day (PD) 20 (prepuberty), PD30 (puberty onset in females), PD40 (puberty onset in males), and PD60 (young adult) using a laser microdissection system. We then examined the mRNA levels of *Esr1*, *Esr2*, *Cyp19a1*, and *Ar* in these brain regions. In the AVPV, *Esr1* mRNA levels were greater in females than males during PD20–60. *Esr2* and *Ar* mRNA expressions did not differ between sexes. *Ar* mRNA levels were higher at PD30 than PD20. *Cyp19a1* mRNA was not detected in the AVPV at PD20–60. In the BNSTp, *Esr1* and *Esr2* mRNA levels were higher in females than in males during PD20–60, although the mRNA levels of *Cyp19a1* and *Ar* did not differ between sexes. Additionally, we revealed that orchiectomy at PD20 induced a failure of normal formation of the male BNSTp and testosterone replacement in the prepubertal period rescued the effect of orchiectomy at PD20. Taken together, it is suggested that pubertal testosterone transported to the AVPV is not converted to estradiol there and does not act *via* ESR1 and ESR2. By contrast, the formation of the male BNSTp may be affected by testicular testosterone during puberty *via* AR and/or *via* ESR1 after conversion to estradiol by CYP19A1.

Keywords: aromatase, puberty, sex steroids, sexual differentiation, sexually dimorphic nucleus, sex steroid receptor, anteroventral periventricular nucleus, principal nucleus of the bed nucleus of the stria terminalis

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *28 November 2017*
Accepted: *16 April 2018*
Published: *02 May 2018*

Citation:
Kanaya M, Morishita M and Tsukahara S (2018) Temporal Expression Patterns of Genes Related to Sex Steroid Action in Sexually Dimorphic Nuclei During Puberty. Front. Endocrinol. 9:213. doi: 10.3389/fendo.2018.00213

INTRODUCTION

Sexually dimorphic nuclei underlie sex- and gender-specific functions in the brain. The anteroventral periventricular nucleus (AVPV) of the hypothalamus in rodents is a sexually dimorphic nucleus that is larger in size and contains more neurons in females than in males (1–4). Specifically, the female AVPV contains a larger number of neurons expressing tyrosine

hydroxylase (TH) (3) and kisspeptin (5, 6). TH neurons in the AVPV promote parental behavior in female mice (7). Although TH neurons in the AVPV do not promote parental behavior in male mice, they suppress inter-male aggression (7). Kisspeptin neurons in the female AVPV appear to participate in the estradiol-induced surge of luteinizing hormone (8, 9). The principal nucleus of the bed nucleus of the stria terminalis (BNSTp) is a sexually dimorphic nucleus in the forebrain that is larger and contains a greater number of neurons in male rodents (10–13). Males have a greater number of vasopressin neurons in the BNSTp, which are involved in anxiety, aggressive behavior, and stress responses (14), and which project to the lateral septum (15–17). These differences in vasopressin neurons may underlie behavioral sex differences, particularly as male rodents exhibit more anxiety-related behavior than females (18, 19).

In male rodents, sexually dimorphic nuclei are formed by the influence of testicular testosterone during the perinatal period (20, 21). Like the AVPV in male rats, the AVPV in female rats treated with testosterone postnatally is smaller and contains fewer TH neurons and kisspeptin neurons in adulthood (6, 22). We previously showed that the volume and number of neurons in the AVPV are increased in male mice with deletion of the estrogen receptor-α (*Esr1*) and aromatase (*Cyp19a1*) genes, but are not affected by deletion of the estrogen receptor-β (*Esr2*) or androgen receptor (*Ar*) gene (23). These previous findings suggest that *Esr1* and *Cyp19a1* are essential to the formation of the male AVPV, but *Esr2* and *Ar* are not necessary. In support of this notion, *Esr1* and *Cyp19a1* mRNAs are expressed in the AVPV in perinatal mice (23). Postnatal treatment with estradiol, as well as testosterone, reduces the number of neurons in the AVPV in adult female rats (24). Thus, testicular testosterone in the perinatal period might be converted by CYP19A1 in the AVPV to estradiol, which signals through ESR1 to defeminize the morphology of the AVPV.

Perinatal testicular testosterone also affects the BNSTp, although the effects are opposite to those on the AVPV. The volume and number of neurons in the BNSTp in adult male rats are decreased by neonatal orchiectomy (12). Postnatal treatment with testosterone or estradiol increases the number of neurons in the BNSTp of adult female mice (25). The volume and number of neurons in the BNSTp in male mice are reduced to levels similar to those in females by deletion of *Esr1* or *Cyp19a1*, but not *Esr2* (26). *Esr1* and *Cyp19a1* are expressed in the BNSTp in perinatal mice (23). These findings suggest that the formation of the male BNSTp involves the action of estradiol (produced by the aromatization of testosterone) *via* ESR1 during the perinatal period. Additionally, the AR plays an important role in the formation of the male BNSTp, because the volume and number of neurons in the BNSTp are smaller in male *Ar* knockout (KO) mice, compared with wild-type males, similar to females (23). *Ar* is not expressed in the murine BNSTp prenatally, but begins expression early in the postnatal period (23, 27). The formation of the male BNSTp might require not only estradiol, formed from the aromatization of testosterone, acting *via* ESR1 in the perinatal period, but also testosterone acting *via* AR postnatally.

Accumulating evidence shows that testicular testosterone during the perinatal period is essential for the sexually dimorphic formation of brain structures. However, the formation of sexually dimorphic nuclei is affected by pubertal gonadal hormones as well (28, 29). In male mice, the BNSTp contains a comparatively greater number of calbindin neurons (30). This sex difference in calbindin neurons emerges before puberty, and increases further as calbindin neurons continue to increase in males and decrease in females during the pubertal and adolescent periods (31, 32). Testicular hormones during puberty also contribute to the formation of the male BNSTp, because the increase in calbindin neurons in the male BNSTp is perturbed by prepubertal orchiectomy, although the decrease in the female BNSTp is not altered by prepubertal ovariectomy (32). The sexually dimorphic formation of the AVPV might be influenced by pubertal ovarian hormones. Prepubertal ovariectomy decreases the volume and number of neurons in the AVPV in female rats (33). Thus, gonadal hormones during puberty play an important role in the sexually dimorphic formation of the AVPV and BNSTp; however, it remains unknown whether genes related to gonadal hormone actions are expressed in the sexually dimorphic nuclei during puberty.

In this study, we aimed to determine whether the AVPV and BNSTp of pubertal mice express genes involved in gonadal hormone actions, and whether the expression, if any, varies by sex, age, and brain-region. First, using tissue samples isolated precisely and accurately from the AVPV and BNSTp of peripubertal mice, we evaluated the mRNA expression patterns of *Esr1*, *Cyp19a1*, and *Ar*, which play an essential role in the sexually dimorphic formation of the AVPV and/or BNSTp (23, 26), as well as that of *Esr2*. As a result, the mRNA of *Cyp19a1* was expressed in the BNSTp, but not in the AVPV of peripubertal mice, suggesting that the BNSTp of male mice is affected by estradiol, which is converted from testicular testosterone by CYP19A1 locally there during puberty, but the AVPV is not. Next, to determine whether testicular testosterone during puberty contributes to the formation of the male BNSTp, we investigated the effects of prepubertal orchiectomy and testosterone replacement on the morphology of the BNSTp in adulthood with reference to calbindin expression.

MATERIALS AND METHODS

Animals

Adult male and female C57BL/6J mice for breeding were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). Offspring derived from mating in our facility were housed with dams in the same cages until weaning on postnatal day (PD) 21 (PD0 = day of birth). All animals were bred and housed in a room with a controlled temperature (22°C) and a 12-h light/12-h dark cycle (lights on: 08:00–20:00). Standard diet and tap water were available *ad libitum*. All animal experimental procedures were approved by the Animal Care and Experimentation Committee of Saitama University and were conducted in accordance with the Guidelines for the Care and Use of Experimental Animals of Saitama University.

Gene Expression Analysis of the AVPV and BNSTp in Peripubertal Mice

Brain Tissue Collection

Male and female mice of different ages {PD20, prepuberty; PD30, puberty onset in females; PD40, puberty onset in males; and PD60, young adult [see review by Piekarski et al. (34)]} were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (64.8 mg/kg body weight) and decapitated. Fresh brains were quickly frozen in hexane chilled to −80°C and stored at −80°C until further processing. There are several studies showing that the levels of *Esr1* mRNA and protein in the preoptic area (35, 36) and the number of ESR1- and AR-immunoreactive cells in the BNSTp (37, 38) are affected by circulating estradiol. Therefore, we compared the mRNA levels of *Esr1*, *Esr2*, *Cyp19a1*, and *Ar* in the AVPV and BNSTp of diestrous and proestrous female mice. When collecting brains from females on PD40 or PD60, vaginal smears were performed starting on the day of vaginal opening, and animals in the diestrous or proestrous phase were sacrificed between 12:00 hours and 14:00 hours on PD40 or PD60. As a result, unexpectedly, diestrous and proestrous female mice did not differ with regard to the mRNA levels of *Esr1*, *Esr2*, and *Ar* in the AVPV and BNSTp (data not shown). *Cyp19a1* mRNA was not detected in the AVPV on PD40 and PD60. *Cyp19a1* mRNA in the BNSTp did not differ between diestrous and proestrous females on PD40 and PD60 (data not shown). In this study, diestrous and proestrous female mice of the same age were therefore combined into a single group. The numbers of animals used for this study were as follows: PD20 male, $n = 6$; PD20 female, $n = 4$; PD30 male, $n = 4$; PD30 female, $n = 5$; PD40 male, $n = 4$; PD40 female, $n = 6$; PD60 male, $n = 4$; PD60 female, $n = 8$.

Isolation of the AVPV and BNSTp

The AVPV and BNSTp were dissected using a laser microdissection (LMD) system (Leica LMD 7000; Leica Microsystems, Wetzlar, Germany) in accordance with the procedure reported previously, with slight modification (23). Briefly, frozen brains were coronally cut at a thickness of 30 μm on a cryostat. Brain sections containing the AVPV and BNSTp were obtained in the coronal plane 0.38–0.02 mm rostral to the bregma and 0.10–0.34 mm caudal to the bregma, respectively, according to the mouse brain atlas (39). Brain sections containing the AVPV or BNSTp were mounted on PEN membrane slides (Leica Microsystems), fixed with ice-cold 5% acetic acid in ethanol for 3 min, stained with ice-cold 0.2% cresyl fast violet for 1 min, rinsed in 100% ethanol for 1 min, and dried with cool air. The AVPV and BNSTp were then dissected out from cresyl fast violet-stained brain sections by the LMD system and collected in a tube containing 70 μL RNA extraction buffer and 5 μL carrier RNA working solution (4 ng/μL) from the RNeasy Micro Kit (Qiagen, Valencia, CA, USA). To confirm whether the AVPV and BNSTp were precisely collected, we measured the volume of the nuclei. If the AVPV and BNSTp were correctly isolated, a sex difference in the volume of the nuclei could be found. The areas of the dissected tissue fragments were recorded by the LMD system. We calculated the volume of the acquired tissues by multiplying total of the areas of the dissected tissue fragments by the thickness of brain sections (30 μm) for each sample.

Quantification of mRNA Levels

Total RNA was extracted and purified using the RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized using the TaKaRa Prime Script RT reagent kit (TaKaRa Bio, Otsu, Japan). For each sample, total RNA (approximately 160 ng) was reverse-transcribed to first-strand cDNA in a final volume of 15 μL Prime Script buffer containing Prime Script RT Enzyme Mix I (0.75 μL) and random hexamers (75 pmol). Standard samples for *Esr1*, *Cyp19a1*, *Ar*, and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), a housekeeping gene, were prepared by mixing an equal amount of each cDNA sample and serially diluting in EASY Dilution solution (TaKaRa Bio). To prepare standard samples for *Esr2*, cDNA obtained from the hypothalamus of adult mice was serially diluted.

Quantitative polymerase chain reaction (qPCR) was performed using a Light Cycler ST300 (Roche Diagnostics, Mannheim, Germany). A 2-μL aliquot of standard or unknown sample was amplified in a 20-μL reaction mixture containing 200 nM of each gene-specific primer [*Gapdh* forward primer: CACTGCCACC CAGAAGA, *Gapdh* reverse primer: TCCACGACGGACACATT; other genes were designed according to our previous study (23)] and 10 μL of 2× SYBR Premix Ex Taq (TaKaRa Bio). The thermocycling parameters for qPCR were 30 s at 95°C, followed by 40 cycles of 95°C for 5 s and 60°C for 20 s. After qPCR, melting curve analysis was performed to assess the specificity of the PCR products. This analysis showed that the melting curves for the PCR products all had a single peak (data not shown).

qPCR analysis for *Gapdh* mRNA in the AVPV and BNSTp revealed that *Gapdh* mRNA levels in these nuclei did not differ significantly by sex or age (data not shown). Therefore, the amount of *Esr1*, *Esr2*, *Cyp19a1*, and *Ar* mRNAs was normalized by dividing by the amount of *Gapdh* mRNA for each sample.

Morphological Analysis of the BNSTp in Male Mice

Prepubertal Orchiectomy and Testosterone Replacement

At PD20, male mice were orchiectomized and subcutaneously implanted with a Silastic tube [1.02 mm inner diameter, 2.16 mm outer diameter, 10.9 mm in total length (effective length: 6.9 mm); Dow Corning Corporation, Midland, MI, USA] containing testosterone (Sigma-Aldrich, St. Louis, MO, USA; $n = 5$) or cholesterol (Wako Pure Chemical Industries, Osaka, Japan; $n = 5$). The testosterone implantation was designed to produce the levels of serum testosterone in young male mice (Tsukahara, unpublished data). Surgery was performed under isoflurane inhalational anesthesia (concentration, 1.5% in air; flow rate, 1 L/min). Additionally, we prepared gonadally intact males ($n = 5$).

Tissue Preparation

At 10 weeks of ages, all animals were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (64.8 mg/kg body weight) and transcardially perfused with 0.05 M ice-cold

phosphate-buffered saline (PBS; pH, 7.4) followed by ice-cold 4% paraformaldehyde in 0.05 M phosphate buffer (pH, 7.4). Brains were postfixed with the same fixative at 4°C overnight and then immersed in 30% sucrose in 0.05 M phosphate buffer at 4°C for 2 days. Fixed brains were quickly frozen, coronally sectioned at a thickness of 30 μm using a cryostat, and collected at 60-μm intervals.

Immunohistochemistry for Calbindin

Brain sections were rinsed in 0.05 M PBS containing 1% Triton X-100 (PBST) and placed in 0.6% H_2O_2 in PBST for 30 min at room temperature. The sections were then placed in 5% normal goat serum in PBST for 1 h at room temperature and were reacted with a mouse monoclonal anti-calbindin antibody (1:15,000; C9848; Sigma-Aldrich; RRID: AB_476894) in 5% normal goat serum-containing PBST at 4°C over two nights. After rinsing in PBST, the sections were reacted with peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulin (K4001, Dako, Carpinteria, CA, USA) for 1 h at room temperature. Calbindin immunoreactivity was visualized with a chromogenic substrate, 3,3′-diaminobenzidine (liquid DAB plus substrate chromogen system, Dako). Immunostained sections were mounted on gelatin-coated glass slides, air dried, dehydrated in ascending ethanol, cleared with xylene, and cover-slipped with a mounting medium.

Morphometric Analysis

The volumes of the BNSTp and the number of calbindin-immunopositive (calbindin-ir) cells in the BNSTp were measured using a light microscope equipped with a charge-coupled device camera (CX9000; MBF Bioscience, Williston, VT, USA) and a computer running Stereo Investigator software (MBF Bioscience). The outlines of the BNSTp on the left of the midline were traced to measure the volume. Calbindin-ir cells in the BNSTp were then counted using the optical fractionator method. Detailed parameters of the stereological analyses are as follows: section thickness, 30 μm; section interval, 60 μm; sampling grid size, 150 μm × 150 μm; counting frame size, 50 μm × 50 μm; dissector height, 13–16 μm; and guard zone height, 1.5 μm.

Statistical Analysis

Two-way analysis of variance (ANOVA) was used to assess the effects of sex and age and the interaction between main factors on AVPV and BNSTp volume and the mRNA levels of target genes. The test of simple main effects was conducted when the interaction between main factors was significant in the two-way ANOVA. If there was a significant effect of age, but no significant interaction between sex and age, Bonferroni *post hoc* test was used to compare the mRNA levels of target genes between the different age groups. Differences in BNSTp volume and the number of calbindin-ir cells in the BNSTp among intact males, males subjected to prepubertal orchiectomy and cholesterol-implantation, and males subjected to prepubertal orchiectomy and testosterone implantation were analyzed with one-way ANOVA followed by Bonferroni *post hoc* test where appropriate. IBM SPSS Statistics v20.0 (IBM, Armonk, NY, USA) was used for data analyses. $P < 0.05$ was considered statistically significant. A *post hoc* power analysis was carried out after data has been collected to determine the power in this study using G*Power 3 (40), a free power analysis program.

RESULTS

AVPV and BNSTp Volume on PD20–60

Tissues from the AVPV were collected from cresyl fast violet-stained brain sections using the LMD system for accuracy and precision (**Figure 1A**). Two-way ANOVA indicated that the volume of the AVPV was significantly larger in females than in males [$F_{1, 32} = 13.86$, $p < 0.005$, power $(1 - \beta) = 0.98$; **Figure 1B** inset], and that it increased significantly with age from PD20 to PD60 [$F_{3, 32} = 4.70$, $p < 0.01$, power $(1 - \beta) = 0.93$; **Figure 1B**], although there was no significant interaction between sex and age [power $(1 - \beta) = 0.10$]. AVPV tissue volumes in PD30 mice were significantly greater ($p < 0.05$) than PD20 mice and did not differ from those in PD40 and PD60 mice.

Tissues from the BNSTp were isolated from cresyl fast violet-stained brain sections using the LMD system (**Figure 2A**). BNSTp volume was significantly greater in PD20–60 males than in

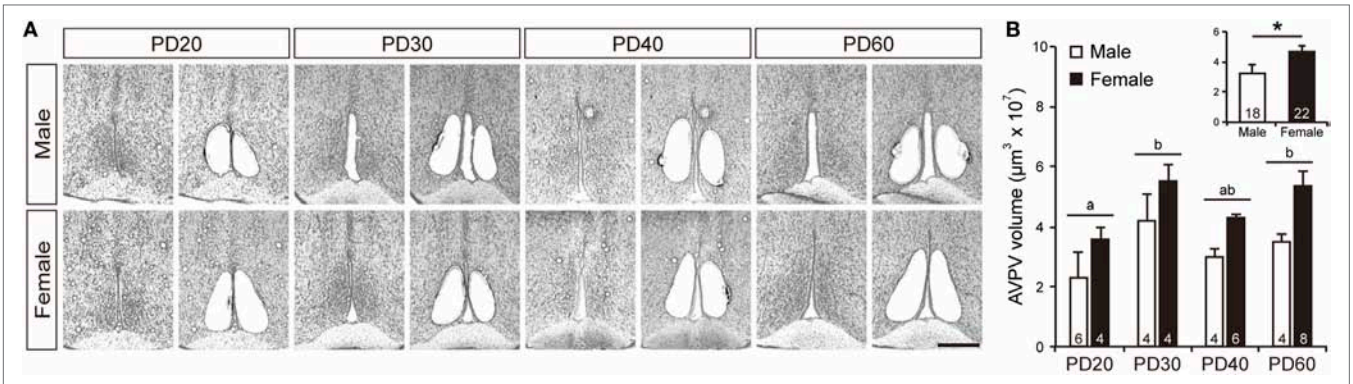

FIGURE 1 | Isolation of the AVPV. **(A)** Representative photographs showing brain sections before and after the AVPV was isolated in male and female mice on PD20–60. Scale bar indicates 400 μm. **(B)** The volume of AVPV tissues isolated from the brain of male and female mice on PD20, PD30, PD40, and PD60. The inset graph in panel **(B)** indicates the average volume of the AVPV in all ages. The numbers in the columns indicate the numbers of animals. Values are the mean ± SEM. Differences in values that are significantly ($p < 0.05$) different from each other are indicated with letters. *$p < 0.05$.

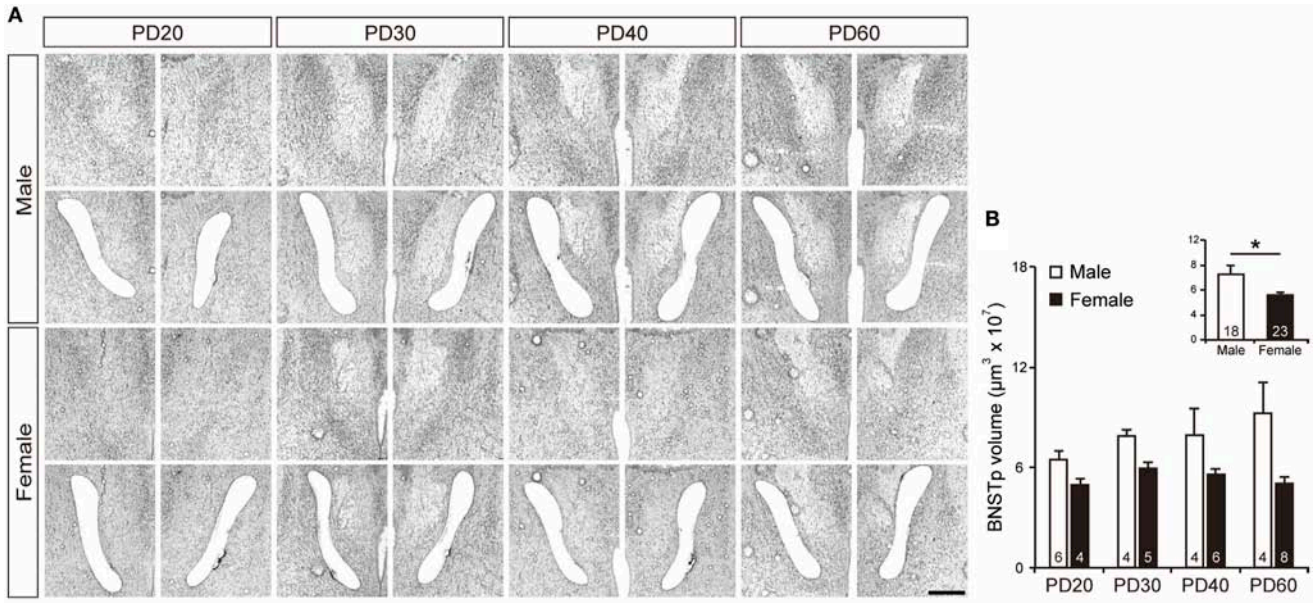

FIGURE 2 | Isolation of the BNSTp. **(A)** Representative photographs showing brain sections before and after the BNSTp was isolated in male and female mice on PD20–60. Scale bar indicates 400 μm. **(B)** The volume of BNSTp tissues isolated from the brain of male and female mice on PD20, PD30, PD40, and PD60. The inset graph in panel **(B)** indicates the average volume of the BNSTp in all ages. The numbers in the columns indicate the numbers of animals. Values are the mean ± SEM. *$p < 0.05$.

PD20–60 females [$F_{1, 33} = 16.33$, $p < 0.0001$, power $(1 - \beta) = 0.99$; **Figure 2B** inset]. The main effect of age and the interaction between sex and age on BNSTp volume were not significant [power $(1 - \beta) = 0.68$ and 0.58, respectively; **Figure 2B**].

Expression of *Esr1*, *Esr2*, *Cyp19a1*, and *Ar* mRNA in the AVPV on PD20–60

Although the effect of age and interaction between sex and age on *Esr1* mRNA levels were not significant [power $(1 - \beta) = 0.68$ and 0.63, respectively], *Esr1* mRNA levels in the AVPV at PD20–60 were significantly higher in females than in males [$F_{1, 32} = 35.75$, $p < 0.0001$, power $(1 - \beta) = 1.00$; **Figure 3A** inset]. *Esr2* mRNA levels in the AVPV were not significantly affected by sex, age, and their interaction [power $(1 - \beta) = 0.56$, 0.25 and 0.20, respectively; **Figure 3B**]. *Ar* mRNA levels in the AVPV was affected significantly by age [$F_{3, 32} = 3.03$, $p < 0.05$, power $(1 - \beta) = 0.76$], but not by sex [power $(1 - \beta) = 0.53$] and the interaction between sex and age [power $(1 - \beta) = 0.34$]. *Ar* mRNA levels in the AVPV of PD30 mice were significantly higher ($p < 0.05$) than those of PD20 mice and comparable with those of PD40 and PD60 mice (**Figure 3C**). *Cyp19a1* mRNA in the AVPV on PD20–60 was at an undetectable level in most animals in either sex. *Cyp19a1* mRNA was detectable in one female each on PD20, PD30, and PD40, in two males at PD30, and in two males and two females at PD60, but at the limit of detection by qPCR (data not shown).

Expression of *Esr1*, *Esr2*, *Cyp19a1*, and *Ar* mRNA in the BNSTp on PD20–60

There was a significant effect of sex [$F_{1, 33} = 51.56$, $p < 0.0001$, power $(1 - \beta) = 1.00$] on *Esr1* mRNA levels in the BNSTp during PD20–60 (**Figure 4A** inset). However, the *Esr1* mRNA levels were not significantly affected by age [power $(1 - \beta) = 0.12$] and by interaction between sex and age [power $(1 - \beta) = 0.51$]. *Esr2* mRNA levels in the BNSTp were significantly higher in females than in males at PD20–60 [$F_{1, 33} = 4.35$, $p < 0.05$, power $(1 - \beta) = 0.62$; **Figure 4B** inset], although they did not significantly change with age [power $(1 - \beta) = 0.07$] and were not affected by the interaction between sex and age [power $(1 - \beta) = 0.08$] (**Figure 4B**). Unlike the AVPV, the BNSTp expressed *Cyp19a1* mRNA at PD20–60 (**Figure 4C**). *Cyp19a1* mRNA levels in the BNSTp did not significantly change with sex and age [power $(1 - \beta) = 0.22$ and 0.24, respectively]. The interaction of sex and age was also not significant on the *Cyp19a1* mRNA levels [power $(1 - \beta) = 0.22$]. *Ar* mRNA levels in the BNSTp during PD20–60 were not significantly impacted by sex, age, and their interaction [power $(1 - \beta) = 0.24$, 0.73 and 0.40, respectively] (**Figure 4D**).

Effects of Prepubertal Orchiectomy and Testosterone Replacement on the BNSTp in Male Mice

One-way ANOVA revealed that BNSTp volume [$F_{2, 12} = 7.07$, $p < 0.01$, power $(1 - \beta) = 0.92$] and the number of calbindin-ir cells in the BNSTp [$F_{2, 12} = 6.98$, $p < 0.05$, power $(1 - \beta) = 0.92$] of adult male mice were significantly different among the experimental groups. BNSTp volume in males that were orchiectomized and implanted with cholesterol at PD20 was significantly smaller ($p < 0.05$) than that in gonadally intact males (**Figures 5A,B**). However, BNSTp volume in males that were orchiectomized and implanted with testosterone at PD20 was similar to that in

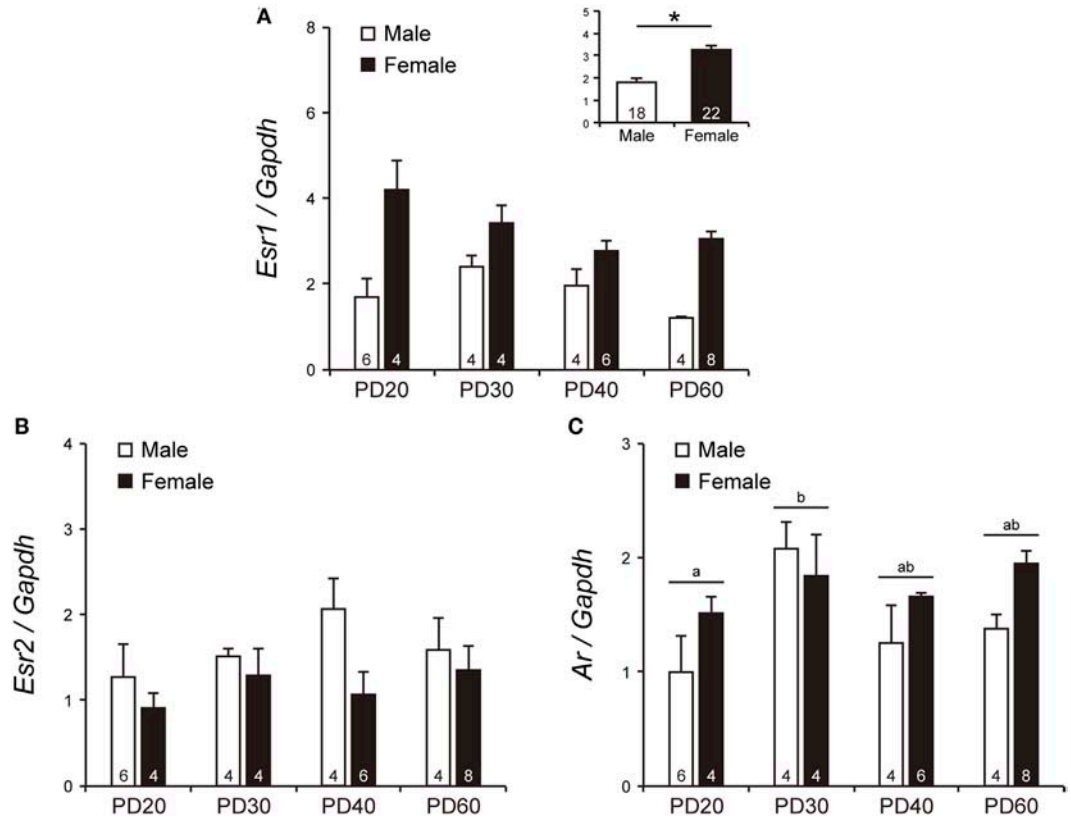

FIGURE 3 | The mRNA levels of *Esr1*, *Esr2*, and *Ar* in the AVPV of peripubertal mice. The mRNA levels of *Esr1* **(A)**, *Esr2* **(B)**, and *Ar* **(C)** in male and female mice on PD20, PD30, PD40, and PD60. The inset graph in panel **(A)** indicates the average mRNA level of *Esr1* in all ages. The numbers in the columns indicate the numbers of animals. Values are the mean ± SEM. Differences in values that are significantly ($p < 0.05$) different from each other are indicated with letters. *$p < 0.05$.

gonadally intact males and was significantly greater ($p < 0.05$) than that in the orchidectomzied and cholesterol-implanted males (**Figures 5A,B**). Likewise, the number of calbindin-ir cells in the BNSTp was significantly smaller ($p < 0.05$) in the orchiectomized and cholesterol-implanted males than in gonadally intact males (**Figure 5C**). The cell number in the orchiectomized and testosterone-implanted males was significantly larger ($p < 0.05$) than that in the orchiectomized and cholesterol-implanted males and was similar to that in gonadally intact males.

DISCUSSION

Here, we examined the mRNA levels of *Esr1*, *Esr2*, *Cyp19a1*, and *Ar* in the AVPV and BNSTp of peripubertal mice. To the best of our knowledge, this is the first study to examine these mRNA expressions to investigate the role of gonadal hormones during puberty in the formation of sexually dimorphic structures in the AVPV and BNSTp. We revealed that these genes are expressed in a sex-, age-, and region-specific manner. Especially, *Cyp19a1* mRNA was expressed in the BNSTp but not in the AVPV on PD20–60. This suggests that the AVPV and BNSTp respond differentially to testicular testosterone, which might contribute to the sexually dimorphic development of these brain structures.

Role of Sex Steroids in the Development of the AVPV

We previously showed that the volume and neuron number in the AVPV of male mice lacking *Esr1* and *Cyp19a1* are larger than those of wild-type males and similar to those of females, although the volume and neuron number in the AVPV of male mice lacking *Esr2* are comparable to those of wild-type males (23). We further showed that the AVPV expresses *Esr1* and *Cyp19a1* in the perinatal period (23). Additionally, it has been reported that administration of testosterone to postnatal female rats reduces AVPV volume and the number of TH and kisspeptin neurons in this structure in adulthood (6, 22). In this study, we found that the AVPV expressed *Esr1* and *Esr2*, but not *Cyp19a1* during PD20–60. This finding suggests that testicular testosterone in the peripubertal period is not converted to estradiol locally in the AVPV and does not affect the AVPV of male mice *via* ESR1 and ESR2, although it is not ruled out that estradiol produced elsewhere can affect the AVPV. Taken together, it is suggested that the morphology of the male AVPV might be organized by estradiol, which is synthesized from testicular testosterone by CYP19A1 (aromatase) expressed locally in the AVPV, which signals *via* ESR1 in the perinatal, but not pubertal period. Here, we found that *Ar* mRNA was expressed in the AVPV during puberty. However, AR-mediated testosterone signaling might

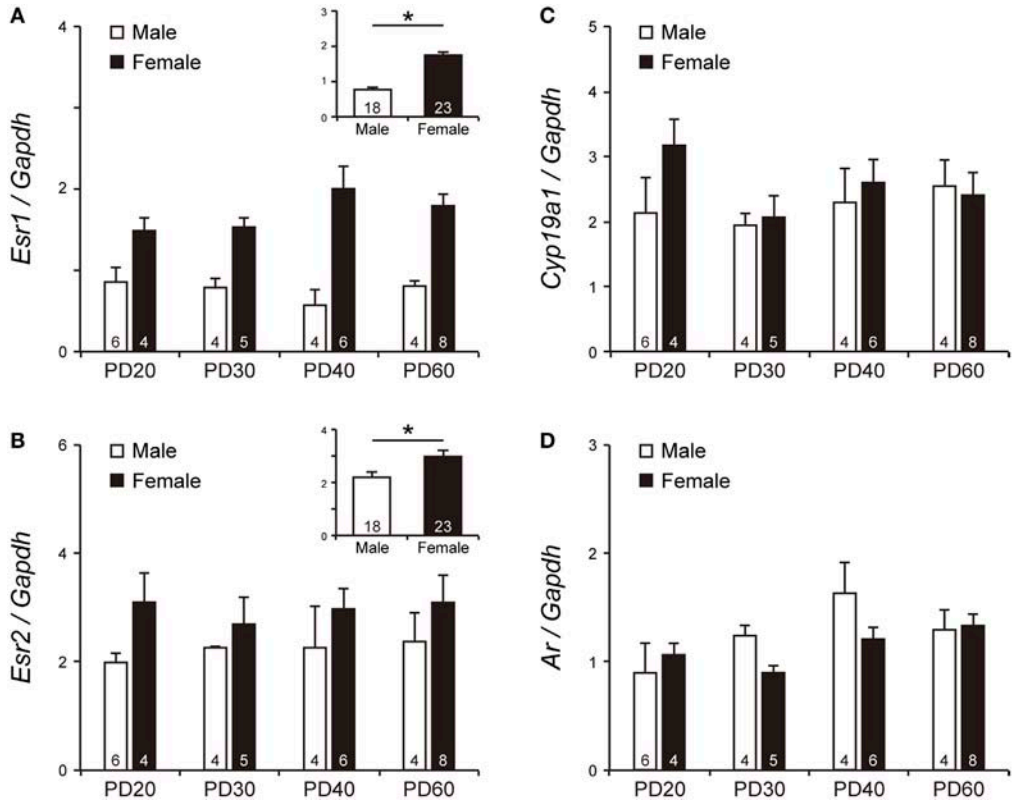

FIGURE 4 | The mRNA levels of *Esr1*, *Esr2*, *Cyp19a1*, and *Ar* in the BNSTp of peripubertal mice. The mRNA levels of *Esr1* **(A)**, *Esr2* **(B)**, *Cyp19a1* **(C)**, and *Ar* **(D)** in male and female mice on PD20, PD30, PD40 and PD60. The inset graphs in panels **(A,B)** indicate the average mRNA level of *Esr1* and *Esr2* in all ages, respectively. The numbers in the columns indicate the numbers of animals. Values are the mean ± SEM. *$p < 0.05$.

not be necessary for the organization of the AVPV in male mice, because the volume and neuron number in the AVPV of *Ar* KO male mice is comparable to those of wild-type male mice (23). Additionally, the AVPV in male rats appears to be not affected by prepubertal orchiectomy (33), suggesting that testicular hormones during puberty contribute minimally to the organization of the AVPV in male mice.

Esr1 and *Esr2* were expressed in the female AVPV from PD20 to PD60. Given that ovaries begin to produce estradiol on PD7 (41), ovarian estradiol could act on the AVPV through ESR1 and ESR2 during the peripubertal period. We found here that AVPV volume increased from PD20 to PD30, and remained high on PD60, and that it was greater in female mice compared with male mice. During puberty in rats, newborn cells are incorporated into the AVPV, more so in females than in males, and this incorporation of new cells is suppressed by ovariectomy on PD20 (33). Thus, ovarian estradiol, signaling through ESR1 and/or ESR2 during puberty, might promote the generation of AVPV cells, which could contribute to the sexually dimorphic development of the AVPV.

Role of Sex Steroids in the Development of the BNSTp

We previously reported that the masculinization of the BNSTp is disrupted in male mice lacking *Esr1*, *Cyp19a1*, or *Ar*, but not *Esr2* (23). We further found that the BNSTp expresses mRNAs of *Esr1* and *Cyp19a1* in the perinatal period and *Ar* mRNA in the postnatal period (23). Neonatal orchiectomy decreases the volume and number of neurons in the BNSTp of adult male rats (12), and early postnatal treatment with testosterone or estradiol increases the number of neurons in the BNSTp of adult female mice (25). These findings suggest that testosterone, acting through AR, during the postnatal period, and aromatized testosterone (estradiol), acting through ESR1, during the perinatal period help to masculinize the morphology of the BNSTp. In addition, it is thought that pubertal gonadal hormones affect the formation of the BNSTp. The BNSTp in prepubertal male mice has a greater volume and contains a larger number of calbindin neurons than the BNSTp of female mice of the same age (31, 32), and these sex differences become more pronounced over time, with a gradual increase in these parameters in males and a gradual decrease in females during puberty (31, 32). The increase in the volume and calbindin neuron number of the male BNSTp is suppressed by orchiectomy on PD20 (32). In this study, we confirmed that orchiectomy at PD20 reduced BNSTp volume and calbindin-ir cell number of the BNSTp in adult male mice. Moreover, we revealed that a compensatory treatment with testosterone could rescue the effects of orchiectomy at PD20 on the BNSTp in male mice. These findings suggest that testicular testosterone during puberty plays an important role in the formation of the male

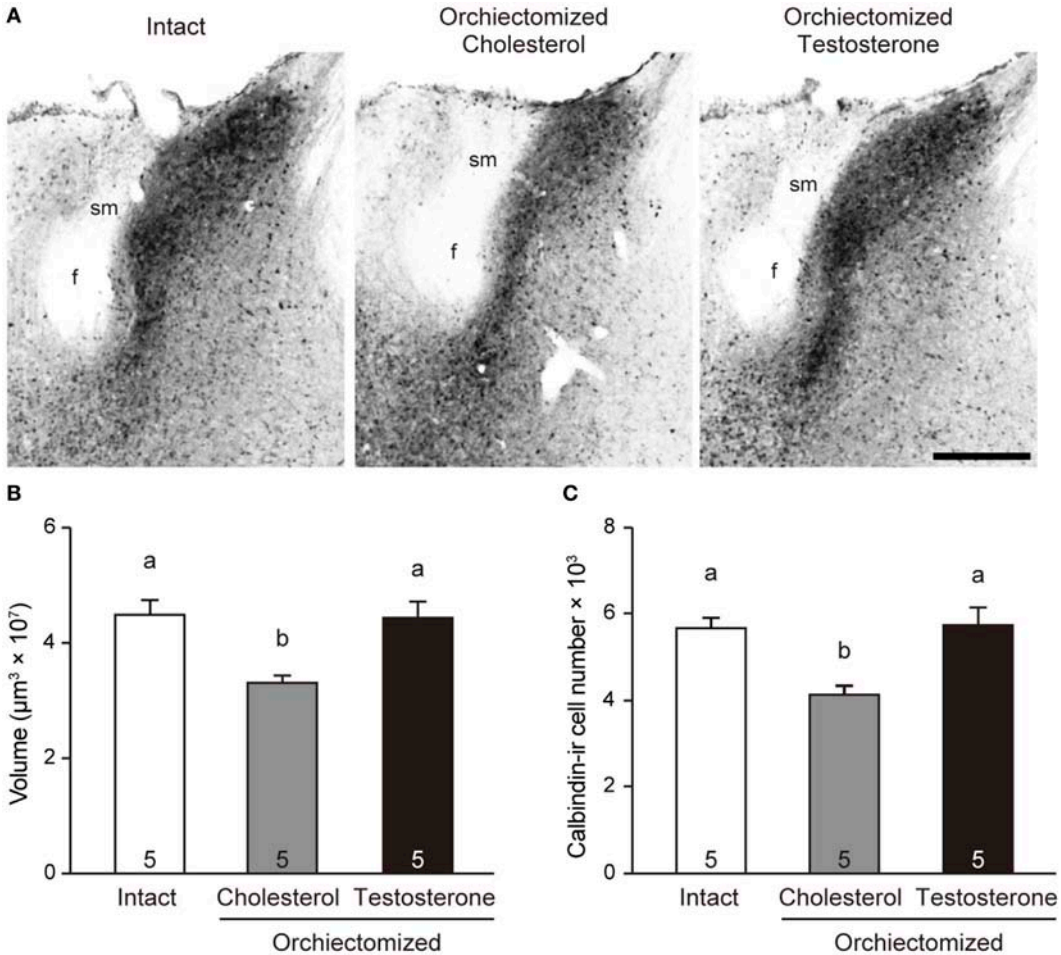

FIGURE 5 | Effects of peripubertal testicular testosterone on the BNSTp in male mice. **(A)** Representative photomicrographs of brain sections from a gonadally intact adult male mouse, an adult male mouse subjected to orchiectomy and cholesterol implantation at PD20, and an adult male mouse subjected to orchiectomy and testosterone implantation at PD20. Scale bar indicates 300 μm. f, fornix; sm, stria medullaris of the thalamus. **(B)** The volume of the BNSTp in adult male mice. **(C)** The number of the calbindin-ir cells in the BNSTp of adult male mice. The numbers in the columns indicate the numbers of animals. Differences in values that are significantly ($p < 0.05$) different from each other are indicated with letters.

BNSTp. It was reported that the volume of the BNSTp in adult male rats is reduced by orchiectomy in adulthood (42), whereas orchiectomy in adulthood has no effect on BNSTp volume in guinea pigs (43). Therefore, the results of our current study do not exclude the notion that the formation of the male BNSTp requires testicular testosterone in the postpubertal period as well as the pubertal period. Considering that the BNSTp from PD20 to PD60 expressed the mRNAs of *Esr1*, *Cyp19a1*, and *Ar*, we can deduce that the masculinization of the BNSTp is affected both by testicular testosterone acting *via* AR and by estradiol, which is synthesized locally in the BNSTp by CYP19A1 from testicular testosterone and acts *via* ESR1, during the pubertal period. Thus, unlike the defeminization of the AVPV, the masculinization of the BNSTp is likely established by testicular testosterone and estradiol not only in the perinatal period, but also in the pubertal period. In comparison, the volume and number of calbindin neurons in the BNSTp are lower in adult females than in PD20 females, and ovariectomy on PD20 does not affect the reduction in BNSTp volume or neuron number during puberty (32). Unlike the formation of the male BNSTp, the formation of the female BNSTp may be independent of ovarian hormones during puberty, despite the mRNA expression of *Esr1* and *Esr2* in this brain structure.

On PD4, the mRNA levels of *Esr1* and *Cyp19a1* in the BNSTp of *Ar* KO male mice are higher than those in wild-type male mice (23). This suggests that testosterone acting *via* AR suppresses the mRNA expression of *Esr1* and *Cyp19a1* in the male BNSTp, thereby reducing estradiol signaling in the early postnatal period. In male mice, the testosterone concentration in blood is gradually increased before puberty and it is higher levels during puberty (PD35 and PD40) (44). Therefore, if the inhibitory action of testosterone on *Esr1* and *Cyp19a1* mRNA expressions persists until puberty, the action of a large amount of testosterone *via* AR and lower ESR1 expression in the male BNSTp during puberty might stimulate male-specific development of this structure, as more testosterone would be available to bind the AR (as a lower

amount would be converted to estradiol, which would also have fewer receptors to signal through). Nonetheless, it was reported that *Cyp19a1* mRNA and aromatase activity in the bed nucleus of the stria terminalis (BST) including the BNSTp of adult male rats are enhanced by testosterone actions *via* AR (45, 46). If this mechanism is functional during puberty as well as adult period, pubertal testosterone may facilitate estrogen production, followed by enhancement of estrogen signaling *via* ESR. Indeed, a marked less ESR1 in the male BNSTp than in the female BNSTp on PD20–60 may be due to a downregulation of ESR1 by a large amount of aromatized testosterone. Taken together, further experiments are required to determine the relevance between testosterone action *via* AR and estradiol action *via* ESR1 in the BNSTp during puberty in the sexually dimorphic formation of the BNSTp.

Microscopic Isolation of Target Tissues

In this study, we isolated the AVPV and BNSTp from the brain using the LMD system. The LMD system enables precise and accurate isolation of target regions in sample tissues. This allowed us to detect sex-, age-, and region-dependent differences in the mRNA expression of genes related to sex steroid signaling. In the AVPV, we found that *Esr1* mRNA levels were greater in females at PD20–60. However, in one study, the expression of ESR1 protein in the AVPV on PD25 did not differ between the sexes (47). There might be a sex difference in the posttranscriptional stability of *Esr1* mRNA and/or the post-translational stability of ESR1 protein. In the BNSTp, one study reported that greater *Esr1* mRNA expression in female mice is not observed at PD20, but appears by PD60 (48). Another study reported that the murine BNSTp on PD25 contains a greater number of ESR1-immunoreactive cells in females compared with males (47). In this study, although we did not find a significant difference in *Esr1* mRNA levels between sexes at each age, we observed higher mRNA levels of *Esr1* in female mice at PD20–60. Our current study showed no sex difference in *Cyp19a1* mRNA expression in the BNSTp. Another study showed that *Cyp19a1* mRNA expression in the BST is greater

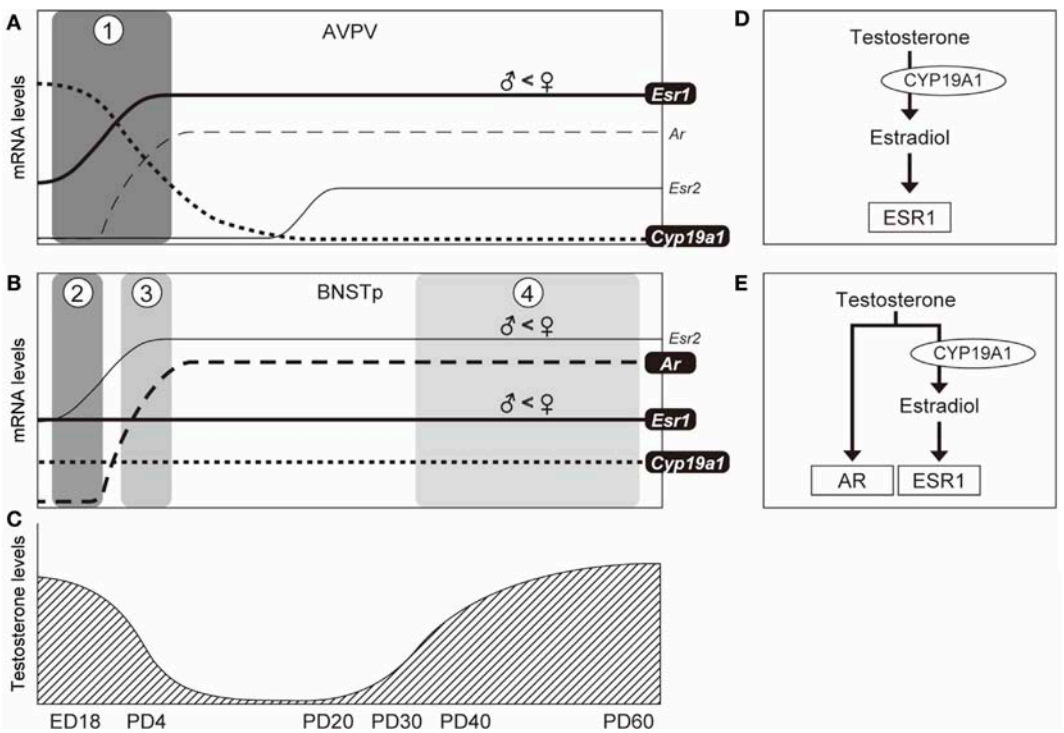

FIGURE 6 | Schematic illustration of the temporal expression patterns of genes in the AVPV and BNSTp and possible testicular testosterone actions on the formation of the AVPV and BNSTp in male mice. In the AVPV [*panel* **(A)**], based upon our previous (23) and current studies, *Esr1* mRNA expression (*solid line*) increases from embryonic day 18 (ED18) to PD4 and continues in the pubertal period. *Cyp19a1* mRNA level (*dotted line*) decreases from ED18 to PD4 and is not expressed by PD20. *Esr1* and *Cyp19a1* are essential to the formation of the male AVPV, but *Esr2* and *Ar* are not necessary (23). Testicular testosterone production reaches a peak level at ED18 and decreases after birth (50), and again is increased from PD35 (44) [*panel* **(C)**]. The temporal patterns of *Esr1*, *Cyp19a1*, and testicular testosterone may indicate that the male AVPV is organized by estradiol (formed by the aromatization of testosterone) signaling through ESR1 during the perinatal period [*boxed phase 1 in panel* **(A)** and *panel* **(D)**]. In the BNSTp [*panel* **(B)**], based upon our previous (23) and current studies, *Esr1* mRNA (*solid line*) is expressed during the perinatal period, and is also expressed during the pubertal period. *Cyp19a1* mRNA level (*dotted line*) is stably expressed in the perinatal and pubertal periods. *Ar* mRNA (*broken line*) is not expressed in the late fetal period (ED18), but expressed in the postnatal (PD4) and pubertal periods. *Esr1*, *Cyp19a1*, and *AR* are requisite for the sexual differentiation of the male BNSTp (23, 26). The patterns of *Esr1*, *Cyp19a1*, *Ar*, and testicular testosterone levels may indicate that the male BNSTp is formed by the action of estradiol (formed by the aromatization of testicular testosterone) *via* ESR1 in the fetal period [*boxed phase 2 in panel* **(B)** and *panel* **(D)**], and both the action of estradiol *via* ESR1 and testicular testosterone *via* AR in the postnatal [*boxed phase 3 in panel* **(B)** and *panel* **(E)**] and pubertal [*boxed phase 4 in panel* **(B)** and *panel* **(E)**] periods.

in male mice than in female mice (49). This discordance may be due to a difference of target area. Target area in the previous study was the BST that includes some sub-nuclei (lateral division–dorsal, intermediate, posterior and ventral parts; and medial division–anterior, ventral parts, etc.). By contrast, our current study targeted only the BNSTp (the principal nucleus of the BST posterior division).

In this study, the volume of the AVPV and BNSTp was measured. We found that volume of the AVPV was larger in female mice than that in male mice through PD20 to PD60, although the sex difference was no found at each age. It was previously reported that the sexual dimorphism in AVPV volume in rats emerges between PD30 and PD40 (2). Considering species differences, it is plausible that we correctly isolated AVPV tissues enough to detect sex- and age-related alterations. Regarding the BNSTp, we found a male-biased difference in the volume on PD20–PD60. This sex difference may be resulted from an increase in the volume of the male BNSTp during puberty as reported previously (28, 31, 32). In this study, there was a tendency that the volume of the male BNSTp increased with age. Taken together, the results that sex differences and age-related alterations in the volume of the AVPV and BNSTp could be acquired in the LMD system may guarantee the accuracy of mRNA expression analysis in the current study.

In conclusion, pubertal testosterone is not converted to estradiol locally in the AVPV because of the absence of *Cyp19a1* mRNA. This suggests that estradiol synthesized in the AVPV itself is not able to act on the AVPV. Our previous study showed that *Esr1* and *Cyp19a1* are necessary genes for the defeminization of the AVPV, but *Esr2* and *Ar* are not, and that *Esr1* and *Cyp19a1* mRNAs are expressed in the AVPV in the perinatal period (23). Collectively, the male AVPV might be generated by estradiol (formed by the aromatization of testosterone) signaling through ESR1 during the perinatal period, but not during puberty (**Figure 6**). Additionally, our previous study showed that *Esr1*, *Cyp19a1*, and *Ar* are necessary for the masculinization of the BNSTp, but *Esr2* is not (23, 26). Furthermore, we previously reported that the BNSTp expresses *Esr1* and *Cyp19a1* mRNAs in the perinatal period and *Ar* mRNA in the postnatal period (23). In this study, we found that the BNSTp expresses *Esr1*, *Cyp19a1*, and *Ar* in the pubertal period. Additionally, the results of the histological analysis of the BNSTp suggest that testicular testosterone during puberty affects the formation of the BNSTp in male mice. The findings of our current study may indicate that the masculinization of the BNSTp is affected by testosterone, *via* AR, and by estradiol (formed by the aromatization of testosterone) *via* ESR1 during puberty. Thus, the sexually dimorphic development of the BNSTp in males might be accomplished by testicular testosterone in the postnatal and pubertal periods as well as by estradiol derived from testosterone in the perinatal and pubertal periods (**Figure 6**).

ETHICS STATEMENT

All animal experimental procedures were approved by the Animal Care and Experimentation Committee of Saitama University and were conducted in accordance with the Guidelines for the Care and Use of Experimental Animals of Saitama University.

AUTHOR CONTRIBUTIONS

MK performed the experiments, analyzed the data, and prepared the manuscript. MM performed the experiments and analyzed the data. ST designed the experiments, analyzed the data, and prepared the manuscript.

FUNDING

This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 17K08567 to ST).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 07 May 2018
doi: 10.3389/fendo.2018.00212

Reduced Luteinizing Hormone Induction Following Estrogen and Progesterone Priming in Female-to-Male Transsexuals

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Anatomical studies have suggested that one of the brain structures involved in gender identity is the bed nucleus of the stria terminalis, though this brain structure is probably not the only one to control gender identity. We hypothesized that, if this brain area also affected gonadotropin secretion in humans, transsexual individuals might produce different gonadotropin levels in response to exogenous stimulation. In the present study, we examined whether estrogen combined with progesterone might lead to a change in luteinizing hormone (LH) secretion in female-to-male (FTM) transsexual individuals. We studied female control subjects (n = 9), FTM transsexual subjects (n = 12), and male-to-female (MTF) transsexual subjects (n = 8). Ethinyl estradiol (50 μg/tablet) was administered orally, twice a day, for five consecutive days. After the first blood sampling, progesterone (12.5 mg) was injected intramuscularly. Plasma LH was measured with an immunoradiometric assay. The combination of estrogen and progesterone resulted in increased LH secretion in female control subjects and in MTF subjects, but this increase appeared to be attenuated in FTM transsexual subjects. In fact, the %LH response was significantly reduced in FTM subjects ($P < 0.05$), but not in MTF subjects ($P > 0.5$), compared to female control subjects. In addition, the peak time after progesterone injection was significantly delayed in FTM subjects ($P < 0.05$), but not in MTF subjects ($P > 0.5$), compared to female control subjects. We then compared subjects according to whether the combination of estrogen and progesterone had a positive (more than 200% increase) or negative (less than 200% increase) effect on LH secretion. A χ^2 analysis revealed significantly different ($P < 0.05$) effects on LH secretion between female controls (positive n = 7, negative n = 2) and FTM transsexual subjects (positive n = 4, negative n = 8), but not between female controls and MTF transsexual subjects (positive n = 7, negative n = 1). Thus, LH secretion in response to estrogen- and progesterone priming was attenuated in FTM subjects, but not in MTF subjects, compared to control females. This finding suggested that the brain area related to gender identity in morphological studies might also be involved in the LH secretory response in humans. Thus, altered brain morphology might be correlated to altered function in FTM transsexuals.

Keywords: transsexual, gender dysphoria, gender identity, estrogen, progesterone, luteinizing hormone, bed nucleus, human

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Edited by:
Takayoshi Ubuka, Monash University Malaysia, Malaysia

Reviewed by:
Hitoshi Ozawa, Nippon Medical School, Japan
Lance Kriegsfeld, University of California, Berkeley, United States

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *10 December 2017*
Accepted: *16 April 2018*
Published: *07 May 2018*

Citation:
Funabashi T, Sakakibara H, Hirahara F and Kimura F (2018) Reduced Luteinizing Hormone Induction Following Estrogen and Progesterone Priming in Female-to-Male Transsexuals. Front. Endocrinol. 9:212. doi: 10.3389/fendo.2018.00212

INTRODUCTION

It is generally agreed that the rodent central nervous system is sexually differentiated. This includes the hypothalamus and the anterior pituitary, which control luteinizing hormone (LH) secretion. Female rodents, but not males, exhibit a surge of LH secretion that drives ovulation, which results from a "positive feedback" effect of estrogen (1). Androgen exposure during the neonatal period is thought to be critical for the development of sexual differentiation (2–5). Thus, the presence of a sex difference, or sexual dimorphism, is found in many brain structures, due to the organizational effects of sex steroids, which produce male- or female-like morphology.

The bed nucleus of the stria terminalis (BST) is one of several structures that show sexually dimorphic regions (6, 7). Moreover, neuronal fibers that project from the BST to various brain regions (8–10) are involved in many different types of motivational behaviors (11–13). In addition, the BST was shown to be involved in gonadotropin secretion (14–17). Indeed, estrogen-binding cells were found in the BST (18), where gonadal steroid receptors were expressed (19–21). The rat lateral subdivision of the BST is homologous to the human central BST (22, 23), which is thought be involved in gender identity in humans.

Gender identity disorder, or gender dysphoria (GD), is defined as a strong gender identification with the opposite sex; for example, female-to-male (FTM) transsexuals have a strong male gender identity, and thus, they want to reassign their physical sex (24–26). Although GD is a highly complex clinical situation, its etiology has been described by several environmental, genetic, and anatomical theories (27–30). Currently, there is morphological evidence that, in humans, the BST is related to gender identity (31–33). Studies have shown that the number of neurons in the BST (34) and the central BST size (35) in male-to-female (MTF) transsexual individuals corresponded to the respective norms for biological females. Conversely, FTM transsexual individuals exhibited a BST size that corresponded to the norm for biological males (35). It is unknown whether these structural features are causally related to sexual identity or whether they are indirectly correlated.

Taken together, findings from previous studies have led to the hypothesis that, if the same area of the central nervous system that controls LH secretions is also involved in gender identity, or conversely, if the causal factor(s) (as yet undetermined) that affects brain structure in GD can also account for LH secretion, then the LH secretory profile in subjects with GD might be different from the typical profile expected for a given sex. Gooren and colleagues studied this hypothesis in detail (36, 37) and concluded that the neuroendocrine regulation of LH secretion did not differ between transsexual and non-transsexual individuals, either males or females (24, 28). This result may be reasonable, since the neuroendocrine mechanism that regulates the cyclic release (surge) of LH in primates is not thought to be different between the sexes (5, 38). Indeed, estrogen was equally capable of inducing surge-like LH secretions in male rhesus monkeys (5, 38–40) and female monkeys (1). In humans, it was reported that a similar surge-like LH secretion could be induced by estrogen in gonadectomized men (41, 42).

However, androgen exposure during the neonatal period, which is essential for brain differentiation in rodents (2, 3, 38), may alter the LH secretory response later in life, even in female primates (39, 43–46). It was reported that, in estrogen-primed ovariectomized female rhesus monkeys, progesterone had a stimulatory effect on LH secretion (47, 48). Importantly, this stimulatory effect was unremarkable in orchidectomized male rhesus macaques pretreated with 17-β estradiol (49). Although one report suggested that a surge-like LH secretion could be induced in men with progesterone after estrogen priming (50), the response was not directly compared with responses in women subjects; thus, the amplitude of the effect was unclear. Therefore, we hypothesized that estrogen combined with progesterone might lead to a change in LH secretion in patients with GD.

SUBJECTS AND METHODS

Subjects

For the present study, we recruited healthy Japanese volunteers who had undergone either FTM or MTF transsexual procedures. All FTM subjects were diagnosed with GD by psychiatrists, independent of the present study. All MTF subjects self-reported that they were bilaterally orchidectomized. Although, ideally, MTF subjects should be compared to male control subjects with bilateral orchidectomies, we could not recruit these control subjects. Thus, we compared the MTF transsexual data to female control data. One month before the sampling experiment, MTF subjects were asked to stop their cross-gender hormone treatments. All subjects self-reported their sexual orientation.

Treatment

Ethinyl estradiol (50 μg) tablets were administered to all subjects orally twice per day (100 μg total per day) for five consecutive days (days 3 to 7). Day 1 of the experiment was the day that menstruation began in female and FTM subjects. In previous studies, this dose of estrogen alone did not induce LH secretion, but when combined with progesterone, it induced a surge-like LH secretion (51–53). The estrogen treatment period was chosen, based on previous reports (51–53) and on our previous experience with rats (54). Blood samples were drawn on day 7. After the blood sample was taken, progesterone (12.5 mg) was injected at the University Hospital, between 0830 and 0930 h. Then, sequential blood samples (1.0–1.5 ml each) were collected on the same day, at 1200, 1500, 1800, and 2100 h. The final blood sample was drawn at 0900 h the next day. All blood samples were drawn through a median cubital vein. Plasma was separated from the blood samples at 4°C and stored at −20°C.

Ethics

This study was conducted in accordance with the recommendations of the Ethical Guidelines for Medical and Health Research Involving Human Subjects, established by the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labor and Welfare. We obtained written

informed consent to participate in the study from all volunteers, in accordance with the Declaration of Helsinki. The protocol was approved by the institutional Ethics Committee of the Yokohama City University School of Medicine. Subjects were interviewed to obtain data on their general health condition, sexual orientation, and menstrual cycles.

Analytical Methods and Statistical Analyses

Plasma LH was measured with immunoradiometric assay kits (Daiichi Radioisotope Institute). The intra- and inter-assay coefficients of variation, estimated at the mean LH level of 7.6 mIU/ml, were 3.0 and 8.0%, respectively. We tested statistical significance, as appropriate, with the one-way ANOVA, followed by Fisher's Least Significant Differences (LSD) *post hoc* comparison; with the Kruskal–Wallis test, followed by Dunn's multiple comparison test; or with the χ^2 test. Significance was accepted at $P < 0.05$.

RESULTS

Subjects

A total of nine female control subjects, 12 FTM subjects, and 8 MTF subjects, aged 28.7 ± 2.7, 27.4 ± 1.5, and 39.3 ± 2.3 years, respectively, participated in blood sampling experiments. We found that the baseline LH levels (before injection) were higher in MTF subjects (**Figure 1**) than in the other subjects. This finding suggested that no negative feedback was present, consistent with the removal of the testes. All female control and 10 FTM subjects self-reported regular menstrual cycles (regular was defined as a menstrual cycle of 25–38 days). Sexual orientations were self-reported; all female control subjects were attracted to men. All FTM subjects were attracted to women. Among the MTF subjects, one was attracted to women, four were to men, and three were attracted to both.

Effects of Estrogen Combined With Progesterone on LH Secretion

The LH response to estrogen and progesterone was tentatively defined as positive, when LH levels were greater than 200% of the control level. This threshold was based on data from previous studies (55, 56). According to this criterion, 77.7% of control females and 33.3% of FTM subjects showed positive responses (**Table 1**, $P < 0.05$, χ^2 test). In addition, seven out of eight MTF subjects displayed positive responses, a similar proportion to that observed among control females ($P > 0.1$).

Representative changes in LH profiles over time are shown in **Figure 1**. The estrogen and progesterone treatment appeared to stimulate LH release in both female control and MTF subjects, but not in FTM subjects. The mean LH concentrations at each time point are shown in **Figure 2A**. We analyzed the %change in LH secretion. This analysis showed that the LH response in FTM subjects was significantly smaller than in female controls (**Figure 2B**, ANOVA $P < 0.05$, Fisher's LSD $P < 0.05$). On the other hand, the LH response in MTF subjects was not different from that observed in female controls (Fisher's LSD $P > 0.5$). The mean peak time of LH secretion in FTM subjects was significantly delayed compared to that observed in female controls (**Figure 2C**, Kruskal–Wallis $P < 0.01$, Dunn's multiple comparison $P < 0.05$). The mean peak time of LH secretion in MTF subjects was not different from that observed in female controls (Dunn's multiple comparison, no significant difference).

DISCUSSION

In the present study, FTM subjects were attracted to women, but the MTF subjects were attracted to women, men, or both sexes. This result was in good accord with previous reports (57–59). For example, Auer et al. (59) described an MTF group that comprised individuals attracted to women (gynephilic, 51%) or men (androphilic, 26%), and an FTM group that were attracted to women (gynephilic, 73%). These similarities to our data suggested that our sampling population was not biased.

By examining the LH response to estrogen combined with progesterone, we found that the neuroendocrine response was different between FTM subjects and female controls. We should consider the physiological meaning of this phenomenon. Progesterone is certainly an important factor for induction of the LH surge in the rodent (1, 60), the ewe (61), and the primate (47, 48). However, the physiological role of progesterone in the ovulation-induced LH surge has not been fully understood (62). For example, a primary question is, what is the source of progesterone? Progesterone in the follicular fluid in the ovary exhibits higher than in the blood of pregnant women and thus source of progesterone may be follicle cells (62). Progesterone may come from the central nervous system (63). Basically, in the menstrual cycle, a gonadotropin surge and follicular rupture occur, followed by the luteinization of granulosa cells. Granulosa cells then establish the corpus luteum and progesterone secretion increases. Thus, we speculated that, with the current understanding, it might be difficult to distinguish FTM subjects from control subjects, based solely on the LH response to estrogen, and suggest the importance of determining the LH controlling mechanism by means of progesterone treatment in transsexuals.

The majority of FTM subjects exhibited a regular menstrual cycle. Consequently, like female control subjects, they could reproduce the menstrual cycle, as shown by Gooren (24). We confirmed that some FTM subjects displayed a preovulatory LH surge (Funabashi and Kimura, unpublished observations), but we did not examine ovulation directly in this study. However, our previous studies suggested that there was a discrepancy in reproductive abilities; female rats exposed to a low dose of testosterone during the neonatal period displayed a surge-like LH secretion in response to the combination of estrogen and progesterone, but the number of gonadotropin-releasing hormone neurons that expressed Fos was attenuated (54). Since these rats exhibit a regular estrous cycle, these results are consistent with FTM in this study having a regular menstrual cycle, but exhibiting attenuated LH secretion in response to estrogen combined with progesterone. Thus, the discrepancy between the mechanism for controlling menstrual

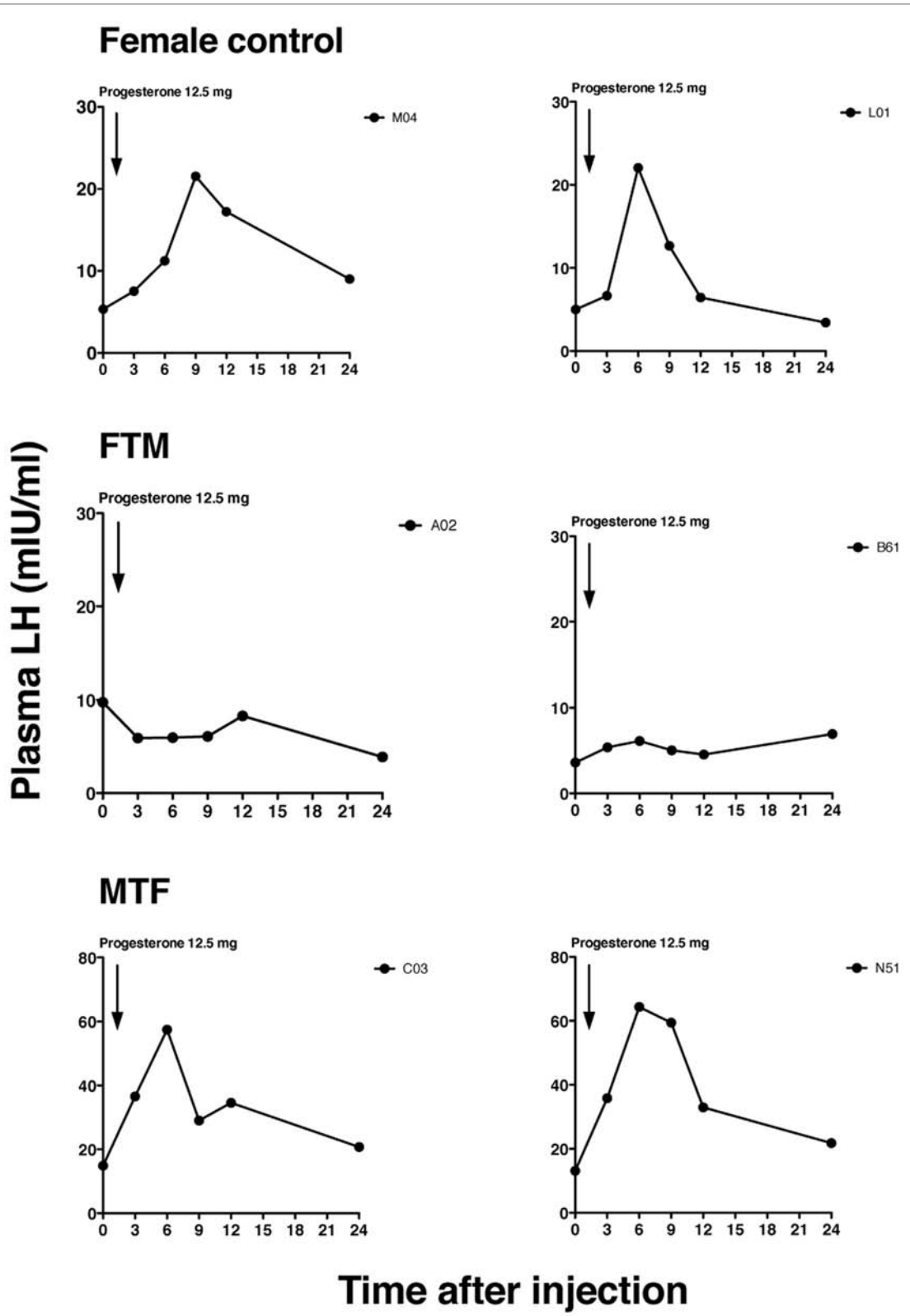

FIGURE 1 | Representative luteinizing hormone (LH) secretory patterns in transsexual individuals. Profiles of the LH secretory pattern in two female controls *(upper panels)*, two female-to-male transsexual (FTM) subjects *(middle panels)*, and two male-to-female transsexual (MTF) subjects *(lower panels)*, after estrogen priming (ethinyl estradiol, 100 μg for 5 days), and before (time 0) and after the progesterone injection. Each point indicates the plasma LH level. The arrow indicates the time of progesterone injection (12.5 mg).

TABLE 1 | Luteinizing hormone (LH) response to estrogen and progesterone stimulation, in control females and transsexual subjects.

Subjects	Number	LH response[a]		*P*-value
		Positive	Negative	
Control	9	7	2	
Female-to-Male	12	4	8	*
Male-to-Female	8	7	1	NS

[a]*The LH responses were classified as positive (more than 200% increase) or negative (less than 200% increase), compared to baseline. *P < 0.05 vs. female control subjects, based on the χ^2 test. NS = not significant.*

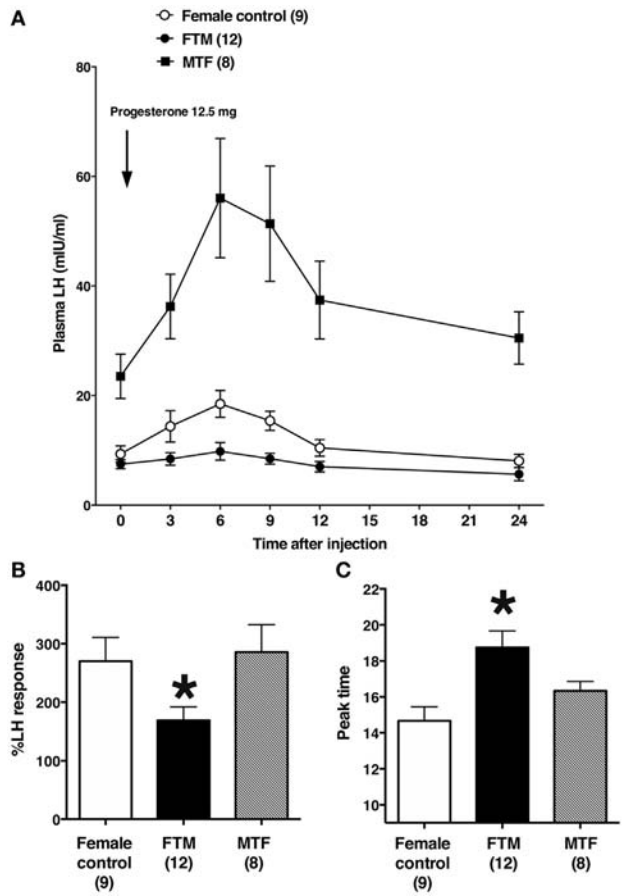

FIGURE 2 | Summary of the effects of estrogen and progesterone treatment on luteinizing hormone (LH) secretion in transsexuals. **(A)** Effects on LH secretion over time in female controls (open circles), female-to-male transsexual (FTM) subjects (closed circles), and male-to-female transsexual (MTF) subjects (closed squares). Each point and vertical line indicate the mean and SE, respectively. The LH level after 5 days of estrogen is shown at time 0. The arrow indicates the time of progesterone injection (12.5 mg). **(B)** The peak %changes in plasma LH are shown after estrogen and progesterone treatment, in female controls (*open bars*), FTM transsexual subjects (*filled bars*), and MTF transsexual subjects (*hatched bars*). **(C)** The time of peak plasma LH levels in subjects treated with estrogen and progesterone. Numbers in parentheses are the number of subjects in each group. Each bar and vertical line indicate the mean and SE, respectively; *$P < 0.05$ vs. female control.

cycle and the steroid-induced LH surge may be reasonable but LH response by an exogenous stimulus is attenuated in FTM subjects.

We would like to discuss whether the LH controlling mechanism is different between the sexes in humans. A previous report found that this mechanism was not different between transsexual individuals and controls; therefore, they concluded that it was not sexually differentiated (24). In the present study, the majority of MTF subjects showed an increase in LH secretion in response to estrogen combined with progesterone, similar to female control subjects. This result suggested that the LH controlling mechanism was not sexually differentiated. This finding was supported by a previous report that suggested that a surge-like LH secretion could be induced in men by injecting progesterone after estrogen priming (50); however, that response was not compared with a response in women subjects in that report; therefore, sexual differentiation could not be ruled out. If it is reasonable to compare MTF subjects to FTM subjects, due to the opposite genetic backgrounds and sexual identities, different conclusions are drawn. Since LH responses in transsexuals were different, the alteration in sexual differentiation may be related to the GD etiology (25, 57). Thus, gender identity, sexual orientation, and reproduction might be sexually differentiated through different brain mechanisms. In previous studies, different brain areas were responsible for sexual orientation and gender identity; the suprachiasmatic nucleus and the bed nucleus of the stria terminalis, respectively (25, 28).

The main limitation of this study was the lack of a male control without testes, which prevented us from drawing conclusions. This study was also limited by the small number of GD subjects; thus, we lacked firm evidence to support our hypothesis. Consequently, our findings remain to be confirmed in future studies with larger numbers of subjects.

ETHICS STATEMENT

This study was conducted in accordance with the recommendations of the Ethical Guidelines for Medical and Health Research Involving Human Subjects, established by the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labor and Welfare. We obtained written informed consent to participate in the study from all volunteers, in accordance with the Declaration of Helsinki. The protocol was approved by the institutional Ethics Committee of the Yokohama City University School of Medicine. Subjects were interviewed to obtain data on their general health condition, sexual orientation, and menstrual cycles.

AUTHOR CONTRIBUTIONS

TF and FK designed the study, analysis and interpretation of data, and assisted in the preparation of the manuscript. TF wrote the initial draft of the manuscript. All other authors have contributed to data collection. FK critically reviewed the manuscript. All authors approved the final version of the

manuscript, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ACKNOWLEDGMENTS

The present study was supported by JSPS KAKENHI, Grant Numbers 16659059 (to FK) and 17K08577 (to TF). The authors thank the Yokohama City University Hospital nursing staff for implementing the study protocol. The authors appreciate the support of A. K. for technical assistance.

FUNDING

The present study was supported by Grant-in-Aid for Exploratory Research (FK: 16659059) and Grant-in-Aid for Scientific Research (TF: 17K08577).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 09 May 2018
doi: 10.3389/fendo.2018.00230

Brain Aromatase Modulates Serotonergic Neuron by Regulating Serotonin Levels in Zebrafish Embryos and Larvae

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Teleost fish are known to express two isoforms of P450 aromatase, a key enzyme for estrogen synthesis. One of the isoforms, brain aromatase (AroB), *cyp19a1b*, is highly expressed during early development of zebrafish, thereby suggesting its role in brain development. On the other hand, early development of serotonergic neuron, one of the major monoamine neurons, is considered to play an important role in neurogenesis. Therefore, in this study, we investigated the role of AroB in development of serotonergic neuron by testing the effects of (1) estradiol (E_2) exposure and (2) morpholino (MO)-mediated AroB knockdown. When embryos were exposed to E_2, the effects were biphasic. The low dose of E_2 (0.005 μM) significantly increased serotonin (5-HT) positive area at 48 hour post-fertilization (hpf) detected by immunohistochemistry and relative mRNA levels of tryptophan hydroxylase isoforms (*tph1a*, *tph1b*, and *tph2*) at 96 hpf measured by semi-quantitative PCR. To test the effects on serotonin transmission, heart rate and thigmotaxis, an indicator of anxiety, were analyzed. The low dose also significantly increased heart rate at 48 hpf and decreased thigmotaxis. The high dose of E_2 (1 μM) exhibited opposite effects in all parameters. The effects of both low and high doses were reversed by addition of estrogen receptor (ER) blocker, ICI 182,780, thereby suggesting that the effects were mediated through ER. When AroB MO was injected to fertilized eggs, 5-HT-positive area was significantly decreased, while the significant decrease in relative *tph* mRNA levels was found only with *tph2* but not with two other isoforms. AroB MO also decreased heart rate and increased thigmotaxis. All the effects were rescued by co-injection with AroB mRNA and by exposure to E_2. Taken together, this study demonstrates the role of brain aromatase in development of serotonergic neuron in zebrafish embryos and larvae, implying that brain-formed estrogen is an important factor to sustain early development of serotonergic neuron.

Keywords: estradiol, brain aromatase, biphasic manner, serotonergic neuron, zebrafish, early development

OPEN ACCESS

Edited by:
Takayoshi Ubuka, Monash University Malaysia, Malaysia

Reviewed by:
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *23 February 2018*
Accepted: *23 April 2018*
Published: *09 May 2018*

Citation:
Ulhaq ZS and Kishida M (2018) Brain Aromatase Modulates Serotonergic Neuron by Regulating Serotonin Levels in Zebrafish Embryos and Larvae. Front. Endocrinol. 9:230. doi: 10.3389/fendo.2018.00230

INTRODUCTION

Biosynthesis of estrogen is catalyzed by the action of cytochrome P450 aromatase, a product of *cyp19a1* gene (1, 2). Contrary to mammals, zebrafish and many other teleosts have two isoforms of aromatase gene, *cyp19a1a* and *cyp19a1b*, encoding ovarian and brain aromatase, respectively (3, 4), and their predominant expression in respective tissues indicates differential regulation and

functions. Fish brain is characterized by having much higher aromatase expression in brain compared to mammals (5). At the same time, fish brain has been reported to exhibit elevated neuroregenerative capacity compared to mammals (6–8). Widespread proliferation zones are detected in zebrafish brain (6, 9), while only limited areas such as subependymal and subgranular zones exhibit proliferation in mammals (7). Such high neurogenic activity in teleost fish may be attributed to increased synthesis of estrogen due to the elevated expression of brain aromatase. Indeed, expression of brain aromatase is localized in radial glial cells (RGCs), which differentiate into neurons and other glial cells contributing to adult neurogenesis as well as developmental neurogenesis (10–12). Developmental studies in zebrafish show that expression of brain aromatase in embryos increases rapidly after 12 hour post-fertilization (hpf), and is regulated by positive feedback loop through its own product, estrogen, acting on estrogen response element of *cyp19a1b* (3, 13, 14). Therefore, the zebrafish model expressing elevated levels of brain aromatase in early development is suitable to investigate the functional significance of aromatase and neural estrogen in developing brain.

Serotonin (5-HT), a neurotransmitter produced by multiple enzymatic steps including a rate-limiting action of tryptophan hydroxylase (TPH), plays a major role in a number of physiological processes and pathological conditions, such as depression (15, 16), stress (15, 17), cardiac function (18), reward seeking behavior (19), and anxiety (15, 20). In addition, serotonergic neuron is known to be involved in neurogenic activities (21). It has been reported that 5-HT is critically involved in the brain plasticity, neural trafficking, synapse formation, and network construction during development (22, 23). Serotonergic neurons in raphe nuclei extend their axons to the forebrain possibly modulating the differentiation of neuronal progenitors (24). Early ontogeny of serotonergic system may further suggest its role in brain development (25). Raphe 5-HT populations in human brain are considered as the earliest to be identified (24, 26).

Serotonergic neurons in mammalian brain are localized mainly in raphe nuclei of brain stem, which project into accumbens, hypothalamus, substantia nigra, and periaqueductal gray (22, 23). On the other hand, 5-HT-positive cell bodies are detected mainly in three populations in adult fish brain: pretectal area, posterior tuberculum/hypothalamus, and raphe (27, 28). Interestingly, distributions of serotonergic populations and their fibers overlap with highly proliferative areas of fish brain, which may indicate serotonergic regulation in adult neurogenesis in fish (27). In adult zebrafish, serotonin has been shown to promote regeneration of motor neurons by acting on progenitor cells (29).

It is well documented that serotonergic neuron is one of the targets of estrogen in mammals (30, 31). In macaques, estrogen increases gene expression and protein contents for TPH (32), and decreases gene expression of the serotonin reuptake transporter and the 5HT1A autoreceptor (33, 34). In mammals, both ERα and ERβ are expressed in 5-HT neurons with differential distributions depending on species and sex (35–37). ERβ has been shown to regulate *tph2* expression in serotonergic neurons (38, 39). Similarly in teleost fish, effects of ovarian steroids on serotonin system have been reported in some species. In tilapia, the response of 5-HT content in brain to E_2 treatment was dependent on developmental stages. Treatment between days 7 and 10 posthatching decreased 5-HT content, while the treatment at later stages increased it (40). Similar result was obtained in Japanese sea bass, which shows a significant decrease in brain 5-HT content in fingerlings after E_2 treatment, while the content increased in juvenile group (41). Indeed, overlapping distributions of ER with raphe 5-HT innervation in telencephalon and diencephalon of adult zebrafish brain implies close association of ER and serotonergic neurons (27, 42). It has been reported that ERβ exhibits broad distribution along the brain ventricles of telencephalon and diencephalon in adult zebrafish (43), though co-localization of ER in serotonergic neurons has yet to be documented in fish.

Therefore, in this study, we tested the hypothesis that brain aromatase modulates serotonergic neuron in early development of zebrafish. In order to elucidate a possible role of brain-formed estrogen, we first examined the effects of exogenous E_2 and then MO-mediated knockdown of brain aromatase on parameters such as 5-HT contents, relative *tph* expression levels, heart rate, and thigmotaxis in zebrafish embryos and larvae.

MATERIALS AND METHODS

Fish Maintenance and Embryo Culture

Adult zebrafish (*Danio rerio*) were obtained from the local pet shop and reared in a 60-L tank. Water temperature was maintained at 26–30°C, and the light regime was 14 h of light starting at 10:00 followed by 10 h of dark. Fish were fed with TetraMin (Tetra Japan Inc.) twice a day. Fertilized eggs were collected within 15 min after fertilization and washed in embryo medium (EM) (0.004% $CaCl_2$, 0.163% $MgSO_4$, 0.1% NaCl, and 0.003% KCl) to remove debris. Embryos were transferred to a 6-well plastic plate (30 embryos in 8 mL of EM per well), and incubated at 28 ± 0.5°C. The medium was changed daily. All experimental procedures and maintenance of fish were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health.

Exposure Experiments

Stock solutions of 17β-estradiol (E_2) (Sigma-Aldrich) at 10 mM, ICI 182,780 (ICI) (Tocris Bioscience) at 10 mM, and dexamethasone (DEX) (Wako) at 100 mM were prepared in dimethyl sulfoxide (DMSO), and diluted with EM to the final concentrations indicated in the experiments. Quipazine maleate salt (Q) (Sigma-Aldrich) and fluoxetine hydrochloride (FLX) (Wako) were dissolved in ethanol at 100 and 10 mM, respectively, which were further diluted with EM to the final concentrations used in the experiments. Control embryos were cultured in 0.1% DMSO or ethanol. Exposure started at 2 hpf and continued till embryos and larvae were subjected to the assays. The media were changed daily.

Morpholino (MO) Microinjection

Morpholino antisense oligos were purchased from Gene Tool. MO sequences are shown in **Table 1**. MOs were dissolved in distilled water to 50 mg/mL and stored at −20°C. Before injection,

MO solution was heated at 65°C for 5 min and further diluted to the working concentrations (2.5 and 5 ng/nL) with deionized H_2O containing rhodamine B (Wako). The final concentration of rhodamine B was 0.08%. MO was injected into embryos at one to four cell stages using a glass microcapillary injection needle attached to the automatic nanoliter injector (Drummond Scientific). Injection volume was set at 2.3 nL per embryo. After the injection, embryos were observed under the fluorescence microscope (Leica M165 FC), and embryos that did not exhibit red fluorescence were discarded. To examine the effect of MO-mediated AroB knockdown, AroB MO designed to block translation was injected (2.5 and 5 ng/nL). Uninjected embryos (C), embryos injected with 5 ng/nL of standard control MO (Std MO), and inverted AroB MO (InvB MO) served as control groups. MO to block translation of *cyp19a1a*, ovarian aromatase (AroA MO) was also tested for 5-HT immunohistochemistry. As it has been reported that MO injection will cause off-target effect such as apoptosis through activation of *p53* gene (44, 45), MO to block translation of p53 (p53 MO) at 2 ng/nL was co-injected with AroB MO at 5 ng/nL.

For rescue experiments, the AroB mRNA (30 pg/nL) was co-injected with AroB MO (5 ng/nL). The full length AroB cDNA was obtained by One Step PrimeScript RT-PCR Kit (Takara) using total RNA from 7-dpf zebrafish larvae and AroB primers (**Table 2**). Amplified products were purified with NucleoSpin Gel and PCR Clean-up (Machery-Nagel) and subcloned into pGEM-T Easy Vector (Promega). Nucleotide sequences and orientation of the inserts were verified by DNA sequencing analysis carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI 3130 xl genetic analyzer (Applied Biosystems). Plasmid DNA was linearized with *SalI* and the full length AroB mRNA was transcribed *in vitro* by MAXIscript T7 Kit (Ambion).

TABLE 1 | Morpholino (MO) sequences.

Name	Sequence	Reference
AroA MO (ovarian aromatase MO) Genebank Acc. No.: AF226620	GGAGCAGATCACCTGCCATAAGAAC	This paper
InvA MO (inverted ovarian aromatase MO)	CAAGAATACCGTCCACTAGACGAGG	This paper
AroB MO (brain aromatase MO) Genebank Acc. No.: AF226619	ATCCTTTACCACATGCTCCATCATC	This paper
InvB MO (inverted brain aromatase MO)	CTACTACCTCGTACACCATTTCCTA	This paper
Std MO (standard control MO)	CCTCTTACCTCAGTTACAATTTATA	Gene tools
p53 MO Genebank Acc. No.: NM 131327	GCGCCATTGCTTTGCAAGAATTG	(44, 45)

Western Blot and Dot Blot Analysis

The antiserum to brain aromatase was produced in a rabbit against the synthetic peptide, CNSNGETADNRTSKE of zebrafish AroB (Sigma-Genosys). This peptide sequence has been used to raise the specific antibody as previously described (47). To confirm the specificity of the antiserum, Western blot of brain extract was conducted. Adult female zebrafish were exposed to E_2 (5 and 25 ng/L) or vehicle alone (0.00025% DMSO) for 24 h (three fish per group). Brains were pooled and homogenized in HBST buffer (100 mM NaCl, 10 mM HEPES, 0.5% Triton-X 100, 0.01% TPCK, and 0.01% TLCK). After centrifugation at 10,000 *g* for 10 min, protein concentrations in supernatant were measured using BCA protein assay kit (Thermo Scientific). Extracts (30 µg protein/sample) were separated on 12.5% SDS-PAGE and transferred to a PVDF membrane. Precision Plus Protein Unstained Standards (Bio-Rad) were used for size reference. After blocking by 1% skim milk in PBS for 1 h, the membrane was incubated with the AroB antiserum (1:500) for 2 h, and then with the secondary antibody

TABLE 2 | PCR primers and conditions.

Gene	Primer sequence (5′ → 3′)	Size of PCR product (bp)	Amplification profile
tph1a	F: TTCAAGGACAATGTCTATCG R: GGGAGTCGCAGTGTTTGATG Genebank Acc. No.: AF548566 (46)	214	94°C—30 s 55°C—30 s 72°C—60 s (35 cycles)
tph1b	F: TACCTGCAGAACCTGCCTCT R: AGAGAAGACCAGCCCCGTAT Genebank Acc. No.: BC154120 (46)	430	94°C—30 s 55°C—30 s 72°C—60 s (35 cycles)
tph2	F: GTGTGAACTCCAAAGCAGCA R: TGGTATTCCTTCCCCATCTG Genebank Acc. No.: AB125219 (46)	684	94°C—30 s 55°C—30 s 72°C—60 s (35 cycles)
cyp19a1b (AroB)	F: TTAAAGAGGTGTGTCTGTATGTGAGGTG R: GGAATTTACTCTGTGCGCCTTTAAATGT Genebank Acc. No: BC076104	1,435	42°C—10 min 94°C—30 s 60°C—15 s 72°C—90 s (40 cycles)
β-Actin	F: GGTATGGGACAGAAAGACAG R: AGAGTCCATCACGATACCAG Genebank Acc. No: AF025305 (3)	330	94°C—30 s 58°C—30 s 72°C—60 s (34 cycles)

conjugated with alkaline phosphatase (AP) (Abcam) (1:1,000) for 1 h. After washing, the membrane was incubated in AP buffer (0.1 M Tris–HCl, pH 9.5, 0.1 M NaCl, 1 M MgCl) for 1 min. Signals were developed for 2–3 min in BCIP/NBT substrate (Roche) diluted at 1:50 in AP buffer, and the reaction was stopped by 0.5 M EDTA. All the incubation steps were done at RT. The antiserum to ovarian aromatase was raised in a rabbit using a synthetic peptide, CKPDVYFRLDWLHKKHKRD of zebrafish AroA (Sigma-Genosys). Similarly, Western blot with the antiserum (1:500) was performed using the ovarian extract prepared with HBST buffer from three adult fish (30 μg/lane).

To examine the effect of MO-mediated AroB knockdown, dot blot analysis using 120 larvae at 6 dpf collected from 4 separate MO injection experiments were pooled and extracted similarly as described for the brain extract. Extracts containing 40 μg protein (3 μL) were spotted onto nitrocellulose membrane (GVS Life Science). The membrane was treated similarly as in Western blot except for the concentration of the secondary antibody at 1:2,000. Density of the blots were analyzed with NIH ImageJ software. Blots of embryo extracts treated with the pre-immunized rabbit serum were used as a negative control to subtract from the density obtained with the AroB antiserum. No changes were observed among controls (uninjected, standard control MO, and inverted AroB MO) (data not shown). The effect of MO-mediated AroA knockdown was also examined by dot blot analysis. Briefly, pooled 120 embryos at 2 dpf collected from 5 separate embryo cohorts were extracted. Extracts containing 30 μg protein (3 μL) were spotted onto nitrocellulose membrane and subjected to immunostaining using AroA antiserum at 1:500. No changes were observed among controls including inverted AroA MO (data not shown).

5-HT Immunohistochemistry

Whole-mount immunohistochemistry for 5-HT was carried out according to the previous studies (48, 49). 2-dpf embryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C. Fixed embryos were rinsed in PBS, bleached in 3% H_2O_2 for 30 min and stored in methanol at −20°C until use. For immunostaining, embryos were washed in PBS containing 0.1% Tween-20 and 0.5% Triton X-100 (PBSTX), and then permeabilization was achieved by incubation in deionized H_2O for 60 min at RT followed by 100% acetone for 8 min at −20°C. Non-specific binding was blocked by incubation in 10% normal goat serum (NGS) and 3% BSA for 3 h at RT. After several washes with PBSTX, embryos were incubated in rabbit polyclonal anti 5-HT (ImmunoStar) diluted at 1:500 in 10% NGS/PBS containing 0.3% Triton-X 100 for 2 days at 4°C. After rinsing in PBSTX for 4 h, embryos were incubated in the goat anti-rabbit IgG Alexa Fluor 488 (Molecular Probes Invitrogen Detection Technologies) diluted at 1:100 in 10% NGS/PBS overnight at 4°C. After thorough washing in PBSTX, embryos were mounted in 0.5% agarose and observed under the fluorescence microscope (Leica M165 FC). Negative controls processed by omitting incubation with the primary antibody or by replacing the primary antibody with normal rabbit serum showed no positive signals. For measurement of 5-HT-positive area, focus was adjusted on the field with the largest positive area, and NIH ImageJ software was used to quantify manually outlined areas. Immunostaining was performed using five to eight embryos per group, and the experiments were done in triplicate.

RT-PCR

Total RNA was extracted from larvae at 4 and 7 dpf (25 larvae/group) using ISOGEN II (Nippon Gene) and treated with DNase free (Ambion). cDNA was synthesized from 1 μg total RNA using Reverse Transcription System (Promega). A total reaction volume of 25 μL containing 2× GoTaq Green Master Mix (Promega), 10 μM of each primer, and 1 μL cDNA was subjected to PCR using Program Temp Control System PC708 (Astec). β-Actin was used as an internal control. Amplification conditions and primer sequences are listed in **Table 2**. The amplified products were separated on a 2% agarose gel. Levels of mRNAs expression were analyzed by NIH ImageJ software and normalized by the expression level in the control group at each developmental time. Experiments were done in triplicate.

Heart Rate Measurement

Embryos at 2 dpf were individually placed in a well of a 12-well culture plate containing 500 μL of corresponding experimental medium and kept for 15 min to allow heartbeats to resume a steady rate. Heart beats were counted manually for 15 s under a stereo microscope (Leica 58APO). Ten embryos were used for each group. Experiments were repeated three times with eggs collected from different spawns.

Thigmotaxis Assay

Assay was performed according to the protocols described previously (49, 50). Briefly, zebrafish larvae at 6 dpf were transferred into a 6-well tissue culture plate with one fish per well containing 4 mL EM. The bottom of each well was divided into two portions designated as inner and outer zones. After habituation at 28°C for 2 h followed by acclimation under the video camera for 5 min, swimming activity was recorded for 5 min. For each group, 12 larvae were used. Data from 36 larvae from three different spawns were pooled and analyzed to express % of time a fish spent in the outer zone.

Statistical Analysis

Data are presented as mean ± SEM. Statistical differences between groups were evaluated by one-way ANOVA followed by Tukey's or least significant difference *post hoc* test using IBM SPSS statistics version 19. Unpaired Student's *t*-test was used for the dot blot analysis. Kruskal–Wallis test and Mann–Whitney *U* test were used for thigmotaxis assays, as the data did not meet the assumptions required for parametric testing. Significant differences were accepted when $p < 0.05$.

RESULTS

Effect of E_2 Exposure on Serotonergic Neuron

5-HT-positive neurons were detected in the embryos at 2 dpf by whole-mount fluorescent immunohistochemistry (**Figure 1A**). Positively stained neurons were located in pretectal and thalamic

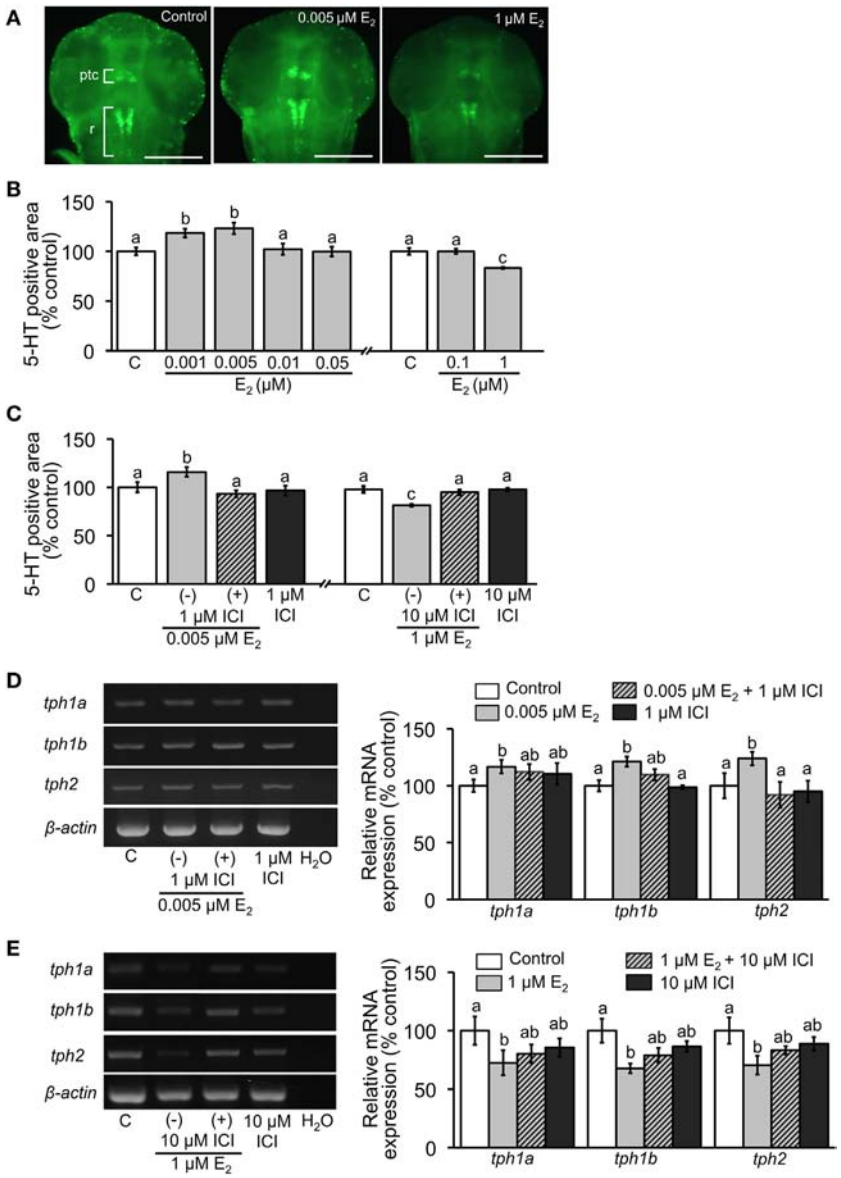

FIGURE 1 | Effect of E_2 on 5-HT-positive area and relative expression of *tph* isoforms. **(A)** Representative images of ventral view of head region showing 5-HT-positive cells in pretectal and thalamic complex (ptc) and raphe (r) at 2 dpf. Scale bar: 200 μm. **(B,C)** Measurements of the area of 5-HT-positive neurons in the experiment of E_2 exposure and co-incubation of E_2 and ICI, respectively. **(D,E)** Semi-quantitative PCR for expression of *tph* isoforms in 4 dpf larvae in the experiments of exposure to low and high doses of E_2 and co-incubation with ICI, respectively. Data are presented as a mean ± SEM. Different letters in each graph indicate significant differences ($p < 0.05$).

complex and raphe as reported previously (48, 51–54). 5-HT-positive areas were significantly increased when exposed to low doses E_2 (0.001 and 0.005 μM) but decreased in high dose (1 μM) exposure (**Figure 1B**). Effects of both low and high doses E_2 were significantly reversed by addition of 1 or 10 μM ICI, respectively (**Figure 1C**).

Relative expression levels of *tph* isoforms at 4 dpf were analyzed by semi-quantitative PCR. Significant increase in expression was detected in all *tph* isoforms when embryos were exposed to low-dose E_2 (0.005 μM). Addition of 1 μM ICI completely reversed the decreased expression of *tph2*, while expressions of *tph1a* and *tph1b* were reversed partially (**Figure 1D**). Conversely, high-dose E_2 exposure significantly decreased expression levels of all isoforms, which was partially reversed by addition of 10 μM ICI (**Figure 1E**).

When the embryos were exposed to E_2, the heart rate at 2 dpf was significantly increased in the 0.005 μM group, while significant decrease was found in the 0.1 and 1 μM groups (**Figure 2A**). Addition of 1 and 10 μM ICI significantly reversed the effects caused by low or high dose of E_2, respectively (**Figure 2A**). To verify the role of serotonergic signaling in regulation of heart rate, effects of Q (5-HT agonist) and FLX (5-HT selective re-uptake

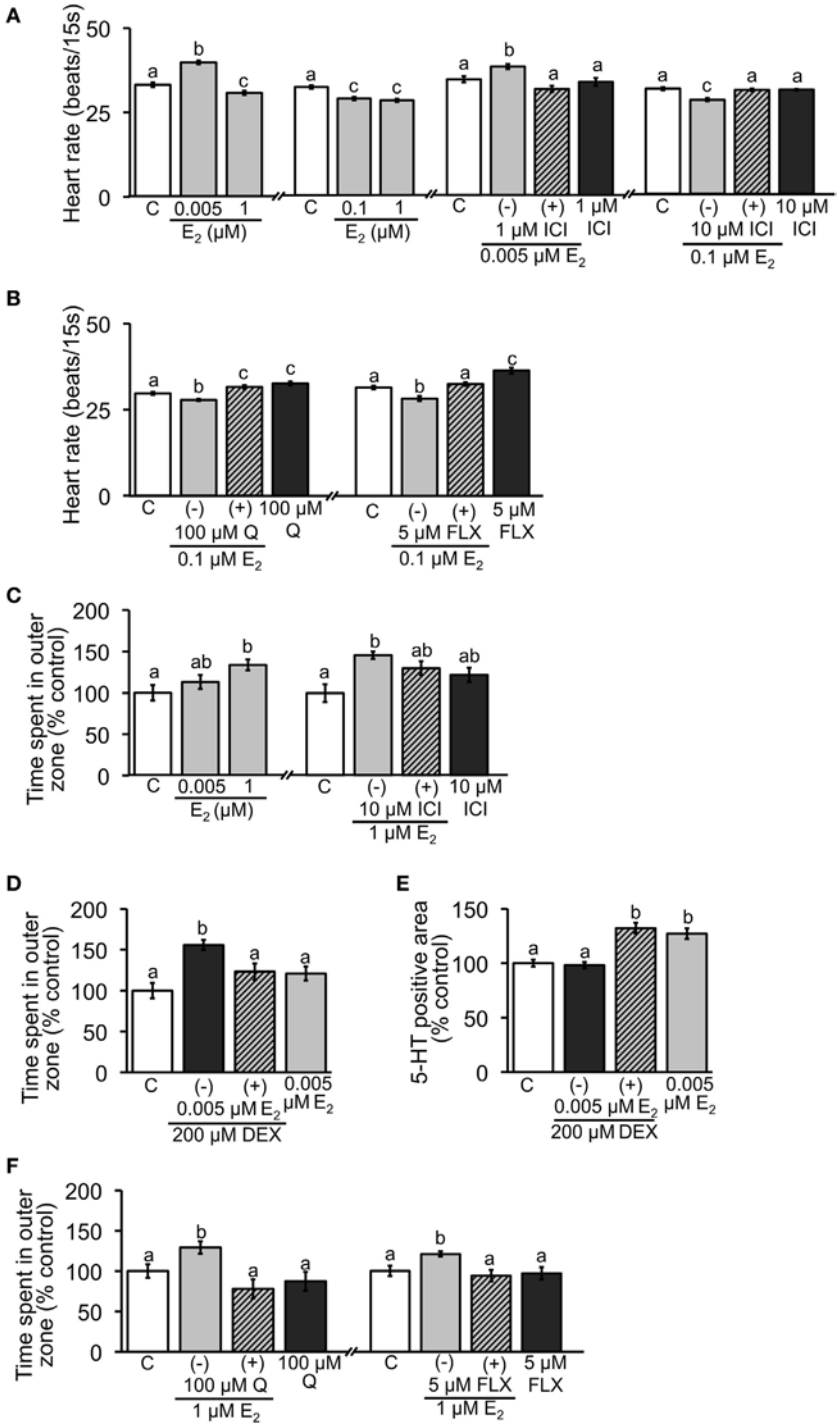

FIGURE 2 | Effect of E_2 on heart rate and thigmotaxis. Heart rate was measured in 2-dpf embryos; **(A)** exposure to low and high doses of E_2 and co-incubation with ICI; **(B)** co-incubation of E_2 and Q or FLX to examine involvement of serotonin signaling. Thigmotaxis assay was performed to evaluate anxiety level in 6-dpf larvae; **(C)** exposure to low and high doses of E_2 and co-incubation with ICI; **(D)** anxiolytic effect of low dose of E_2 when larvae were exposed to 200 μM DEX. **(E)** Measurements of 5-HT-positive neuron area in 2-dpf embryos exposed to DEX and low dose of E_2. **(F)** Effects of co-incubation of E_2 and Q or FLX on thigmotaxis to examine involvement of serotonin signaling. Data are presented as a mean ± SEM. Different letters in each graph indicate significant differences ($p < 0.05$).

inhibitor) were tested. Co-incubation with 0.1 μM E_2 significantly reversed the decreased heart rate caused by E_2. Heart rate was significantly increased when exposed to Q or FLX alone (**Figure 2B**).

Thigmotaxis assay was performed using 6-dpf larvae. Exposure to 1 μM E_2 increased the time fish spent in outer zone, suggesting that anxiety was increased, but no significant difference was

observed in 0.005 μM E_2 group (**Figure 2C**). Addition of 10 μM ICI partially reversed the increase caused by 1 μM E_2 (**Figure 2C**). To further examine the effect of low-dose E_2, embryos were exposed to 200 μM DEX and subjected to the assay. DEX alone increased the time, but co-incubation with 0.005 μM E_2 significantly reduced the increase (**Figure 2D**). Immunostaining for 5-HT showed that co-incubation of low-dose E_2 and DEX increased the positive staining, although DEX alone had no effect (**Figure 2E**). To verify the role of serotonergic signaling in thigmotaxis assay, effects of Q and FLX were tested. Both Q and FLX significantly decreased the time fish spent in outer zone caused by 1 μM E_2 exposure (**Figure 2F**).

Validation of MO-Mediated Knockdown Using the Specific Antisera

Specificity of the antisera to AroB and AroA was examined by Western blot. The anti-AroB revealed a single band at the expected size of 50 kDa in brain extract from the fish exposed to E_2 at 25 ng/L (47) (**Figure 3A**). The anti-AroA detected a single band at the expected size of 75 kDa in the ovarian extract (**Figure 3A**), which is in agreement with the previous study (55). In addition, immunohistochemistry of the ovary showed similar localization of AroA as previously described (56) (data not shown).

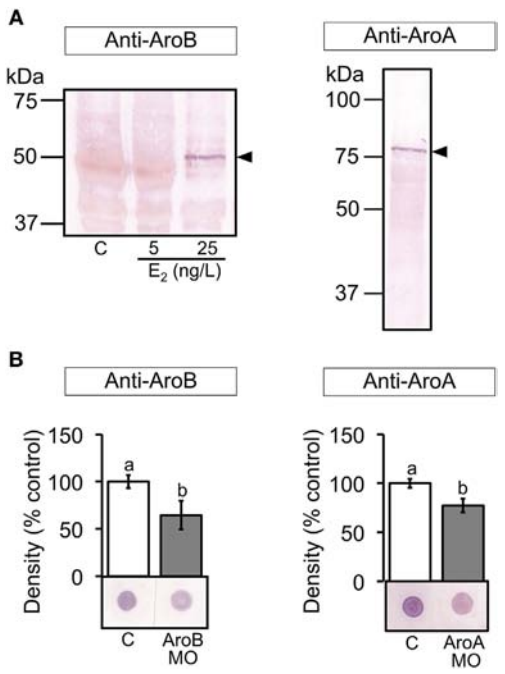

FIGURE 3 | Validation of MO-mediated knockdown on translation. **(A)** Western blots of brain extracts from control and E_2 exposed fish and ovarian extract from untreated fish were stained with the antiserum to AroB and AroA, respectively, showing a positive band at the expected size for each aromatase as indicated by arrowheads. **(B)** Dot blots of 6 and 2 dpf larval extracts using the antiserum to AroB and AroA, respectively, were analyzed. Representative blots are shown in each graph. Data are presented as a mean ± SEM. Different letters in each graph indicate significant differences ($p < 0.05$).

The dot blot analysis of the larval extracts showed that both AroB and AroA MO injections significantly decreased immunoreactivity compared to the uninjected control, indicating decreased translation of AroB and AroA, respectively (**Figure 3B**). Std MO, InvB, or InvA MO did not show any significant difference compared to the uninjected control (data not shown).

Effect of MO-Mediated Knockdown of AroB on Serotonergic Neuron

When AroB MO was injected, 5-HT-positive area was significantly decreased in the 5 ng/L group and partially decreased in the 2.5 ng/nL group compared to the uninjected control (**Figure 4A**). Injections of Std MO and InvB MO did not show any significant difference in 5-HT-positive areas compared to the uninjected control (**Figure 4A**). Moreover, the injection of AroA did not show any changes (**Figure 4A**). The decrease in 5-HT-positive area caused by AroB was completely rescued by co-injection of 30 pg/nL AroB mRNA (**Figure 4B**) and partially rescued by E_2 exposure at 0.1 μM (**Figure 4C**). When p53 MO was co-injected with AroB MO to examine off-target effect, no significant difference in 5-HT-positive area was observed, suggesting that decrease in 5-HT-positive area caused by AroB MO is not due to apoptosis caused by *p53* activation (**Figure 4D**).

The effect of AroB MO injection on relative expression of *tph* isoforms was evaluated by semi-quantitative PCR using 7-dpf larvae. While expression levels of *tph1a* and *tph1b* showed no significant changes, expression of *tph2* isoform was significantly decreased and partially rescued by E_2 exposure at 0.1 μM (**Figure 4E**).

Heart rate of AroB MO injected embryos was significantly decreased compared to the uninjected or Std MO and InvB MO injected controls (**Figure 5A**). The decrease caused by AroB MO was rescued either by co-injection of AroB mRNA or by exposure to 0.1 μM E_2 (**Figures 5B,C**). Exposure to 100 μM Q as well as to 5 μM FLX (**Figure 5D**) reversed the decrease to the control level.

In thigmotaxis assay, AroB MO injection caused significant increase in time fish spent in outer zone compared to the uninjected, Std MO injected, and InvB MO injected controls (**Figure 5E**). This effect of AroB MO was rescued either by co-injection of AroB mRNA or by exposure to 0.1 μM E_2 (**Figures 5F,G**). Exposure to 100 μM Q as well as to 5 μM FLX reversed the time fish spent in outer zone increased by AroB MO injection (**Figure 5H**).

DISCUSSION

The aim of this study is to elucidate the role of estradiol and brain aromatase in modulation of serotonergic neurons in early development of zebrafish, as the early ontogeny of serotonergic system may be one of the important factors for neuronal growth and brain development. We demonstrated that exogenous administration of E_2 biphasically affected parameters such as 5-HT-positive areas, relative expression of *tph* isoforms, heart rate and thigmotactic behavior with stimulation and suppression of serotonin system at the low dose and the high dose,

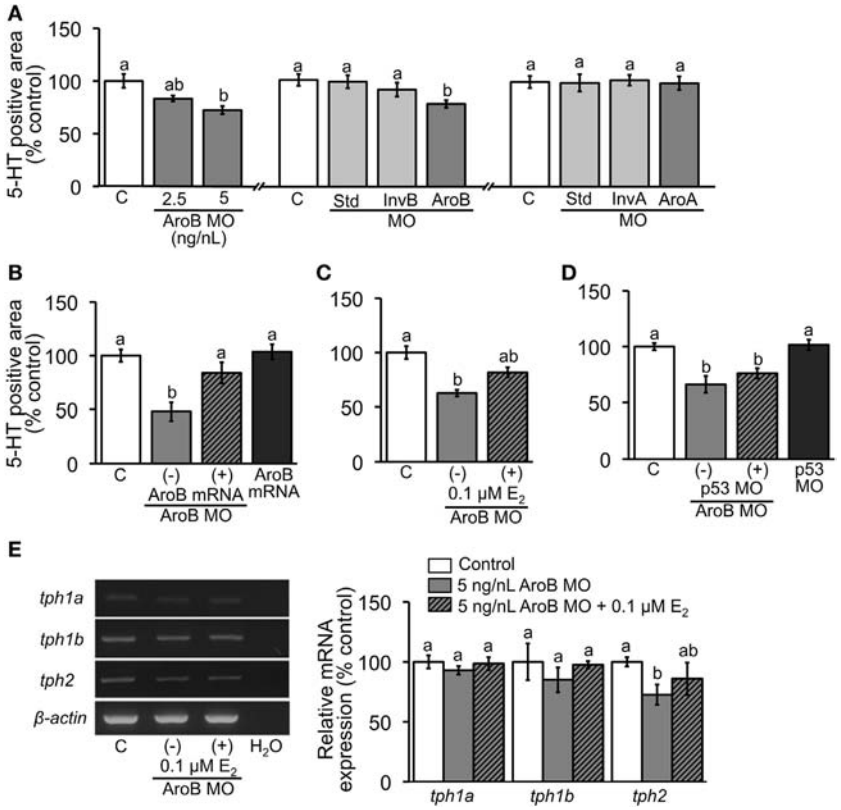

FIGURE 4 | AroB MO-mediated effect on serotonergic neuron. 5-HT-positive neuron area was measured in 2-dpf embryos; **(A)** injection of AroB MO and control MOs (Std MO, InvB MO and AroA MO); **(B,C)** co-injection of AroB mRNA and exposure to E_2, respectively, to rescue the effect of AroB MO; **(D)** co-injection of p53 MO to test off-target effect of AroB MO. **(E)** Semi-quantitative PCR measurement of expression of *tph* isoforms in 6-dpf larvae injected with AroB MO with and without exposure to E_2. Data are presented as a mean ± SEM. Different letters in each graph indicate significant differences ($p < 0.05$).

respectively, through acting on ER. On the other hand, activities of serotonergic neurons were suppressed by AroB MO-mediated knockdown, suggesting that brain-formed E_2 in early development stimulates serotonergic neurons, which is in accordance with the results of the low-dose E_2. Recent study shows that MO-mediated brain aromatase knockdown results in a significant decrease in E_2 concentration in 48 hpf embryos (57), which supports that our MO experiments reflect the reduction of estrogen production.

Non-monotonic dose responses of hormones and endocrine-disrupting chemicals have been widely documented (58). Estrogen among other hormones is known to exhibit biphasic dose-dependent effects in various physiological processes (59–66). However, only limited information is available in regards to serotonin system. There is one study in fish showing that low dose of E_2 stimulated monoamine oxidase activity and decreased 5-HT content in hypothalamus in ovariectomized catfish, while the result was opposite for high dose (67). Our study demonstrates that biphasic dose-dependent effects of E_2 on serotonergic neuron in fish, and shows that the effects or both low and high doses are mediated through ER, indicating physiological relevance. The effect of the low dose of E_2, stimulating serotonergic neuron, is likely to reflect the role of endogenous E_2 in embryos, as AroB MO-mediated effects demonstrate that brain-formed estrogen is necessary to maintain activity of serotonergic neuron in embryos. Mechanisms of biphasic responses are complex, but may be in part controlled by downregulation and desensitization of receptors (57, 68). Thus, effects of high doses of E_2 on serotonergic neuron in this study may be due to downregulation/desensitization of ERs. Sequence analysis of the promoter region of zebrafish *tph* isoforms shows the presence of 1/2 ERE in the upstream of transcription start site in all isoforms, suggesting possible nuclear action of estrogen, though their functional analysis is yet to be reported. In human serotonergic cell line, binding of E_2 and ERβ has been shown to directly interact with 1/2 ERE of *tph2* promoter to elicit gene expression (39). In addition to the classical action of E_2 on nuclear receptors membrane ERs plays an important role in brain (69, 70). Interaction between membrane ERs and the metabolic glutamate receptor in the brain provides a rapid and transient E_2 action (71, 72). Membrane bound G-protein-coupled ER, GPER/GPR30, also known to be involved in modulating rapid non-genomic action of E_2, plays a role in several brain areas (73). Estrogen action through GPR30 has been suggested in regulation of serotonergic neuron in mammals (74). Further studies are required to elucidate the mechanisms by which estrogen regulates serotonergic neuron in zebrafish.

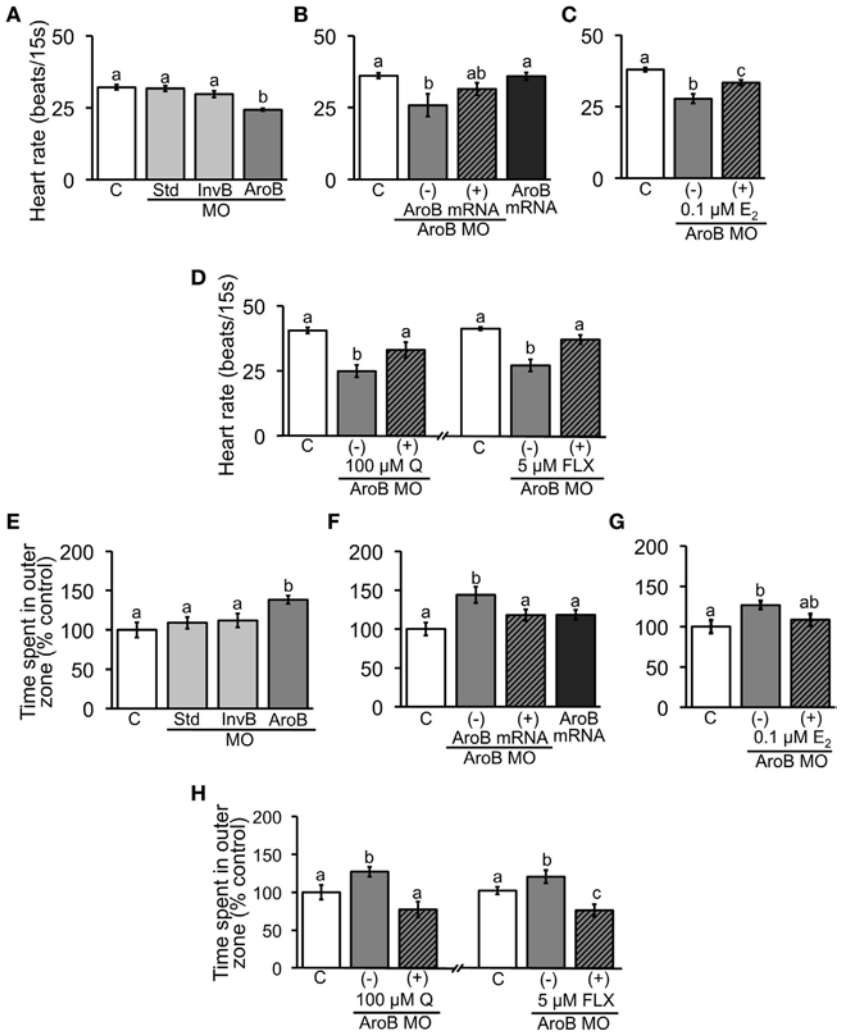

FIGURE 5 | AroB MO-mediated effect on heart rate and thigmotaxis. Heart rate was measured in 2-dpf embryos; **(A)** injection of AroB MO and control MOs; **(B,C)** co-injection of AroB mRNA and exposure to E_2, respectively, to rescue the effect of AroB MO; **(D)** injection of AroB MO with and without exposure to Q and FLX to examine involvement of serotonin signaling. Thigmotaxis assay was conducted using 6-dpf larvae; **(E)** injection of AroB MO and control MOs; **(F,G)** co-injection of AroB mRNA and exposure to E_2, respectively, to rescue the effect of AroB MO; **(H)** injection of AroB MO with and without exposure to Q and FLX to examine involvement of serotonin signaling. Data are presented as a mean ± SEM. Different letters in each graph indicate significant differences ($p < 0.05$).

Attenuation of serotonergic neuron by AroB MO-mediated knockdown clearly demonstrated that brain-formed estrogen is necessary to maintain the serotonin system to control heart rate and anxiety behavior in early development of zebrafish. Validity of AroB knockdown was supported by several lines of evidence. Immunoreactivity to the antiserum specific to AroB was decreased in AroB MO injected embryos. In addition to no significant effects found in the controls including standard MO, inverted AroB MO and AroA MO-injected embryos, AroB MO-mediated effects were rescued by co-injection of AroB mRNA and exposure to E_2. Off-target effect of MO injection was also examined by knockdown of *p53*, showing that the decreased 5-HT-positive area caused by AroB MO is not through activation of *p53*. The decrease in 5-HT-positive area by AroB MO injection indicates that brain-formed estrogen stimulates 5-HT synthesis, which is in accordance with the stimulatory effect of low-dose E_2. When the relative expressions of *tph* isoforms were examined in AroB MO injected embryos, only *tph2* expression was significantly decreased by AroB MO, which is well supported by the previous studies showing *tph2* but not *tph1* is expressed in raphe 5-HT neurons (54, 75, 76). Expression of *tph2* in 5-HT neurons in pretectal and hypothalamic complex starts to appear at 60 hpf (76). On the other hand, whereas in the exposure experiments, expressions of all isoforms were affected by E_2; increased by low dose and decreased by high dose. The results support the previous studies reporting that *tph2* expressed in brain is responsible for 5-HT synthesis in the zebrafish (27, 28, 54). Thus, we provide the evidence that brain-formed estrogen stimulates *tph2* expression to maintain 5-HT content in the serotonin neuron. The effects of E_2 exposure on *tph* isoforms

indicate E_2 also modulates serotonin biosynthesis in tissues outside the brain. 5-HT has been reported to be produced in various organs including intestine which is the major source of 5-HT in the body and TPH1 is responsible for its synthesis (54, 77). Investigation of estrogen regulation of serotonin production in intestine during development would be a future research interest.

The parameters of physiological functions of serotonin system, heart rate, and thigmotactic behavior were measure to verify the activity of serotonergic neuron. The results were in accordance to the changes in 5-HT levels in the neurons; the increased 5-HT levels are accompanied by the increased heart rate and decreased thigmotactic behavior, while the contrary was true for the decreased 5-HT levels. Serotonin is known to be involved in cardiovascular function, and the effect of central serotonergic neuron is mediated through autonomic nervous system in mammals (18). Our result of the low-dose (0.005 μM) E_2 which increased heart rate corroborates the effect of MO-mediated AroB knockdown, indicating that nanomolar level of brain-formed estrogen, or even lower level in the tissue, stimulates serotonergic neuron to increase the heart rate. Exposure to quipazine (serotonin agonist), or fluoxetine (selective serotonin reuptake inhibitor, SSRI) completely reversed the decreased heart rate caused by the high dose (0.1 μM) E_2, or AroB MO injection confirming that heart rate is under the control of serotonin signaling. Taken together with a recent study showing that GPER in the pituitary of zebrafish embryo regulates heart rate through thyroid hormone (78), estrogen in brain centrally regulates heart rate through various mechanisms. On the other hand, cardiac functions are directly regulated by estrogen (79) and aromatase has been detected in the heart tissues such as myocardium in mice (80–82). Therefore, it is possible that AroB MO injection may affect aromatase expression in the heart and locally produced estrogen modulates heart rate. In some teleost fish, both ovarian and brain aromatases are expressed in the heart (83–85), but in ricefield eel only brain aromatase is detected (86), while only ovarian aromatase is present in spotted scat (87). These difference may be due to technical difference as well as differences in species and developmental and physiological status. Our preliminary analysis indicated the expression of ovarian aromatase but not the brain form in adult zebrafish heart (data not shown), suggesting that our result of MO injection is likely to be mediated through knockdown of brain aromatase expressed in the brain not in the heart. However, expression of aromatase in the heart during development needs to be verified.

Thigmotaxis is an evolutionally conserved behavior associated with fear and has been shown to be affected by anxiolytic and anxiogenic compounds (88); thus, it has been used to measure anxiety levels in animals including fish (89–92). Our present study shows the high dose E_2 (1 μM), which decreased the 5-HT level, significantly increased anxiety (increased time spent in outer zone), and this increase was abolished by addition of 5-HT agonist (Q) or SSRI (FLX), indicating the effect of high dose E_2 is mediated through serotonin signaling. Similarly, increased anxiety by AroB MO was also abolished by Q or FLX, which supports our hypothesis that brain-formed estrogen modulates serotonergic neuron. Despite our expectation, the low-dose E_2 (0.005 μM), which increased the 5-HT level, did not cause reduction of anxiety. Therefore, we further examined to see if the low-dose E_2 exerts anxiolytic effect in the larvae exposed to DEX to induce stress, and indeed, low-dose E_2 decreased the anxiety level. Thus, our data demonstrate a negative correlation between anxiety behavior and 5-HT level, which is in accordance with previous studies. In mammals, depletion of 5-HT level in rat brain induces anxiety (93) and acute reduction of tryptophan increases the anxiety level in patients of a social anxiety disorder (94). The role of 5-HT in anxiety is also reported in zebrafish (20, 95). Buspirone, partial agonist for 5-HT1A receptor, exerts anxiolytic-like effect in zebrafish (96). The phenotype of zebrafish leopard strain, which is characterized by increased anxiety-like behavior, is rescued by acute treatment with FLX (97). Taken together, we provide the evidence that brain-formed E_2 has an important role in modulating anxiety through serotonergic transmission.

In contrast to mammalian brain, where aromatase is expressed in both neuron and glia (98, 99), it is well documented that brain aromatase in fish is exclusively expressed in RGCs along the ventricles of forebrain, midbrain, and hindbrain serving as neural progenitors (10, 11, 47). While most RGCs are transformed into astrocytes by the time of adulthood in mammalian brain (100), presence of RGCs persists throughout the lifespan of zebrafish, which is considered to be one of the contributing factors for high capacity of neuronal proliferation (101). On the other hand, serotonin is known to play a role in neurogenesis (102). In adult zebrafish, it has been reported that projection of 5-HT neurons in raphe to ventricular surface of the brain, where highly proliferative cells are found. In addition, expression of 5-HT receptors are localized in ventricular surface in larval and adult zebrafish (27, 103). Thus, it may be possible that RGCs in ventricular surface are innervated by 5-HT neurons in raphe and modulated for neurogenesis. Interestingly, it has been reported that AroB-positive RGCs in PVO area in adult zebrafish has an ability to differentiate into serotonergic neuron (104). Taken together with our present study providing the evidence that brain-formed estrogen is necessary to maintain the levels of 5-HT in neurons in raphe, we can hypothesize that differentiation of AroB-expressing RGCs in serotonin neurons is regulated by serotonin neuron in raphe, whose activity is modulated by estrogen produced by AroB. It has been shown that placenta aromatase activity and expression are stimulated by serotonergic 5-HT2A receptor signaling (105). In goldfish, AroB expression in RGCs *in vitro* is upregulated by dopamine with modulation by E_2 (106). Nonetheless, estrogen biosynthesis and homeostasis in CNS are regulated and fine-tuned by multiple factors like neurotransmitters and hormones, so that diverse functions of estrogen can be coordinated.

In conclusion, this study demonstrates that estradiol exhibits a biphasic effect on serotonergic neuron, and that brain aromatase, thus brain-formed estrogen plays a significant role in modulating serotonin levels to sustain appropriate development and functions of serotonergic neurons which regulate heart rate and anxiety behavior in zebrafish embryos and larvae. Considering the role of serotonergic neurons in neural

development and neurogenesis, it is possible to postulate that one of the mechanisms of brain aromatase and brain-formed estrogen to regulate neurogenesis in teleost brain may be through modulation of serotonergic system, which awaits future investigation.

ETHICS STATEMENT

All experimental procedures and maintenance of fish were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health.

AUTHOR CONTRIBUTIONS

ZU and MK designed the experiments. ZU performed the experiments and analyzed the data. ZU and MK wrote the paper.

ACKNOWLEDGMENTS

ZU was supported by MEXT (Ministry of Education, Culture, Sports, Science and Technology, Japan) scholarship. We would like to thank Prof. Akiyoshi Takahashi and Assoc. Prof. Kanta Mizusawa for helping ZU to generate AroB mRNA, and Prof. Kazufumi Takamune for his support with western blot experiments.

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Conflict of Interest Statement: The authors declare that there is no conflict of interest regarding the publication of this article.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 16 May 2018
doi: 10.3389/fendo.2018.00243

The Role of Estrogen Receptor β in the Dorsal Raphe Nucleus on the Expression of Female Sexual Behavior in C57BL/6J Mice

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OPEN ACCESS

Edited by:
Ishwar Parhar, Monash University Malaysia, Malaysia

Reviewed by:
Hirotaka Sakamoto, Okayama University, Japan
Ignacio Camacho-Arroyo, Universidad Nacional Autónoma de México, Mexico

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *16 March 2018*
Accepted: *27 April 2018*
Published: *16 May 2018*

Citation:
Sano K, Morimoto C, Nataka M, Musatov S, Tsuda MC, Yamaguchi N, Sakamoto T and Ogawa S (2018) The Role of Estrogen Receptor β in the Dorsal Raphe Nucleus on the Expression of Female Sexual Behavior in C57BL/6J Mice. Front. Endocrinol. 9:243. doi: 10.3389/fendo.2018.00243

17β-Estradiol (E_2) regulates the expression of female sexual behavior by acting through estrogen receptor (ER) α and β. Previously, we have shown that ERβ knockout female mice maintain high level of lordosis expression on the day after behavioral estrus when wild-type mice show a clear decline of the behavior, suggesting ERβ may be involved in inhibitory regulation of lordosis. However, it is not identified yet in which brain region(s) ERβ may mediate an inhibitory action of E_2. In this study, we have focused on the dorsal raphe nucleus (DRN) that expresses ERβ in higher density than ERα. We site specifically knocked down ERβ in the DRN in ovariectomized mice with virally mediated RNA interference method. All mice were tested weekly for a total of 3 weeks for their lordosis expression against a stud male in two consecutive days: day 1 with the hormonal condition mimicking the day of behavioral estrus, and day 2 under the hormonal condition mimicking the day after behavioral estrus. We found that the level of lordosis expression in ERβ knockdown (βERKD) mice was not different from that of control mice on day 1. However, βERKD mice continuously showed elevated levels of lordosis behavior on day 2 tests, whereas control mice showed a clear decline of the behavior on day 2. These results suggest that the expression of ERβ in the DRN may be involved in the inhibitory regulation of sexual behavior on the day after behavioral estrus in cycling female mice.

Keywords: adeno-associated virus-mediated RNA interference, estrogen receptor β, estrogen receptor α, lordosis, dorsal raphe nucleus, estrogen, progesterone, serotonin

INTRODUCTION

The expression of female sexual behavior undergoes cyclic change during an estrus cycle. An ovarian hormone, 17β-estradiol (E_2) plays an essential role in this cyclic regulation of the behavior. In rodents, lordosis, a stereotypical female sexual behavior is expressed at high levels only during the time around ovulation, the period called as "behavioral estrus" occurring subsequently to the exponential elevation of circulating E_2. E_2 is known to act through at least two receptor subtypes, estrogen receptor (ER) α and β. Studies using knockout mouse models have shown that the level of sexual behavioral expression is greatly reduced in ERα knockout (αERKO) females (1–3) whereas in the ERβ knockouts (βERKO), such alteration is not observed (4, 5). It has also been reported that the selective ERα

agonist, propyl-pyrazole triol facilitates female sexual behavior in ovariectomized female rat whereas the selective ERβ agonist, diarylpropionitrile (DPN) is unable to do so (6). Furthermore, Musatov et al. (7) demonstrated that the site-specific knockdown of ERα in the ventromedial nucleus of the hypothalamus (VMN) leads to a complete disappearance of female sexual behavior in mice. These studies collectively suggest that E_2 action through ERα, particularly in the VMN, is necessary for normal display of female sexual behavior whereas ERβ may not be involved in facilitatory regulation of female sexual behavior by E_2. However, close observation of gonadally intact βERKO mice has revealed an intriguing behavioral phenotype that the period of behavioral estrus may be extended in βERKO females (4). In these mice, the level of sexual receptivity was reported to stay high until the day after behavioral estrus when their respective wild-type (WT) mice show a clear decline of the behavior. In addition, except the day of behavioral estrus and the day after, βERKO females express very little receptive behavior similar to WT females (4). To replicate this finding in more hormonally controlled setting, we performed a preliminary experiment in ovariectomized βERKO females treated with exogenous ovarian steroids. We found that these females continuously showed elevated levels of lordosis expression 72 h after estradiol benzoate (EB) and 24 h after progesterone administration that mimic typical hormonal condition of the day after behavioral estrus (unpublished data). These findings collectively suggest that E_2 is not only promoting sexual behavior in females during the behavioral estrus with ERα dependent mechanisms but also involved in active inhibition of lordosis expression on the day after behavioral estrus by acting through ERβ. To further test this hypothesis and elucidate possible neural mechanisms of ERβ-mediated inhibitory regulation of lordosis, we aimed to identify the responsible brain site(s) in this study.

The dorsal raphe nucleus (DRN) is one of the brain areas that possibly act inhibitory on the expression of female sexual behavior. Lesions of this area are reported to increase lordosis expression in both EB-treated ovariectomized female rat and EB-treated castrated male rat (8–11) whereas the electrical stimulation of the DRN can lead to marked and immediate suppression of lordosis in ovariectomized female rat hormonally primed with EB and progesterone (12). In addition to its possible inhibitory role in the regulation of female sexual behavior, the DRN is also known for high levels of ERβ expression that is about two-fold of that of ERα within the nucleus (13, 14). Thus, it is reasonable to hypothesize that the DRN may be the brain site responsible for ERβ-mediated inhibitory action of E_2 in female sexual behavior. To test this hypothesis, we employed a virally mediated RNA interference method and examined the effects of site-specific knockdown of ERβ in the DRN on the expression of lordosis behavior.

MATERIALS AND METHODS

Subjects

Adult C57BL/6J female mice originally purchased from a commercial breeder (CLEA, Japan) and maintained in a breeding colony at the University of Tsukuba. All mice were housed under standard conditions (23 ± 2°C) with a 12:12-h light/dark cycle (lights off at 12:00). Food and water were provided *ad libitum*. All procedures were approved by the Animal Care and Use Committee and the Recombinant DNA Use Committee at the University of Tsukuba and conducted strictly in accordance with the National Institutes of Health guidelines. All efforts were made to minimize the number of animals and their suffering.

Design of Small Hairpin (sh) RNA for ER β Silencing

Adeno-associated virus (AAV) vectors expressing an shRNA against either the sequence specific for the ERβ gene (AAV-shERβ: 5′-GATCCCCGCCACGAATCAGTGTACCATCTTCCTGTCAATGGTACACTGATTCGTGGCTTTTTTGGAAT-3′ and 5′-CTAGATTCCAAAAAAGCCACGAATCAGTGTACCATTGACAGGAAGATGGTACACTGATTCGTGGCGGG-3′) or the sequence specific for luciferase (LUC) as control (AAV-shLUC: 5′-GATCCCCCCGCTGGAGAGCAACTGCATCTTCCTGTCAATGCAGTTGCTCTCCAGCGGTTTTTGGAAT-3′ and 5′-CTAGATTCCAAAAACCGCTGGAGAGCAACTGCATTGACAGGAAGATGCAGTTGCTCTCCAGCGGGGG-3′) were used. The nucleotides specific for ERβ or LUC are underlined. These vectors also express enhanced green fluorescent protein (GFP) as a reporter to visually detect transduced neurons.

Stereotaxic Surgery

Adult female mice (11.4 ± 2.2 weeks old) were assigned to either vector treatment (i) AAV-shERβ [ERβ knockdown (βERKD)] or (ii) AAV-shLUC (Control). They were anesthetized with sodium pentobarbital (60 mg/kg) and placed in a stereotaxic frame. A 5 µl Hamilton syringe inclined at 25° was aimed at the DRN [anteroposterior, −4.96 mm, mediolateral, 0.00 mm; dorsoventral, −3.40 mm] that was determined based on The Mouse Brain Stereotaxic Coordinates (15). Each mouse was injected with 0.5 µl of either AAV-shERβ or AAV-shLUC (10^{12} packaged genomic particles) using a micropump injector (World Precision Instruments Inc., USA). The injection lasted 5 min, and the needle was left in place for an additional 10 min following the end of infusion. Mice were then group housed with their littermates (4–5 mice/cage) until they were used for behavioral studies.

Ovariectomy and Hormone Treatment

Two weeks after stereotaxic surgery, all mice were ovariectomized (OVX) under isoflurane inhalation anesthesia. Mice were then individually housed in plastic cages (19 cm × 29 cm × 19 cm). All female mice were hormonally treated with weekly subcutaneous injections of EB (5 µg/0.1 ml sesame oil) followed by progesterone (P; 250 µg/0.1 ml sesame oil) at 44–46 h later, to mimic hormonal conditions in cycling females.

Sexual Behavior Test

Starting 1 week after OVX, all mice were weekly tested for sexual behavior against a sexually experienced male (gonadally intact ICR/Jcl) mouse in the males' home cages on two consecutive days, for a total of six trials during 3 weeks. For each week, mice were first tested 4–5 h after P injection which mimic the day of behavioral estrous (day 1), then tested again 24 h later (day 2)

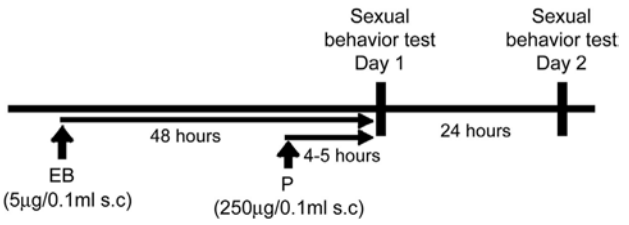

FIGURE 1 | Experimental paradigm.

(**Figure 1**). Each test was performed during the dark phase (starting 2–3 h after lights off) of the light/dark cycle under red light illumination. Each test lasted until females received 15 mounts or intromissions. Male intromissions were terminated (after about 8 thrusts) by the experimenter after female's behavioral responses were scored. Lordosis quotient (LQ) was calculated by dividing the number of lordosis responses by 15, the number of mounts or intromissions. In addition, each lordosis posture was given a score of 1–3 depending on the degree of dorsiflexion of the vertebral column and behaviors. For calculation of Lordosis Quality Scores, only lordosis responses that were given a score between 1 and 3 were included and averaged in each mouse. Mice with no lordosis response were excluded from the analysis in each test.

Tissue Preparation for Immunohistochemistry

Two weeks after the completion of behavioral testing, all mice were given subcutaneous injection of EB (5 μg/0.1 ml sesame oil). Forty-eight hours later, the time point matching the hormonal condition of day 1 tests, they were deeply anesthetized with a solution of 1:1 mixture of sodium pentobarbital (60 mg/kg) and heparin (1,000 U/kg) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS; pH 7.2) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.2). Brains were removed and post-fixed overnight at 4°C in 4% PFA in 0.1 M PB. They were then rinsed with 0.1 M PB and cryoprotected in 0.1 M PB containing 30% sucrose.

Immunohistochemistry

Free-floating coronal sections (35 μm thickness) prepared on freezing microtome were split into four series (140 μm apart). One series of sections from each mouse was incubated in a rinse buffer, PBS-X (PBS and 0.2% Triton X-100), containing 1% hydrogen peroxide for 20 min to inhibit endogenous peroxidase activity, and then blocked in an incubation buffer (3% skim milk and 3% bovine serum albumin in PBS-X) for 2 h at room temperature. After the blocking process, they were incubated in a goat polyclonal anti-GFP antiserum (1:5,000; ab6673, Abcam) dissolved in the incubation buffer overnight at 4°C. Sections were then treated with a 1:250 dilution of biotinylated rabbit anti-goat secondary antibody (Vector Laboratories) in the incubation buffer for 2 h at room temperature, followed by 1 h incubation with avidin-biotin complex (Vectastain ABC Elite kit, Vector Laboratories) in TBS. After the completion of antiserum reaction procedures, sections were visualized with 0.02% diaminobenzidine and 0.003% hydrogen peroxide in TBS. A few sections from each group were also processed for immunohistochemical staining for ERβ. They were incubated in a rinse buffer, PBS-X, containing 1% hydrogen peroxide for 20 min, and then blocked in an incubation buffer (5% bovine serum albumin in PBS-X) for 2 h at room temperature. After the blocking process, they were incubated in a goat polyclonal anti-ERβ antiserum (1:1,000; Z8P, lot 10766190, Zymed Laboratories) dissolved in the incubation buffer for three days at 4°C. Sections were then treated with a1:250 dilution of biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) in the incubation buffer for 4 h at room temperature, followed by 1 h incubation with avidin-biotin complex (Vectastain ABC Elite kit, Vector Laboratories) in TBS. After the completion of antiserum reaction procedures, sections were visualized with 0.03% diaminobenzidine, 0.15% $NiNH_4SO_4$, and 0.003% hydrogen peroxide in TBS.

All sections were mounted in gelatin-coated slides, air-dried and dehydrated through ascending alcohol series, which were cleared with xylene, and cover slipped with Permount (Fisher Scientific, USA). To verify the specificity of the immunohistochemical procedures, we included negative controls in which the primary antiserums were omitted from the staining procedure. In these conditions, neither cells nor fibers were stained.

The seven sections containing the DRN [Bregma −4.24 to −5.20 mm (15)] were obtained and photographed at 40× magnification with a digital camera mounted on an Olympus microscope (DP21, Olympus, Japan). To verify successful infusion of shRNA in the DRN, the distribution of GFP immunoreactive cells was evaluated on these seven sections.

Statistics

Lordosis quotients in the Control and βERKD groups were analyzed by a three-way analysis of variance (ANOVA) for repeated measurements for the main effects of vector treatment, day, week, and their interactions. LQs in the missed βERKD group were analyzed by a two-way ANOVA for repeated measurement for the main effect of day and week, and their interactions. ANOVAs were followed by Bonferroni *post hoc* test when it was appropriate. Lordosis Quality Scores were analyzed and compared by Mann–Whitney *U* test between day 1 and day 2 in each week in each vector treatment group. All data were presented as mean ± SEM. Statistically significant differences were considered at $p < 0.05$ (two-tailed). LQs were analyzed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA) statistical package, and Lordosis Quality Scores were analyzed using StatView 5.0.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Verification of Transfection

Similar to previous reports in the medial amygdala and medial preoptic area (16), the efficacy of the vector in silencing the expression of ERβ site-specifically in the DRN was confirmed as shown in **Figure 2**.

Successful infusion of shRNA in the DRN was confirmed in 12 females injected with AAV-shLUC (Control) and 10 females with AAV-shERβ (βERKD) (**Figure 3**). In addition to these mice in

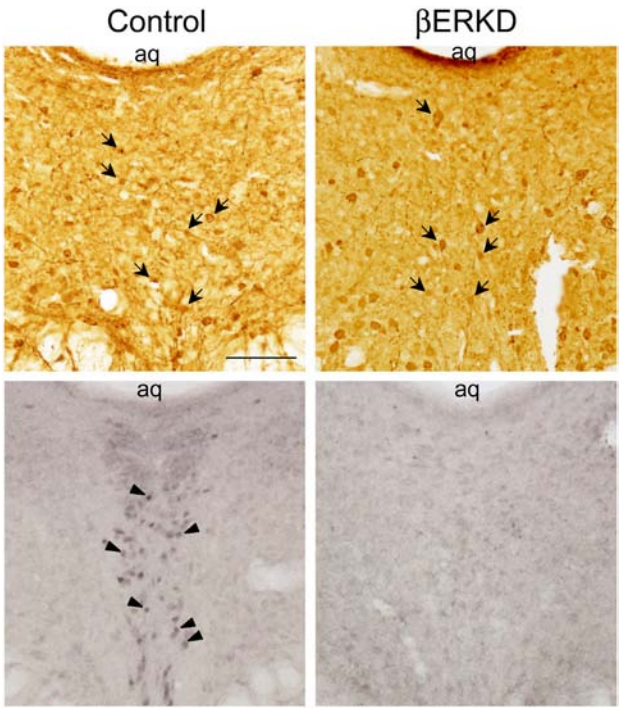

FIGURE 2 | Representative photomicrographs of immunohistochemically labeled cells for green fluorescent protein (GFP) in brain sections of control and ERβ knockdown (βERKD) groups (top panels), and those for estrogen receptor (ER) β in the adjacent sections (bottom panels). GFP and ERβ immunoreactive cells were detected by the methods described in the present and previous studies (16). The scale bar in the panel represents 100 μm. Arrows in the top panels indicate GFP stained cells, and arrow heads in the bottom panel indicate ERβ stained cells.

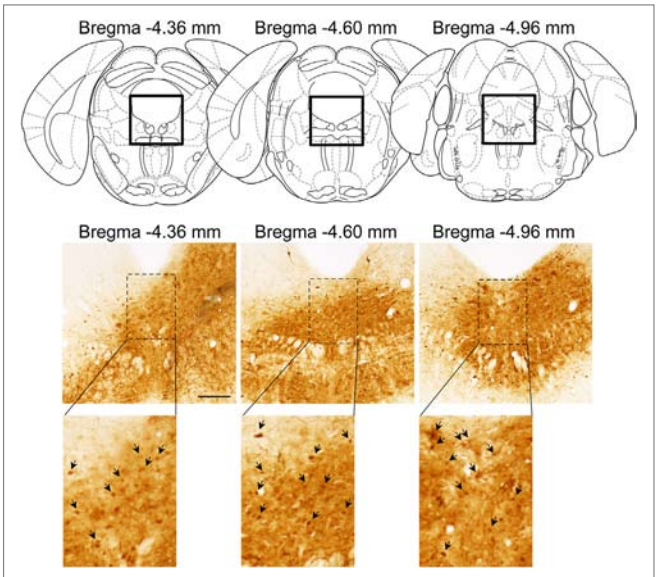

FIGURE 3 | Representative distribution pattern of adeno-associated virus within the dorsal raphe nucleus determined based on green fluorescent protein immunohistochemistry. The scale bar represents 200 μm.

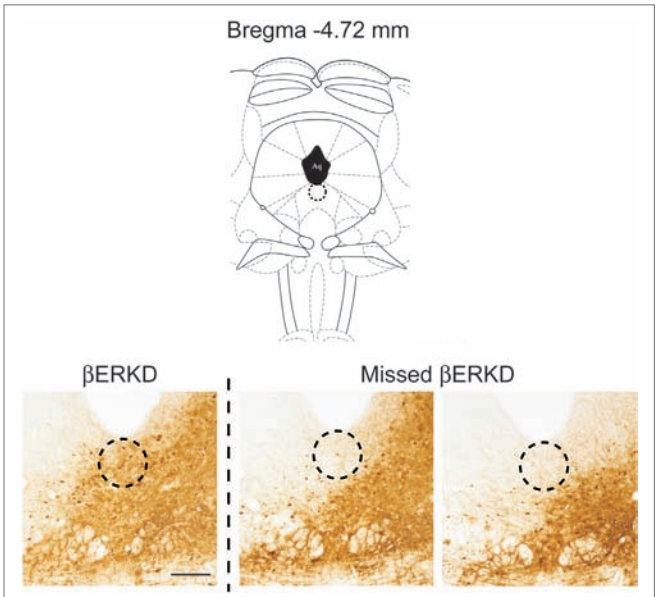

FIGURE 4 | Comparison of distribution pattern of adeno-associated virus within the mediodorsal area of dorsal part of the dorsal raphe nucleus (Bregma −4.72 mm) determined by green fluorescent protein immunohistochemistry in successful ERβ knockdown (βERKD) (left) and missed βERKD (middle and right) mice. The scale bar represents 200 μm.

which AAV-shERβ was successfully infused into the entire DRN, there were five females in which AAV-shERβ was not spread in the mediodorsal area of dorsal part of the nucleus (**Figure 4**). These animals were classified as a missed βERKD group, and their behavioral data were analyzed separately. The mediodorsal area of dorsal part of the DRN was defined as the area within a 0.2 mm diameter circle placed on right below the bottom edge of the aqueduct and ranging from Bregma −4.48 to −4.84 mm (15) (**Figure 4**).

Effect of ERβ Knockdown in the DRN on the LQ

Knocking down of ERβ in the DRN dramatically affected LQ in day 2, but not in day 1. As shown in **Figure 5**, Control and βERKD showed similar levels of LQ on day 1 test in each week. In the Control group, the LQs were greatly decreased on day 2 compared with day 1 in all three weekly tests, whereas βERKD showed similar levels of LQ on day 2 as those on day 1 in all three tests. Three-way repeated measures ANOVA revealed significant effects of Day [$F(1, 20) = 22.247$, $P < 0.0001$], Treatment [$F(1, 20) = 4.881$, $P < 0.05$], and Week [$F(2, 40) = 37.487$, $P < 0.0001$]. There were significant interactions between Treatment and Day [$F(1, 20) = 5.518$, $P < 0.05$], Treatment and Week [$F(2, 40) = 3.876$, $P < 0.05$]. There were no statistically significant interactions between Day and Week [$F(2, 40) = 0.390$, ns] or Treatment, Day, and Week [$F(2, 40) = 0.381$, ns]. *Post hoc* analysis revealed that LQ on day 2 was lower than day 1 in the Control group alone (the day 1 vs. the day 2 in each test, $P < 0.01$, for control) in each test. However, there was no significant difference between day 1 and day 2 in LQ of βERKD in each test.

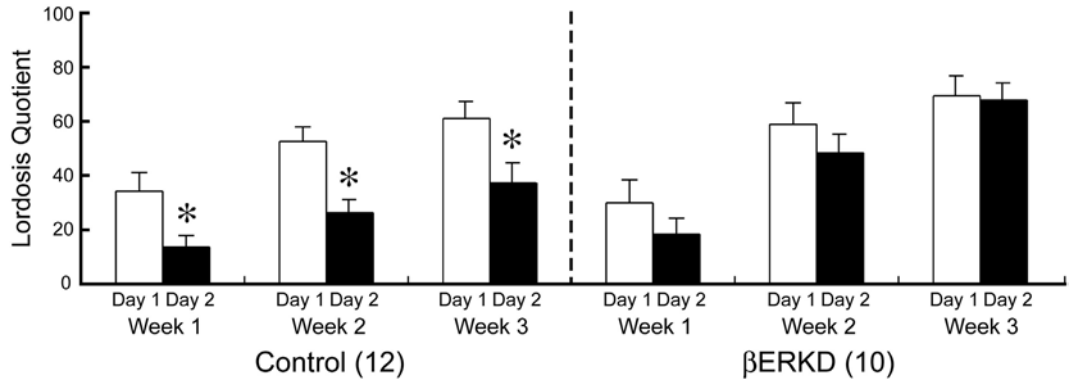

FIGURE 5 | Effect of estrogen receptor (ER) β knockdown in the dorsal raphe nucleus on Lordosis quotient (LQ). LQ of control (left) and ERβ knockdown (βERKD) (right) in each test. *$P < 0.05$ vs. day 1. Data presented as mean ± SEM.

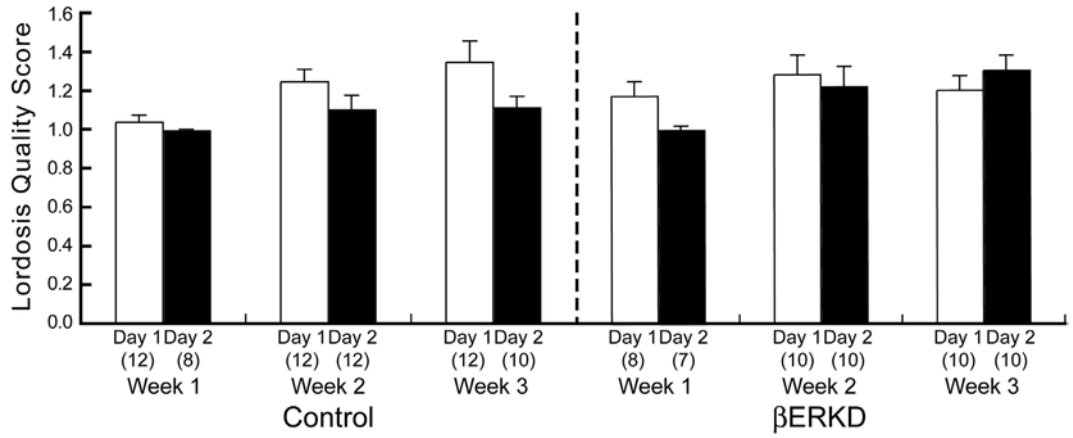

FIGURE 6 | Lordosis Quality Score of control (left) and ERβ knockouts (right) mice in each test. There was no difference between day 1 and day 2 in each week regardless of treatment group. The number in parentheses showed the number of mice which expressed lordosis (i.e., lordosis score of higher than 1) in each test.

Effect of ERβ Knockdown in the DRN on the Lordosis Quality Score

Estrogen receptor β knockdown did not affect the Lordosis Quality Score (**Figure 6**). Mann–Whitney *U* test revealed that there was no difference between day 1 and day 2 of Lordosis Quality Score in each week regardless of treatment group (Control: Week 1; $U = 44.000$, Week 2; $U = 40.500$, Week 3; $U = 33.500$, βERKD: Week 1; $U = 16.000$, Week 2; $U = 43.000$, Week 3; $U = 33.000$, all ns).

LQ of Missed βERKD Group

Behavioral data shown above included only mice in which the range of vector infusion covered the entire targeted area, but not elsewhere. These βERKD mice maintained the LQ that was equivalent to day 1 in day 2. On the other hand, such effect was absent if the virus did not spread into the mediodorsal area of dorsal part in the DRN. Two-way repeated measures ANOVA revealed significant effects of Day [$F(1, 4) = 21.574$, $P < 0.05$] and Week [$F(2, 8) = 7.704$, $P < 0.05$]. There were no statistically significant interactions between Day and Week [$F(2, 8) = 0.141$, ns].

DISCUSSION

In this study, we demonstrated that knocking down of ERβ in the DRN altered female sexual behavior on the day after behavioral estrus without affecting the behavior on the day of behavioral estrus. Our results showed that LQ between βERKD and Control mice was at a similar level on day 1 test performed at 44–46 h after EB and 4–6 h after progesterone injection that mimicked hormonal states of the day of behavioral estrus. By contrast, on day 2 test which corresponds to the day after behavioral estrus, βERKD mice still showed similar levels of LQ in comparison with their LQ on day 1 in each of three test. Control mice showed greatly decreased levels of LQ on day 2 compared with day 1 (**Figure 5**). These findings are consistent with previous studies using βERKO mice in which both gonadally intact and hormonally manipulated ovariectomized βERKO females continuously showed elevated levels of receptivity on the day after behavioral estrus [(4); unpublished data]. Thus, our finding suggests that the DRN is one of the brain sites responsible for the ERβ-mediated inhibitory regulation of female sexual behavioral expression on the day after behavioral estrus. On the other hand, Lordosis

Quality Score that indicates the quality of each lordosis was not different between day 1 and day 2 in both βERKD and Control groups (**Figure 6**). The brain sites responsible for the qualitative aspect of lordosis posture and possible involvement of gonadal steroids are not known. Our data indicate that ERβ expressed in the DRN may not be involved in the qualitative regulation of lordosis responses.

Furthermore, the knockdown effect was absent if the virus did not spread into the mediodorsal area within the dorsal part of the DRN even though the infusion was successful elsewhere within the nucleus (**Figures 4** and **7**). This finding may indicate that even within the DRN, the mediodorsal area of dorsal part is the most critical area for the ERβ-mediated inhibition of female sexual behavior on the day after behavioral estrus.

The exact mechanism of this ERβ-mediated inhibitory regulation of female sexual behavior has yet to be elucidated. However, it is possible to hypothesize that ERβ in the DRN may be involved in inhibition of female sexual behavior by intervening the serotonergic systems. Serotonin is known to act inhibitory on the expression of female sexual behavior through its binding to 5HT-1A receptors (17). The DRN is the largest serotonin synthesizing nucleus whose projections directly innervate the VMN and MPOA where 5HT-1A receptors are highly abundant (18–20). Selective lesions of serotonergic neurons projecting from the DRN to the VMN have been reported to facilitate lordosis (21). On the other hand, site-specific activation of 5HT-1A receptors either in the VMN or MPOA by local infusion of selective agonist, 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) reduces the expression of lordosis in hormonally primed ovariectomized and gonadally intact proestrus female rats (22–24). Within the DRN, more than 95% of ERβ expressing neurons are serotonergic (14), and the majority of serotonergic neurons that project into the MPOA express ERβ (25). Furthermore, the administration of 17β-estradiol or ERβ-specific agonists, DPN to ovariectomized female rats are reported to enhance mRNA expression of tryptophan hydroxylase 2 (TPH 2), the rate-limiting enzyme for serotonin synthesis (26). In addition, the TPH 2 mRNA expression is greatly reduced in the DRN of βERKO female mice (14). Thus, ERβ in the DRN is possibly mediating the inhibition of female sexual behavior by upregulating the activity of the serotonergic system within the DRN that further activates 5HT-1A receptors expressed in hypothalamic regions such as the VMN and MPOA. The expression of 5HT-1A receptors in these regions seems to be also upregulated by estrogen (20, 27). Interestingly, a recent study by Spiteri et al. (28) reported that the site-specific knockdown of ERα in the MPOA, the region also known for its inhibitory role on the expression of female sexual behavior, increase the lordosis expression in response to a low dose EB treatment that generally fails to stimulate lordosis in ovariectomized females. Thus, the inhibitory action of the MPOA on the female sexual behavior seems to be ERα mediate although the crucial role of ERα in the VMN in the facilitation of the behavior is well known (7).

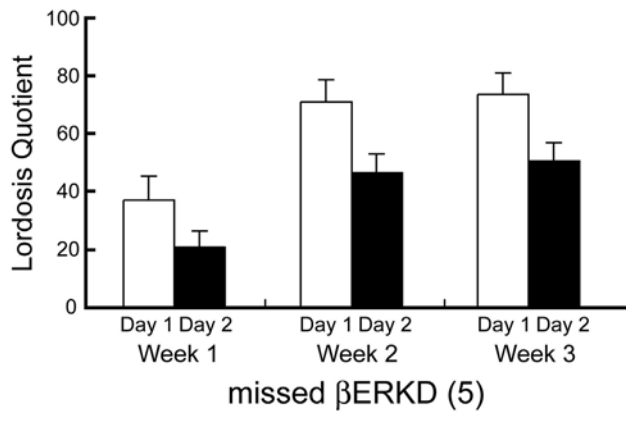

FIGURE 7 | Lordosis quotient of missed ERβ knockdown (βERKD) mice. Data presented as mean ± SEM.

Collectively with these studies, our present findings provide a significant piece to lay out the possible mechanism underling the cyclic regulation of female sexual behavior. Thus, on the day of behavioral estrus, high levels of circulating estradiol simultaneously (1) facilitate behavioral expression *via* ERα-mediated genomic action in the VMN, (2) upregulate 5HT-1A receptor expression *via* ERα in the hypothalamic areas such as the VMN and MPOA, while (3) upregulate TPH 2, the rate-limiting enzyme for serotonin synthesis in the DRN *via* ERβ. On the day after behavioral estrus, serotonin release by serotonergic neurons in the DRN may activate 5HT-1A receptors in the VMN and/or the MPOA and inhibit lordosis expression.

In addition to serotonin, progesterone is also known to inhibit the expression of female sexual behavior in its long-term effect while its short-term effect is facilitatory. Progesterone acts through progesterone receptor (PR), and the expression of the receptor is highly dependent on estrogen action. In female mice, the expression of PR in the VMN is known to be upregulated by estrogen *via* ERα (7). Whereas in the DRN, it seems to be ERβ dependent since EB administration still upregulate PR expression in the DRN of ovariectomized αERKO female mice (29). Moreover, estrogen-induced PR expression in the DRN found in αERKO female is suspected to be on the serotonergic neuron since the majority of PR immunoreactive cells coexpressed TPH immunoreactivity in these females (29). Therefore, it is reasonable to hypothesize that PR upregulation by E_2 in the DRN is mediated ERβ by and this progesterone–PR signaling pathway, in turn, may contribute the activation of serotonergic system that inhibits the expression of female sexual behavior on the day after behavioral estrus.

In this study, we focused on lordosis that reflects more of consummatory aspects. However, we cannot exclude the possible effect of ERβ knockdown in the DRN on behavioral components other than lordosis itself. Ogawa et al. (4) reported that gonadally intact βERKO female mice exhibited slightly higher levels of proceptivity throughout the estrous cycle compared with WT mice. Thus, in future experiments, it will be important to investigate the site-specific involvement of ERβ in the DRN in the appetitive aspects of female sexual behavior. It is also important to directly monitor or manipulate neuronal activity of ERβ- and/or PR-expressing cells and the serotonergic system within the DRN during both estrus and non-estrus phase to further elucidate neuronal and intracellular mechanisms of ERβ-mediated inhibitory regulation of female

sexual behavior. Moreover, it is our great interest to investigate site-specific involvement of ERβ in brain regions other than the DRN such as the MPOA, the region also involved in the inhibitory regulation of female sexual behavior and known to highly express ERβ.

CONCLUSION

The main aim for this study was to elucidate site-specific involvement of ERβ expressed in the DRN in the inhibitory regulation of sexual behavior in female mice. This study demonstrated that βERKD mice continuously showed elevated levels of receptivity even on the day after behavioral estrus, whereas control mice showed greatly reduced receptivity on the day after behavioral estrus compared with the day of estrus. Therefore, the findings in this study suggest that ERβ in the DRN may be involved in the inhibition of female sexual behavior on the day after behavioral estrus. This is the first study to demonstrate the site-specific involvement of ERβ in the inhibitory regulation of female sexual behavior.

ETHICS STATEMENT

All procedures were approved by the Animal Care and Use Committee and the Recombinant DNA Use Committee at the University of Tsukuba and conducted strictly in accordance with the National Institutes of Health guidelines. All efforts were made to minimize the number of animals and their suffering.

AUTHOR CONTRIBUTIONS

KS, CM, and SO designed research and wrote the paper. KS, CM, MN, SM, MT, NY, and TS performed research and analyzed data. SM and SO contributed new reagents/analytic tools; KS and CM equally contributed to the study. SM designed and prepared the viral vectors expressing shRNAs used in this study.

FUNDING

This work was supported by Grant-in-Aid for Scientific Research 15H05724 to SO.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 25 May 2018
doi: 10.3389/fnins.2018.00245

Estradiol and the Development of the Cerebral Cortex: An Unexpected Role?

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The cerebral cortex undergoes rapid folding in an "inside-outside" manner during embryonic development resulting in the establishment of six discrete cortical layers. This unique cytoarchitecture occurs via the coordinated processes of neurogenesis and cell migration. In addition, these processes are fine-tuned by a number of extracellular cues, which exert their effects by regulating intracellular signaling pathways. Interestingly, multiple brain regions have been shown to develop in a sexually dimorphic manner. In many cases, estrogens have been demonstrated to play an integral role in mediating these sexual dimorphisms in both males and females. Indeed, 17β-estradiol, the main biologically active estrogen, plays a critical organizational role during early brain development and has been shown to be pivotal in the sexually dimorphic development and regulation of the neural circuitry underlying sex-typical and socio-aggressive behaviors in males and females. However, whether and how estrogens, and 17β-estradiol in particular, regulate the development of the cerebral cortex is less well understood. In this review, we outline the evidence that estrogens are not only present but are engaged and regulate molecular machinery required for the fine-tuning of processes central to the cortex. We discuss how estrogens are thought to regulate the function of key molecular players and signaling pathways involved in corticogenesis, and where possible, highlight if these processes are sexually dimorphic. Collectively, we hope this review highlights the need to consider how estrogens may influence the development of brain regions directly involved in the sex-typical and socio-aggressive behaviors as well as development of sexually dimorphic regions such as the cerebral cortex.

Keywords: aromatase, subventricular zone, cortical plate, neurogenesis, migration, sexual dimorphism, 17β-estradiol, brain synthesized

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience

Received: *30 December 2017*
Accepted: *28 March 2018*
Published: *25 May 2018*

Citation:
Denley MCS, Gatford NJF, Sellers KJ and Srivastava DP (2018) Estradiol and the Development of the Cerebral Cortex: An Unexpected Role? Front. Neurosci. 12:245. doi: 10.3389/fnins.2018.00245

INTRODUCTION

The complex neuronal organization and architecture of the cerebral cortex is thought to be responsible for the higher cognitive function bestowed upon mammals. The unique anatomical compartmentalization and lamination of discrete neurons arranges into horizontal layer identifiable with specific molecular markers (Kriegstein and Parnavelas, 2006; Molyneaux et al., 2007; Greig et al., 2013). This organization is established during embryonic development, and is achieved in an "inside–outside" fashion, with the "deep" or "inner" layers

developing first and the outer layers developing last. Critically, this unique cytoarchitecture is the basis by which the correct assembly of synaptic connectivity and therefore, functional cortical circuitry is established (Marín et al., 2010). Mechanistically, the inside-outside organization of the cerebral cortex is established throughout development and controlled by coordinating processes in neurogenesis, cell migration (Götz and Huttner, 2005; Taverna et al., 2014) as well as responses to extracellular cues and activation of intracellular signaling pathways (Hippenmeyer, 2014; Hansen et al., 2017). The phenotypic display of this coordination can be seen in **Figure 1**. Whilst our understanding of the molecular events that underlie these processes is ever growing, understanding the mechanisms and signals that exert influences over corticogenesis remains a major challenge. This is particularly emphasized by increasing evidence that abnormal corticogenesis may contribute to a range of neurodevelopment and psychiatric disorders (Hoerder-Suabedissen et al., 2013; Ishii et al., 2016).

BACKGROUND

Neurons forming the basic unit of nervous tissue architecture was a notion first proposed and described by Ramón y Cajal (1911). Connectivity between these units underlies the normal brain functions of neural circuitry in cognition and behavior. Therefore, understanding the developmental formation of neural circuitry is vital to understanding how these structures respond to environmental, physiological and pathological stimuli. Steroid hormones, such as estrogen, have long been the focus of research and review (Alcaraz et al., 1969; Dominique Toran-Allerand, 1976; Hammond and Rowe, 1976), and have long been suggested to be key signals orchestrating the development of sexual dimorphism of many cerebral regions. However, previous research has also indicated that estrogen can have wildly polarized effects in terms of behavior and physiology (Alcaraz et al., 1969; Gillies and McArthur, 2010). Research has also suggested that this response is due to specific estrogen receptor

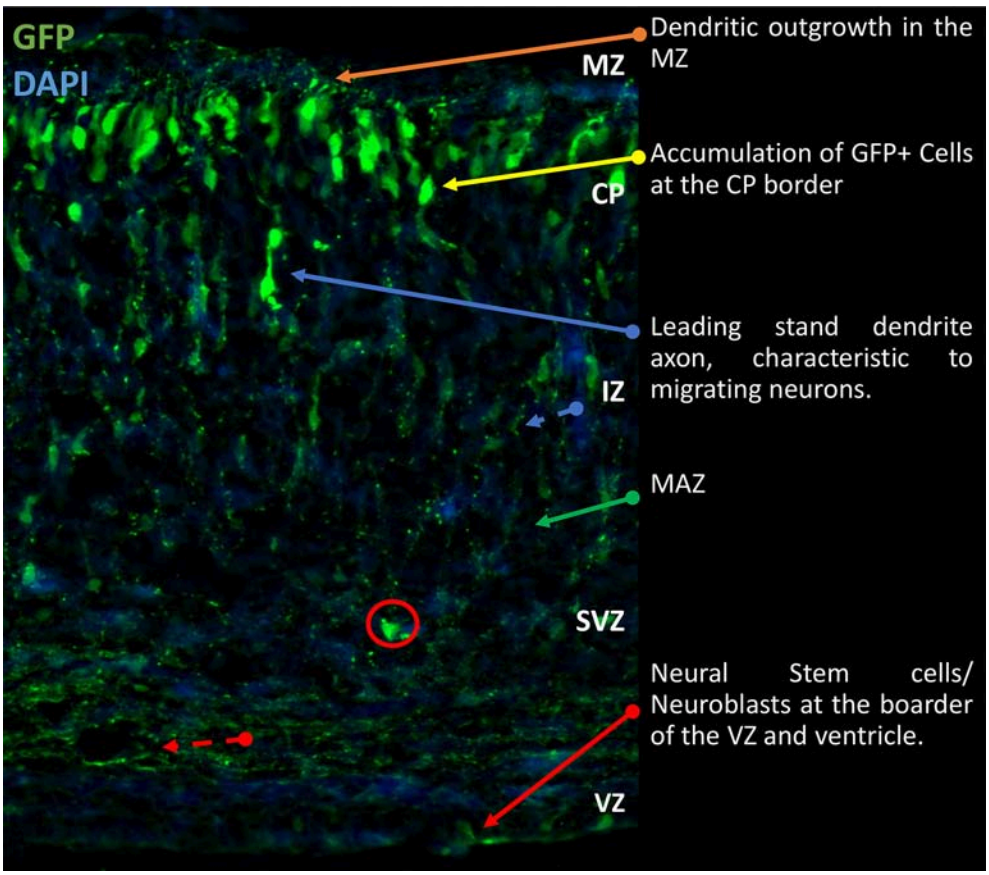

FIGURE 1 | Gross morphological schematic of sub-compartments in the developing rodent cortex. Representative image of developing cortex. Electroporation of eGFP was performed at E14.5 and brains collected at P0 as previously described (Srivastava et al., 2012a,b). The cortex is comprised of four morphologically distinct regions, the VZ, SVZ, IZ, and CP. Further to this there are the MAZ and MZ, located in the IZ and CP respectively. Located on the basal surface of the cortex proximal to the cerebral ventricles is the VZ responsible for generation of NSCs. Beyond the VZ, the SVZ contains proliferating and early differentiating neural progenitors. Between the SVZ and IZ, the MAZ is a point of accumulation of polarizing cells. After which the cells migrate through the IZ to the CP where terminal translocation takes place. This brief outline is the general schematic throughout development of the cortex. Cells migrate to the outmost layer and continually build on top of each other in a sedimentary manner. IZ, intermediate zone; MAZ, multipolar cell accumulation zone; CP, cortical plate; GFP, green fluorescence; NSCs, Neural Stem Cells; VZ, ventricular zone; SVZ, subventricular zone; MZ, marginal zone.

subtypes and, thus, is dependent on the expression in the discrete location of the receptor in specific brain regions. However, response to estrogen exposure is impeccably sensitive to factors such as dose and stage of life cycle (Katzenellenbogen et al., 2000; Sánchez-Criado et al., 2004). More recent research has made attempts to explain the mechanism behind sexual dimorphic response to estrogen during development (Grassi D. et al., 2012; Grassi S. et al., 2012; Liu et al., 2017). Interest in estrogen is further emphasized because of the demonstrable ability to affect psychiatric and neurodegenerative disorders (Arevalo et al., 2011; Srivastava et al., 2013b; Gobinath et al., 2017). This includes, for example, Autism Spectrum Disorder, which shows a strong male bias (Werling and Geschwind, 2013) and therefore suggests sexual dimorphic underlying physiology (Adhya et al., 2018).

Sexual dimorphism is not wholly dependent on the action and mechanism of hormones. Indeed, sex hormones and other gonadal secretions do have sex-specific effects on the brain, but gene expression in individual cells may have a larger role in the dimorphic phenotype. Increasing evidence has suggested a role for developmental mediation by X and Y chromosome-linked genes (Arnold, 2004). These revelations have led to a neurodevelopmental debate: epigenetics or genetics? This debate suggested that development of specific areas in the brain may be hormone-dependent, whilst others are hormone-independent (Reisert and Pilgrim, 1991). Here, we specifically discuss the hormone-dependent roles in neurodevelopment.

Estrogens are a class of steroids of which 17β-estradiol (often referred to as estradiol or just E2) is the main biologically active form (Blaustein, 2008). This class of steroid has long been known to exert powerful effects on development of the nervous system, as well as its function and plasticity (McEwen and Alves, 1999; McCarthy, 2008; Brinton, 2009; Srivastava et al., 2013b). Importantly, increasing evidence suggests that in addition to their actions within the hypothalamus (Kelly et al., 2005), estrogens exert effects within the neocortex and hippocampus (McEwen and Alves, 1999; Srivastava et al., 2013b). Moreover, the actions of estrogens during development and in the mature brain are not limited to those described in females. Effects of estrogens have also been consistently reported in males, albeit in a sexually dimorphic manner in many cases (Gillies and McArthur, 2010; McCarthy and Nugent, 2013; Gobinath et al., 2017). However, our current understanding of the role estrogens such as estradiol play in regulating the cellular or molecular processes critical for corticogenesis is limited. In this review, we will discuss the evidence that implicate a role for estrogens during the development of the cortex. Furthermore, we will highlight specific molecular pathways involved in developing the complex neuronal organization of the neocortex and emphasize how estrogens, particularly, estradiol signaling, interacts with these processes.

THE CASE FOR ESTROGEN IN THE DEVELOPING CORTEX

Canon has long established that the main source of estrogen production is the female sex organs. However, there is growing evidence that the key enzyme that converts androgens into estrogens, aromatase, is highly expressed in nervous tissue (Yague et al., 2006; Ish et al., 2007; Hojo et al., 2009; Boon et al., 2010; Saldanha et al., 2013). Aromatase has been identified in the hypothalamus, hippocampus, visual cortex, and temporal cortex in avian, mammalian, and human brain (Rune and Frotscher, 2005; Yague et al., 2006; Boon et al., 2010; Azcoitia et al., 2011; Saldanha et al., 2011).

The abundance of information on aromatase expression hints toward its fundamental importance. Aromatase expression has been evolutionarily conserved in nervous tissue, from chordates and early teleost. Complete removal of sex organs demonstrates that the expression is indeed sourced from within the nervous tissue, as removal does not lead to a complete loss-of-function (Yague et al., 2006; Ish et al., 2007; Hojo et al., 2009; Boon et al., 2010; Konkle and McCarthy, 2011; Saldanha et al., 2011). Indeed, Callard et al. (1978) highlighted that aromatase expression is highly concentrated to the forebrain in vertebrates. Furthermore, goldfish and toadfish demonstrate a high concentration of aromatase in the hypothalamus and preoptic areas (Pasmanik and Callard, 1985). Interestingly, work in toadfish demonstrated that expression of aromatase in certain neural areas accounted for sexually dimorphic behaviors (Pasmanik and Callard, 1985). Estrogens and aromatase expression in the limbic system as an evolutionarily conserved "ancient" function is further reinforced by the presence of both steroid and enzyme in non-mammalian vertebrate reptiles (Callard et al., 1977).

The evolutionary conservation model fits within the evolution of "higher cognition" in mammals, such as humans. Humans express aromatase and estrogens in the limbic system, and frontal cortex where they play roles in cognitive ability (Sasano et al., 1998). As the human neocortex has develop, so too has our ability to utilize estrogens and the "Aromadition—the system under aromatase control" to alter structures depending on stress/learning interactions, exploiting a combination of rapid and sustained cellular mechanisms (Konkle and McCarthy, 2011; Saldanha et al., 2011; Srivastava et al., 2011). Furthermore, the discovery of neuroactive steroidal involvement in clinical investigation has highlighted a very real application of estrogens as pharmaceutical agents (Stoffel-Wagner, 2001).

Estrogens have shown the ability to enact change in a very short time scale. Through modulation of spinogenesis, synaptogenesis, and synaptic connectivity, estrogen is able to enact these rapid changes in neural circuits (Saldanha et al., 2011; Srivastava et al., 2013b; Sellers et al., 2015a,b). For examples, when rat cortical neurons were treated with 10 nM Estradiol, structural changes (spinogenesis) were observed within 30 min. Studies into the ability of estrogens to influence behavior also demonstrate a rapid influence of estradiol and estrogen receptor agonists (Srivastava et al., 2013b; Luine, 2014; Sellers et al., 2015a; Gobinath et al., 2017). However, these studies present a clear inverted U-shaped curve. Previous studies have suggested that either 1–2 or 5 μg/kg dose of estradiol receives a better effect (Inagaki et al., 2010), reflecting the optimal level of receptor dynamic activation (Srivastava et al., 2013b; Luine, 2014). However, this dose response may be limited to enhancement of behavioral and cognitive tasks.

Studies *in vivo* have shown that estrogen receptors have an integral function in the development of cerebral architecture. Specifically, the pyramidal cells of the hippocampus and the cortical laminae II-VI demonstrate expression of ER mRNA and ER protein (Shughrue and Merchenthaler, 2000). Studies in this area began to elucidate further evidence of the large scale of nuclear ERs spread across the cortex, particularly in laminae III-V (Shughrue et al., 1999). Evidence for the implication of estrogen receptors in organization of the cortex has been the discovery of extra-nuclear ERα in dendritic spines and astrocytes (Milner et al., 2000). ERß mRNA and protein has also been detected in the cortex of rats (Shughrue and Merchenthaler, 2001).

These data indicate that ERs are indeed expressed through the cortical laminae of rodents. However, much less is known about the expression of ERs and estrogen-related genes, transcription factors and proteins during development. Our preliminary studies examining the expression of aromatase has begun to illuminate potential pathways linking estrogen and development, particularly sexually dimorphic development.

Aromatase has been found to be highly expressed in pyramidal neurons as well as glial cells (Kretz et al., 2004; Yague et al., 2006, 2008). Consistent with this, there is increasing evidence that estradiol is produced within the neocortex even in the absence of sex organs in both male and female animals (Ish et al., 2007; Hojo et al., 2009). Critically, estradiol, as well as other steroids, have been measured in embryonic brains of male and female rats within the cortex (Konkle and McCarthy, 2011). These findings are mirrored by previous findings of aromatase activity in the cortex and hippocampus in perinatal animals (Tobet et al., 1985; MacLusky et al., 1994). These studies highlight the possibility that the *de novo* synthesis of estradiol, mediated by aromatase, represents a major source of estrogens within the brain (Cornil et al., 2006; Azcoitia et al., 2011; Saldanha et al., 2011; Srivastava et al., 2013b; Balthazart and Ball, 2017), and may influence the development of the cortex (Tobet et al., 1985; MacLusky et al., 1994; Konkle and McCarthy, 2011). It is also important to note that the three major estrogen receptors (ERs): ERα, ERβ, and G-protein coupled estrogen receptor 1 (GPER1), were found to be expressed in multiple brain regions, including the cortex (Mitra et al., 2003; Milner et al., 2005). Using the BrainSpan transcriptomic atlas of the developing brain (http://www.brainspan.org/), we found several of the key molecular players involved in estrogenic signaling are expressed in the developing human brain, namely *CY19A1* (aromatase), ESR1 (ERα), ESR2 (ERβ), and GPER1 (GPER1). Specifically, these genes were found to be expressed during the developmental period spanning embryonic to late prenatal in the dorsal frontal cortex (DFC), ventral frontal cortex (VFC), and the hippocampus (HIP). CYP19A1 expression was found to increase throughout development, within these regions. Similarly, ESR1 trends toward an increase during over development, whereas ESR2 seems to be highly expressed during early corticogenesis before decreasing slightly then plateauing until late prenatal stage (Gatford, Denley and Srivastava, unpublished observations). Interestingly, the recently identified estrogen sensitive G-protein coupled receptor, GPER1 (also known as GPR30) (Srivastava and Evans, 2013) increases in its expression throughout corticogenesis. Combined, these data support the notion that the molecular machinery required for estrogenic signaling is expressed throughout early development within the cortex, and further suggests that estrogens may play a role during corticogenesis. Consistent with these data, we find that aromatase is expressed in multiple cell types within the brain of post-natal day 0 mice (Bakker et al., 2002; Karolczak et al., 2008). It should be noted that during brain development, estradiol has an organizational role and is central to the sexually dimorphic development and regulation of the neural circuitry underlying sex-typical and socio-aggressive behaviors in males and females (Nelson and Trainor, 2007; McCarthy and Arnold, 2011; Ubuka and Tsutsui, 2014). Interestingly, these effects seem to be in part mediated by the active repression of DNA methylation in the POA and hypothalamic ventromedial nucleus (Nugent et al., 2015; Mosley et al., 2017). Ultimately, whether such mechanisms are also important for the development of cortical circuitry is currently unknown. Nevertheless, taken together, these data suggest that estrogens, and aromatase, are not only present, but also actively regulated during key phases of the developing cerebral cortex.

Another line of evidence that estrogens play an important role in the development of the cortex in a sex specific manner comes from studies examining the detrimental effects of Bisphenol A (BPA). BPA is a Xenobiotic∗ and antagonist to estrogens via ERs. It has been noted that BPA alters sexual differentiation in early development. Males, but not females, exposed to BPA at 400 μg/kg altered the number of neurons and glial cells in the deep layers of the medial prefrontal cortex (Sadowski et al., 2014). Altered expression of ERs, which has shown to be sex-specific may account for this biased and unexpected mechanism (Kundakovic et al., 2013). It should be noted that BPA also binds androgen receptors (Kuiper et al., 1998; Sohoni and Sumpter, 1998), which may confer some of these effects. Sexually dimorphic neuron progeny may be attributed to the divisive effects of BPA among sexes, as specific neurons comprising sexually dimorphic regions are produced at markedly different timespans (Jacobson and Gorski, 1981). Nevertheless, the effects of BPA on neuronal and glial cell volumes within the cortex (Kubo et al., 2003) provides a strong case for estrogenic signaling in cortical development, and suggests that the steroid hormone may account for some sexual dimorphism seen in this area. However, one additional caveat of using BPA as an example to demonstrate a role for estrogens during corticogenesis, is its universal effects on other tissues in the body. The thyroid is one such example; environmental contamination by BPA results in up-regulation of thyroid hormone-responsive genes in the dentate gyrus (Zoeller et al., 2005), which is an area that has shown sexual dimorphic characteristics (Roof, 1993; Tabibnia et al., 1999). As highlighted above, BPA also strongly inhibits the activity of androgen receptors (Sohoni and Sumpter, 1998), which will have a considerable impact on the development and function of the cerebral cortex (Clark et al., 1988; Nuñez et al., 2003). A more comprehensive summary of BPAs effects can be found in Wetherill et al. (2007).

Maternal consumption and/or exposure to xenobiotic compounds can enter into the amniotic environment of the fetus and effect neurodevelopment. Exposure to xenobiotic compounds at physiological levels and from the environment

can be detected in the amniotic fluid, which introduces it to the fetus (Nikaido et al., 2004; Engel et al., 2006). An area of increasing concern is that regarding dietary phytoestrogens. This area also provides further evidence to support the argument of estrogen and aromatase as key regulators of cortical development. In numerous animal models, manipulation of estrogen function by dietary phytoestrogens during gestation can lead to disrupted brain organization (Gorski, 1985; Lindzey and Korach, 1997). It has been suggested that dietary phytoestrogens are easily diffusible across the placenta and may then interfere with development (Patisaul and Jefferson, 2010). One must be cautious to draw conclusions from this, as absorption and concentration may differ wildly, especially, considering other dietary factors. In rodent studies, phytoestrogens such as polyphenols, flavonoids, and isoflavonoids have been shown to readily cross into the placenta and into the fetus brain (Doerge et al., 2001). Furthermore, during the prenatal period, systemic circulation of phytoestrogens is considerably more efficient compared to adults (Chang et al., 2000). Three anatomical locations have been particularly prominent in researching estrogen and epigenetics, the preoptic area (POA), locus coeruleus (LC), and the hypothalamic-pituitary axis (HPA), specifically the ventromedial nucleus. The POA and LC show sexual dimorphic areas, with ERα playing the more dominant role over ERß in both cases (Shughrue et al., 1997; Pérez et al., 2003; Patchev et al., 2004). Yet this raises the question, whether there are sexual dimorphic areas, hitherto undiscovered, that ERß maintains dominance?

The rodent POA has shown to be heavily sensitive to estradiol aromatized from gonadal androgens (Gorski, 1985). This is reflected in the greater volume of the male POA in comparison to the female POA. Furthermore, the expression of aromatase is variable not just in areas of sexual dimorphism, but also in response to testosterone and estrogen exposure (Roselli and Stormshak, 2010). Within the POA, the phytoestrogen genistein acts as an agonist to estrogen receptors. Exposure to genistein results in increased volumes in male but not female calbindin labeled sexually dimorphic areas (Scallet et al., 2004). This particular example highlights the importance of androgen synthesis and pharmacological dynamics, which are unfortunately beyond the scope of this literature review. Conversely, in the pre- and postnatal LC, exposure to the phytoestrogen resveratrol demasculinizes the male brain resulting in sexual dimorphism to volume and cell density (Kubo et al., 2003). It is pertinent to add that androgens are chiefly responsible for masculinizing the male brain. However, it would be irresponsible to not give weight to the estrogen (perhaps more so of ERα) mediated changes in organizational structure of the brain, with this example showing the ability of estrogen and aromatase to change both cerebral volume and cell densities in discrete areas.

CORTICAL DEVELOPMENT

Episodic processes of progenitor proliferation, neural differentiation, polarization, neuronal migration and lamination underpin the cortical development pattern (Ohtaka-Maruyama and Okado, 2015). Through selective expression of transcription factors and interaction with extra-cellular protein receptors, cortical layers are formed "inside-outside" (**Figure 1**; Angevine Jun and Sidman, 1961). Late-borne progenitors will ascend to the pial layers, whereas early-borne progenitors remain in deeper layers, which is modulated and regulated through transcription at the various episodes. Below, we highlight some of the key cell types and events that are critical for normal corticogenesis.

Proliferation and Progenitor Maintenance

In proliferative and neurogenic states during development, progenitors are produced through division and proliferation of neural stem cells (NSCs) in the ventricular zone (VZ) (**Figure 2**) and sub-ventricular zone (SVZ) (**Figure 3**). Basal progenitors of the SVZ, identified by the expression of Svet1 and Tbr2, of the SVZ undergo symmetrical division and produce two neurons. Vimentin and Sox2 staining can highlight radial glial cell (RGC) (**Figure 2**) progenitors of the outer SVZ (Lancaster et al., 2013). Ngn3 contributes to maintaining progenitor oligodendrocytes in the SVZ (Ivanova et al., 2003) (**Figure 3**). Only progenitors of the VZ expressed Par complex rich domains, which develop into apical progenitors (express: Hes1, Pax6, Ki67, Phospho-vimentin, and Sox2; Knoblich, 2008) (**Figures 2**, **3**). RC2, GLAST, BLBP, Nestin, and GFAP staining can also highlight RGC progenitors of the VZ (Rakic, 1971; Hartfuss et al., 2001; Liu et al., 2017; **Figure 2**). Hes1 gene transcription regulates VZ progenitor pools and proliferation. However, RGCs typically divide asymmetrically, and thus, increase the diversity of daughter cells (Noctor et al., 2004). However, during highly proliferative episodes, symmetrical division is favored to increase the progenitor supply pool. Mechanistically, this process is mediated through Wnt signaling, leading to an increase in progenitor pool (ß-catenin-dependent) (Galceran et al., 2000). Interestingly, Par inheritance is directly linked to inherited Notch signaling capability (Kon et al., 2017; Bultje et al., 2018), furthermore, Notch signaling is required for neurogenesis and the maintenance of VZ NSCs (Imayoshi et al., 2010). Progenitors in the SVZ inherit or express high levels of Cux1 and Cux2, which signals them as apical progenitors (Nieto et al., 2004; **Figure 3**). G1 phase mitosis is a key regulatory point in proliferation (Dehay and Kennedy, 2007). CDK inhibitors, p27 and p57 coordinate corticogenesis and cell cycle exit. Prior to differentiation, p57 is responsible for cell cycling (Bilodeau et al., 2009). BM88 influences NSCs to exit the cell cycle and is expressed by RGCs (Koutmani et al., 2004).

Asymmetric inheritance is also seen in Pax6 and Ngn2 positive RGCs (Kawaguchi et al., 2004). Pax6 regulates neurogenesis and proliferation in the VZ and SVZ (Gan et al., 2014) and maintains the SVZ progenitor pool (Wong et al., 2015). Pax6 activity has shown to be ß-catenin-dependent. A dynamic relationship between Sox1 and Pax6 controls transgression through periodical cortical development states (Suter et al., 2009; Wong et al., 2015). EphA4 binds to fibroblast growth factor (FGF), which is a potent stimulator of cortical proliferation (Collette et al., 2017). EphA4 regulates FRS2α (Yokote et al., 2005), which activates

downstream RAS-MAPK and PI3K signaling pathways (Ornitz and Itoh, 2015).

Differentiation and Polarization

Differentiation of progenitors causes morphological changes, which allows migration and maturation. Differentiation and migration require dynamic cytoskeleton changes; extracellular and intrinsic signaling tightly regulates these events. For example, cadherins alter cellular dynamics (Nagafuchi et al., 1987). N-glycan-dependent attachment of cells recruits cadherin, causing greater cell-cell interaction (Hall et al., 2014). Nestin signals morphological changes within RGCs localized to axonal projections extending from the VZ (Xu et al., 2015; Vinci et al., 2016). Tbr2 generates transcriptional changes to cause cell to select a leading (axonal) strand (Sessa et al., 2008) (**Figure 2**). Ngn2 is downregulated by Hes1, which shifts a cell toward differentiation by modulating Notch signaling (Niwa et al., 2009; **Figures 2**, **3**). Ngn3 is upregulated to stimulate dendritogenesis and synaptogenesis. CRM1 mediates the nucleo-cytoplasmic shuttling of Ngn3, allowing transcriptional change (Simon-Areces et al., 2013) (**Figures 2**, **3**). In addition to its effect on proliferation, Wnt signaling utilizes ß-catenin-dependent pathways to differentiate intermediate progenitors into neurons (Clevers, 2018). Interestingly, cadherins also employ this pathway to mediate cell cycling (Zhang et al., 2013). Indeed, cadherins activate Wnt and Akt to switch from proliferation to migration (Ajioka and Nakajima, 2005).

Switching mechanisms and alterations in morphology are crucial to poising the cell to undergo polarization. The subplate of the IZ is a multipolar cell accumulation zone (MAZ) (**Figure 4**). Beyond the MAZ cells are mostly bipolar or unipolar neurons. Ngn2 regulates the transition of polarity (Ohtaka-Maruyama and Okado, 2015). Ngn2 inhibits RhoA, causing a cascade that downregulates MAPK (Zeidan et al., 2014) (**Figure 4**). RP58 regulates Ngn2-mediated morphological changes (Ohtaka-Maruyama et al., 2018). Interaction between ABP/cofilin/myosin inhibits RhoA through Rnd3 causing filopodial and lamellipodial growth and retraction (Pacary et al., 2011; Gomez and Letourneau, 2014). GDP-bound and GTP-bound oscillation regulates lamellipodium formation that orientates cells within the MAZ (Sailland et al., 2014). Par complex proteins (Par3, Par6, aPKC) cause apical base neurons to undergo polarization through Cdc42 coupling (Cappello et al., 2006). A compressive review of polarization can be found in Polleux and Snider's paper Polleux and Snider.

Migration and Lamination

Glial-guided neurons use cellular locomotion to migrate to the CP. Once in position, neurons attach their leading strand to the marginal zone (MZ) and switch to somal translocation.

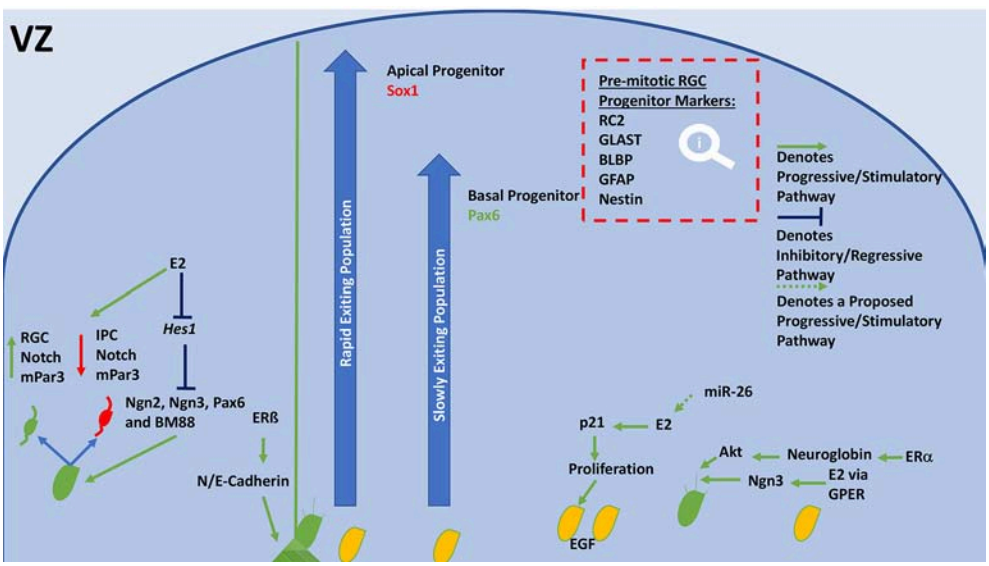

FIGURE 2 | General schematic diagram of molecular events that regulate neurogenesis and proliferation in the VZ. Neuroblasts, or NSCs divide asymmetrically, their progeny inherits either high notch and mPar3 or low notch and mPar3. RGCs receive higher notch signaling, their counterpart receives lower notch signaling and becomes an IPC. E2 inhibits Hes1 expression, resulting in increased division by inhibition of neurogenins, Pax6, and BM88. ERß is able to modulate N and E-Cadherin levels, which stabilizes end-feet of radial scaffolds and mediates adhesion. Depending on the expression of Sox1 or Pax6 a progenitor will join the rapidly or slowly ascending pool. Sox1-expressing populations (Apical progenitors) are released rapidly but remaining in the deeper layers of the CP. Pax6-expressing populations (Basal progenitors) are released slowly and comprise the superficial neurons of the CP. E2 has also shown to increase proliferation of NSCs through stimulation of p21 and increasing EGF expression. miR-26 may increase E2 synthesis or ER expression to meet this end. E2 is also able to increase neuritogenesis, leading to the formation of an axon. It accomplishes this via GPER and ERα, which are able to stimulate neuritogenesis through Ngn3 and Neuroglobin/Akt, respectively. E2, Estradiol; VZ, ventricular zone; RGC, radial glial cell; NSCs, neural stem cells; IPC, intermediate progenitor cell; Ngn, neurogenin; GLAST, astrocyte-specific glutamate transporter; BLBP, brain-lipid binding protein; GFAP, glial fibrillary acidic protein; ERα, estrogen receptor alpha; ERß, estrogen receptor beta; GPER, G-protein estrogen receptor; EGF, epidermal growth factor; CP, cortical plate.

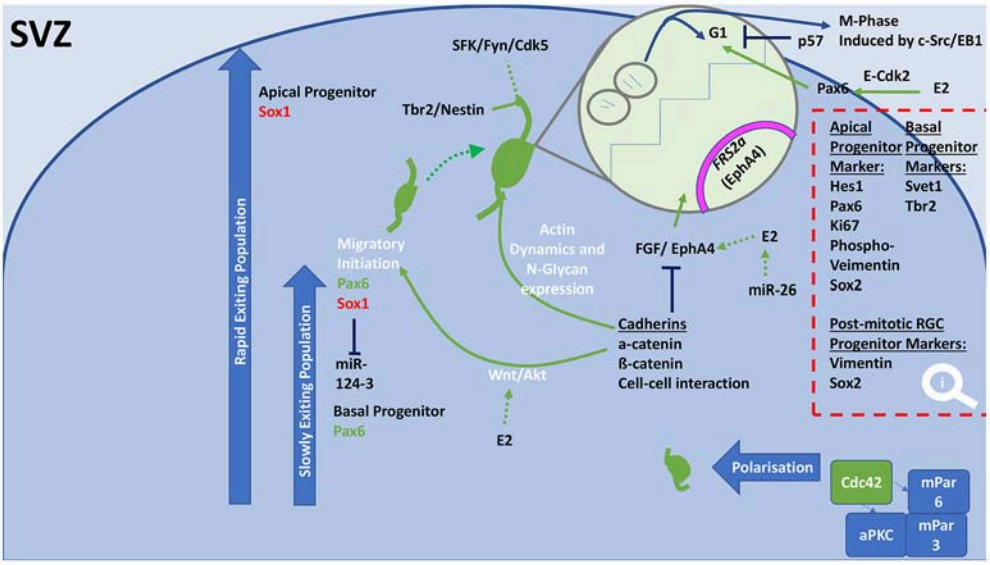

FIGURE 3 | General schematic diagram of molecular events that regulate proliferation, differentiation and the initiation of migration in the SVZ. Progenitor populations in the SVZ and outer SVZ undergo proliferation, differentiation, polarization, which are controlled to achieve either neural population increases or migration. In both these populations, collections of cellular processes are regulated to shift balance between proliferation and differentiation. Cadherins cause cell-cell interactions through Wnt/Akt signaling, which result in altered levels of Pax6 or Sox1 expression. Cadherins increase expression of actin dynamic molecules and N-glycan. Resulting in axonal growth and migration, which involves incorporation of Tbr2, Nestin, and the SFK/Fyn/Cdk5 pathway. Cadherins influence the cell cycle through inhibition of the growth factor FGF and EphA4, which results in decreased proliferation and increased differentiation to allow migration. E2 is able to increase FGF/EphA4 action leading to greater proliferation. This action is achieved through E2 stimulating Pax6 through E-Cdk2. Polarization of migrating neurons in the SVZ can be accomplished by the coupling of Cdc42 to an aPKC/mPar6/mPar3 complex. SVZ, subventricular zone; miR, microRNA; E2, Estradiol; ERα, estrogen receptor alpha; ERß, estrogen receptor beta; GPER, G-protein estrogen receptor; aPKC, atypical protein kinase C; FGF, fibroblast growth factor; FRS2a, fibroblast growth factor receptor substrate 2a; G1, Gap 1 phase; SFK, Src family kinase; EphA4, Ephrin type-A receptor 4; Fyn, Src tyrosine-protein kinase fyn; NSCs, neural stem cells.

Chemotaxis is mediated through membrane-bound receptors and extracellular matrix proteins, including Rac, brain-derived neurotropic factor (BDNF) and TrkB signaling (**Figure 4**; Zhou et al., 2018). Calcium-dependent mechanisms recruit BDNF (Zhou et al., 2018). BDNF binding to TrkB stimulates axonal growth and dendritic morphological change (Cohen-Cory and Fraser, 1995; Wang et al., 2015) (**Figure 4**). The selection and elongation of a leading (axonal) strand stimulates migration. Migration can be induced by Tiam1/Rac1/ERK signaling (Xiao et al., 2013). Interaction between the SFK Fyn and serine/threonine kinase family member Cdk5 are evidenced to play a leading role in progenitor cell axon guidance and dendritic orientation (Sasaki et al., 2002; **Figure 4**). Reelin and Dab1 alter cytoskeleton morphology through CLASP2 (Dillon et al., 2017). Cux1 and Cux2 regulate dendritic morphology through Pax6 and p27 in pyramidal neurons (Nieto et al., 2004).

The lamination process relies on signaling pathways that halt migration and trigger neuronal maturation through terminal and somal translocation. Reelin, Dab1, Wnt/Fizzled, and SPARC-like1 (SC1) are essential for this process. Wnt/Frizzled and reelin signaling regulates boundary formation during lamination (Augustine et al., 2001; Brault et al., 2001; Franco et al., 2018). SPARC-like1 (SC1) signals detachment of RGCs from the membrane. Terminal translocation in neurons is signaled by Dab1 and Cullin5 interaction (Feng et al., 2007; **Figure 5**). Migrating neurons interact with reelin through RapGEF2 (Ye et al., 2014). Neurons extend dendrites into the CP, which requires elongator to acetylate α-tubulin resulting in microtubule reassembly (Creppe et al., 2009; Heng et al., 2010). Interaction between reelin and VLDLR and ApoER2 causes Dab1 phosphorylation, which recruits PI3K and Lis1 (Bock et al., 2003; Chai and Frotscher, 2016; Lane-Donovan and Herz, 2017). Nectin1 and Nectin3 show capability of facilitating and mediating somal translocation (Hirota and Nakajima, 2017; **Figure 5**). N-cadherin interacts through the Nectin's cytoplasmic domain region.

CYTOARCHITECTURE-SPECIFIC MECHANISM INVOLVED IN SEXUAL DIFFERENTIATION

Sexual differentiation in the central nervous systems occurs throughout vertebrate evolution, including human beings (Jazin and Cahill, 2010). Hypothalamic nuclei are profoundly sensitive to sex-dependent change. The anteroventral periventricular (AVPV) nuclei in the hypothalamus is typical of the sexual dimorphism. Due to caspase-dependent apoptotic signaling induced by perinatal exposure to estradiol (Waters and Simerly, 2009), males typically have a smaller AVPV than their female peers (Simerly et al., 1985). The phenotype of this is a decreased cellular volume (Forger et al., 2004). Estradiol has shown to be a

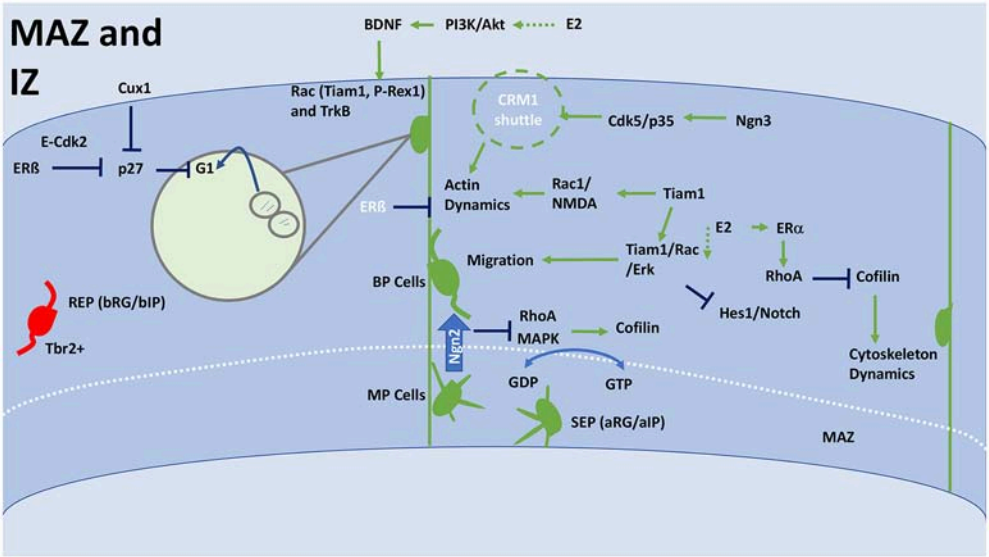

FIGURE 4 | General schematic of corticogenesis in the IZ and MAZ, including differentiation and migration. The IZ facilitates cytoskeleton changes that affect the morphology of the migrating cell, much of this will occur in the MAZ. The MAZ is an accumulation ground for multipolar cells that are forming into mature bipolar neurons, which can then enter into the CP. The MAZ is comprised of two populations SEP and REP. Whereas the REP population passes straight through the MAZ, the SEP remains in the MAZ for much longer. Ultimately, the SEP forms the more superficial layer of the CP. REP can be distinguished from SEPs as they are Tbr2+. Crossing from the MAZ to IZ requires a transition from MP to BP, which is initiated by Ngn2, which inhibits RhoA and MAPK that have a downstream effect on Cofilin. Cofilin interacts with RGCs to increase cytoskeleton dynamics and affect a neuronal morphology. Cofilin can also be inhibited by RhoA, which is modulated through ERα. Oscillation between GDP and GTP control orientation and polarization of cells within the MAZ. This is achieved through lamellipodium formation and interaction with the cofilin system. Upregulation of Tiam1 or E2 can result in activation of Tiam1/Rac/Erk signaling, which stimulates BP cells to migrate through the IZ. Tiam1 also activates Rac1/NMDA to alter actin dynamics, which results in increased elongation, synaptogenesis and dendritogenesis. This process can be negatively regulated by ERβ or actively upregulated by Ngn3 through Cdk5/p35 activating a CRM1 shuttle. RGCs are stimulated to move toward the CP by interaction with BDNF. E2 stimulation via PI3K/Akt signaling can cause BDNF expression. BDNF binds to TrkB and upregulates Rac/Tiam1/P-Rex1 activity. Through Cux1 and E2 (ERβ), proliferation is inhibited at this stage allowing for migration to take place. Both Cux1 and E2 inhibit p27, which stops proliferating cells at the G1 phase. E2, estradiol; IZ, intermediate zone; MAZ multipolar cell accumulation zone; CP, cortical plate; RGC, Radial glial cell; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; BP, bipolar; MP, multipolar; SEP, slow exiting population; REP, rapidly exiting population; BDNF, brain-derived neurotrophic factor; NMDA, N-methyl-D-aspartate; TrkB, Tropomyosin receptor kinase B; MAPK, mitogen-activated protein kinase; PI3K, Phosphoinositide 3 kinase; Akt, Protein Kinase B; bRG/bIP, basal radial glia/intermediate progenitor; aRG/aIP, apical radial glia/intermediate progenitor; G(D/T)P, guanosine (di/tri)-phosphate; CRM1, chromosomal maintenance 1/exportin 1; Cux1, cut like homeobox 1; RhoA, Ras homolog gene family, A; EphA4, Ephrin type-A receptor 4; Tiam1, T-cell lymphoma Invasion And Metastasis 1; Erk, extracellular signal-regulated kinases; Tbr2, T-box brain 2; E-Cdk2, G1 phase specific Cyclin E, Cyclin-dependent kinase 2; NSCs, neural stem cells.

crucial hormone in regulating and mediating the development of the neocortex (Beyer, 1999; McEwen and Alves, 1999). For example, ERß knockout mice (ERβKO) have revealed that ERβ is necessary for cortical lamination, which indicates a potential role for estradiol in orientation through actin dynamics (Wang et al., 2003), although the mechanisms by which this occurs is poorly understood. Ultimately, it is not clear if this mechanism may lead to sexually dimorphic phenotypes. A number of studies have shown that estradiol increases the proliferative status in embryonic NSCs (Fried et al., 2004), which involves ERs but is limited to neurogenesis in embryonic development (Brännvall et al., 2002). Estradiol-induced proliferation of NSCs is mediated through the classic estradiol receptors, ERα and ERβ (Okada et al., 2010). Hitherto, the literature has not provided a comprehensive summary of the finer biochemical interactions of estradiol, which can be seen in many pathways mentioned above and throughout the cortex. In the proceeding sections, we review the evidence that indicates specific molecular players involved in corticogenesis and how these may be regulated in a sexually dimorphic manner. In addition, data that link estradiol-signaling with the regulation of key molecular players involved in the distinct and carefully orchestrated events described in the previous sections (**Figure 6**).

Markers of Proliferation in Sexual Dimorphic Areas

Sexual dimorphism during development can be attributed to physiological factors such as hormonal exposure, as well as cell-intrinsic mechanisms that lead to differentiation (Swaab et al., 1995; Tsukahara, 2009). Cell-specific differentiation can be met through numerous factors, some of which will be discussed here.

The cellular state of neural progenitors can provide a comprehensive depiction on the condition of neurogenesis. As a marker of proliferation Ki67 expression in the discrete developing areas, can provide evidence to draw conclusions about total cell volumes at maturity. Infant rat preoptic area and hypothalamus show higher expression of Ki67, when compared to adults (He et al., 2013). Furthermore, colocalisation of

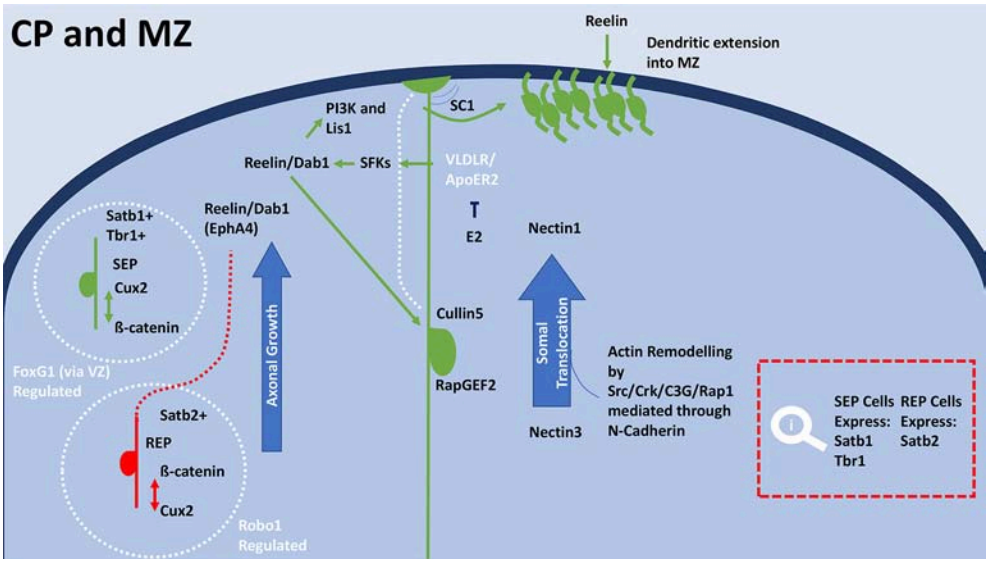

FIGURE 5 | General schematic of terminal translocation and differentiation in the CP during development. Within the CP SEP neurons (green) have high expression of Cux2 and low expression of β-catenin, whereas REP neurons (red) have high expression of β-catenin and low expression of Cux2. Satb1 positive cells express in the superficial layers of the MZ, which overlap with Tbr1 expression. Terminal translocation and therefore lamination rely on regulation by FoxG1 for the SEP neurons and radial distribution of Satb2 expressing REP neurons is regulated by Robo1. FoxG1 on deep-layer progenitors through transcription switches their progeny to upper-layer neurons through repression of Tbr1. After exiting the cell cycle, Satb2-expressing cells immediately migrate to the upper layers of the cortical plate. Satb2 expressing cells are much more reliant on the reelin/Dab1 and Ephrin-A pathways for cortical migration. Reelin binds to the RGCs by VLDLR and Apoer2 receptors (Lane-Donovan and Herz, 2017), which causes the adaptor protein Dab1 to become phosphorylated. Upon phosphorylation by SFKs, Dab1 recruits various downstream molecules including PI3K and Lis1. E2 is able to inhibit VLDLR/ApoER, modulates reelin's mechanisms in cortical migration. Reelin's interaction with cadherin is also essential for the termination of migration. Through regulating terminal translocation, the reelin/Dab1/Rap1/N-Cadherin signaling pathway leads to the inside-out lamination of the cortex. Nectin molecules expressed in the Cajal-Retzius cell (Nectin1) and the migrating neuron (Nectin3) are also necessary for somal translocation. The initiation of detachment is signaled by SC1, which is expressed on the surface at the top and bottom of RGCs surfaces. The anti-adhesive signal is crucial to proper cortical development, as the absence of SC1 results in failure of neurons to detach and properly position. Dab1 interacts with Cullin5 in the migrating cell to accumulate in the appropriate cortical layer. Termination of polarization upon reaching the appropriate location is met by the degradation of reelin receptors, N-cadherin and Dab1 by exocytosis and endocytosis. E2, Estradiol; IZ, intermediate zone; MAZ, multipolar cell accumulation zone; CP, cortical plate; RGC, Radial glial cell; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; BP, bipolar; MP, multipolar; SEP, slow exiting population; REP, rapidly exiting population; SFK, Src family kinases; PI3K, Phosphoinositide 3 kinase; SC1, SPARC-like1; VLDLR, very low-density lipoprotein receptor; ApoER2, apolipoprotein E receptor 2; Cux2, cut like homeobox 2; FoxG1, Forkhead Box G1; Dab1, Disabled-1; Tbr1, T-box brain 1; Lis-1, Lissencephaly-1; Crk, (p38/adaptor molecule crk); C3G, CRK SH3-binding GNRP; Rap1, Ras-like GTPase; Satb(1/2) Special At-rich sequence binding protein (1/2); Robo1, Roundabout Guidance Receptor 1; EphA4, Ephrin type-A receptor 4; NSCs, neural stem cells.

Ki67+ cells and estradiol is noted in the preoptic area and hypothalamus.

Nestin has also been implicated in generating larger cells volumes in sexually dimorphic ventricular areas, such as the POA (He et al., 2013). Estradiol treatment on rat telencephalon cultures did not have an effect on the proportion of nestin-positive cells (Okada et al., 2010). However, estradiol did affect the cell fate of progenitor cells from the telencephalon of rats, which caused an increase in the ratio of oligodendrocytes being produced (Okada et al., 2008). The rostral third ventricle is the site of stem cell niches that strongly express nestin, which supplies the frontal and medial neocortex with neuroblasts (He et al., 2013). Estradiol has significantly strong proliferation-promoting effects in the 3rd ventricle stem cell niche (He et al., 2015). The effect of estradiol on nestin-expressing stem cell niches may serve to further reinforce the proliferative proprieties of estradiol in the developing cortex.

Ki67 and nestin expression colocalized with estradiol in sexually dimorphic areas is suggestive of a possible mechanism. The inclusion of a proliferative marker and intermediate filament protein is suggestive that estradiol increases the proliferative state and leads to greater migration through radial axon growth (Scholzen and Gerdes, 2000; Xu et al., 2015).

Glial Fibrillary Acidic Protein (GFAP)

GFAP is a robust astrocytic marker of RGC progeny (Berman et al., 1997). Immunoreactive GFAP has been detected in neural circuits that display sexual dimorphism. These circuitries can be manipulated through introduction of exogenous steroid hormones (Martinez et al., 2006). In studies primarily focusing on the cortex, ovariectomised rats showed higher rates of cortical proliferation resulting from treatment with estradiol (Malinowska-Kolodziej et al., 2009). The study also noted co-localization of nestin and GFAP in the cortex, which can be suggested as a mark of increased radial glial migration resulting from estradiol treatment. The substantia nigra of ovariectomised parkinsonian model mice did not respond to estradiol in the same manner, with GFAP remaining unchanged (Yi et al., 2016).

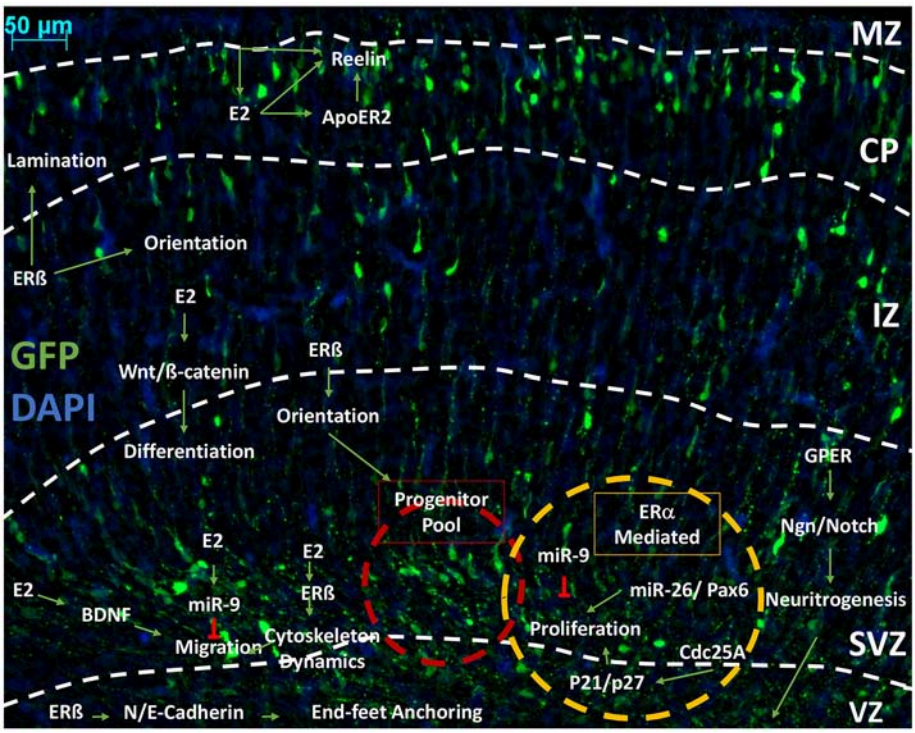

FIGURE 6 | An outline of the pathways influenced by estrogenic signaling to drive cortical development. Representative image of developing cortex. Electroporation of eGFP was performed at E14.5 and brains collected at P0 as previously described (Srivastava et al., 2012a,b). Representative image of a coronal rodent brain slice at P0 during cortical development. Estrogen (Estradiol/17ß-estradiol) is shown interacting in pathways outlined in **Figures 1–4**. that are involved in neurogenesis, proliferation, differentiation and migration. We propose that Estradiol may be able to affect the proliferative status of the progenitor pool through Pax6 and kinase inhibitors through ERß or ER. Through ERß, Estradiol can affect migration by marking terminal translocation points, therefore setting lamination boundaries. Estradiol is able to influence orientation through a number of mechanisms, which has been shown specifically in GFAP+ RGCs and mediated through ERß. Through ERß, Estradiol also alters the cytoskeleton dynamics to initiate migration. Cytoskeleton dynamics are also affected by GPER to increase neuritogenesis, which will later form axons and dendrites. Estradiol also alters differentiation state by increasing Wnt/ß-catenin signaling, which furthers dendritogenesis. Estradiol is able to negatively regulate migration and proliferation by increasing miR-9, which inhibits these processes. In regards to proliferation, it is hypothesized that ERα is primarily responsible for mediating the action of estrogenic signaling. Estradiol also interacts with reelin signaling in the CP and MZ, both directly by increasing expression and indirectly by stimulation of ApoE gene expression, which increases the activity of ApoER to affect the morphology of the cortex and cytoarchitecture of the CP. E2, estradiol; IZ, intermediate zone; MAZ, multipolar cell accumulation zone; CP, cortical plate; ERß, estrogen receptor beta; ERα, estrogen receptor alpha; GPER, G-protein estrogen receptor; miR-x, microRNA; ApoER2, apolipoprotein E receptor 2; Wnt, Wingless-type; BDNF, brain-derived neurotropic factor; p21, cyclin-dependent kinase inhibitor 1A/Cip1; p27, cyclin-dependent kinase inhibitor 1B/Kip1; Ngn, neurogenin; Cdc25A, Cell division cycle 25 homolog A; VZ, ventricular zone; SVZ, subventricular zone.

However, Yi et al.'s team used BDNF as a marker to show an estradiol-dependent increase in net migration of neurons in the midbrain. These results suggest that estradiol within the cerebral cortex is able to increase proliferative status. The interaction between GFAP and estradiol is likely mediated through ERß. Indeed, Zsarnovszky et al. (2002) showed that estradiol via ERß is able to directly regulate GFAP expression in the interpeduncular nucleus, an important component of the limbic midbrain circuitry. Furthermore, agonists of ERß, namely LY3201, caused immunoreactive GFAP in hippocampal and cortical astrocytes to increase (Tan et al., 2012). This study also found that dendritic spines could be reduced using an ERß agonist (Tan et al., 2012). This suggests the mechanism is a result of downstream ER signaling and not directly related to estradiol expression. Taken together, these data suggest interaction between GFAP and estrogen results in altered astrocytic expression and has implications in the proliferation of RGCs.

Paired Box Protein—Pax-6

The transcription factor Pax-6 is essential in neurogenesis. Mutations in the *PAX6* gene can cause serious developmental disorders such as aniridia, autism spectrum disorder, and intellectual disability (Davis et al., 2008). Pax-6 and estradiol have been targeted in a large portion of breast cancer research. Concordantly, suppression of *PAX6* gene expression inhibits cell growth, which appeared to have a knock-on effect from or to ERK1/2, p38 and cyclin D1 (Zhao et al., 2014). In ER-positive cancer cell lines, MCF-7, Pax-6 knock-down interrupts cell cycling at G1 phase and reduced proliferation (Zong et al., 2011). Estradiol's stimulation of *PAX6* has been noted in MCF7 cell lines. Estradiol is able to stimulate the transcription activity of 187 transcription factors (Li et al., 2014b), which impresses its involvement in proliferation, migration and cell cycle regulation. Based on these data, it is plausible that Pax-6 may be the facilitator for estradiol-mediated proliferation, which can be seen in the increased proliferative status of GFAP+ NSCs (**Figure 6**).

One might expect that Pax-6 knockdown models may be rescued by simulation of ERß during neurodevelopment. However, one must be cautious about the interpretation of this data. Purely due to the availability the authors have used evidence and data from MCF7 (Cancer) cultures, which are not directly comparable to neural circuits and cultures. However, it is the opinion of the authors that the MCF7 lines still suggest an interesting case for the interaction of estrogen and Pax-6.

Neurogenin (Ngn)

Intracellular progenitor Notch signaling plays a pivotal role in progenitor maintenance, cell fate and differentiation (Ohtaka-Maruyama and Okado, 2015). Balancing between Hedgehog and Notch signaling maintains correct cortical formation (Dave et al., 2011). Estradiol is able to mediate the regulators of Notch signaling via ERs (Bender et al., 2010). Through the utilization of Ngn3 and GPER1, estradiol promotes neuritogenesis in the mouse hippocampus (**Figure 6**), which also incurred the activation of PI3K signaling (Ruiz-Palmero et al., 2013). Ngn3 is promoted through downregulation of Notch signaling (Arevalo et al., 2011). Furthermore, neurogenins could be partially responsible for sex-dependent dimorphisms. Ngn3 activity has been suggested to influence sex-dependent differentiation in neurons, which may result from epigenetic regulation of sex-linked genes (Scerbo et al., 2014). Ngn3 is also repressed by estradiol-mediated inhibition of Hes-1, which is regulated through Notch signaling (Salama-Cohen et al., 2006). Ngn2 and Ngn3 have a role in division within the VZ, which is mediated by *Hes-1*. Through which, estradiol may act as a mediator control asymmetric division. Furthermore, as Ngn3 is able to stimulate the growth and migration of cells toward the CP, it is plausible that estradiol works through Ngn3 during neurodevelopment.

Kinases

Estradiol targets CDK Cdc25A to promote growth during cell cycling, which also influences expression of p21 and p27 (Foster et al., 2001). In cultured cortical neurons, activation of ERβ resulted in the phosphorylation of kinase proteins: p21-activated kinase and ERK1/2 Kinase proteins are responsible for regulation of actin cytoskeleton dynamics (Srivastava et al., 2010). Rapid estrogenic signaling mediated through ERα (Sanchez et al., 2009) activates Src/Rho/Cdk5, WAVE1 pathway and a RhoA/ROCK-2/Moesin cascade, which cause the protrusion of dendritic spines (Polleux and Snider, 2010; Srivastava et al., 2013b). It has been suggested that estradiol is a modulator of these processes (Srivastava et al., 2010; Sellers et al., 2015b; Zhao et al., 2017). Other CDKs have been implicated in cell cycle regulation. E-Cdk2 has been identified as a central component of estradiol's regulatory system during G1 to S phase of the cell cycle progression (Doisneau-Sixou et al., 2003). Upregulation of CDK inhibitor p21 can stimulate proliferation of embryonic stem cells induced by epidermal growth factor, which is activated by estradiol (Brännvall et al., 2002). Interaction with CDK inhibitors such as p27 can regulate ERs and *vice versa* to mediate transcription and gene expression (Prall et al., 1997). In the MCF-7 cell line ERα is mediated by p27 to regulate nuclear localisation and therefore transcription (Jeon et al., 2012). Estradiol is also able to utilize the ubiquitin proteasome system (UPS) to degrade p27, after phosphorylation by MAPK/ERK (Singer et al., 1999; Huang et al., 2012). This may serve as a regulatory mechanism for proliferation. Estradiol elicits rapid responses, which can be attributed to kinase phosphorylation (Srivastava et al., 2013b). These rapid actions can be attributed to involvement of tyrosine kinases (Karthikeyan and Thampan, 1996). Furthermore, by binding to cytoplasmic or membrane-bound receptors, estradiol is able to activate phosphorylation cascades by ERK, PI3K and MAPK (Singh et al., 1999; Fox et al., 2009).

Small Non-coding RNAs

In MCF-7 cell lines, estradiol's influence on small non-coding microRNA (miR) is well documented (Klinge, 2009; Jiang et al., 2016). Estradiol-stimulated proliferation can be induced by miR-26 in MCF-7 cell lines (Tan et al., 2014). Conversely, this process can be negatively regulated by estradiol through upregulation of miR-9, which inhibits proliferation and migration (Fang et al., 2015). Interestingly, whereas Tan's group (Tan et al., 2014) showed miR-26 was ER-dependent to induce proliferation, Fang et al.'s group (2015) showed miR-9 inhibited proliferation and migration independently of ERs (**Figure 6**). Current literature shows ERα induces proliferation and ERß induces apoptosis, which is reflected in miR interaction with ERα and ERß leading to polarizing effect (Helguero et al., 2005). miR-22 (Pandey and Picard, 2009), miR-221-222 (Di Leva et al., 2010), miR-206 (Adams et al., 2007; Kondo et al., 2008) are generally correlated with a reduction or inhibition of ERα and its effects. However, some miRNAs have shown to be an exception to this. miR-342 (He et al., 2013) overexpression upregulated MCF-7 cells to induce apoptosis and inhibited proliferation, which possibly highlights the interdependence of the two ERs in conditions such as extreme overexpression, which would coincide with the "ying-yang" relationship ERs have been suggested to possess (Lindberg et al., 2003).

In a clinical setting, estradiol may act on miR to account for the gender disparity seen in the diagnosis of schizophrenia (Mellios et al., 2012). Specifically, in the frontal cortex of schizophrenic male mice, miR-30b was expressed in greater quantity than in females. Many of the studies cited in this section are from cancer cell lines, which leave conclusions drawn little more than conjecture. The strength of the individual results coupled with the correlative observations, implicate miR as a player during cortical development.

Using miRs in studying cortical development will require further development of laboratory techniques. Currently, processes involved in analyzing miRs are costly and the literature has shown to be divided in agreeing upon various sequences and expression patterns (Guo et al., 2016).

Wnt

Wnt signaling coordinates the progeny of progenitor cells during differentiation (Kriska et al., 2016), which could serve as a mechanism used by estradiol to mediate differentiation. Through Wnt3A, estradiol receptor signaling is able to promote differentiation in mesenchymal progenitor cells (Gao et al., 2013). Wnt signaling is also known to regulate neuronal differentiation

of cortical intermediate progenitors (Munji et al., 2011). Cross talk between Wnt and estradiol is facilitated by interaction with ß-catenin (Kouzmenko et al., 2004). A recent review highlights the importance of estradiol in Wnt signaling as a chief mediator in maintaining the balance of Wnt antagonist Dkk1 and Wnt/ß-catenin (Scott and Brann, 2013).

RhoA

As a key regulator of cell orientation in the cortex, RhoA activity is essential in cellular migration. Consequently, excess and deficit can cause impairment, however, ERα may serve as a regulator to RhoA activity and stability. Inactivation of ERα by siRNA increased RhoA protein expression, which resulted in decreased migration (Sailland et al., 2014). It is worth noting that PCR showed this process was independent of Wnt11 and N-Cadherin expression, which are established mechanisms leading to ERα activation (Dwyer et al., 2010). RhoA was also shown to decrease the transcriptional activity of ERα via RhoGDI increasing transactivation by Rho GTPase (Su et al., 2001).

Cadherin

Cadherins are used to anchor the end-feet of RGCs to the ventricular surface, which is a key aspect of maintaining polarization in the developing cortex (**Figure 6**). In ERßKO mice, migration, morphology and polarization are defective. Furthermore, it was shown that these processes could occur as ERßKO lacked the modulation of ERß over E-Cadherin and N-Cadherin (Xu et al., 2015).

BDNF and TrkB

The steroid hormone estrogen and the neurotrophin BDNF have been observed sharing mutual functions, in a co-dependent or even synergistic manner (Scharfman and Maclusky, 2005; Numakawa et al., 2010; Srivastava et al., 2013a). Respectively, they are established as two extracellular signaling molecules that work to meet numerous physiological functions. Estrogen and BDNF are both able to influence signaling cascades, synaptic structures and neuronal physiology in multiple and mutually inclusive areas (Greenberg et al., 2009; Waterhouse and Xu, 2009; Srivastava et al., 2010, 2011).

The connection between BDNF and estrogen is likely through a novel signaling system completely removed from the traditional trkB pathway. This is evidenced by no significant change in trkB mRNA or protein noted in gonadectomized or estrogen-replaced animals (Solum and Handa, 2002). Solum and Handa (2002) concluded that due to co-localisation of ERα and BDNF in pyramidal cells of the CA1 and CA3 hippocampal sub-regions. Since the publication of the Solum paper, other teams have uncovered a similar mechanism using GPER in the same cerebral region (Briz et al., 2015). Both groups concluded that estrogen and BDNF are in some way used to regulate cerebral function.

A possible mechanism for the interaction between BDNF and estrogen could be met through intracellular phosphorylation cascades. Estrogen has been documented providing restorative effects to ovariectomised mice. Estrogen (0.1 mg/kg) worked through PI3K/Akt signaling pathway to provide short-term neuroprotection of neurons in the midbrain by upregulating BDNF (Yi et al., 2016). Cultured dentate gyrus neurons *in vitro* reacted in comparable manner, as significant BDNF upregulation occurred after pharmacological exposure to estradiol (100 mM−1 μM) (Sato et al., 2007).

A comprehensive review of the literature in this area can be found in the references list (Srivastava et al., 2013a).

Neuroglobin

ERα regulates neuroglobin expression through genomic transcript region regulation (Guglielmotto et al., 2016). Estradiol is capable of upregulating neuroglobin through activation of ERß (De Marinis et al., 2013; Fiocchetti et al., 2015). Estradiol may utilize neuroglobin to regulate the formation of neurites during migration. Neuroglobin is found within the SVZ and IZ (Shang et al., 2006) and increases the phosphorylative state of Akt leading to neurite outgrowth (Li et al., 2014a).

Reelin and Dab1

In hippocampal slice cultures, exogenous application of estradiol causes reelin expression to increase from Cajal-Retzius cells, which is facilitated by ERs. Furthermore, aromatase activity contributes to reelin expression. Impairment of aromatase activity causes reelin expression to reduce (Bender et al., 2010). If the aromatase gene is knocked out in male mice, specific areas in the brain have been shown to change in size (Pierman et al., 2008). Moreover, this morphological impairment can somewhat be saved through estrogen and testosterone treatments. As shown in reeler mice, estradiol upregulates reelin mRNA in the Purkinje cells (PC) of the cerebellum, with a particularly notable effect in males. Reelin protein bands (420, 310, and 180 kDa) were also shown to be upregulated. Furthermore, *reeler* mice show an abnormal steroid hormone profile with testosterone and estradiol increased but dihydrotesterone decreased (Biamonte et al., 2009).

Apolipoprotein

Estradiol down-regulates ApoER2 in differentiating osteoblasts, which is reflected in other LDLR family members. Leading to the promotion of differentiation in osteoclasts (Gui et al., 2016). Estradiol may alter apolipoprotein expression through genomic transcript mediation. Estradiol is able to upregulate ApoE gene expression in BL6 mice (Srivastava et al., 1997). ERα mRNA expression during development correlates with regulation of differentiation of osteoblasts (Bodine et al., 1998). Differentiation of apical papilla stem cells is also regulated by estradiol via the activation of MAPK signaling (Li et al., 2014c). Furthermore, ApoER2 interacts with reelin to govern morphology and cytoarchitecture (Stranahan et al., 2013).

Estradiol and Gene Expression

The cortical transcriptome can be manipulated epigenetically by estradiol, including functions ranging from memory (Frick et al., 2011), transcription factor regulation (Jadhav et al., 2015), and cell cycling (Couse et al., 1995). Estradiol has shown to significantly alter at least 88 genes in relation to the control of the cortex (Humphreys et al., 2014). Genes associated with synaptic activity, myelination, synthesis and metabolism, neurotransmission and kinase signaling

were all shown to alter expression following introduction of estradiol after ovariectomy. Removing the expression and synthesis of extra-nervous estrogen in males, would likely further exacerbate the effects and may highlight sex-dependent gene expression in cortical development. Introduction of estradiol in ovariectomised rats induces genomic changes that influence the dopaminergic and peptidergic neural networks (Sárvári et al., 2010). This may be as a result of estradiol's role in neurotransmission. It is known that aromatase is localized to the synapse and can be activated by calcium phosphorylation (Balthazart and Ball, 2017).

ERα has also shown to mediate subtle epigenetic mechanism that result in sex-specific differences in the prefrontal cortex in adulthood (Westberry and Wilson, 2012). It is plausible that this sex-specific difference is met through alteration in the inhibitory GABAergic signaling pathway. Pyramidal neurons of the prefrontal cortex rely on phosphorylation-dependent mechanisms for cellular trafficking and signaling. G protein-gated inwardly rectifying potassium channels modulate excitability and were shown to be susceptible to sex-specific neurochemistry (Marron Fernandez de Velasco et al., 2015). The extent to which estradiol is able to regulate these mechanisms remains to be seen. Estradiol affects the genetic transcriptome of 88 genes (likely more) within the cerebral cortex. However, questions remain: which mechanisms does estradiol utilize to interact with these genes and are these genes affected in different sexes? Are males more susceptible to estrogen transcriptome deficits during cortical development? Does the knockout of aromatase impact estrogenic signaling? Does aromatase knockout affect males and females unequally? Does the aromatase knockout affect the transcriptome? Answering these questions would prove to further elucidate the pathways involved in cortical development. Furthermore, answering these questions would further elucidate the mechanisms estrogen adopts to control sexual dimorphism and to what extent it is able to control sexual dimorphism. Delivering a clear mechanism for sexual dimorphism would also aid in better understanding of neurodevelopmental disorders that show biased sexual distribution, such as autism spectrum disorder.

SUMMARY AND CONCLUSIONS

In summary, many lines of evidence exist that suggest estradiol has many critical roles in corticogenesis (**Figure 6**). Hitherto, no clear mechanism has been established. Substantial evidence purports to show a connection between estradiol, migration, and neurogenesis. A strong body of evidence exists showing that estradiol influences the proliferative body within the subventricular zone, which increases the availability of NSCs. The mechanism underlying this action has not been established but may be associated with Pax-6, neurogenins, and nestin. Estradiol influences many regulators of estrogenic-signaling, which suggests that this maybe a primary function. Mechanisms underlying estradiol's influence in regulating migration has been discussed in detail. The interaction with Wnt, Cadherin as well as various kinases produce dynamic actin changes, which has been suggested as mechanism for migration. Estradiol has also shown to associate greatly with the reelin/Dab1 system. Collectively, this investigation suggests estradiol as a proliferative regulator and migratory stimulator. Disruption to estrogenic signaling may result in an enlarged SVZ from impaired migration and un-regulated proliferative cycle. Alternatively, cells may accumulate within the CP after being ejected too early from the cell cycle. Understanding these mechanisms will further benefit developmental disorders.

AUTHOR CONTRIBUTIONS

MCSD and DPS wrote the manuscript. NJFG and KJS edited and contributed to the writing of the manuscript. KJS and DPS oversaw the project.

FUNDING

This study was funded by the Medical Research Council (MRC) grant MR/L021064/1 awarded to DPS.

ACKNOWLEDGMENTS

We thank Dr. George Chennell and the Wohl Cellular Imaging Centre for help with image acquisition.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 05 June 2018
doi: 10.3389/fendo.2018.00279

Androgen Regulation of the Mesocorticolimbic System and Executive Function

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *16 February 2018*
Accepted: *11 May 2018*
Published: *05 June 2018*

Citation:
Tobiansky DJ, Wallin-Miller KG, Floresco SB, Wood RI and Soma KK (2018) Androgen Regulation of the Mesocorticolimbic System and Executive Function. Front. Endocrinol. 9:279. doi: 10.3389/fendo.2018.00279

Multiple lines of evidence indicate that androgens, such as testosterone, modulate the mesocorticolimbic system and executive function. This review integrates neuroanatomical, molecular biological, neurochemical, and behavioral studies to highlight how endogenous and exogenous androgens alter behaviors, such as behavioral flexibility, decision making, and risk taking. First, we briefly review the neuroanatomy of the mesocorticolimbic system, which mediates executive function, with a focus on the ventral tegmental area (VTA), nucleus accumbens (NAc), medial prefrontal cortex (mPFC), and orbitofrontal cortex (OFC). Second, we present evidence that androgen receptors (AR) and other steroid receptors are expressed in the mesocorticolimbic system. Using sensitive immunohistochemistry and quantitative polymerase chain reaction (qPCR) techniques, ARs are detected in the VTA, NAc, mPFC, and OFC. Third, we describe recent evidence for local androgens ("neuroandrogens") in the mesocorticolimbic system. Steroidogenic enzymes are expressed in mesocorticolimbic regions. Furthermore, following long-term gonadectomy, testosterone is nondetectable in the blood but detectable in the mesocorticolimbic system, using liquid chromatography tandem mass spectrometry. However, the physiological relevance of neuroandrogens remains unknown. Fourth, we review how anabolic-androgenic steroids (AAS) influence the mesocorticolimbic system. Fifth, we describe how androgens modulate the neurochemistry and structure of the mesocorticolimbic system, particularly with regard to dopaminergic signaling. Finally, we discuss evidence that androgens influence executive functions, including the effects of androgen deprivation therapy and AAS. Taken together, the evidence indicates that androgens are critical modulators of executive function. Similar to dopamine signaling, there might be optimal levels of androgen signaling within the mesocorticolimbic system for executive functioning. Future studies should examine the regulation and functions of neurosteroids in the mesocorticolimbic system, as well as the potential deleterious and enduring effects of AAS use.

Keywords: 3β-hydroxysteroid dehydrogenase, aromatase, cognition, Cyp17a1, estradiol, neurosteroid, dehydroepiandrosterone, LC–MS/MS

INTRODUCTION

Berthold first reported the masculinizing effects of a bloodborne "substance" produced by the testes in male chicks (1). This substance is now known to belong to a class of steroids called androgens, which are synthesized by the male gonads and released into the circulatory system to regulate development, physiology, and behavior. Endogenous androgens are 19-carbon (C_{19}) steroids and include testosterone (T) and its metabolite 5α-dihydrotestosterone (DHT), which have the most pronounced androgenic effects. Other C_{19} steroids include T precursors such as dehydroepiandrosterone (DHEA) and androstenedione (**Figure 1**). Androgen synthesis from cholesterol occurs in the Leydig cells of the testes, stromal, and thecal cells of the ovaries, and the zona reticularis of the adrenal cortices in some mammalian species (2, 3).

Numerous studies examining the effects of gonadectomy (GDX), androgen receptor (AR) antagonists, androgen synthesis inhibitors, androgen replacement, and administration of supraphysiological amounts of androgens [i.e., anabolic-androgenic steroids (AAS)] demonstrate that androgens are critical for reproductive behavior [reviewed in Ref. (7)] and aggressive behavior [reviewed in Ref. (8, 9)]. However, recent research has revealed that more complex behaviors and cognitive processes, such as executive function, are also regulated by androgens. We will review research that examines the role of androgens in regulating the neural circuitry that mediates executive function and behaviors associated with executive function.

In the first section "The mesocorticolimbic system and executive function," we give a brief overview of the mesocorticolimbic system and its involvement in various executive functions. In the second section, we describe evidence for the presence of sex steroid receptors in the mesocorticolimbic system. In the third section, we summarize recent work that provides strong evidence for local synthesis of androgens and estrogens within the mesocorticolimbic system. In the fourth section, we discuss how AAS modulate the mesocorticolimbic system. In the fifth section, we explore how androgens alter neurochemical signaling and cytoarchitecture in nodes of the mesocorticolimbic system. Finally, in the last section, we review preclinical and clinical studies demonstrating that GDX, AAS, and perhaps local production of androgens influence executive functions such as behavioral flexibility and inhibitory control. Like many other neuromodulator systems, androgen signaling levels are likely maintained at particular levels within different brain regions to achieve optimal executive function.

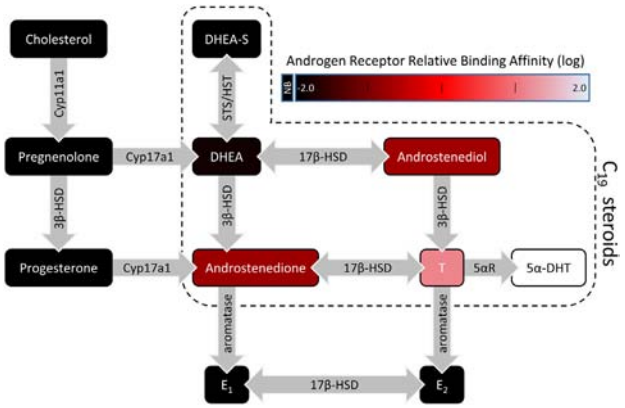

FIGURE 1 | A simplified illustration of the steroidogenic pathway with a focus on C_{19} steroids. For C_{19} steroids, fill color represents the relative binding affinity to the androgen receptor (4–6). Steroidogenic enzymes are represented by the gray arrows. Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase/isomerase; 5αR, 5α-reductase; 5α-DHT, 5α-dihydrotestosterone; 17β-HSD, 17β-hydroxysteroid dehydrogenase; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; E_1, estrone; E_2, 17β-estradiol; HST, hydroxysteroid sulfotransferase; STS, steroid sulfatase; NB, no binding.

THE MESOCORTICOLIMBIC SYSTEM AND EXECUTIVE FUNCTION

Executive functions are a collection of cognitive operations that interact to facilitate selection and implementation of behaviors to attain chosen goals. More basic operations include selective attention, inhibitory control, and working memory (i.e., temporary maintenance and manipulation of information). These operations work in concert with those processed by other mnemonic, affective, and motivational systems to regulate more complex processes such as cognitive flexibility and cost/benefit decision making. It is well established from lesion and functional imaging studies in humans and non-human animals that various aspects of executive functioning are critically dependent on different regions of the prefrontal cortex (PFC) and its interactions with striatal regions, including the nucleus accumbens (NAc; **Figure 2**).

The PFC and the NAc receive dopamine (DA) input from the ventral tegmental area (VTA) in the midbrain, and DA transmission within these regions plays a key role in facilitating both basic and more complex functions mediated by these circuits. Thus, the seminal findings of Brozoski et al. (10) revealed that DA depletion in the frontal lobes of monkeys markedly impairs

Abbreviations: 17β-HSD, 17β-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase/isomerase; 5αR, 5α-reductase; AAS, anabolic androgenic steroids; ACC, anterior cingulate cortex; ADT, androgen deprivation therapy; AR, androgen receptor; CPP, conditioned place preference; D_1R, dopamine receptor D_1; D_2R, dopamine receptor D_2; DA, dopamine; DAT, dopamine transporter; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; DHT, 5α-dihydrotestosterone; E_2, 17β-estradiol; ER, estrogen receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; GABA, γ-aminobutyric acid; $GABA_A$R, Type A γ-aminobutyric acid receptor; GDX, gonadectomy; GPER1, G protein–coupled estrogen receptor 1; GPRC6A, G protein–coupled receptor family C group 6 member A; HST, hydroxysteroid sulfotransferase 2A1; i.c.v., intracerebroventricular; i.v., intravenous; IGT, Iowa Gambling Task; ir, immunoreactivity; mAR, membrane-associated androgen receptor; mER, membrane-associated estrogen receptor; mPFC, medial prefrontal cortex; mPFC-PL, prelimbic medial prefrontal cortex; NAc, nucleus accumbens; NAcC, nucleus accumbens core; NAcS, nucleus accumbens shell; NMDA-R, *N*-methyl-D-aspartate receptor; OFC, orbitofrontal cortex; PCR, polymerase chain reaction; PFC, prefrontal cortex; POA/HYP, preoptic area/hypothalamus; PPI, prepulse inhibition; qPCR, quantitative polymerase chain reaction; StAR, steroidogenic acute regulatory protein; T, testosterone; TH, tyrosine hydroxylase; TSA, tyramide signal amplification; VTA, ventral tegmental area; ZIP9, Zrt- and Irt-like protein 9.

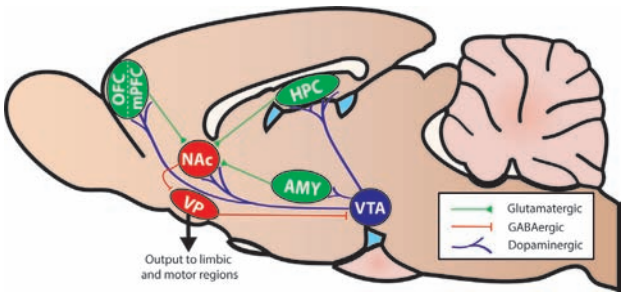

FIGURE 2 | A simplified diagram of the mesocorticolimbic system and associated structures in the rodent brain (sagittal view). Abbreviations: AMY, amygdala; HPC, hippocampus; NAc, nucleus accumbens; mPFC, medial prefrontal cortex; OFC, orbitofrontal cortex; VP, ventral pallidum; VTA, ventral tegmental area.

working memory, and subsequent psychopharmacological studies revealed that these functions are dependent primarily on PFC D_1 receptor (D_1R) activity [reviewed in Ref. (11)]. Different forms of cognitive flexibility are also dependent on DA activity within the frontal lobes and/or striatal regions. For example, shifts between strategies, rules, or attentional sets are dependent on DA transmission in both the medial prefrontal cortex (mPFC) and NAc. D_2 receptors (D_2R) in the PFC facilitate suppression of old strategies, whereas D_1R in the PFC and NAc facilitate establishment and maintenance of new strategies (11–14). In comparison, reversal learning is a simpler form of cognitive flexibility, entailing a shift between stimulus–reinforcement associations (i.e., use the same basic strategy, but approach a different stimulus). The orbitofrontal cortex (OFC) plays a key role in mediating reversal learning in both primates and rats (15, 16). Reversal learning is generally unimpaired by global depletion of PFC DA (17), and DA input to dorsal striatal regions appears more crucial to this form of flexibility (18, 19).

Dopamine transmission in prefrontal–striatal circuitry also mediates evaluative functions entailing a choice between a smaller, readily available reward vs. a larger/more palatable reward associated with some form of cost, which can diminish the subjective value of objectively larger or more-preferred rewards. These forms of decision making are exquisitely sensitive to manipulation of DA transmission, in that systemic treatment with DA antagonists reduces preference for larger rewards associated with a greater effort cost or uncertainty (20–22). However, the mechanisms through which DA regulates choice behavior can vary across different nodes of the mesocorticolimbic circuit. For example, blockade of D_1R, but not D_2R, in the NAc reduces risky choice (23), whereas blockade of either receptor in the NAc diminishes preference for more preferred rewards associated with a greater effort cost (24). Likewise, blockade of D_1R, but not D_2R, in different subregions of the mPFC shifts preference away from more costly rewards (25, 26) and also makes animals more risk-averse (27). Yet, blockade of PFC D_2R impairs modifications of decision biases in response to changes in risk/reward contingencies (27, 28). Collectively, these studies indicate that DA transmission within different nodes of the mesocorticolimbic system helps to refine different types of decision making by promoting choice toward larger, yet more costly, rewards, and modifying decision biases when cost/benefit contingencies change. The critical involvement of DA in various executive functions suggests that other signals that can influence DA signaling, such as sex steroids, may also influence these functions.

THE MESOCORTICOLIMBIC SYSTEM CONTAINS SEX-STEROID RECEPTORS

Multiple lines of evidence indicate that receptors for sex steroids are present in the VTA, NAc, mPFC, and OFC. Here, we focus on the classical AR, the estrogen receptors (ER)α and ERβ, and more recently discovered membrane-associated androgen receptors (mAR) and ER (mER). We briefly discuss androgen metabolites that can act *via* allosteric binding sites on neurotransmitter receptors.

Androgens can act on target cells by binding to intracellular AR. Of the endogenous androgens, T and DHT have the highest binding affinities for AR, while DHEA, androstenedione, and androstenediol have weak binding affinities for AR [(4–6); **Figure 1**]. AAS have a wide range of binding affinities for AR, and users select different AAS according to the balance of desired anabolic (myotrophic) actions and unwanted side-effects (e.g., gynecomastia).

Androgens are lipophilic and non-polar, and thus they can pass through the blood–brain barrier and then the plasma membrane of cells to bind with AR in the cytosol. This ligand–receptor complex then dimerizes, is phosphorylated, and translocates to the cell nucleus, where the DNA-binding domain binds to a specific sequence of DNA called the hormone response element and acts as a transcription factor (29). Such genomic effects are responsible for many of the peripheral effects of androgens, such as enhancing muscle growth (30). ARs are also found in multiple brain regions. Generally, ARs are found in the highest concentrations in hypothalamic and limbic regions that regulate homeostatic functions, reproductive behaviors, and aggressive behaviors (31). For example, male mice with reduced AR in the nervous system show decreases in mating and aggression (32).

One way in which androgens might influence executive function is through direct actions on the mesocorticolimbic system. ARs are expressed in regions of the mesocorticolimbic system, albeit at lower levels than in the hypothalamus. In particular, the VTA, NAc, and mPFC express low to moderate levels of AR in male and female rodents (33–37), non-human primates (38, 39), and humans (40). Using microdissected tissue from mesocorticolimbic nodes, we recently demonstrated AR mRNA in the VTA, NAc, and mPFC using sensitive and specific probe-based quantitative polymerase chain reaction (qPCR) assays (36). The presence of AR protein immunoreactivity (AR-ir) in these regions has also been reported; however, the number of AR per cell is low, which results in immunohistochemical staining that is faint, challenging to quantify, and easy to overlook (41). One reason is that, in extrahypothalamic regions, androgen receptor immunoreactivity (AR-ir) is often located in neuronal processes and not concentrated in neuronal nuclei. Nonetheless, there are

many processes and nuclei that express AR in the cerebral cortex, which has been verified by immunoelectron microscopy (35, 41). By adding a Tyramide Signal Amplification (TSA) step in the immunohistochemistry protocol, we recently showed that AR-ir cells are present in the VTA, NAc, mPFC, and OFC [(33); **Figures 3** and **4**]. Double-label immunofluorescence coupled with confocal microscopy demonstrates that AR-ir cells in the PFC are neurons (**Figure 3**). In the VTA, AR-ir cells express tyrosine hydroxylase (TH), a marker of DA-synthetic neurons (42). Furthermore, perikarya in the VTA that project to the NAc and mPFC express AR (43). Of the VTA neurons that project to the prelimbic mPFC (mPFC-PL), the proportion of DAergic (TH-positive) efferents containing AR is higher in male rats than female rats (~30 vs <5%), but the proportion of TH-negative efferents containing AR is similar between males and females (44). Thus, androgens can influence the male mPFC *via* actions on these DAergic projection neurons (42). Taken together, these data suggest that AR are well positioned to modulate executive function.

In addition, T can be locally aromatized to E_2 and bind to ER in the mesocorticolimbic system (**Figure 1**). Many brain regions contain aromatase, the enzyme that catalyzes the conversion of androgens to estrogens (36). Aromatase expression is high in the hypothalamus (46, 47), and aromatase is also present in other regions including the mesocorticolimbic system (see below). The VTA, NAc, and mPFC contain some cells that express ERα or ERβ in female and male rats (48, 49). However, the VTA neurons that project to the NAc do not express ERβ. Instead, in both sexes, VTA neurons that express ERβ project principally to the ventral caudate putamen and amygdala (43). VTA neurons that project to mPFC-PL (TH-positive and TH-negative) lack ERα and less than 10% contain ERβ (44). In general, in female and male rodents, the NAc also has little intracellular ERα and ERβ (49–51).

In addition, androgens can modulate the mesocorticolimbic system through other mechanisms. First, hypothalamic nuclei that have high concentrations of AR, ERα, and ERβ directly innervate

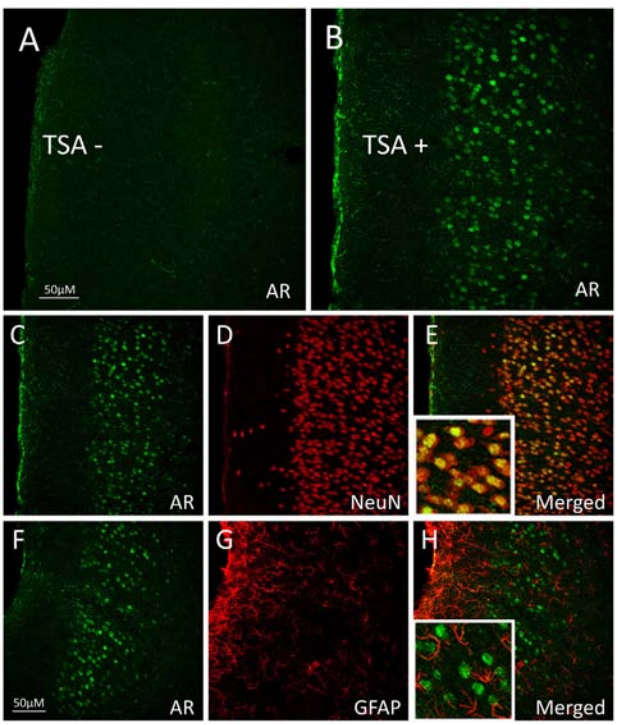

FIGURE 3 | Androgen receptor (AR), neuronal nuclei (NeuN, neuronal marker), and glial fibrillary acidic protein (GFAP; glial marker) immunoreactivity (ir) in the medial prefrontal cortex (mPFC) of adult male rats. **(A,B)** Pseudocolored confocal photomicrographs of androgen receptor immunoreactivity (AR-ir) in coronal hemisections of the mPFC **(A)** without tyramide signal amplification (TSA–) and **(B)** with tyramide signal amplification (TSA+). TSA-enhanced detection of AR in the mPFC of male rats. **(C–E)** Confocal photomicrographs of mPFC with **(C)** AR-ir cells (green), **(D)** NeuN-ir cells (red), and **(E)** AR-ir and NeuN-ir cells merged. Cells that co-express AR-ir and NeuN-ir appear orange–yellow, suggesting that AR is primarily expressed in neurons. **(F–H)** Confocal photomicrographs of **(F)** AR-ir cells (green), **(G)** GFAP-ir cells (red), and **(H)** AR-ir and GFAP-ir cells merged. AR-ir is not co-expressed with GFAP. Adapted from Ref. (33); Reprinted by permission of SAGE Publications.

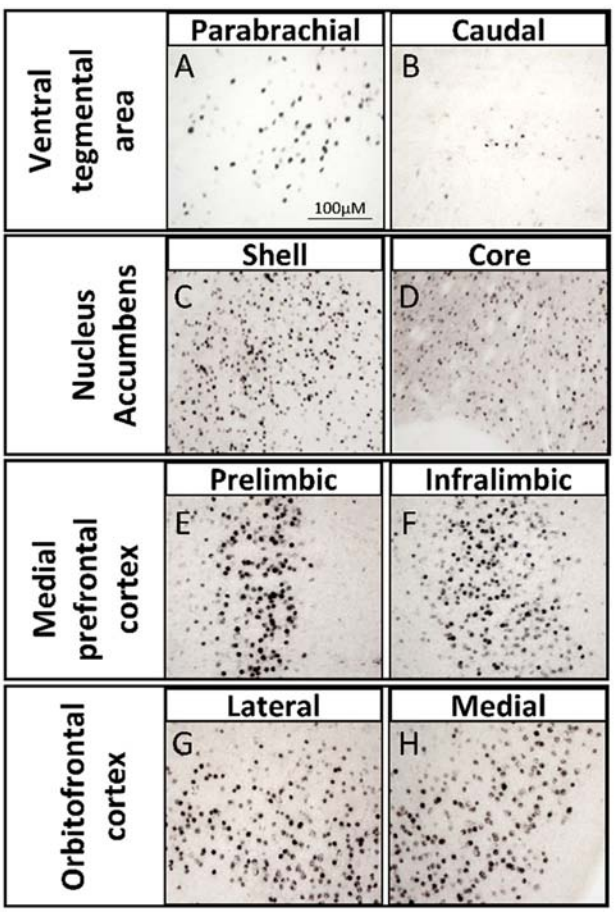

FIGURE 4 | Brightfield photomicrographs depicting androgen receptor immunoreactivity (AR-ir) with tyramide signal amplification in nodes of the mesocorticolimbic system of adult male rats. AR-ir in the **(A)** parabrachial pigmented nucleus of the ventral tegmental area (VTA), **(B)** caudal VTA, **(C)** shell of the nucleus accumbens (NAc), **(D)** core of the NAc, **(E)** prelimbic subregion of the medial prefrontal cortex (mPFC), **(F)** infralimbic subregion of the mPFC, **(G)** lateral subregion of the orbitofrontal cortex (OFC), and **(H)** medial subregion of the OFC. Adapted from Ref. (45).

mesocorticolimbic nodes and influence DA release. For example, the medial preoptic area is rich in AR and ERs and projects to the VTA and modulates DAergic neurons (52–54). Second, mAR and mER might mediate the rapid, nongenomic effects of androgens in the mesocorticolimbic circuit. Two possible candidates for mAR are ZIP9 and GPRC6A (55–57). However, no studies have examined ZIP9 or GPRC6A in mesocorticolimbic nodes, and whole-brain analyses have not reported either transcript in the VTA, NAc, or mPFC in mice (58, 59) or humans (40). In addition, AR variants have been found in neuronal lipid rafts (60). Alternatively, the G protein–coupled estrogen receptor 1 (GPER1; formerly known as GPR30) is present in the VTA, NAc, and, to a lesser extent, the PFC in rats and humans (61–63). Thus, systemic and locally synthesized estrogens could act on the mesocorticolimbic system *via* GPER1. Third, some C_{19} steroids can rapidly (milliseconds to seconds) modulate neuronal excitability *via* allosteric binding sites on neurotransmitter receptors, voltage-gated channels, and neurotrophin receptors [reviewed in Ref. (64)]. For example, the γ-aminobutyric acid (GABA)-gated chloride channel $GABA_A$ receptor ($GABA_AR$) and the glutamate-gated sodium/calcium channel *N*-methyl-D-aspartate receptor are sensitive to allosteric regulation by DHEA, DHEA-S, and 3α-androstanediol (65, 66).

THE MESOCORTICOLIMBIC SYSTEM LOCALLY SYNTHESIZES ANDROGENS

Our understanding of the role of androgens in the brain changed dramatically with the first suggestion of steroid synthesis in the rodent brain. Baulieu, Robel, and colleagues (67, 68) originally suggested that levels of DHEA and pregnenolone and their sulfoconjugates were higher in grossly dissected regions of the male rat brain (i.e., divided into the "anterior" and "posterior" brain) than in the serum. Moreover, GDX and adrenalectomy did not eliminate these steroids in the brain. Later, Liere and colleagues described how these findings were actually artifacts resulting from sample preparation, including oxidation of cholesterol in brain tissue (69). Recent studies, however, have shown that androgens are present at higher levels in several brain regions than in the blood in male rats [e.g., (36)], are directly synthesized in the brain in female and male rats [e.g., (70, 71)], or metabolized in the brain [e.g., (72)]. Local production of neurosteroids serves to influence gene expression or neuron excitability in an intracrine, paracrine, autocrine, or synaptocrine manner under normal physiological conditions (73) or as a compensatory mechanism when circulating steroid levels are low (74).

The steroidogenic capacity of the brain is further corroborated by studies demonstrating that steroidogenic enzymes are present in the brain. In many of the initial studies, the lower sensitivity of Northern blots, *in situ* hybridization, immunohistochemistry, and even PCR was insufficient to detect some steroidogenic enzymes in the brain. For example, Goascogne and colleagues (75) attempted to detect Cyp17a1 (**Figure 1**), which catalyzes conversion of progestins into androgens, in the rodent brain *via* immunostaining, but it was not until 1995 that several groups detected *Cyp17a1* transcripts and protein in the brain (76–78), and even then only at very low levels or only in embryonic brains. Several labs did find other steroidogenic enzymes in the brain, including Cyp11a1 (76, 79) and aromatase (78). Guennoun and colleagues (80) detected mRNA and protein of 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) in the hippocampus (HPC), hypothalamus, cerebellum, and cerebral cortex. Current techniques, particularly PCR, can detect all the enzymes necessary for androgen synthesis and metabolism in multiple regions in the male and female rat brain (81–84) and human brain (85, 86).

Little is known about the androgenic capacity of the mesocorticolimbic system, and even less about the physiological relevance of these locally produced steroids. Most studies have measured steroidogenic enzymes in gross neuroanatomical regions (e.g., cerebral cortex, HPC), without specific attention to mesocorticolimbic regions [e.g., (81, 87, 88)]. Specifically, Cyp11a1, Cyp17a1, and aromatase have been reported in the frontal cortex and midbrain or tegmentum of birds (89, 90), rodents (79), and humans [reviewed in Ref. (85)]. However, these reports have low spatial resolution, so steroidogenic enzyme levels specifically in the mPFC or VTA are unclear. Raab and colleagues (91) detected aromatase mRNA in the VTA of male and female rats, but only during early development. More recently, one study showed a behavioral effect of Cyp11a1 overexpression in the VTA, but not in the NAc (92). These results suggest that, if present in the VTA, Cyp11a1 affects reward-seeking behavior, but they did not demonstrate the importance of endogenous Cyp11a1 in the VTA. In the NAc, 3α-HSD and 5α-reductase type I, both involved in synthesizing DHT, are present in GABAergic medium spiny neurons of male mice (93). The steroidogenic acute regulatory protein (StAR), which is essential for *de novo* steroid synthesis, is also present in the NAc of mice (84).

We have recently shown expression of *Cyp17a1*, *Cyp19a1* (aromatase), and *Hsd3b1* (3β-HSD type I) mRNA in microdissected mesocorticolimbic nodes in the adult male rat using exon-spanning, probe-based qPCR assays that are specific and sensitive [(36); **Figure 5**]. The VTA, NAc, and mPFC contained low levels of *Cyp17a1* mRNA, compared to the preoptic area/hypothalamus (POA/HYP). In the VTA, GDX decreased *Cyp17a1* mRNA at the 2 weeks time point. Compared to the VTA, the NAc and the mPFC contained much higher levels of aromatase mRNA. While GDX decreased aromatase mRNA in the POA/HYP, GDX had no effect on aromatase in VTA, NAc, or mPFC. 3β-HSD type I was expressed in trace amounts in the VTA, but was nondetectable in the NAc and mPFC. This is further evidence that the mesocorticolimbic system can synthesize androgens *de novo* from cholesterol or from circulating steroids (DHEA, progesterone).

Using the contralateral side of the brain of the same subjects described above, we also examined steroid concentrations in mesocorticolimbic nodes *via* specific and ultra-sensitive liquid chromatography tandem mass spectrometry. Several results suggested local T synthesis. First, in sham-operated animals, T levels were 2–4× higher in the VTA, NAc and mPFC than in the blood (**Figure 5B**). Second, in all GDX subjects, T was nondetectable in the blood at 2 and 6 wks postoperatively (**Figure 6**). In the VTA, NAc and mPFC, T levels were lowered by GDX but nonetheless still detectable in ~50% of GDX subjects at 2 and 6 wks postoperatively (**Figures 6A–D**). Third, in subjects with detectable T, VTA T levels

were similar in sham-operated and GDX subjects. Fourth, in GDX subjects, local T levels in the VTA might be driven by 3β-HSD type I, as *Hsd3b1* mRNA was positively correlated with T levels ($r = 0.316$). We did not detect other significant correlations between local T concentrations and steroidogenic enzymes in GDX animals, but we did not examine all androgenic enzymes (e.g., 17β-HSD, 3β-HSD type 2). Overall, these data suggest that androgen synthesis occurs in mesocorticolimbic nodes and partially compensates for the loss of circulating T in GDX animals. Moreover, the fact that T remains at physiologically relevant levels long after GDX suggests that it exerts a significant physiological effect. Future studies should examine how other androgenic enzyme isoforms may contribute to regulation of local androgen synthesis, as well as the physiological relevance of neurally produced androgens.

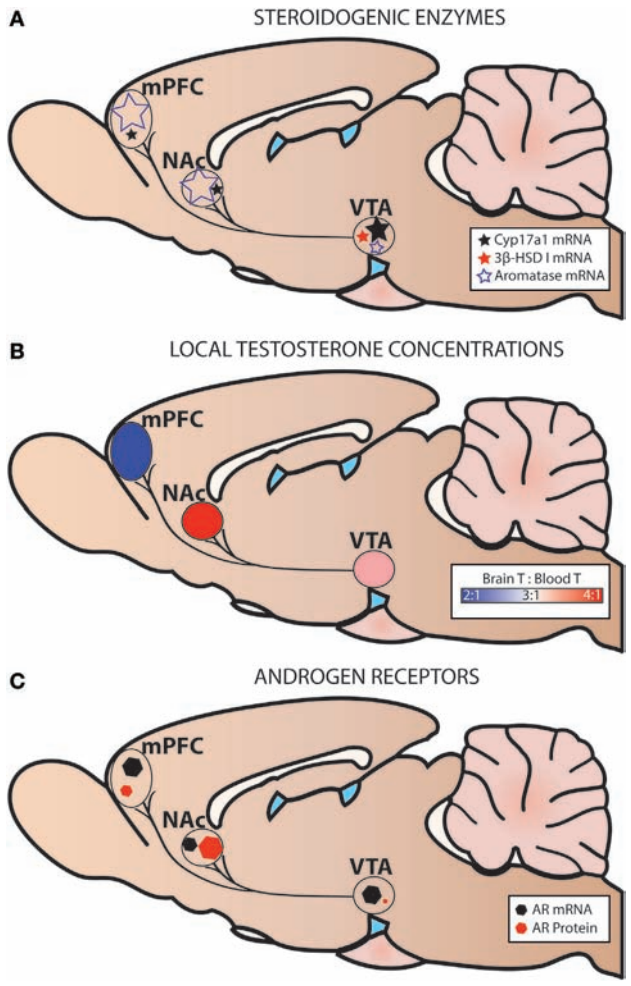

FIGURE 5 | Steroidogenic enzymes, local testosterone concentrations, and androgen receptors (AR) in the mesocorticolimbic system of adult male rats. **(A)** Levels of steroidogenic enzyme mRNA are based on probe-based quantitative polymerase chain reaction (qPCR) assays (36). **(B)** Local testosterone (T) concentrations are based on Brain T: Blood T ratios in intact adult male rats [fed *ad libitum* or calorie restricted (36)]. **(C)** Levels of AR mRNA are based on probe-based qPCR assays (36), and levels of AR protein are based on immunohistochemistry (33). Levels of AR mRNA and AR protein are not shown relative to one another.

The presence of steroidogenic enzyme mRNA or protein does not necessarily indicate steroidogenic enzyme activity. Few studies have demonstrated steroidogenic enzyme activity in brain cells *in vitro* or *in vivo*. In male rats, T in the cerebral cortex and tegmentum (including the VTA) is metabolized

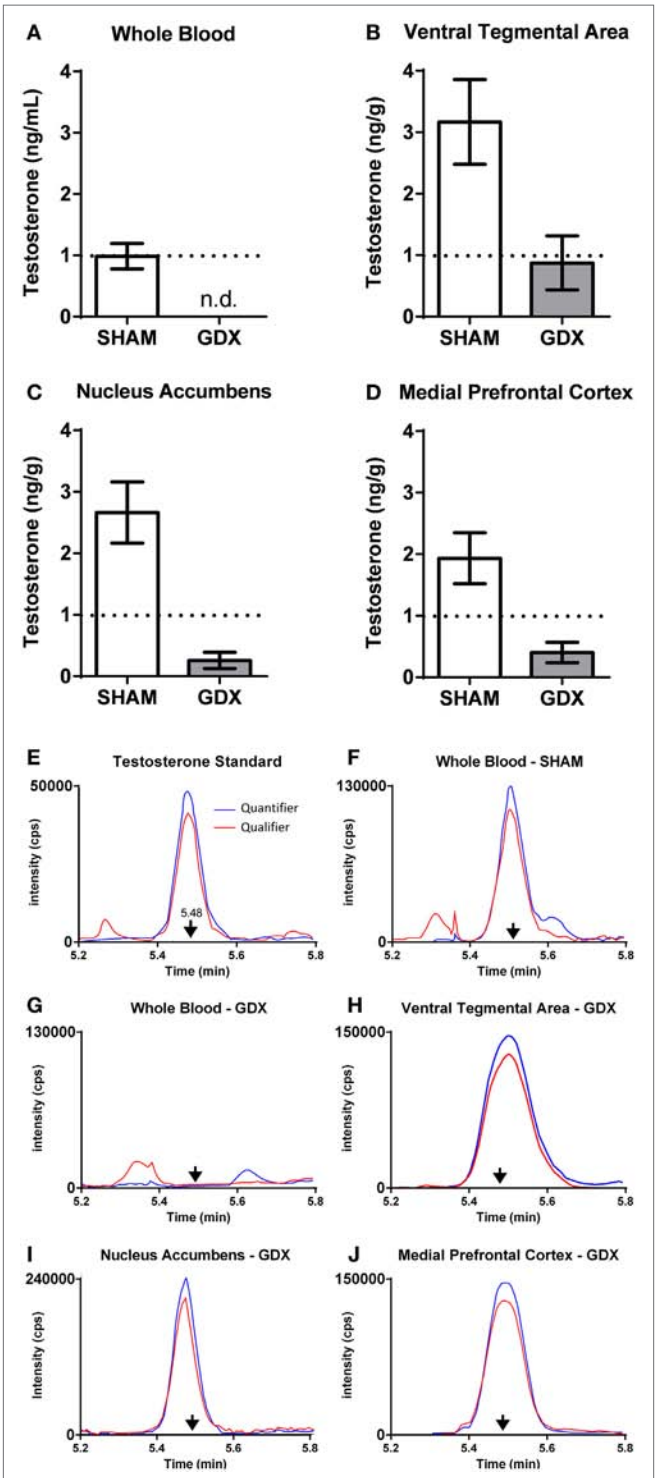

FIGURE 6 | **Continued**

FIGURE 6 | Testosterone is present in microdissected nodes of the mesocorticolimbic system of adult male rats at 6 weeks after GDX using LC–MS/MS. **(A–D)** Testosterone concentrations at 6 weeks after either SHAM surgery (n = 18–20) or GDX (n = 18–20) in the **(A)** whole blood, **(B)** ventral tegmental area (VTA), **(C)** nucleus accumbens (NAc), and **(D)** medial prefrontal cortex (mPFC). Values presented as mean ± SEM. **(E–J)** Representative chromatograms of testosterone quantifier ion (blue) and qualifier ion (red) for **(E)** testosterone standard (2 pg), **(F)** whole blood in a SHAM subject, **(G)** whole blood in a GDX subject, **(H)** VTA in a GDX subject, **(I)** NAc in a GDX subject, and **(J)** mPFC in a GDX subject. Arrows denote the retention time for testosterone. Note the differences in the intensity (counts per second, cps) on the y-axes. In **(G–J)**, samples are from different subjects, as not all GDX subjects had detectable testosterone in all brain regions. Adapted from Ref. (36). Abbreviations: GDX, gonadectomy; SHAM, sham surgery; n.d., nondetectable.

into 5α-androstanolone *in vitro* (94). Zwain and Yen (95) established that neonatal rat astrocytes and neurons synthesize pregnenolone, DHEA, androstenedione, T, and E_2 from precursors *in vitro*. Furthermore, steroidogenesis was reduced when the steroidogenic enzymes were pharmacologically inhibited or when transcription was inhibited. In humans, adult and fetal brains are capable of metabolizing T and androstenedione *in vitro* to E_2 and T, respectively (96, 97). More recently, studies demonstrate that androgens and estrogens are synthesized *de novo* in male and female rat hippocampal slices (70, 71, 98). Steroidogenic enzyme activity, the mesocorticolimbic system, however, has not yet been examined. What is more, whether these neurally-produced steroids modulate behavior remains largely unexplored.

AAS AFFECT BEHAVIOR *VIA* ACTION ON THE MESOCORTICOLIMBIC SYSTEM

Recent studies have explored the consequences of androgen supplementation at supraphysiological (pharmacological) doses. This is relevant to the problem of AAS abuse. Importantly, when administered at pharmacological doses, AAS may act *via* different mechanisms from those under physiological conditions. AAS are performance-enhancing substances derived from T (99). The media focuses on AAS use among elite athletes and on steroid detection to ensure "fairness" in sport. In reality, use of AAS is far more widespread, and potential risks are only now becoming evident (100). As many as 3 million Americans have used AAS, which includes use in high schools, fitness centers, and "rejuvenation" clinics. A typical AAS user is a young man in his late teens or early 20s (100). Among U.S. high school students, 4–6% of boys have used AAS vs 1–2% of girls (101). This is comparable to the rates of crack cocaine or heroin use (101). It is estimated that AAS use among men in their 20s is even higher (100).

Commonly abused AAS include both aromatizable and non-aromatizable androgens (102). Elite athletes choose T because it is challenging to differentiate exogenous from endogenous sources (103). Rank-and-file users choose T because of its low cost and easy availability. Furthermore, most AAS users do not limit themselves to a single dose or type of steroid (104). Instead, users combine different steroids ("stacking") in cycles of increasing and decreasing concentrations ("pyramiding"). AAS users take steroids orally, transdermally, or by intramuscular injection (105).

Recent research highlights a range of adverse health effects from chronic AAS abuse, including cardiovascular, hepatic, reproductive, and psychiatric dysfunction (105). However, the dangers of AAS abuse are not limited to the medical consequences of high-dose steroids themselves, but also result from risk-taking in non-social [e.g., drinking and driving (106)] and social contexts [e.g., aggression, sexual violence (107–110), and risky sex (106, 111, 112)]. Understanding the interplay of AAS and social behavior in risk-taking is particularly important in adolescents and young adults. This age group is strongly influenced by peer interactions (113, 114), exquisitely sensitive to rising levels of endogenous gonadal steroids (115), less risk-averse (116), and especially vulnerable to substance abuse (117). In part, this stems from adolescent immaturity in mPFC development (118).

Because it is not ethical to administer supraphysiological doses of AAS to normal volunteers, most of our knowledge of the behavioral effects of these drugs comes from studies of illicit users in the field and from animal studies. Furthermore, animal studies can explore consequences of AAS in an experimental context, where appearance and athletic performance are irrelevant. These studies have revealed that AAS appear to be rewarding and have potential to cause dependence. Rodents will voluntarily self-administer AAS orally (119) and by i.v. or i.c.v. injection (120). Moreover, they demonstrate tolerance, withdrawal, and fatal overdose with self-administration (121). T self-administration (i.c.v.) is blocked by the AR antagonist flutamide (121), although it appears that classical AR are not required for androgen reinforcement (122). The behavioral and physiological effects of supraphysiological doses of T resemble those of opioid overdose and are rapidly reversed by opioid antagonists (121). Likewise, many human AAS users meet DSM criteria for psychoactive substance dependence, including continued use despite negative side-effects, and withdrawal symptoms when steroids are discontinued (123).

The effects of AAS on reward and reinforcement strongly implicate involvement of the mesocorticolimbic system, since drugs of abuse act, in part, *via* DA release in NAc (124). Male rats form conditioned place preference (CPP) in response to intra-NAc infusion of T (125) or its metabolites (126), similar to the effects of DA-releasing drugs (127). Conversely, systemic or intra-NAc treatments with D_1R and D_2R antagonists block T-induced CPP (128, 129). Nonetheless, the manner in which androgens modulate DA release and signaling is still unclear. For example, acute administration of T does not induce NAc DA release (130), and in fact, AAS can reduce cocaine- or amphetamine-evoked DA release in NAc (131, 132). This latter finding is consistent with the observation that the acquisition of T self-administration is slow compared with cocaine or other addictive drugs (119). On the other hand, T upregulates the Fos protein, a marker of cellular activity, in regions of the mesolimbic DA system (133). Thus, the reinforcing effects of exogenous T may be due to its ability to modulate neural activity and DA signaling within the mesocorticolimbic circuit, but may do so without directly affecting DA release. Indeed, chronic AAS administration alters $GABA_A$R subunit expression throughout the brain (including

mesocorticolimbic regions), thus altering the physiological response to DA-independent GABAergic signaling (134, 135).

ANDROGENS MODULATE THE NEUROCHEMISTRY AND STRUCTURE OF THE MESOCORTICOLIMBIC SYSTEM

Many androgen-dependent behaviors are mediated by neurochemical changes and neuronal activity in the mesocorticolimbic system. In several mammalian species, GDX of adult males diminishes expression of copulatory behavior, which can be restored by chronic T treatment (136). Copulatory behavior, particularly ejaculation, is correlated with a T-dependent increase in DA release in the NAc (137, 138). In this section, we will discuss how androgen deprivation, AAS, and androgen synthesis influence the neurochemistry and structure of the mesocorticolimbic system.

Most studies examining T regulation of the mesocorticolimbic system have focused on DAergic transmission in the NAc and mPFC. GDX alters DA tone in the mPFC of male and female rats (139). In the mPFC, GDX decreases basal DA after 4 days but increases it after 28 days. This is likely a result of GDX increasing bursting of VTA DA neurons, altering activity of mPFC efferents to the VTA, and gradually increasing TH in the VTA (140, 141). In contrast, in the NAc, basal DA is unchanged after GDX, but the DA metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid are increased after GDX (142). This finding suggests that GDX increases DA turnover in the NAc, which might indicate faster clearance of DA from the synapse and higher rates of DA signaling at baseline. GDX also modulates evoked electrophysiological and DAergic responses in the mPFC and NAc. In superfused striatal tissue, K^+-simulated DA release was higher in GDX compared to GDX + T adult male mice (143). In the same study, reserpine, a drug that depletes DA, had the opposite effect, whereby DA release was higher in GDX + T male mice. This is in line with studies demonstrating that GDX affects storage, uptake, and/or synthesis of catecholamines in mesocorticolimbic nodes (142, 144) and helps to maintain NAc DA levels when exposed to methamphetamine (145).

Androgen-mediated structural plasticity and alterations in neurotransmitter receptor densities in the mesocorticolimbic system are other potential mechanism through which these hormones may alter cognitive/behavioral functions of this system. GDX decreases and high doses of T increase dendritic spine density in limbic regions, including the amygdala and HPC, in male rats (146, 147) and male monkeys (148). In a recent study, male rats were treated chronically with high-dose T, and brains were stained by Golgi–Cox to analyze neuronal morphology in medium spiny neurons of nucleus accumbens shell (NAcS) (149). T decreased spine density throughout the dendritic tree in the NAcS. However, T treatment did not affect total spine number, dendritic length, or arborization. Similarly, in the mPFC, GDX reduces and DHT increases dendritic spine formation in male mice (150). The effect of DHT on dendritic spine formation was reduced, but not absent, in GDX testicular feminization mutant male rats (a naturally occurring mutant with severely attenuated AR binding capacity), which suggests both androgenic and non-androgenic influence on synaptic remodeling.

Androgens can influence the function of the mesocorticolimbic nodes by their local metabolism to more potent androgens (e.g., T→DHT), further metabolism to weak androgens (e.g., T→DHT→3α-androstanediol), or metabolism to estrogens (T→E_2). 3α-androstanediol, for example, has weak androgenic effects but also acts as a robust and rapid neuromodulator *via* allosteric binding to $GABA_AR$ (66, 151). Indeed, 3α-androstanediol in the NAc facilitates CPP (a DA-dependent behavior) in rodents, likely through allosteric agonism of $GABA_AR$ in GABAergic medium spiny neurons (152). Concurrently, the aromatization of T into E_2 may also influence activity in the mesocorticolimbic system. E_2 decreases striatal DA transporter density, enhances DA synthesis and degradation (153, 154), and downregulates DA binding to D_2R in the NAc (155). In contrast, systemic treatment with the aromatase inhibitor letrozole decreases basal DA turnover in the mPFC of male and female adult rats (156). The regulation of DA turnover in the brain may be directly related to changes in phasic DA signaling. Indeed, direct pulsatile application of E_2 to striatal slices induces DA release (157) and enhances K^+-mediated DA release (158). Patch clamp analysis of ion transfer across the membrane in dissociated NAc medium spiny neurons demonstrated that there is a prompt diminution of Ca^{++} currents in response to acute E_2 (159). Taken together, these data suggest that local production of E_2 in males and females modulates DA signaling and postsynaptic neural excitability in the mesocorticolimbic system.

Few studies have examined whether neurosteroids produced in the mesocorticolimbic system influence neurochemistry. Pharmacological inhibition of Cyp17a1 regulates a DA-dependent behavior [prepulse inhibition (PPI)], but the study did not examine the direct effect on DA signaling (160). Inhibition of Cyp17a1 would decrease both androgen and estrogen signaling. DHEA, a product of Cyp17a1, is present in human, but not in laboratory rat or mouse, circulation. DHEA has a wide range of neurochemical effects, but the source of DHEA is rarely determined (161). The mesocorticolimbic system is sensitive to DHEA. For example, DHEA decreases the activity of monoamine oxidase, an enzyme necessary for the degradation of monoamines, in the NAc in male rats *in vivo* and *in vitro* (162). Pharmacological inhibition of 5αR suggests that DHT influences neurochemistry, particularly DA signaling, in the mesocorticolimbic system (163–165). Overall, these data suggest neurosteroids regulate DA turnover and DA signaling in the mesocorticolimbic system, which is important for regulating executive functions.

ANDROGENS REGULATE EXECUTIVE FUNCTION

Clinical and preclinical evidence suggest that hypogonadism and GDX have deleterious effects on executive functioning, which can often be ameliorated with androgen replacement. Furthermore, excessive androgen exposure (e.g., AAS) during adolescence and/or adulthood has detrimental effects on executive functioning. We also examine evidence that the brain compensates for a

decrease in circulating androgens by increasing local androgen synthesis in the mesocorticolimbic system to mitigate deficits in executive functioning. These studies support the hypothesis that there is an optimal level of androgen signaling within the mesocorticolimbic system for proper executive functioning.

Low Androgen Signaling

Andrew and Rogers (166) were among the first to demonstrate that androgens affect executive function. In a foraging paradigm, young male chicks treated with T pecked grains of a familiar color and ignored unfamiliar, novel-colored grains, while vehicle-treated chicks demonstrated behavioral flexibility and pecked both grain colors without bias (166). The authors used the term "persistence" (also called "perseveration") to describe the inability to stop using a response strategy when it is no longer relevant or advantageous. Rogers (167) then showed that antiandrogen treatment or GDX decreased persistence in adult male chickens, whereas systemic T replacement in GDX chickens reinstated persistence. Subsequent studies in adult male rodents revealed that GDX or an antiandrogen reduced persistence, supporting the initial findings in birds (168, 169). GDX also decreases male persistence during social investigation of female conspecifics, suggesting that T increases male persistence in gaining access to potential mates (170).

T increases perseveration in operant conditioning tasks that require behavioral flexibility. Using a reversal learning task, van Hest and colleagues (171) demonstrated that GDX male rats perseverated less on the previously reinforced lever, while administration of T to GDX subjects displayed the highest rates of perseveration. Additionally, GDX male rats exposed to a conditional discrimination task in a T-maze made fewer errors during the reversal phase (i.e., decrease in perseverative errors) compared to intact subjects (172). On a delayed spatial alternation test, GDX subjects made less perseverative errors than intact subjects, but only after a delay of 6 sec or more, suggesting a concurrent deficit in working memory (173).

In men, declines in executive functioning and visuospatial ability are the most commonly reported adverse cognitive effects of androgen deprivation therapy [ADT (174)]. ADTs are administered to nearly 50% of prostate cancer patients (175) and include GnRH analogs (e.g., Histrelin), AR antagonists (e.g., flutamide), and androgenic enzyme inhibitors (e.g., abiraterone, a Cyp17a1 inhibitor) [see (176) for review]. Many ADTs decrease systemic T but might not decrease local androgen synthesis equally across tissue types (e.g., GnRH analogs). If a clinical study includes only subjects on ADTs that inhibit androgen synthesis and/or signaling in the brain, which is often not the case, then the effects of ADTs on executive function might be more clear (see below).

There is contradictory evidence on the effect of ADTs on executive functioning. For example, ADT is associated with deficits in attention and cognitive control [i.e., Trail Making B task, Stroop Interference Test (177)]. Furthermore, ADT is associated with decreases in gray matter volume in the dorsolateral and frontopolar PFC (178) and decreased neural activity and connectivity in the mPFC during tasks requiring inhibitory control (179). ADT is also associated with changes in impulsivity, emotional lability, and working memory, compared to matched non-ADT subjects (180). These findings suggest that ADT disrupts PFC function, an area particularly sensitive to androgens in males (33, 39).

However, other studies have not found an association between ADT and executive function (181–183). A recent meta-analysis of the effects of ADT on a variety of cognitive functions found that only visuospatial ability was reliably affected by ADT, whereas assays of executive functioning (e.g., Trail Making Test B, Stroop Interference Task) did not detect any significant differences (184). These findings are in line with a study in menopausal women with low T who were then treated with T and did not show changes in a range of executive functions compared to untreated subjects (185). However, the assessment tools for these studies might not be sensitive enough to detect small, yet important, changes in executive functions [e.g., (186)]. In addition, studies on ADT and executive function may lack statistical power, neglect confounding variables (183, 187), or include subjects who have not received ADT for enough time [ADT is usually administered for 2–3 years (181)]. These issues reduce the ability to detect effects of ADT on executive functioning. For example, Alibhai and colleagues (181) conducted a small study that regularly administered a battery of neuropsychological tests to ADT patients for 36 months, and ADT was not associated with deficits in cognitive flexibility or working memory. However, this study might not have utilized cognitive tests sensitive enough to detect PFC-specific deficits in executive functioning [e.g., Iowa Gambling Task (IGT)]. Moreover, 95% of ADT subjects in this study were using GnRH analogs as their sole ADT, which may not affect androgen synthesis in the brain, as would androgenic enzyme inhibitors.

High Androgen Signaling

Supraphysiological levels of androgens typically seen with AAS use also impair executive function. This has been explored in rats treated with supraphysiological levels of T and trained to work for food reward (sugar pellets) in an operant chamber (**Figures 7** and **8**). T-treated rats display deficits in different forms of cognitive flexibility, including reversal learning and extra-dimensional set-shifting (188). These rats take longer to shift their behavior when stimuli associated with rewards are reversed, or when rats must employ a novel discrimination strategy to obtain rewards. Importantly, set-shifting behavior is dependent upon D_1R in NAc (12), and AAS reduce NAc D_1R (189).

Anabolic-androgenic steroids also alter different forms of cost/benefit decision making in operant discounting tasks. In these tasks, rats choose between two retractable levers, one of which is associated with a smaller, easily obtainable reward (1 pellet) and the other with a larger reward (3 or 4 pellets) associated some cost. These costs can include effort, delay, punishment, and probability, which results in discounting of the larger reward (i.e., making it less desirable; **Figure 8**). AAS and DA have site- and task-specific effects on discounting behavior. In particular, AAS do not cause impulsivity with a consistent preference for small rewards, nor do they produce a "win-at-all-costs" strategy that always favors the large reward. Instead, there is a selective effect of AAS. Specifically, AAS-treated rats are less sensitive to effort (188), punishment (192), and delay (191), but are more sensitive to uncertainty (190). In particular, AAS may diminish sensitivity

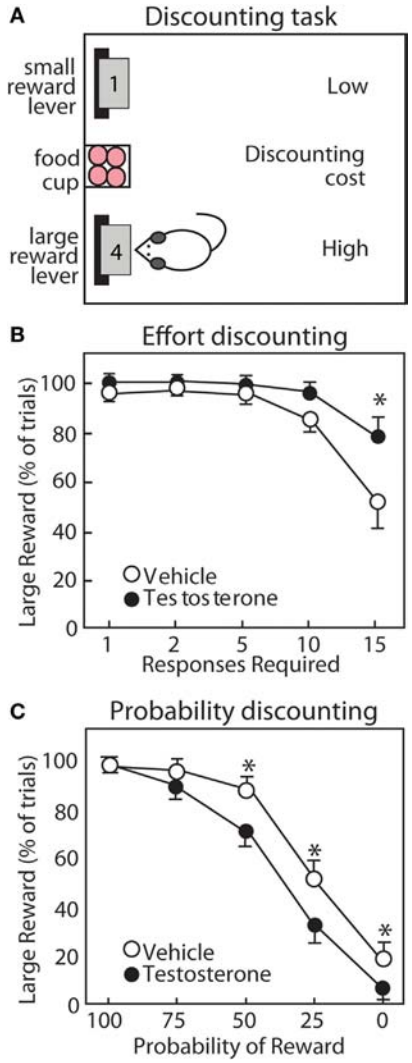

FIGURE 7 | Testosterone influences discounting behavior of male rats. **(A)** Operant task for discounting behavior. Rats choose between two levers. The small reward lever delivers 1 pellet with minimal cost. The large reward lever delivers four pellets with increasing cost throughout the session. **(B)** For effort discounting behavior, the response requirement (number of lever presses) increases. **(C)** For probability discounting, the large reward is delivered with decreasing probability. Compared with vehicle controls (open circles), testosterone (closed circles) increases preference for the large reward lever in effort discounting, but reduces preference for the large reward lever in probability discounting. Adapted with permission from Ref. (190). Values presented as mean ± SEM. $^*p \leq 0.05$.

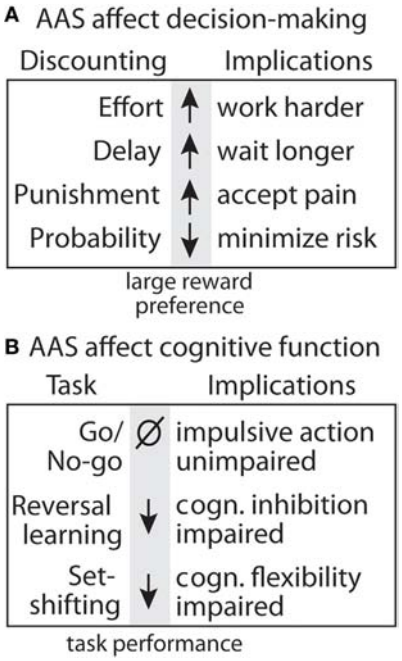

FIGURE 8 | Summary of effects of anabolic-androgenic steroids (AAS) on decision making and cognitive function. **(A)** Testosterone effects on discounting behavior for effort (190), delay (191), punishment (192), and uncertainty (190). **(B)** Testosterone effects on cognitive function and motor impulsivity in the go/no-go task (192), cognitive inhibition in the reversal learning task, and cognitive flexibility in the set-shifting task (188).

to future negative consequences, even as they render users more sensitive to unpredictable outcomes.

A wealth of studies has mapped the neurotransmitters and brain regions responsible for discounting behavior using systemic treatment with neurotransmitter receptor agonists and antagonists and selective inactivation of NAc subregions. As discussed previously, D_1R and D_2R each promote preference for the large reward in effort discounting (delivery of the large reward requires more lever presses) and probability discounting [delivery of the large reward is uncertain (193)]. Studies using inactivation of NAc subregions have revealed that effort discounting is regulated by the NAc core (NAcC), while probability discounting is regulated more prominently by the NAc shell [NAcS (194, 195)]. These findings align with modulation of D_1R and D_2R in NAc subregions by the AAS nandrolone (189) and with recent studies of effort and probability discounting in response to high-dose T (190). T reduces preference for larger reward during probability discounting (190), and AAS reduce DA receptors in NAcS (189). Conversely, T treatment increases preference for the large reward during effort discounting (190), and nandrolone increases D_2R in NAcC (189). Thus, AAS might reduce sensitivity to effort during effort discounting by increasing D_2R in NAcC.

In animal studies, it is interesting that AAS selectively alter elements of risk-taking and impulsivity. In probability discounting, risk is reflected by the potential for reward omission, and T makes rats more risk-averse, an effect that may be driven by a reduction in NAc D_1R. At the same time, they are less risk-averse in punishment discounting, whereby they risk a footshock with delivery of the large reward (190). Together, these results reveal a nuanced effect of supplemental T to increase sensitivity to reward omission and simultaneously decrease responsiveness to punishment. A similar picture emerges in assessment of how T alters different aspects of impulsivity. T has no effect on impulsive actions as measured in a go/no-go task (192), wherein rats must switch between initiating and inhibiting a response to obtain rewards. However, the same study showed that T reduces impulsive choice, assessed with a delay discounting task, in that it increases the subjects' willingness

to wait for a large, delayed reward. Given the complex manner in which increasing androgen activity can influence various forms of decision making, it is unlikely that the effects of these treatments are driven by uniform increases or decreases in mesolimbic DA activity. Rather, these findings suggest that the manner in which T influences the behavioral functions depends in part on the specific costs that are being evaluated and the underlying corticostriatal circuitry that is recruited in guiding these decisions.

Studies of executive function in human AAS users are limited and restricted to male subjects. AAS abusers show impaired visuospatial working memory compared to non-users, similar to deficits seen in ADT (184), and the level of impairment is correlated with lifetime AAS use (196). A variety of evidence further implicates androgens and AAS in risk-taking behavior in humans. In a study of American high school students, AAS use was associated with risky sex, drinking and driving, carrying a weapon, and not wearing a helmet or seat belt (106). Psychological evaluations of human users have also implicated AAS in impaired decision making, which may stem from feelings of invincibility (197). Deaths among AAS users show high rates of homicide, suicide, and drug overdose (110). These possible effects of AAS abuse on risk-taking in humans might be similar to punishment discounting in rats, in that androgens increase the appetite for reward despite a risk of punishment.

Risk taking induced by AAS has a potentially dangerous social dimension as well. AAS use has been implicated in several violent murders (107–110). In surveys of current AAS users and in studies of human volunteers, increased aggression is the most consistent behavioral effect of high-dose AAS exposure in humans (103, 105). Compared with non-users, AAS users report increased sex drive (198) and increases in risky sexual behaviors [i.e., increased numbers of partners, infrequent condom usage (111)], as well as unprotected anal intercourse among HIV-positive gay men (112). Among American high school students, AAS use correlated with not using a condom and a history of sexually transmitted disease (106). Thus, a key danger of AAS abuse is the likelihood that users will engage in behaviors that harm themselves and those around them.

Individual Variation in Circulating Androgen Levels and Executive Functioning

In normal, healthy individuals, circulating levels of androgens vary dramatically, allowing for correlational analyses of androgen levels and executive function. Interestingly, perseveration and risky behavior are positively correlated with endogenous androgens in adolescence and adulthood, similar to findings from animal studies (199). For example, adolescent males that exhibit external signs of high T (e.g., hirsutism) perform better on simple repetitive tasks than those without such external signs, independent of cognitive ability (200). This early study suggested a positive correlation between endogenous androgens and persistence. Furthermore, higher androgen levels in pubertal boys correlate with a greater probability of lifetime ethanol use (201). Therefore, circulating androgens may enhance ethanol effects on behavior, potentially increasing risk-taking in one or both sexes. To address this possibility, a study compared GDX male and female rats with and without hormone replacement in the probability discounting task, to investigate the influence of ethanol and gonadal steroids on the response to uncertainty (202). At baseline, GDX + T males showed a greater preference for the large reward than GDX males. Ethanol further increased large reward preference, but only in males. These results suggest that both ethanol and T at normal physiological levels increase tolerance for a large uncertain reward.

In adults, men with higher T are more likely to choose cards from decks offering large monetary gains paired with larger, infrequent losses in the IGT, a probabilistic, risk-based decision making task (203). This result is similar to patients with damage to the OFC and ventromedial PFC (204, 205). As a result, men with higher T earned less money throughout the session, relative to men with lower T. High levels of endogenous T also correlate with economic risk-taking outside of the lab. In a study of London stock traders, morning T levels predicted risk-taking throughout the day (206). In young and menopausal women, T is not associated with changes in any measure of executive function (207–209). Taken together, such studies suggest that individual variation in systemic T levels in males, but not females, is correlated with specific aspects of executive function.

Neuroandrogens and Executive Function

In addition to acting as endocrine signals, androgens also act as intracrine, paracrine, and autocrine signals. Specific nodes within the mesocorticolimbic system might require a particular androgen concentration to function appropriately. Similarly, McEwen and Wingfield (210) posited that local glucocorticoid signaling is tightly regulated to alleviate allostatic load imposed by high circulating glucocorticoid levels. Studies utilizing extreme changes to circulating androgen concentrations, such as AAS, GDX, or ADT demonstrate the importance of systemic androgens for executive function, but they can not reveal the role of local androgen synthesis. As discussed above, local levels of T in the mesocorticolimbic system vary greatly from circulating levels and from one neural node to another. Levels of T are often two or more times higher in the mesocorticolimbic system than in the blood in intact animals, and T is still present in the mesocorticolimbic system at 6 weeks after GDX (36). These results suggest that local T synthesis is important for neural activity in the mesocorticolimbic system and executive functioning.

There are few data on how the local production of androgens in the mesocorticolimbic system influences executive functioning. Several studies report changes in T precursors or androgenic enzymes in the mesocorticolimbic system of patients with mood disorders or in animal models of depression (211–213). Depression is frequently marked by deficits in executive functions (214, 215). In fact, clinicians refer to a disorder that occurs in geriatric populations as "depression-executive dysfunction syndrome" (214, 216). Low circulating DHEA and DHEA-S levels are correlated with depression in aged human populations, and DHEA has been suggested as treatment for depression (217, 218). In a rodent model of childhood depression, DHEA levels are lower in the VTA and NAc (but not amygdala or hypothalamus), suggesting mesolimbic-specific regulation of androgens (211). Expression of several steroidogenic enzymes are altered in

post-mortem analyses of depressed individuals, which include a decrease of 5α-reductase type I in the PFC (213) and Cyp17a1 in the anterior cingulate cortex [ACC (212)], and an increase in hydroxysteroid sulfotransferase 2A1 (HST, **Figure 1**) in the ACC and StAR in the dorsolateral PFC (212). Changes in the expression of these specific steroidogenic enzymes suggest active androgen synthesis and metabolism in the VTA, NAc, and PFC.

Systemic administration of steroidogenic enzyme inhibitors that cross the blood–brain barrier hint at the role of neuroandrogens in modulating executive function. Using set-shifting and reversal learning tasks, the Soma laboratory has recently found that chronic systemic administration of abiraterone (a Cyp17a1 inhibitor) enhances behavioral flexibility in intact and gonadectomized subjects [unpublished results (219)]. Furthermore, systemic administration of letrozole, an aromatase inhibitor, increases risk-taking behavior in human males (220) and improves working memory in female rats (221). In particular, the study by Goudriaan et al. (220) administered letrozole to healthy men for 1 week and tested executive function and risk-taking before and after treatment. Importantly, this treatment was used to increase circulating T, but potentially influenced steroidogenesis in the mesocorticolimbic system. Letrozole-treated subjects demonstrated an increase in risk-taking on the Balloon Analog Risk-Taking task, but not the IGT or Game of Dice, compared to their baseline and estrogen-treated subjects. These findings highlight the importance of using a variety of sensitive neurocognitive assays to detect changes in executive function. This study, along with the studies of behavioral flexibility in male rats and working memory in female rats, suggests that androgens and local androgen synthesis, and not E_2 or local androgen metabolism, have the most profound effects on executive functioning.

There have been no studies, to our knowledge, that directly (e.g., i.c.v. steroidogenic enzyme inhibitor) manipulated neural androgen synthesis and examined executive functioning. The most relevant studies examined the effects of androgenic enzyme (i.e., Cyp17a1 and 5αR) inhibitors on PPI of the acoustic startle reflex and DA signaling in the NAc (160, 163, 165). Frau and colleagues (160) administered apomorphine (a non-selective DA agonist; i.p.) to male rats to cause a deficit in PPI. The effects of apomorphine on PPI were attenuated by microinjecting (i.c.v.) the Cyp17a1 inhibitor abiraterone. Along with studies using systemic finasteride [5αR inhibitor (163, 164, 222, 223)], these results suggest that local androgen synthesis regulates DA signaling in the mesocorticolimbic system and DA-dependent behaviors. While these studies are informative, there still remains an important gap in our understanding of how neural androgen production specifically influences executive functioning.

CONCLUSION

Androgens influence a variety of behaviors and cognitive functions, which include executive functioning. Converging lines of evidence suggest that androgens can influence executive functioning *via* actions on the mesocorticolimbic system. Multiple nodes of the mesocorticolimbic system (VTA, NAc, mPFC, and OFC) contain AR and ER. Emerging evidence suggests that multiple nodes of the mesocorticolimbic system also locally synthesize androgens, estrogens, and other steroids. However, the physiological role of these neuroandrogens still remains to be determined. Reducing endogenous androgens (GDX, ADT) and administering exogenous androgens (AAS) alter the neurochemistry (e.g., DA signaling) and cytoarchitecture of the mesocorticolimbic system. In animal studies, both a reduction of endogenous androgens and pharmacological administration of exogenous androgens lead to alterations in behavioral flexibility and inhibitory control. In human studies, evidence suggests that ADT or AAS abuse can also lead to deficits in executive functioning. Future studies should investigate the roles of systemic and locally produced androgens in the mesocorticolimbic system and cognition. Taken together, such studies broaden our understanding of androgen regulation of behavior to include decision making and executive function, and also highlight neurosteroid and AAS action within the mesocorticolimbic system.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

The authors thank Ryan Tomm, Jordan Hamden, and Katelyn Low for comments on the manuscript.

FUNDING

This review was supported by a Bluma Tischler Postdoctoral Fellowship to DT; a grant from the National Institute on Drug Abuse of the U.S. National Institutes of Health (R01-DA029613) to RW; grants from the Natural Sciences and Engineering Research Council of Canada and the Canadian Institutes of Health Research (MOP 133579) to SF; grants from the Canadian Institutes of Health Research (MOP 133606), Canada Foundation for Innovation, and British Columbia Knowledge Development Fund to KS.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

PERSPECTIVE
published: 15 June 2018
doi: 10.3389/fendo.2018.00316

Steroids and Brain, a Rising Bio-Medical Domain: a Perspective

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Some newly described steroid-related compounds, also found in the rest of the body, are formed and active in the central nervous system, particularly in the brain. Some are of pharmacological and physiopathological interest. We specifically report on two compounds, "MAP4343," a new neurosteroid very efficient antidepressant, and "FKBP52," a protein component of hetero-oligomeric steroid receptors that we found involved in cerebral function, including in Alzheimer's disease.

Keywords: brain steroids, neurosteroids, Alzheimer's disease, tauopathies, pregnenolone, MAP4343, FKBP52, RU486

OPEN ACCESS

Edited by:
Takayoshi Ubuka, Monash University Malaysia, Malaysia

Reviewed by:
Gianfranco Spalletta, Fondazione Santa Lucia (IRCCS), Italy
Vassilios Papadopoulos, University of Southern California, United States

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *26 December 2017*
Accepted: *25 May 2018*
Published: *15 June 2018*

Citation:
Baulieu E-E (2018) Steroids and Brain, a Rising Bio-Medical Domain: a Perspective. Front. Endocrinol. 9:316. doi: 10.3389/fendo.2018.00316

INTRODUCTION

Some cholesterol found in brain and spinal cord is biologically largely independent of that found in the rest of the body (**Figure 1**). It gives rise to "neurosteroids" (1) and we mention in this short "Perspective" some of their pharmacological and pathophysiological properties. In particular, we report the therapeutic antidepressant activity of a new neurosteroid drug "MAP4343," derivative of pregnenolone (PREG) (2). Very differently, we mention the cloning (3) and the function of a protein, "FKBP52," which is a component of hetero-oligomeric steroid receptors and that, with Chambraud et al. (4), we found involved in the function of the cerebral Tau protein, including in Alzheimer's disease. Thus, we take care of two novel and distinct active steroid-related compounds of medical interest.

First, among "neurosteroids" (1), PREG (6) is rather important quantitatively, and MAP4343, its synthetic derivative 3β-methoxy-Δ5-pregnene-20 one [**Figure 2**; (7)], is remarkably active to treat depressive states and addiction to ethanol (in preparation with G. Koob and O. George); it is currently studied in human beings.

Second, "FKBP52" is a protein complexed with hetero-oligomeric steroid receptors that we found to have an unforeseen interaction (4) with the Tau protein centrally involved in Alzheimer's disease and other dementias. The profound decrease of FKBP52 in several tauopathies (9) suggests its possible therapeutic importance.

NEUROSTEROIDS: MAP4343, A THERAPEUTICALLY ACTIVE DERIVATIVE OF PREG

The synthesis of cholesterol in the nervous system has been described by Bloch (10). Its metabolic steroids synthetized in the central and the peripheral nervous systems (11, 12) are called neurosteroids (1, 13); their syntheses in vertebrate brains (14) occurs in neurons and glial cells, sometimes from some imported steroidal precursors. Neurosteroids include several 3β-hydroxy-Δ5 compounds, such as PREG (6, 15) and dehydroepiandrosterone (DHEA) (16–19), and their sulfate esters (20, 21). Moreover, there are Δ4-3oxo steroid hormones (progesterone is quite abundant) (22), and some of their reduced metabolites, such as one of the tetrahydroderivatives

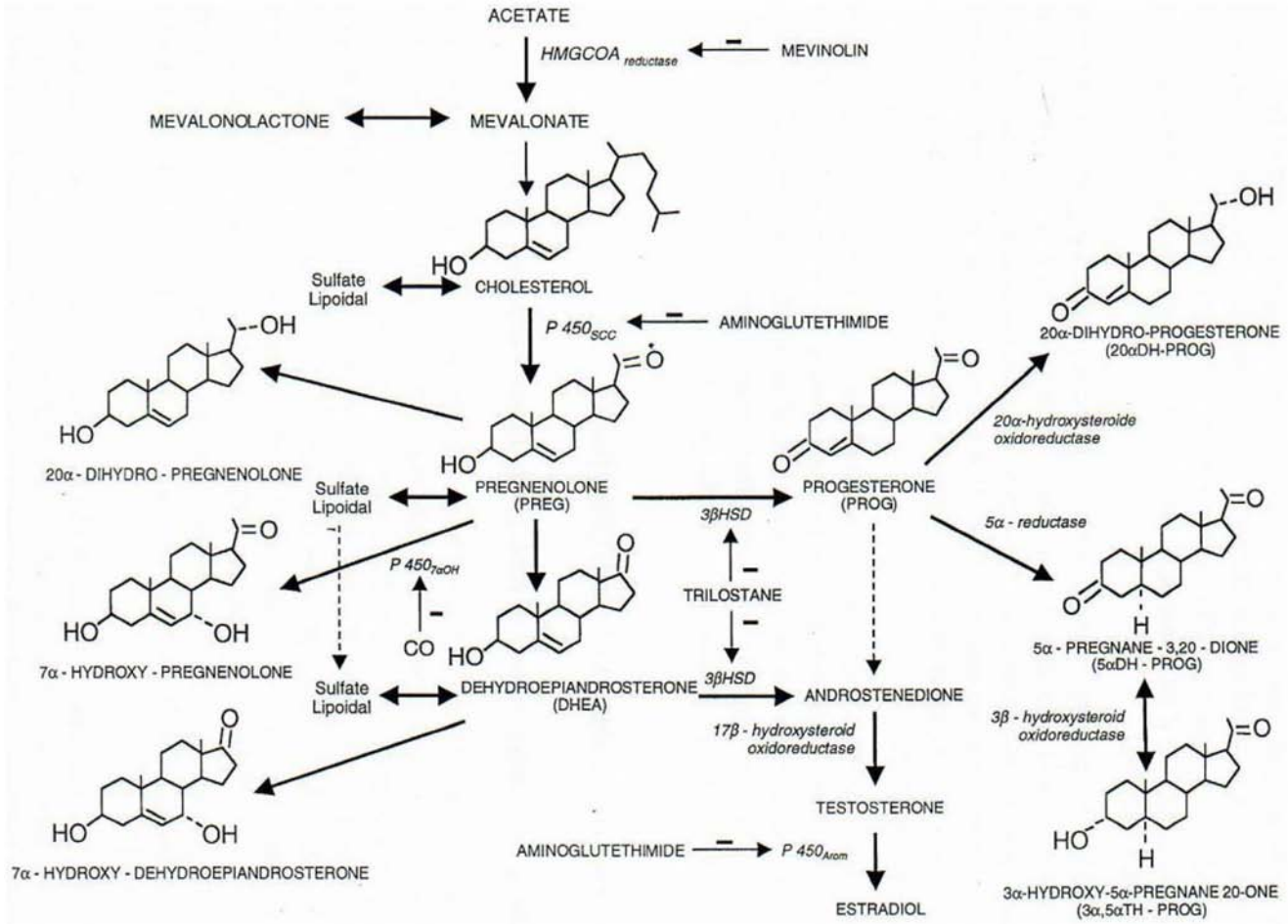

FIGURE 1 | Some metabolic pathways of physiological steroids. All steroids are cholesterol derivatives. The P450scc cytochrome (scc for side chain cleavage) cut the 6 carbon chain of cholesterol, and pregnenolone thus synthetized is precursor of all steroid hormones (3β-HSD, 3β-hydroxysteroid dehydrogenase; 3α-HSOR, 3α-hydroxysteroid oxidoreductase; 17β-HSOR, 17β-hydroxysteroid oxidoreductase). Negative signs (–) indicate the lack of the enzymatic function corresponding to the indicated product. Figure from Baulieu (5).

of progesterone (3αhydroxy-5αpregnan-20one, also called allopregnanolone) (23, 24). Several neurosteroids can act as modulators of neurotransmitter receptors, in particular those of $GABA_A$ (25), NMDA, and sigma-1. Frequently, 3β-hydroxy-Δ5 steroids are metabolized to Δ4-3oxo steroids in brain by 3βHSD (3β-hydroxysteroid dehydrogenase). Δ4-3oxo neurosteroids act *via* classical nuclear steroid receptors (26). Recent studies with progesterone indicate the possibility of distinct hormonal derivatives (27). 3β-hydroxy-Δ5 compounds themselves can also bind to the microtubule-associated protein MAP2 (28). With different locations through the brain, neurosteroids may have several concentrations and/or display diverse activities with various roles on environment and behavior (5).

The oxidative transformation of PREG to progesterone and the metabolism of progesterone to other active neurosteroids (including androgens and corticosteroids) have led us to avoid the metabolism in the brain of 3β-hydroxy-Δ5 neurosteroids to oxydated 3oxo compounds; for this reason, we synthetized and used 3β-methoxy-Δ5 steroids which are not metabolizable to Δ4-3oxo steroids of unnecessary or even pathogenically activity: for instance, an appropriate synthetic derivative of PREG is the methoxylated compound MAP4343 [**Figure 2**; (7)]. Interestingly, both PREG and its 3β-methoxy derivative can bind to protein MAP2 and they display the same activity on the microtubular system through their association to this protein (29). The interaction of MAP2 with PREG (natural neurosteroid) or MAP4343 (synthetic derivative) modifies the function of microtubules in target cells, stimulates their assembly, and is then responsible of positive improvement of behavior: not metabolizable to Δ4-3oxo steroids contrarily to PREG (which therefore cannot be safely administered as such). MAP4343 is very active and therefore medically convenient. Experimentally, MAP4343 increases anxiolytic and anti-depressive activities (30) in rats submitted to a specific psychosocial condition (31), in stressed tree shrews (32), and in Kyoto rats resistant to currently available antidepressants (to be submitted by Villey et al. in 2018). MAP4343 is therapeutically rapid and remarkably safe.

There are other results making 3βhydroxy-Δ5 steroids interesting to study. Their deficit in hippocampus of aged rodents may be responsible for some cognitive alteration (33); however, a similar effect has not yet been demonstrated in human beings. It is also worthwhile to analyze the possible effect of cerebral DHEA and its sulfate (34), unevenly distributed in the human brain.

Progesterone is a steroid difficult to study in the central nervous system (CNS) both qualitatively and metabolically. Progesterone is particularly involved in protection/repair after traumatisms of the CNS (35–37) and it plays a fundamental role in (re)myelination (**Figures 3** and **4**). After cryolesion of peripheral nerves, we have analyzed the function of progesterone synthetized by Schwann cells (38, 39).

A particular transcription factor "Krox-20," expressed in Schwann cells and stimulated by progesterone, plays an important role in myelination of regenerating sciatic nerve and sensory neurons (40, 41). The blockade of progesterone stimulation by RU486 suggests a function as for a classical progesterone receptor. There is also another membrane-associated progesterone-binding protein initially called 25Dx and currently known as PGRMC [progesterone receptor membrane compound; (42)].

Moreover, besides genomic mechanism, membrane actions of progesterone in the CNS have been described (27). Several membrane progesterone receptor(s) have been discovered (27, 43–47). It is also interesting to note that the reduced tetrahydro-metabolite of progesterone (allopregnanolone), at nanomolar concentration, can modify GABA evoked current (45). Several other neurosteroids modify functionally the activity of neuromediator receptors.

It is clear that the pharmacological function of some neurosteroids such as progesterone is diversified and this may be the reason for which it is not easy to rationalize the medical usage of post-menopausal hormone replacement (48). In addition, there are a number of synthetic progesterone analogs, multiplying the variety of compounds displaying some selective function(s) of progestins (49).

There are also a number of different neuroestrogens which, as estradiol (50), include a phenolic nucleus equivalent to the Δ4-3oxo structure of other steroid hormones and have distinct metabolic properties (51).

Δ4-3oxo neuroandrogens, the best known and active being testosterone, are partly derived from neurosteroidal 3β-hydroxy-Δ5 compounds such as DHEA, but androgenic neurosteroids synthetized in the brain have not yet been properly quantified. The androgen receptor is decisive in the spontaneous regeneration of myelin (52).

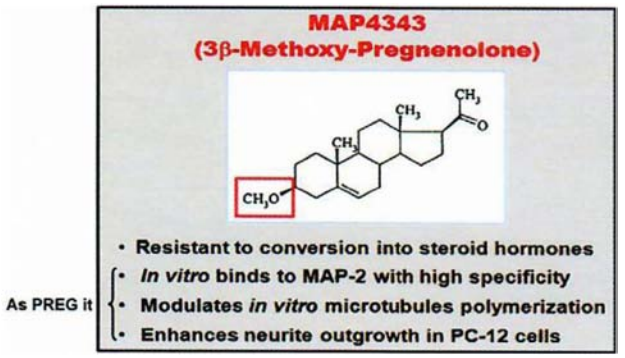

FIGURE 2 | MAP4343, a pregnenolone derivative. Data from Baulieu (8).

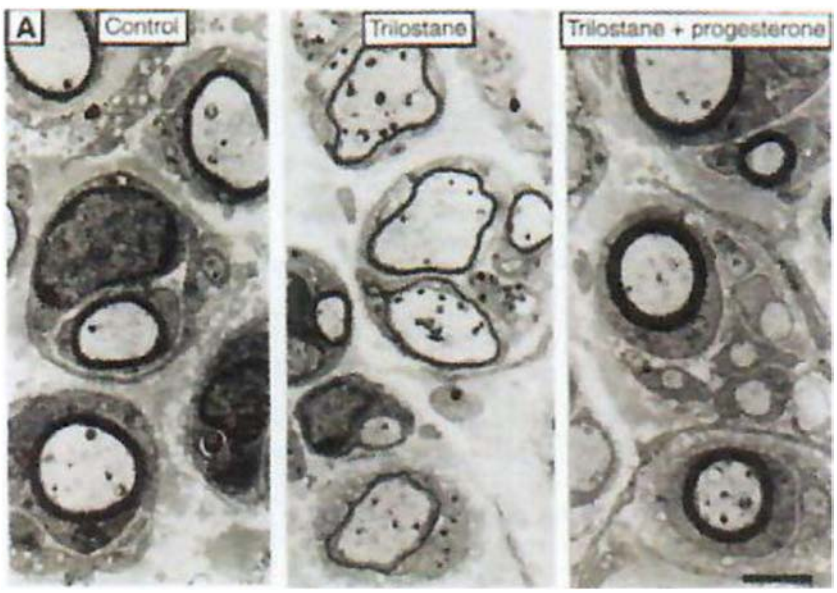

FIGURE 3 | Role of neurosteroids in the formation of myelin sheaths. The thickness of myelin sheaths (number of lamellae) was quantified by electron microscopy of cross section of sciatic nerves from male mice 15 days after cryolesion. Effect of trilostane in the absence (center panel) or presence (right panel) of progesterone on the thickness of myelin sheaths, relative to that in control nerves (left panel). Scale bar, 2 μm. Data from Koenig et al. (38).

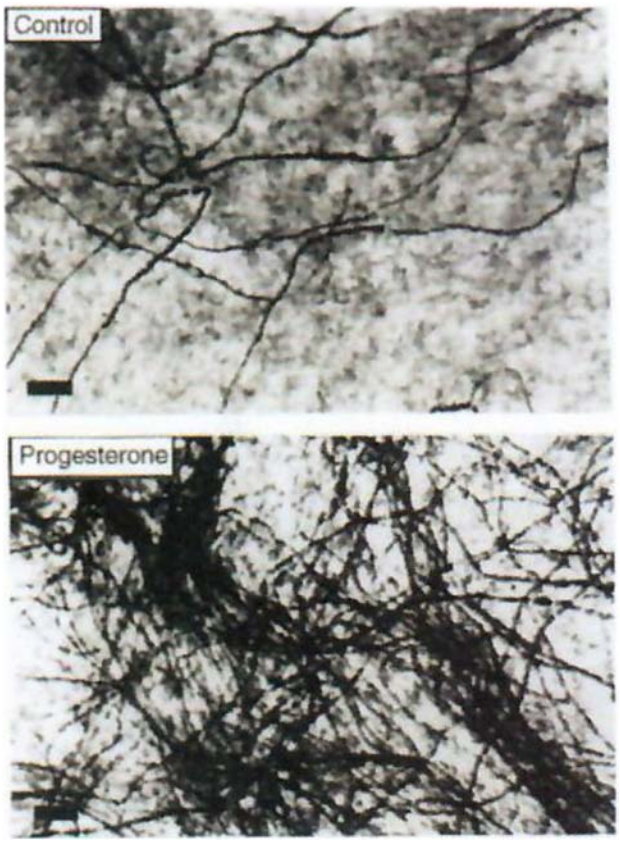

FIGURE 4 | Effect of progesterone on myelin formation in DRG cultures. Cells were cultured for 2 weeks in myelination-promoting medium in the absence (top) or presence (bottom) of 20 nM progesterone. Myelinated fibers were stained with Sudan black and the number of myelin segments was determined. Only myelinated fibers are visible on these photographs. Scale bars, 40 µm. Data from Koenig et al. (38).

It is not yet known if, in the brain, there are neurosteroids with glucocorticosteroid properties active by themselves, or if there are, in the brain, only corticosteroids transferred from the body and participating to the quantitative regulation of their own production.

In summary, not only PREG, DHEA, as well as their sulfates, may by themselves be active in the brain, they also are potential precursors in the CNS of Δ4-3oxo steroids which are biologically active *via* binding to nuclear receptors. There are also, making it even more varied, well-known hormonal steroids certainly imported at least in part into the brain from glands of the body which have a function of physiologically quantitative importance on the development and the activities of neurons and myelination. They are not really "neurosteroids," even if they are intimately linked to the function of the CNS.

Other Pharmacological Compounds With Activities Related to Neurosteroids

An Enantiomeric Form of PREG Sulfate (53–55)

This synthetic compound (56) is curiously much promnesic than PREG sulfate itself, and this effect opens a new field of research to compare activity between natural and enantiomeric structures of hormonal steroids [parenthetically, the natural PREG and PREG sulfate both are themselves active on memory; (21)].

Lithium

It has neuroprotective activity, particularly for remyelination of peripheral nerves (57). The results open perspectives in treatments by an inhibitor of glycogen synthase kinase 3β such as lithium.

Etifoxine (58, 59)

This neurostimulant of estrogens and progestins is active on experimental autoimmune encephalomyelitis, a model of multiple sclerosis (60, 61). However, attempts to decrease post-partum relapses in sick women (62) have not shown a significant success.

RU486 (Mifepristone, an Efficient Anti-Progesterone) (63)

The compound, which is also antiglucocorticosteroid, can be orally administered and has demonstrated anti-neurosteroidal effects on inappropriate neuroprogesterone and neuroglucocorticosteroid pathologies. It also permits protective effects against traumatic neuronal alterations [for example, protection of cerebellar Purkinje cells (64, 65)] and has shown therapeutically activity on some psychotic depressions and meningiomas (unpublished).

ALZHEIMER'S DISEASE: FKBP52, A CONSTITUENT OF HETERO-OLIGOMERIC STEROID HORMONE RECEPTORS, INTERACTS WITH TAU STRUCTURE AND FUNCTION

In human beings, the most frequent and severe human senile dementia, the Alzheimer's disease, currently is not biochemically explained nor treated in order to recover. However, there are several recent progresses for the diagnostic, biomarkers and imagery of the sick brain (66). Since the publication of Alois Alzheimer in 1907, two proteins remain of greatest interest (67). Up to now, the most studied has been "amyloid-β" (Aβ), essentially observed between neurons but its study has still not been successful in any therapeutic approach. The other protein, the structure and the cloning of which have been only precisely analyzed since the 1980 period, is "Tau" (68–70). Besides association to microtubules, the Tau protein is involved in several other cellular functions, including gene regulation (71). In human patients, Tau is present within neuronal cells, and the six isoforms, hyperphosphorylated (72) according to a well-defined pattern (73), are also partly truncated by caspase activity (74). Isomerization and oligomerization of Tau due to FKBP52 are independent processes (75). These modifications are largely responsible for fibrillation and aggregation, and they contribute to establish intracellular neurodegeneration in transgenic animals. It is accepted that modified Tau can be responsible for altered function of nerves, even if details of the mechanism of pathological function of abnormal Tau are not well explained.

Distinct from Alzheimer's disease, there are dementias which are exclusively tauopathy (69), supporting the belief that Tau abnormalities are directly responsible of dementia activity of the brain in Alzheimer's disease (72); the tauopathic diseases without Aβ abnormality could be called "pure tauopathies": some are FTDP17: frontotemporal dementias and parkinsonism linked to chromosome 17; progressive supra-nuclear palsy; Pick diseases, etc.

Consequently, Tau, mostly in aggregated forms, is clearly involved principally in terms of neurodegeneration, and pathological Tau may be most appropriate as potential target for treating Alzheimer (76). The pure tauopathies, human and experimental in animals, are very severe diseases and matters of a large number of studies: in particular, the diseases due to P301L mutation of Tau have been very much studied as convenient models. In genetically P301L mutated zebrafishes, the Tau is much oligomerized and abnormally phosphorylated (77).

In studying steroid receptors in human beings, we had analyzed some of their hetero-oligomeric variants (78, 79). They differ according to diseases, but often include the protein FKBP52. In Alzheimer's disease, this protein colocalizes with the autophagic-endolysosomal system (**Figure 5**). The FKBP structure includes an N-terminal domain (FK1) which harbors a sequence with enzymatic PPIase activity (peptidyl-*cis*/*trans*-prolyl-isomerase), thus classifying FKBP52 among the immunophilins which may bind many small molecular ligands (80, 81). The second domain of FKBP52, FK2, is architecturally similar to the FK1 but does not have a PPIase activity. The rest of the FKBP52 protein includes three TetratricoPeptid Repeats' (TPR) sequences and binds to hsp90, which itself interacts with an hormonal steroid molecule, completing the hetero-oligomeric composition of steroid receptors. After TPR, there is a C terminal sector-binding calmodulin. It is therefore *via* FKBP52 that the hetero-oligomeric steroid hormones could interact functionally with a neuro-protein such as Tau.

Indeed, we had, additionally and very importantly, discovered an interaction of FKBP52 with Tau [(4); **Figures 5** and **6**], completing the description of steroid–FKBP52–Tau sequence(s) never described till now, and that have not been studied in functional terms.

Experiments with zebrafishes under the direction of Dr. Marcel Tawk (77) utilizing transgenic animals expressing the human Tau P301L, severely suffering of abnormal escape behavior and with some change of Tau phosphorylation, indicated, *in vivo* and *in vitro* studies, the functional abnormalities due to the pathogenic mutation of Tau.

This is the basis of a strategy leading to look for a treatment of tauopathies, including Alzheimer's disease and other dementias. FKBP52 may serve for inducing qualitatively and anti-pathological Tau activity and/or directly decreases Tau: considering that Tau is centrally involved in the pathophysiology of Alzheimer, we look for modifying the function of FKBP52 to obtain an actively anti-pathological Tau effect. There is a very profound decrease and/or a reduced function of FKBP52 that we have described in pathological circumstances: in Alzheimer's disease, FTDP17 (9), and other tauopathies. Our hypothesis is based on the concept that the function of Tau is decisively pathological in dementias, and we hypothesize a FKBP 52 induced change quantitatively and/or qualitatively, which could be profitable to the patients (**Figure 7**) treated as early as possible after the beginning of the disease. In summary, appropriate modification of FKBP52 induced by some ligand(s) may help to normalize Tau activity and consequently be able to improve the cerebral function.

It is not excluded that several brain steroids may modify the function of parameters implied in this novel approach for treating

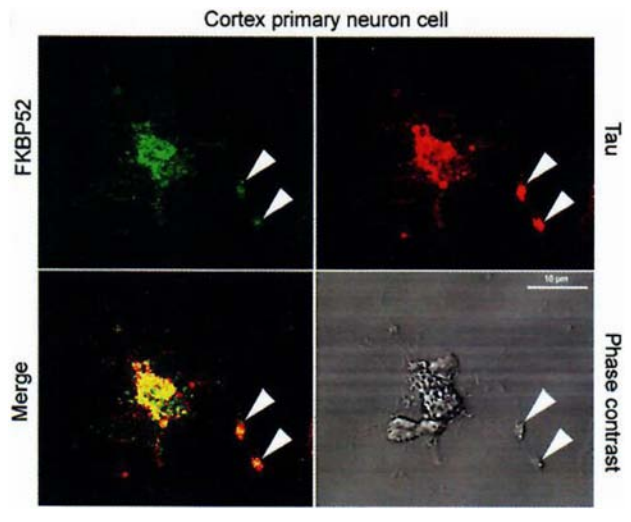

FIGURE 5 | Colocalization of FKBP52 and Tau in primary cortical neurons and PC12 cells. Immunofluorescence staining of primary cortical neurons and PC12 cells treated with 50 nM NGF for 5 days. Double staining for Tau and FKBP52 was performed after cytosol extraction to reveal cytoskeletal association. Arrows indicate preferential colocalization of both proteins in the distal part of the nerve cell axon and at the extremity of PC12 cell neurites. Confocal images of primary cortical neurons. Analysis of 0.5-µm slices confirms the preferential colocalization in the distal part of the axon (arrowheads). Data from Chambraud et al. (4).

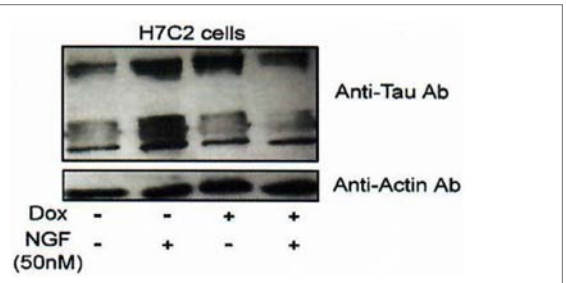

FIGURE 6 | Effect of FKBP52 overexpression on Tau protein accumulation. H7C2 cells: stably transformed PC12 cells by an FKBP52-inducible expression system based on a tetracycline responsive element (82). NGF: 5 days—Dox: 7 days. H7C2 and PC12 cells were treated or not with NGF for 5 days in the presence or absence of DOX for 1 week; 50 µg of extracts was subjected to SDS-PAGE and immunoblotted with anti-Tau (antibody clone DC25). Actin was used as the loading control. Data from Chambraud et al. (4).

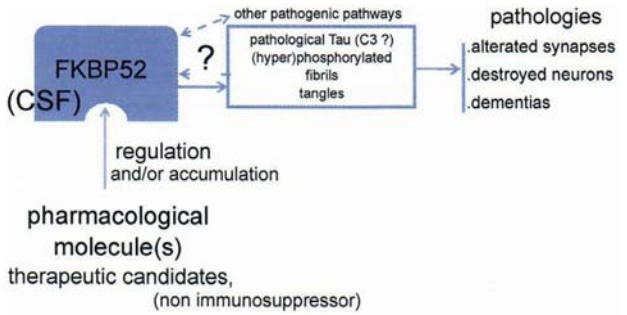

FIGURE 7 | Schema of the working hypothesis and strategy for prevention/treatment of dementias *via* the novel target FKBP52. FKBP52 is measurable in CSF. Data from Baulieu (8).

cerebral abnormality. Other approaches may help to understand alterations of the Tau protein: for instance, studies of synaptic structure (83, 84) and activity (in preparation), and experiments of deep cerebral stimulation can be involved in methods including autophagic-lysosomal protection (85).

Our therapeutic approach centered on effect of FKBP52 on Tau (dys)function may be still far to be operationally favorable to treat dementias (**Figure 7**). Working on Tau with new modified/liganded FKBP52 function and steroid activities, we will obtain novel results. Will they be valuable?

SUMMARY

In this "Perspective," we have treated two distinct examples concerning steroid associated molecules involved in activities of the CNS. They exemplify typical compounds of (1) neurosteroid derivatives and (2) components of hetero-oligomeric steroid receptors. We are happy that, in a number of other laboratories, many publications (not reported in this "Perspective"), have followed our two original studies. This paper is not a review, but we hope to draw attention to the versatile involvement of the steroidal structure and thus in important physiopathological derivatives.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

ACKNOWLEDGMENTS

I thank Christine Broussot-Charbonnier for her remarkable administrative assistance.

FUNDING

No funding for this "Perspective." Support for the work in each cited paper to which I collaborated is detailed in the corresponding reference.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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PERSPECTIVE
published: 18 June 2018
doi: 10.3389/fendo.2018.00270

Brain-Derived Steroids, Behavior and Endocrine Conflicts Across Life History Stages in Birds: A Perspective

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Biological steroids were traditionally thought to be synthesized exclusively by the adrenal glands and gonads. Recent decades have seen the discovery of neurosteroid production that acts locally within the central nervous system to affect physiology and behavior. These actions include, for example, regulation of aggressive behavior, such as territoriality, and locomotor movement associated with migration. Important questions then arose as to how and why neurosteroid production evolved and why similar steroids of peripheral origin do not always fulfill these central roles? Investigations of free-living vertebrates suggest that synthesis and action of bioactive steroids within the brain may have evolved to regulate expression of specific behavior in different life history stages. Synthesis and secretion of these hormones from peripheral glands is broadcast throughout the organism *via* the blood stream. While widespread, general actions of steroids released into the blood might be relevant for regulation of morphological, physiological, and behavioral traits in one life history stage, such hormonal release may not be appropriate in other stages. Specific and localized production of bioactive steroids in the brain, but not released into the periphery, could be a way to avoid such conflicts. Two examples are highlighted. First, we compare the control of territorial aggression of songbirds in the breeding season under the influence of gonadal steroids with autumnal (non-breeding) territoriality regulated by sex steroid production in the brain either from circulating precursors such as dehydroepiandrosterone or local central production of sex steroids *de novo* from cholesterol. Second, we outline the production of 7α-hydroxypregnenolone within the brain that appears to affect locomotor behavior in several contexts. Local production of these steroids in the brain may provide specific regulation of behavioral traits throughout the year and independently of life history stage.

Keywords: territorial aggression, life history stages, aromatase, dehydropepiandrosterone, 7α-hydroxypregnenolone, locomotor activity, neurosteroid

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *26 February 2018*
Accepted: *08 May 2018*
Published: *18 June 2018*

Citation:
Wingfield JC, Wacker DW, Bentley GE and Tsutsui K (2018) Brain-Derived Steroids, Behavior and Endocrine Conflicts Across Life History Stages in Birds: A Perspective. Front. Endocrinol. 9:270. doi: 10.3389/fendo.2018.00270

INTRODUCTION

The life cycles of animals consist of life history stages such as breeding, migration, molt, and non-breeding expressed at appropriate times of year and with specific durations matched to seasonal change [e.g., Ref. (1, 2)]. Each stage has a unique suite of sub-stages in which animals exhibit morphological, physiological, and behavioral adaptations to changing environmental and social conditions.

Some traits may be expressed across multiple life history stages. However, the neuroendocrine and peripheral endocrine regulation of those traits in one stage might be different in another stage. Examples include the regulation of territorial aggression across breeding and non-breeding seasons (3–5), the control of locomotor activity in seasonal migration and facultative movements to avoid perturbations of the environment [e.g., Ref. (6–8)].

There is growing evidence that the neuroendocrine and endocrine cascades involved in regulation of morphology, physiology, and behavior across different life history stages can be altered at many different levels [e.g., Ref. (9); **Figure 1**]. Localized regulatory mechanisms in one life history stage allow trait modulation without the effects associated with signals that act throughout the organism such as hormones secreted into the bloodstream acting on multiple tissues. Thus, conflict between the need for the expression of a particular trait that can be regulated by a particular hormone and the inappropriate co-regulation of other physiological or behavioral responses to that hormone at the wrong season can be avoided. These regulatory processes can be organized into three major categories [see **Figure 1** (10, 11)]:

1. Secretion—cascades of hormone secretion [e.g., the hypothalamo–pituitary–gonad (HPG) axis, **Figure 2**] in which hormones are broadcast throughout the organism's bloodstream and have as many potential targets as cells or tissues that possess receptors for that hormone.
2. Transport—transport systems by which hormones are transported in the blood [e.g., corticosteroid-binding globulins (CBG)], or are selectively taken up by organs and tissues *via* other cellular transport proteins (e.g., the blood–brain barrier).
3. Response—regulated responses of target cells to, and fine-tuned synthesis/availability of, paracrine, neuroendocrine, and endocrine secretions that can facilitate appropriate life history stage changes. These processes may involve alterations to receptor expression within a target cell, actions of metabolizing enzymes that can promote or deactivate a hormone (e.g., steroid metabolizing enzymes in many tissues that regulate local substrate availability for endocrine signaling systems), and a complex system of proteins that can act as enhancers or inhibitors of gene expression. It is possible to take this further downstream to protein synthesis, packaging, and actions, but these are beyond the scope of this paper.

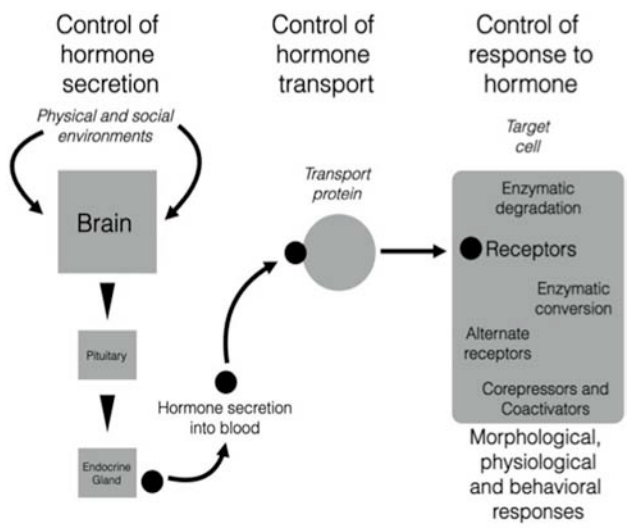

FIGURE 1 | There are three components of the action of hormones that can be regulated in diverse ways. The left-hand part of the figure shows the triggering of neural, neuroendocrine, and endocrine cascades that result in production of a highly specific secreted signal. The central part of the figure shows that after secretion into the blood, many hormones (particularly steroids, thyroid hormones, and some peptides) are transported by carrier proteins. Once the hormonal signal reaches the target cell (e.g., a neuron in the brain), it can act in various ways by binding to membrane or nuclear receptors that trigger extremely complex actions in a cell. Note that all three components are sites of regulation that could change daily or seasonally, or vary among individuals, populations, sex, or in response to unique experience of the environment. It is possible that this scheme is highly conserved across vertebrates and that different species modulate different components and still achieve the same behavioral and physiological end points. See text for more details. From Wingfield (10), courtesy of the American Ornithologist's Union and Wingfield (11), courtesy of Elsevier.

An example of a secretion–transport–response system is the production and action of sex steroids (**Figure 2**), in which the HPG cascade leading to testosterone secretion has multiple levels of possible regulation. Many species of vertebrates have highly specific androgen-binding proteins in the blood, including sex hormone-binding globulin, which is absent in birds, as well as CBG, which binds both glucocorticoids and sex steroids (12–14). The CBG gene has recently been identified in birds (15) and will allow future studies on CBG regulation. Target cell responses can be regulated in various ways, including the transformation of testosterone to estradiol and thus activation of a different array of receptor types [**Figure 2** (16, 17)]. Also, testosterone can be deactivated by 5β-reductase to 5β-dihydrotestosterone that is generally regarded as inert and does not bind to androgen receptors (ARs) [**Figure 2** (18–20)]. Note that peripheral secretion of sex steroids (left-hand side of **Figures 1** and **2**) results in broadcast of hormones to the entire organism through the blood stream, whereas actions at target cells (right-hand part of the **Figures 1** and **2**) allows local adjustment of the responses to a hormone. Steroids produced within the brain also allow paracrine actions at very localized sites without the additional effects on general morphological, physiological, and behavioral responses that occur when the hormone is broadcast through the blood stream [e.g., Ref. (11)]. These flexible points of regulation allow for the fine-tuning of trait expression to meet the specific demands of a stimulus, while keeping the animal within the necessary constraints of its current life history stage.

The secretion–transport–response regulatory model allows for the progressive localization of the effects of blood borne hormones (**Figure 2**; Figure S1 in Supplementary Material). Secretion cascades are generally broadcast to the whole organism whereas transport and especially target cell responses can be tailored to specific situations, environmental change, and life history stage. Environmental cues emanating from changing conditions can have direct effects on the secretion–transport–response system (e.g., temperature-related changes in hormone metabolism) or,

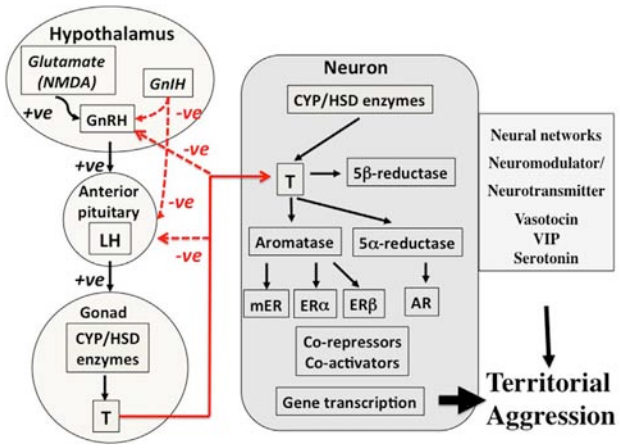

FIGURE 2 | Control mechanisms for territorial aggression in birds in spring. There are three major components to control mechanisms. First, the regulation of hormone secretion from the hypothalamo–pituitary–gonad axis (left part of the figure), transport of hormones such as testosterone in the blood (lines in red), and the mechanisms associated with action of the hormone in the target cell, in this case a neuron in the brain (central part of the figure). The net result is regulation of territorial aggression by neural networks (right-hand part of the figure). This three-part system of control of hormone secretion, transport, and effects on target organs is an important concept because it offers many points of potential regulation. The secretion component on the left summarizes how sensory information, in this case social, in transduced through neurotransmitter, e.g., glutamate (NMDA), and neuroendocrine secretions such as gonadotropin-releasing hormone (GnRH) and gonadotropin-inhibiting hormone (GnIH) into release of gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (not shown) from the anterior pituitary into the blood. LH travels to the gonad where it acts on cells that express steroidogenic enzymes to stimulate secretion of the sex steroid hormone testosterone (T) that is in turn released into the blood. Local actions of T in the testis include regulation of spermatogenesis, but it is also released into the blood in many avian species during breeding. Among many actions of T are effects on territorial aggression, and negative feedback on neuroendocrine and pituitary secretions (red lines). In birds, T circulates bound weakly to corticosteroid-binding globulin before entering target neurons involved in the expression of territorial aggression (center of figure). Once T has entered a target neuron, it has four potential fates. First, it can bind directly to the androgen receptor (AR), a member of the type 1 genomic receptors that become gene transcription factors once they are bound to T. Second, T can be converted to estradiol (E2) by the enzyme aromatase. E2 can then bind to either estrogen receptor alpha (ERα) or estrogen receptor beta (ERβ) both of which are genomic receptors that regulate gene transcription, but different genes from those regulated by AR. Third, T can be converted to 5α-dihydrotestosterone that also binds to AR and cannot be aromatized thus enhancing the AR gene transcription pathway. Fourth, T can be converted to 5β-dihydrotestosterone that binds to no known receptors and also cannot be aromatized indicating a deactivation shunt. The possibility of a rapid acting membrane receptor (non-genomic) should also be considered. A complex system of co-repressors and co-activators of genomic steroid receptor action are also known. Furthermore, it is now known that many neurons in vertebrate brains express all the enzymes required to synthesize T and E2 *de novo* from cholesterol or from circulating inert steroid hormone precursors such as dehydroepiandrosterone, androstenedione, and progesterone. The end result is regulatory action on neural networks that regulate expression of territorial aggression. Several neurotransmitters and neuromodulators such as arginine vasotocin, vasoactive intestinal peptide (VIP), and serotonin as also involved at this level. Evidence suggests that the basic secretory, transport, and action mechanisms are conserved across vertebrates. However, it is also clear that there are very many points at which the expression of territorial aggression may be affected indicating almost limitless combinations of possible regulatory mechanisms. From Wingfield (10), courtesy of the American Ornithologist's Union and Wingfield (11), courtesy of Elsevier.

more commonly, can be signalized *via* neural perception and transduction [Figure S1 in Supplementary Material, see Ref. (21)]. Mechanisms by which environmental cues can affect transport mechanisms and specific target cell responses remain much less well known. For example, it appears that the testes of zebra finches, *Taenopygia guttata*, can integrate and respond to cues of stress directly, rather than relying on a hormonal cascade initiated by the brain (22, 23), but it is not known exactly why the brain is bypassed in this instance. The evolutionary origins of the endocrine axes have seemingly resulted in a remarkable flexibility of response of different tissues (24).

Neurosteroids in Vertebrate Brains

Sex steroids have multiple peripheral and central actions on physiological traits. Sex steroids are not only produced solely by gonads and adrenal glands, but also by the brain [e.g., Ref. (5, 9, 25–31)]. In seasonally breeding vertebrates, neurally derived sex steroids produced in the non-breeding season do not influence reproductive traits *per se*, as very little sex steroid from this source reaches the blood stream and such neurosteroids are synthesized and act in a paracrine fashion in localized areas of the brain (32). Still, neurosteroids can have numerous effects on behavioral traits such as locomotor activity, aggression, reproduction, body temperature, and blood pressure (9, 29, 33–38). In addition, steroids in specific brain nuclei show seasonal changes in concentration, independently of circulating steroids [e.g., Ref. (39)]. These neurosteroids then act in a variety of brain regions to facilitate appropriate behavior for each life history stage (3, 28, 30, 40, 41).

The seasonal regulation of neurosteroid expression is not restricted to sex steroids. In a study on Japanese newts, *Cynops pyrrhogaster*, concentrations of pregnenolone and progesterone in the brain were higher in animals exposed to long spring-like vs. short winter-like day lengths, whereas experimental manipulation of environmental temperature had no effect (28). The newt brain expresses enzymes that synthesize 7α-hydroxypregnenolone *de novo* from cholesterol, and this neurosteroid activates locomotor activity associated with spring breeding. Moreover, the effect of 7α-hydroxypregnenolone on locomotor activity was blocked by dopamine D2-like receptor antagonists (27).

TWO EXAMPLES OF FLEXIBILITY IN STEROID MECHANISMS OF ACTION

We will focus next on two examples in which local production and paracrine actions of steroids in the brain can have major impacts on control of behavior and potentially avoid evolutionary costs of peripheral endocrine secretions at the wrong time.

Androgen/Estrogen Control of Avian Territorial Aggression Across Life History Stages

Although male territorial aggression was classically thought to be associated with circulating testosterone, work over the last 30 years suggests that the neural sensitivity to sex steroids, and the synthesis and action of both androgens and estrogens can modulate such behavior (32, 38, 42, 43). These are particularly

important findings with regards to seasonal animals that express aggression during non-breeding life history stages when high circulating testosterone levels would likely lead to inappropriately timed reproductive behavior. For example, the Pacific Northwest subspecies of the song sparrow (*Melospiza melodia morphna*) shows high levels of aggressive territoriality and circulating testosterone during its breeding life history stage, low levels of testosterone and aggression during the autumnal molt, and high levels of aggression, but very low levels of circulating testosterone during the post-molt, non-breeding period (44). Our three-point secretion–transport–response regulatory model helps to explain how such behavioral expression is achieved across these different life history stages.

Secretion and production of sex steroids that modulate territorial aggression in the song sparrow change seasonally. For example, there is evidence for local production of testosterone and 17β-estradiol in the brain, both *de novo* from cholesterol and from circulating precursors (32, 33, 39). An example of the latter is dehydroepiandrosterone (DHEA), which is released by the adrenal glands, liver, and other peripheral organs, taken up by brain regions, and converted locally to more bioactive steroids (39). Neural steroid production allows for animals to retain actions of sex steroids in regions of the brain relevant to aggression, while avoiding the complication and inappropriate activation of morphology, physiology, and behavior associated with reproduction at the wrong time of year (45). Measurement of circulating DHEA indicates seasonal changes associated with territorial behavior in different life history stages in some species [e.g., Ref. (3, 19)] but not others [e.g., Ref. (46)]. In the song sparrow, circulating DHEA levels are highest during breeding and non-breeding when aggression is highest and lower during the molt, when aggression is also low, suggesting that circulating DHEA acts to promote life history appropriate aggression in this species (39).

The peripheral transport of DHEA and its movement into the brain, especially in birds, is not well characterized. In humans and some other vertebrates, DHEA travels in a hydrophilic sulfated form, DHEAS [e.g., Ref. (47)]. However, in birds, including the song sparrow, DHEA circulates primarily in its non-sulfated form [e.g., Ref. (39)]. Once DHEA arrives at its targets in the brain, it is possible that it has direct effects *via* ERβ, sigma-1, and/or GABAA receptor activation (48–50), though the affinity of DHEA for these receptors can be very low and more work is needed to assess the physiological and behavioral relevance of these possibilities. It is perhaps more likely that DHEA exerts the bulk of its effects on aggression *via* local conversion to testosterone, 5α-dihydrotestosterone, and 17β-estradiol, and subsequent activation of androgen and estrogen receptors.

Although circulating DHEA levels are equally high in breeding and non-breeding song sparrows, its local conversion to testosterone/estrogen in the brain may be more important during the non-breeding life history sub-stage (51). The activity of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD), the enzyme that catalyzes transformation of DHEA to androstenedione, is higher during non-breeding vs. breeding in many brain regions that comprise neural networks underlying social behavior (30). Furthermore, DHEA is rapidly metabolized in such brain regions in dominant male song sparrows during aggressive interactions (42). These same interactions do not alter plasma DHEA levels. Evidence suggests that DHEA is eventually converted to 17β-estradiol to facilitate non-breeding aggression in this species.

Fadrozole, an inhibitor of aromatase, the enzyme that catalyzes the transformation of androgens to estrogens, reduces non-breeding, but not breeding aggression in song sparrows (20). That the precursor, DHEA might play a more prominent role in steroid-dependent, non-breeding aggression makes intuitive sense, as elevated circulating testosterone likely provides ample substrate to brain regions modulating aggression during the breeding period. Furthermore, castration of male song sparrows had no effect on territorial aggression in autumn suggesting that gonadal sources of androgen were not essential for expression of territoriality (52). These findings strengthen the hypothesis that circulating steroid hormone precursors such as DHEA and/or local production of sex steroids in the brain are requisite for expression of aggression in the non-breeding season in some species.

Estrogen synthesis can also be modified to facilitate appropriate behavioral responses. Changes in estrogen synthesis can occur in response to immediate environmental or social cues. For example, aromatase can be rapidly inactivated *via* phosphorylation (53), which may help to quickly alter behavior when an immediate context warrants it, though much more work is needed to address the behavioral relevance of these acute changes (16, 54). Estrogen synthesis can also change seasonally, potentially facilitating life history stage appropriate responses. In the male song sparrow, aromatase activity is highest in the ventromedial telencephalon, home to the medial amygdala, during breeding and non-breeding life history stages, when aggression is also at its height (3). The expression of aromatase mRNA also changes seasonally in multiple brain regions associated with the control of social behavior in the male song sparrow (5). Aromatase expression in the ventromedial nucleus of the hypothalamus (VMH), a brain region known to be involved in aggression-seeking behavior in rats (55), is equally high during breeding and non-breeding, when aggression is also highest, suggesting that the VMH may also mediate aggressive responses in birds (5). Aromatase expression in the preoptic area (POA), a brain region long known to be involved in the regulation of reproductive behavior and physiology, is both elevated during breeding and increased by administration of exogenous DHEA in male song sparrows, suggesting that this androgen/estrogen precursor may mediate different effects in different life history stages (56).

Androgen and estrogen receptor sensitivity may also be modulated to promote life history stage appropriate behavior. For example, neural AR mRNA expression changes seasonally in male song sparrows, with higher levels in the POA, the periventricular nucleus of the medial striatum (pvMSt), and paraHVC, a region of the song control system, during breeding (5). Exogenously administered DHEA increases AR expression in many of the same regions, again suggesting a role for this hormone in the seasonal modulation of social behaviors in this species (56). Current evidence suggests that DHEA, its transport to the brain from peripheral tissues, and its local conversion to

androgens and estrogens helps promote life history appropriate behaviors, including seasonal aggression, in the male song sparrow. Adjustments of AR and aromatase gene expression have also been associated with aggressive responses to simulated territorial intrusions in dark-eyed juncos, *Junco hyemalis* (43), and differences in estrogen receptor alpha (ERα) expression are associated morph-specific variation in aggressive song in white-throated sparrows, *Zonotrichia albicollis* (57). In juncos, there were no changes in ERα expression, however (58), highlighting the importance of considering species-specific differences when assessing steroid responses relating to avian territorial aggression. Changes in sex steroid receptors in specific areas of the brain appear to be a widespread mechanism by which the sensitivity of neural tissue is regulated [e.g., Ref. (59, 60)].

Potential Roles of Neurosteroids on Animal Movement Including Migration

A second example of how the secretion–transport–response regulatory model approach informs hypotheses about how potential conflicts of steroid control of life history stages in different contexts might be avoided, involves the role of the neurosteroid 7α-hydroxypregnenolone in regulation of locomotor activity. Regular avian migrations (e.g., spring and autumn) have well-established endocrine control mechanisms [e.g., Ref. (7, 61)]. However, control mechanisms associated with specific environmental cues in one migration life history stage may have conflicts of action for the other. For example, spring migration toward a breeding area occurs during increasing day length but decreasing temperature and trophic resources as animals move north. By contrast, autumn migration toward a wintering area occurs during decreasing day length but temperatures tend to be higher as the animals move south and trophic resources are good, even increasing [e.g., Ref. (6)]. It is likely that these cues are differently transduced and integrated across these life history stages, even though the resulting migratory behaviors are similar in appearance. Furthermore, other types of animal movements such as facultative movements in response to local conditions may be different again (8). Nevertheless, common mechanisms may exist at fundamental neural levels across life history stages and facultative contexts, and there is preliminary evidence that the paracrine actions of 7α-hydroxypregnenolone may play a key role.

7α-Hydroxypregnenolone has been shown to affect locomotor behavior in newts, fish, and quail [e.g., Ref. (27, 34, 36, 40)]. Prolactin mediates 7α-hydroxypregnenolone action on spring migration in Japanese newts (62) and during upstream migration in chum salmon, *Oncorhynchus keta*, possibly *via* actions on dopaminergic neurons in the magnocellular preoptic nucleus (63). There are also strong circadian effects on 7α-hydroxypregnenolone that are possibly mediated by melatonin (36, 64), setting the stage for circadian control of locomotor behavior.

To assess the distribution and actions of 7α-hydroxypregnenolone in a migratory songbird, the white-crowned sparrow, *Zonotrichia leucophrys gambelii* (see Presentation S1 in Supplementary Material for methods and details), we collected brains from photostimulated birds that were in the spring migratory life history stage and divided them into regions. Left and right hemispheres were analyzed for differences in 7α-hydroxypregnenolone synthesis and concentrations, respectively. We also collected brains from birds in the non-migratory stage and analyzed 7α-hydroxypregnenolone synthesis separately in the cerebrum, diencephalon, mesencephalon, and cerebellum. There were no significant differences in 7α-hydroxypregnenolone synthesis in any brain region between the "migratory phase" and "non-migratory phase" (**Figure 3**).

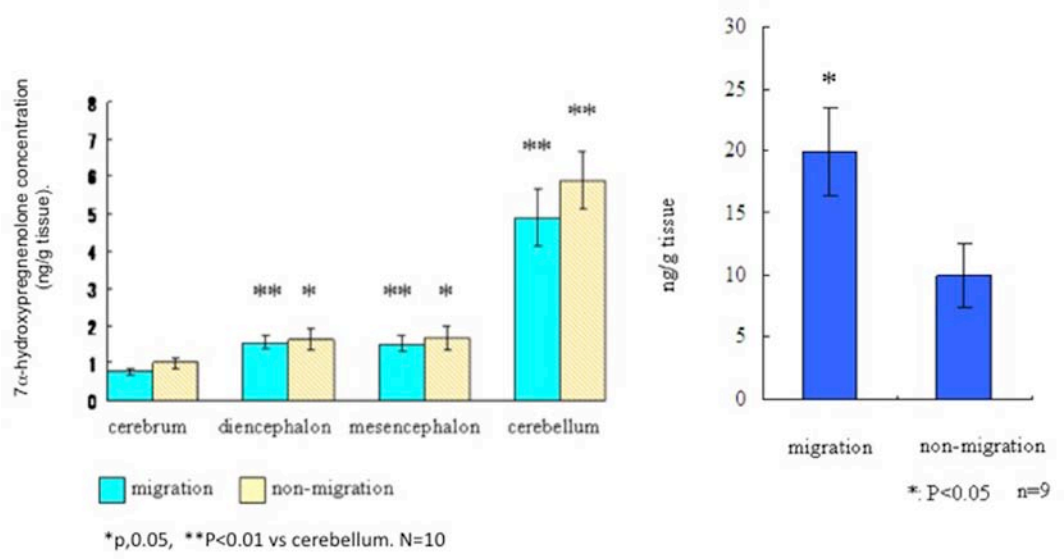

FIGURE 3 | Left panel. Synthesis of 7α-hydroxypregnenolone in different brain regions of the white-crowned sparrow, *Zonotrichia leucophrys gambelii*. Right panel. Whole brain concentrations of 7α-hydroxypregnenolone in white-crowned sparrows, in migratory and non-migratory life history stages.

However, 7α-hydroxypregnenolone synthesis in the cerebellum was higher than those in other brain regions in both males and females and regardless of migratory and non-migratory states (**Figure 3**; Figure S2 in Supplementary Material).

By GC-Ms analysis for 7α-hydroxypregnenolone concentration, we combined different brain regions (cerebrum + diencephalon + mesencephalon + cerebellum) due to the small tissue amounts involved. Results from this analysis suggest that 7α-hydroxypregnenolone concentration during the spring migratory phase is higher than that during the non-migration phase (**Figure 3**, right-hand panel). Note also that white-crowned sparrows in the vernal migratory stage exhibited very high levels of nocturnal migratory restlessness (*Zugunruhe*) compared with photorefractory birds that were molting and not migrating (Figure S3 in Supplementary Material). A higher concentration of 7α-hydroxypregnenolone during the migratory stage may depend on the amount of its precursor pregnenolone. An analysis of P450scc producing pregnenolone may be required in order to fully elucidate the dynamics of 7α-hydroxypregnenolone synthesis during this life history stage.

To determine central behavioral effects of 7α-hydroxypregnenolone infused into the third ventricle of white-crowned sparrows, birds in the molt life history stage were used, i.e., birds not showing migratory behavior (Figure S4 in Supplementary Material). Birds were then video-recorded for 30 min post-infusion, but most of the effects occurred in the first 10 min. 7α-Hydroxypregnenolone significantly inhibited perch-hopping behavior at a dose of 200 ng in 2 μl of vehicle in molting birds, but there was a great deal of variance in the data (top panel of Figure S4 in Supplementary Material). Overall, there was a decrease in perch-hops of approximately 30% compared with activity following saline infusions in the same birds (Figure S4 in Supplementary Material). There was approximately a 90% decrease in some individuals. In a follow-up study, infusion of 7α-hydroxypregnenolone at high and low doses into white-crowned sparrows had no effect on locomotor behavior during the spring migration life history stage (lower panel, Figure S4 in Supplementary Material). These data suggest seasonal and state dependent differences in actions of neurosteroids. Further studies attempting to block the actions of 7α-hydroxypregnenolone in spring and autumn migration will be critical.

PERSPECTIVE

The regulation of morphological, physiological, and behavioral traits specific to life history stages is mediated through endocrine, neuroendocrine, and paracrine secretions. When a specific trait may be expressed in more than one life history stage, the potential for conflict and decreased fitness arises, and the regulation of that trait may differ across those stages. In other words, hormonal regulation appropriate for one life history stage may not be suitable for another. As mentioned earlier, sex steroids of gonadal origin that affect territorial aggression in the breeding season are dependent on the HPG axis and are broadcast throughout an organism *via* the blood stream. In a non-breeding life history stage, such peripheral secretion of sex steroids would also activate morphological, physiological, and behavioral responses that would be inappropriate for that life history stage. Instead, local production of sex steroids in specific areas of the brain regulate territorial aggression during non-breeding, thereby helping to avoid the costs of action of gonadal sources of sex steroids in peripheral tissues.

In other cases, production of neurosteroids in localized, specific, brain regions may regulate behavioral and physiological traits that are expressed throughout the life cycle, and also on facultative bases. The role of 7α-hydroxypregnenolone may fit this scenario, especially in relation to its actions on locomotor activity. Although 7α-hydroxypregnenolone levels in the brain were higher in white-crowned sparrows in migratory vs. non-migratory life history stages (**Figure 3**), the effects of central infusion are difficult to interpret (Figure S4 in Supplementary Material). This may have been because birds were already showing maximum migratory activity, or that other regions of the brain may be important for regulation of migratory behavior. Nonetheless, future investigations will be needed to determine how 7α-hydroxypregnenolone might be important for regulation of migration, and whether this regulation changes across the two migratory life history stages (vernal and autumnal migrations) in the white-crowned sparrow.

Haraguchi et al. (65) have shown that 7α-hydroxyprogesterone can increase locomotor activity following restraint stress in newts, and, along with elevated corticosterone, may regulate behavioral responses to environmental perturbations. The possibility that this neurosteroid is involved in the rapid regulation of facultative movements associated with environmental perturbations during all life history stages is an exciting hypothesis. Facultative movements could occur at any time as part of the corticosteroid-induced emergency life history stage, for example, in response to a storm or increased predator pressure [e.g., Ref. (66, 67)]. Facultative movements would need to cease once suitable alternate habitat is found for the individual to survive the perturbation. 7α-hydroxypregnenolone is one neural paracrine signal that may be involved in both seasonal and facultative behavioral changes. It may also play a role in suppressing activity in molting white-crowned sparrows.

How hormone and neurohormone secretion, transport, and response are integrated to achieve the appropriate morphological, physiological, and behavioral response to life history stage and environmental and social changes requires further integrated studies. Coordination of the biological actions of these newly discovered autocrine and paracrine actions in the brain especially need more investigation in relation to life history stage and other environmental contexts. Understanding each organism's physiological and behavioral requirements across life history stages will allow us to identify the endocrine conflicts each faces as their requirements change. This has the potential to enlighten us as to how novel autocrine and paracrine actions within the brain and other tissues are coordinated and provide insights into the selection pressures that have driven the evolution of these systems.

AUTHOR CONTRIBUTIONS

JW wrote the original review and all other authors contributed equally to edits, addition of relevant references, and conceptual development.

ACKNOWLEDGMENTS

JW is grateful for a series of grant from the National Science Foundation (IBN-9631350 and IBN-0317141), and a subcontract of NIH grant RO1 MH61994-01, Barney Schlinger P.I. U.C.LA. Wingfield also acknowledges the Endowed Chair in Physiology, University of California, Davis. All animal procedures were approved by the University of Washington and University of California, Davis, Institutional Animal Care and Use Committees.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fendo.2018.00270/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer WG declared a past co-authorship with the author JW to the handling Editor.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 20 June 2018
doi: 10.3389/fnins.2018.00332

Estrogen Protects Neurotransmission Transcriptome During Status Epilepticus

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OPEN ACCESS

Edited by:
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Reviewed by:
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Saint Louis University, United States

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience

Received: *27 December 2017*
Accepted: *30 April 2018*
Published: *20 June 2018*

Citation:
Iacobas DA, Iacobas S, Nebieridze N, Velíšek L and Velíšková J (2018) Estrogen Protects Neurotransmission Transcriptome During Status Epilepticus. Front. Neurosci. 12:332. doi: 10.3389/fnins.2018.00332

Women with epilepsy commonly have premature onset of menopause. The decrease in estrogen levels is associated with increased occurrence of neurodegenerative processes and cognitive decline. Previously, we found that estradiol (E2) replacement in ovariectomized (OVX) female rats significantly reduced the seizure-related damage in the sensitive hilar region of hippocampal dentate gyrus (DG). However, the complex mechanisms by which E2 empowers the genomic fabrics of neurotransmission to resist damaging effects of status epilepticus (SE) are still unclear. We determined the protective effects of the estradiol replacement against kainic acid-induced SE-associated transcriptomic alterations in the DG of OVX rats. Without E2 replacement, SE altered expression of 44% of the DG genes. SE affected all major functional pathways, including apoptosis (61%), Alzheimer's disease (47%), cell cycle (59%), long-term potentiation (62%), and depression (55%), as well as synaptic vesicle cycle (62%), glutamatergic (53%), GABAergic (49%), cholinergic (52%), dopaminergic (55%), and serotonergic (49%) neurotransmission. However, in rats with E2 replacement the percentage of significantly affected genes after SE was reduced to the average 11% (from 8% for apoptosis to 32% for GABAergic synapse). Interestingly, while SE down-regulated most of the synaptic receptor genes in oil-injected females it had little effect on these receptors after E2-replacement. Our novel Pathway Protection analysis indicated that the E2-replacement prevented SE-related damage from 50% for GABA to 75% for dopaminergic transmission. The 15% synergistic expression between genes involved in estrogen signaling (ESG) and neurotransmission explains why low E2 levels result in down-regulation of neurotransmission. Interestingly, in animals with E2-replacement, SE switched 131 synergistically expressed ESG-neurotransmission gene pairs into antagonistically expressed gene pairs. Thus, the ESG pathway acts like a buffer against SE-induced alteration of neurotransmission that may contribute to the E2-mediated maintenance of brain function after the SE injury in postmenopausal women. We also

show that the long-term potentiation is lost in OVX rats following SE but not in those with E2 replacement. The electrophysiological findings in OVX female rats with SE are corroborated by the high percentage of long-term potentiation regulated genes (62%) in oil-injected while only 13% of genes were regulated following SE in E2-replaced rats.

Keywords: beta-estradiol, cholinergic synapse, dentate gyrus, dopaminergic synapse, GABAergic synapse, glutamatergic synapse, jun oncogene, serotonergic synapse

INTRODUCTION

Special considerations are necessary for women with epilepsy. Irregularities in ovarian function (reflected in menstrual cycles), may affect the likelihood of seizures (Harden et al., 1999; Herzog et al., 2004; Velíšková and DeSantis, 2013). Especially women with seizures originating in temporal lobe (temporal lobe epilepsy, TLE) often have premature ovarian failure/early menopause (late thirties-early forties), which may affect seizure rate (Klein et al., 2001; Isojarvi, 2003; Pennell, 2009) and further contribute to cognitive decline, dementia, and vulnerability to neuronal dysfunction (Shuster et al., 2010; Rocca et al., 2011). The female sex hormones (especially β-estradiol, E2) have neuroprotective effects including attenuation of seizure-induced hippocampal damage and cognitive decline. The E2 effects involve complex transcriptomic mechanisms that are still poorly understood. Therefore, estrogen effects on seizures and their consequences need to be further investigated to determine beneficial effects of hormone therapy in women with epilepsy.

In this report, we analyze alterations in neurotransmission transcriptome of the hippocampal dentate gyrus (DG) induced by status epilepticus (SE) in ovariectomized (OVX) female rats and compare to changes in OVX rats with E2 replacement. In addition to the standard analyses based on our genomic fabric paradigm approach (Iacobas et al., 2012, 2017; Iacobas, 2016), we used also novel quantifiers termed Weighted Pathway Regulation (WPR) and Pathway Protection (PPR). The new quantifiers are not affected by the arbitrarily introduced cut-offs for the absolute fold-change and *p*-value to consider a gene as significantly regulated. We focused on the interplay between the estrogen signaling (ES) pathway and five types of neurotransmission: glutamatergic (GLU), GABAergic (GABA), dopaminergic (DA), cholinergic (ACH), and serotonergic (5HT). Since estrogen acts through estrogen receptors, which are ligand-induced transcription factors, we wanted to determine the extent of its effects on gene transcription under condition of SE-induced hippocampal damage.

In order to understand the magnitude of the SE-related damage and the protection provided by the E2 we have included also data on several other pathways: apoptosis (APO), cell cycle (CC), synaptic vesicle cycle (SVC), long-term potentiation (LTP), long-term depression (LTD), and Alzheimer's disease (ALZ).

Because estrogen signaling ESG and neurotransmission have several genes in common, we considered these common genes as the main vehicles through which ESG regulates the synaptic connection and as such, the brain circuitry. The common genes were identified using the Kyoto Encyclopedia of Genes and Genomes developed by Kanehisa Laboratories (Kanehisa et al., 2017). These genes are: adenylcyclases (*Adcy2, Adcy3, Adcy5, Adcy6, Adcy7, Adcy8, Adcy9*), oncogenes (*Akt1, Ak3, Fos, Hras, Kras, raf1, Src*), transcription factors (*Atf4, Atf6b*), calmodulins (*Calm1, Calm2, Calm3*), membrane receptors (*Gabbr1, Gabbr2, Grm1, Itpr1, Itpr2*), binding proteins (*Creb1, Creb3l1, Gnai1, Gnai2, Gnai3, Gnao1, Gnaq*), ion channels (*Kcnj3, Kcnj5, Kcnj9*), and kinases (*Map2k1, Mapk1, Mapk3, Pik3ca, Pik3cb*).

In addition, we show the complex transcriptomic regulation of the hippocampal DG neurotransmission by E2 replacement that is functionally important to preserve neuronal plasticity following SE. Results bring essential insights into E2-neurotransmission interactions, which are interesting beyond the seizures for understanding sex-specific and hormonal modulated depression/anxiety, stress processing, memory, and cognition.

MATERIALS AND METHODS

Animals

Experiments were carried out according to the Revised Guide for the Care and Use of Laboratory Animals and approved by the New York Medical College Animal Care and Use Committee. We used 8–9 week old female Sprague-Dawley rats (Taconic Farms) kept on a 12-h light/dark cycle (lights on at 07:00 a.m.) with food and water *ad libitum*. Rats were OVX (=castrated) under ketamine/xylazine (70/10 mg/kg intraperitoneally) anesthesia and, 1 week later, injected subcutaneously with either 17β-estradiol benzoate (2 μg/0.1 ml/day; Sigma-Aldrich, St. Louis, MO) or sterile peanut oil (0.1 ml; controls) daily at 10:00 for 4 consecutive days (Velíšková and Velíšek, 2007), a treatment producing E2 levels within a physiological range (Neal-Perry et al., 2005). Measurements of vaginal impedance confirmed the successful OVX as well as E2-replacement.

SE was induced by injection of kainic acid (12.5 mg/kg intraperitoneally) 24 h following the last oil/E2 injection.

Abbreviations: 5HT, Serotonergic synapse pathway; ACH, cholinergic synapse pathway; ALZ, Alzheimer's disease pathway; APO, apoptosis pathway; CC, cell cycle pathway; CG, common genes; CUT, absolute fold-change cut-off to consider a gene as regulated; DA, dopaminergic synapse pathway; DG, dentate gyrus; E2, β-estradiol; ESG, estrogen signaling pathway; FC, fold-change; GABA, GABAergic synapse pathway; GLU, glutamatergic synapse pathway; KA, kainic acid; LTD, long-term depression pathway; LTP, long-term potentiation pathway; OVX, ovariectomized; PPR, Pathway Protection; SE, status epilepticus; SVC, synaptic vesicle cycle pathway; TBS, theta burst stimulation; TLE, temporal lobe epilepsy; TPR, Transcriptomic Protection; WPR, Weighted Pathway Regulation

Animals were observed for seizure occurrence and only animals with at least2 h of continuous forelimb clonus seizures were included for further experiments. There were no differences in seizure severity or duration between the treatment groups as described previously (Velíšková and Velíšek, 2007). SE was terminated by diazepam (10 mg/kg i.p.) injection. Control animals received saline as well as diazepam injections. Tissue from all groups was collected 24 h after SE. As illustrated in **Figure 1A**, studies were performed on four groups of OVX female rats: CON (castrated, oil-injected, no SE), CEN (castrated, E2-replaced, no SE), COS (castrated, oil-injected, SE), and CES (castrated, E2-replaced, SE). CON and CEN animals were obtained as previously described (Velíšková et al., 2015). All rats were decapitated under light isoflurane anesthesia. Separate groups of identically treated female rats were used for *in vitro* electrophysiology experiments.

Electrophysiology *in Vitro*

After decapitation, the brains were removed. Transverse combined entorhinal cortex-hippocampal slices (400 μm thick) were cut with a vibratome (Leica model DTK-1000) in ice-cold artificial cerebrospinal fluid (aCSF; in mM: NaCl 126, KCl 5, NaH_2PO_4 1.25, $MgCl_2$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, and glucose 10; pH 7.36). Recordings started following 1 h pre-incubation in an interface chamber in a pre-warmed (34–35°C) and oxygenated (5% CO_2/95% O_2) aCSF. Field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass microelectrode (2 M NaCl; 2–5 MΩ) placed in the middle third of the DG molecular layer as described previously (Nebieridze et al., 2012). Medial perforant path axons were stimulated once each 30 s with a bipolar stainless-steel stimulating electrode (FHC Inc.; Bowdoinham, ME). Baseline fEPSPs were recorded using a stimulus intensity that produced ∼30% of maximal fEPSP slope.

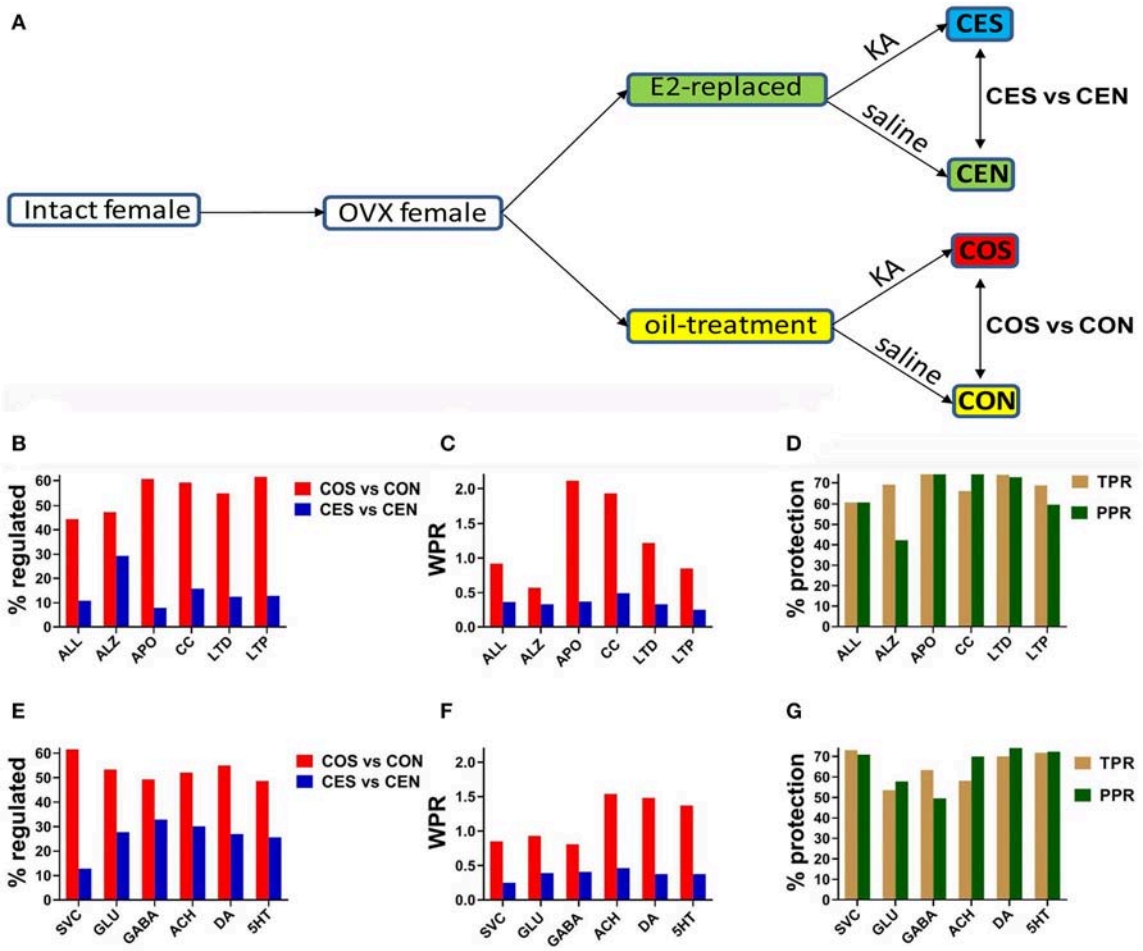
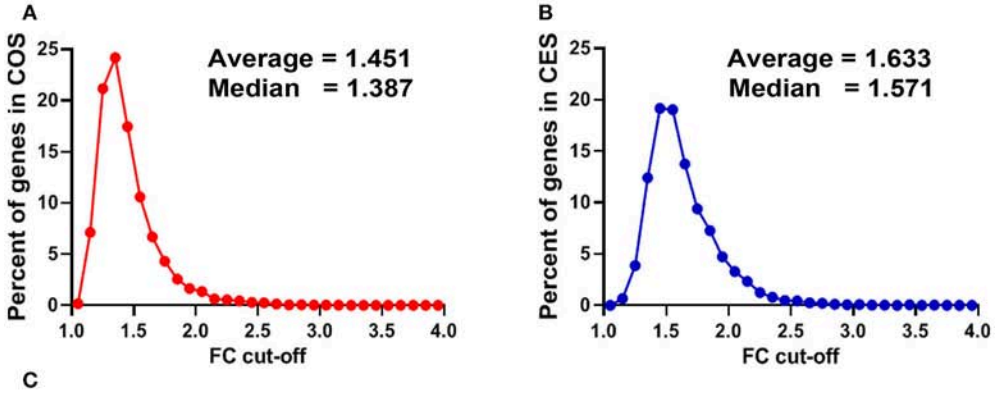


FIGURE 1 | (A) Experimental design. OVX, ovariectomy; KA, kainic acid; saline, KA vehicle. Animals were injected with oil or 2 μg of β-estradiol benzoate for 4 days subcutaneously. **(B,E)** Percentage of the regulated genes within the entire transcriptome (ALL) and included in the analyzed pathways in CES and COS groups with respect to CEN, respectively CON groups. ALZ, Alzheimer's disease, APO, apoptosis, CC, cell cycle, LTD, long-term depression, LTP, long-term potentiation, SVC, synaptic vesicle cycle, GLU, glutamatergic synapse, GABA, GABAergic synapse, ACH, cholinergic synapse, DA, dopaminergic synapse, 5HT, serotonergic synapse. Note the massive effect of SE on the gene expression. **(C,F)** WPR scores for selected pathways. In **(B,C,E,F)** lower is better (less damaging). **(D,G)** Protection against transcriptomic regulation in E2-replaced animals quantified by both TPR (percent reduction of the number of significantly regulated genes) and PPR (percent reduction of WPR). Note that DA had the largest (better) protection as quantified by PPR.



C

GENE	DESCRIPTION	COS	P-VAL	CUT	CES	P-VAL	CUT
Abl1	C-abl oncogene 1, receptor tyrosine kinase	1.417	0.0135	1.298	1.209	0.2329	1.404
Adcy2	Adenylate cyclase 2 (brain)	-1.242	0.0403	1.240	-1.601	0.1721	1.633
Akt1	V-akt murine thymoma viral oncogene homolog 1	-1.258	0.2300	1.390	2.037	0.0282	1.851
Anapc10	Anaphase promoting complex subunit 10	1.359	0.0069	1.217	-1.042	0.7288	1.324
App	Amyloid beta (A4) precursor protein	-1.312	0.1315	1.440	1.471	0.0282	1.405
Atf6	cyclic AMP-dependent transcription factor ATF-6 alpha	1.307	0.0399	1.286	1.430	0.2531	1.869
Atp5c1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	-1.274	0.0706	1.249	1.465	0.0300	1.405
Atp5f1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1	-1.372	0.0103	1.227	-1.153	0.5587	1.618
Atp5g3	ATP synthase lipid-binding protein, mitochondrial	-1.407	0.0235	1.331	-1.244	0.3088	1.575
Atp5h	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	1.352	0.0189	1.262	1.337	0.0210	1.267
Atp6v0d1	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d1	-1.496	0.0147	1.319	-1.106	0.7187	1.763
Atp6v0e1	ATPase, H+ transporting, lysosomal 9kDa, V0 subunit e1	1.482	0.0322	1.423	1.317	0.2341	1.618
Bace2	Beta-site APP-cleaving enzyme 2	1.244	0.0201	1.167	-1.200	0.3373	1.435
Bbc3	Bcl-2 binding component 3	1.308	0.0039	1.169	1.354	0.0929	1.452
Cacna1c	Calcium channel, voltage-dependent, L type, alpha 1C subunit	-1.463	0.0003	1.150	-1.214	0.3446	1.520
Ccnd3	Cyclin D3	1.457	0.0375	1.406	1.691	0.0964	1.851
Ccne1	Cyclin E1	1.289	0.0338	1.228	1.043	0.7747	1.401
Ccnh	Cyclin H	1.296	0.0093	1.183	1.413	0.0719	1.448
Cdc16	Cell division cycle 16 homolog (S. cerevisiae)	1.237	0.0012	1.104	1.160	0.0349	1.154
Cdc25b	Cell division cycle 25 homolog B (S. pombe)	-1.432	0.0224	1.280	-1.092	0.6493	1.503
Cdk5	Cyclin-dependent kinase 5	-1.417	0.0062	1.242	1.665	0.0802	1.769
Cdk7	Cell division protein kinase 7	1.372	0.0075	1.233	-1.261	0.1020	1.295
Chrna7	nicotinic receptor alpha 7 subunit	2.226	0.0473	2.297	1.986	0.0370	1.873
Cox7a2	Cytochrome c oxidase subunit VIIa polypeptide 2	1.381	0.0286	1.332	1.554	0.0017	1.250
Cyp2d4	Cytochrome P450 2D18	-1.439	0.0067	1.264	-1.213	0.4188	1.609
Dab2ip	DAB2 interacting protein	-1.490	0.0181	1.240	-1.073	0.5871	1.345
Gabarap	GABA(A) receptor-associated protein	1.387	0.0078	1.214	-1.047	0.7416	1.358
Grid2	Glutamate receptor, ionotropic, delta 2	1.333	0.0402	1.294	1.400	0.0519	1.411
Grin2a	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	-3.135	0.0165	1.434	1.466	0.0377	1.421
Hras	Harvey rat sarcoma virus oncogene	-1.371	0.0235	1.201	-1.055	0.7105	1.374
Htr2c	5-hydroxytryptamine (serotonin) receptor 2C	-1.880	0.0441	2.309	1.481	0.0356	1.357
Htr7	5-hydroxytryptamine receptor 7	-1.496	0.0231	1.362	5.385	0.0308	1.627
Ide	Insulin degrading enzyme	1.300	0.0112	1.184	1.246	0.1405	1.370
Igf1	Insulin-like growth factor 1	1.380	0.0286	1.305	1.247	0.1595	1.382
Kcnj4	Potassium inwardly-rectifying channel, subfamily J, member 4	-1.442	0.0446	1.266	-1.268	0.2939	1.559
Lmnb2	Lamin B2	1.438	0.0103	1.261	-1.152	0.5359	1.592
Ncstn	Nicastrin	2.498	0.0003	1.422	1.377	0.0072	1.236
Ndufa12	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12	-1.448	0.0118	1.310	1.091	0.4688	1.320
Ndufa8	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8	-1.353	0.0059	1.174	-1.728	0.1440	1.743
Ndufb3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3	-1.482	0.0032	1.246	1.249	0.4350	1.696
Ndufs3	NADH dehydrogenase (ubiquinone) Fe-S protein 3	-1.441	0.0031	1.147	1.171	0.1607	1.282
Ndufs7	NADH dehydrogenase (ubiquinone) Fe-S protein 7	-1.381	0.0009	1.140	-1.093	0.5711	1.410
Ndufs8	NADH dehydrogenase (ubiquinone) Fe-S protein 8	-1.298	0.0070	1.169	1.652	0.0687	1.437
Ndufv2	NADH dehydrogenase (ubiquinone) flavoprotein 2	-1.253	0.0140	1.189	-2.107	0.0485	1.636
Ngf	Beta-nerve growth factor	1.482	0.0434	1.422	1.009	0.9628	1.546
Nsf	N-ethylmaleimide-sensitive factor	-1.795	0.0334	1.393	1.382	0.0383	1.360
Orc3l	Origin recognition complex, subunit 3-like (yeast)	1.811	0.0130	1.400	1.352	0.0391	1.338
Orc4l	Origin recognition complex, subunit 4-like (yeast)	1.439	0.0287	1.367	-3.314	0.0616	2.072
Parp2	Poly (ADP-ribose) polymerase 2	1.349	0.0065	1.212	1.303	0.3259	1.743
Pik3r2	Phosphoinositide-3-kinase, regulatory subunit 2 (beta)	-2.094	0.0112	1.342	1.251	0.0179	1.200
Plcb4	Phospholipase C, beta 4	-1.406	0.0474	1.254	1.129	0.6187	1.665
Plcl1	Phospholipase C-like 1	1.230	0.0332	1.186	-1.052	0.6377	1.289
Prkcb	Protein kinase C, beta	-1.380	0.0051	1.217	1.984	0.0581	1.997
Psenen	Presenilin enhancer 2 homolog (C. elegans)	-1.413	0.0182	1.227	1.179	0.0940	1.197
Raf1	V-raf-leukemia viral oncogene 1	1.363	0.0410	1.344	3.783	0.3266	2.821
Sdhd	Succinate dehydrogenase complex, subunit D, integral membrane protein	1.289	0.0008	1.117	1.003	0.9819	1.341
Shank1	SH3 and multiple ankyrin repeat domains 1	1.365	0.0048	1.208	1.932	0.0208	1.695
Slc1a3	Solute carrier family 1 (glial high affinity glutamate transporter), member 3	-1.270	0.0322	1.251	1.522	0.2295	1.993
Slc6a1	Solute carrier family 6 (neurotransmitter transporter, GABA), member 1	-1.444	0.0050	1.228	-1.583	0.0814	1.425
Stx1b	Syntaxin-1B	-1.464	0.0470	1.349	1.599	0.1610	1.940
Tgfb3	Transforming growth factor, beta 3	1.325	0.0061	1.170	-1.350	0.3318	1.682
Ywhag	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	-1.345	0.4417	1.832	1.251	0.0156	1.194
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	1.249	0.6008	2.211	1.449	0.0278	1.357

FIGURE 2 | Power of the flexible fold-change cut-off. **(A)** Distribution of the fold-change cut-offs for the COS vs. CON comparison. COS, expression ratio in the comparison COS vs. CON, CES, expression ratio in the comparison CES vs. CEN. When all 12,710 quantified unigenes are considered, CUT values range from 1.055 for *Pcyt1a* to 4.062 for *Scgb1c1*, with the average 1.451. **(B)** Distribution of the fold-change cut-offs for the CES vs. CEN comparison. When all 12,710 quantified unigenes are considered, CUT values range from 1.109 for *Mapk1ip1l* to 3.877 for *Ttr*, with the average 1.633. **(C)** "False negatives" and "false positive" regulation hits from the analyzed functional pathways. Note that expression ratio 2.226 was not enough for *Chrna7* to be considered as up-regulated in COS vs. CON comparison (CUT = 2.297) but 1.986 was enough in CES vs. CEN (CUT = 1.873).

LTP was induced by theta burst stimulation (TBS; 10 bursts delivered at interburst interval 200 ms; each burst consisted of 10 stimuli at 100 Hz). The stimulus intensity has not been changed during the TBS. Picrotoxin (50 μM; Abcam, Cambridge, MA, USA) was bath applied during the experiment to enhance the LTP.

Microarray

Two hours after the onset of continuous seizures, under light isoflurane anesthesia the rats were decapitated, brains were removed and hippocampal DG were isolated. Four animals were used from each of the four groups of OVX female rats (CON, CEN, COS, and CES). Previously described experimental protocol (Kravchick et al., 2016) was used for RNA extraction, purification, fluorescent labeling and reverse transcription, and hybridization with Agilent Rat GE 4 × 44k v3 microarrays in the "multiple yellow" design (Iacobas et al., 2006). In this design, differently labeled biological replicates are co-hybridized with each microarray. A non-control microarray spot is valid if the foreground fluorescence is at least twice its background. We normalize the background-subtracted fluorescence of each valid spot to the median of all same-label valid spots in the array, and compute the average for all spots probing the same transcript (thus eliminating the array redundancy). Same label average values for each transcript are then compared across experimental conditions and results averaged for the two colors. An in-house developed iterative algorithm renormalizes all data sets across the fluorescent labels and experimental conditions until the average change of expression ratios between successive iterations falls below 5%.

Functional Pathways

We used Kyoto Encyclopedia of Genes and Genomes (KEGG) maps developed by Kanehisa Laboratories (Kanehisa et al., 2017) to select the genes responsible for estrogen signaling pathway (map04915) and for cholinergic (map04725), glutamatergic (map04724), GABAergic (map04727), dopaminergic (map04728), and serotonergic (map04726) transmissions. KEGG maps were also used for apoptosis (map04210), cell cycle (map04110), synaptic vesicle cycle (map04721), long-term potentiation (map04720), long-term depression (map04730), and Alzheimer's disease (map05010) pathways. Although KEGG designed map04730 for the cerebellar long-term depression the basic molecular mechanisms are likely to occur also in the DG.

Data Analysis

According to our laboratory standard (Lee et al., 2017), a gene was considered as regulated (here by SE in oil-injected/E2-replaced females with respect to the corresponding non-SE animals) if the absolute fold-change (FC) exceeded the combined contributions of the technical noise and biological variability (CUT, cut-off). The regulation was considered significant if the p-value of the heteroscedastic t-test of the equality of the average expression levels in the compared conditions with the Bonferroni-type correction for redundant spots probing the same gene (Iacobas et al., 2005) was below 0.05. This FC cut-off

TABLE 1 | Genes turned on/off by E2 replacement alone (in non-SE rats) and genes turned on by SE (in COS animals) but prevented by E2 replacement (CES rats).

Gene	Symbol
Genes turned on by estradiol replacement alone	
Chemokine (C-C motif) receptor 10	Ccr10
Fibronectin type III domain containing 5	Fndc5
G protein-coupled receptor 39	Gpr39
Short coiled-coil protein	Scoc
Unc-5 homolog C (*C. elegans*)	Unc5c
Genes turned off by estradiol replacement alone	
Leukocyte cell derived chemotaxin 1	Lect1
Genes whose turned on by se was prevented by estradiol replacement	
ARP1 actin-related protein 1 homolog B (yeast)	Actr1b
A disintegrin and metalloprotease domain 4	Adam4
ArfGAP with GTPase domain, ankyrin repeat, and PH domain 1	Agap1
Adenosylhomocysteinase-like 1	Ahcyl1
Aldehyde dehydrogenase family 1, subfamily A7	Aldh1a7
Amyotrophic lateral sclerosis 2 (juvenile) homolog (human)	Als2
ATPase, H+ transporting, lysosomal V0 subunit A2	Atp6v0a2
Dopamine receptor D2	Drd2
Family with sequence similarity 53, member B	Fam53b
Forkhead box C2	Foxc2
Gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)	Gnrh1
HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2	Hecw2
Helicase, lymphoid specific	Hells
Hepatocyte growth factor	Hgf
Minichromosome maintenance complex component 4	Mcm4
Myelin gene regulatory factor	Mrf
Myosin, heavy chain 10, non-muscle	Myh10
Nuclear transcription factor-Y alpha	Nfya
Patatin-like phospholipase domain containing 3	Pnpla3
Surfactant protein C	Sftpc
Sarcoglycan zeta	Sgcz
Seven in absentia 1A	Siah1a
SLAM family member 8	Slamf8
Solute carrier family 39 (zinc transporter), member 14	Slc39a14
Syntaxin 2	Stx2
SMT3 suppressor of mif two 3 homolog 3 (*S. cerevisiae*)	Sumo3
Transcription factor AP-2 beta	Tcfap2b
Tetratricopeptide repeat domain 33	Ttc33
Ubiquitin-associated protein 2	Ubap2
Unc-5 homolog D (*C. elegans*)	Unc5d
Zinc finger, C4H2 domain containing	Zc4h2
Zinc metallopeptidase, STE24 homolog (*S. cerevisiae*)	Zmpste24
Zinc finger, MYM-type 4	Zmym4
Zinc finger protein 286A	Znf286a

computed for every single gene includes in the regulated list the "false negatives" of the traditionally uniform 1.5 × (very stably expressed genes across biological replicates that are probed by very clean spots with 1.5 > FC > CUT). In addition, it eliminates most of the "false positives" from the variably expressed genes that are also probed by not so clean spots (1.5 < FC < CUT).

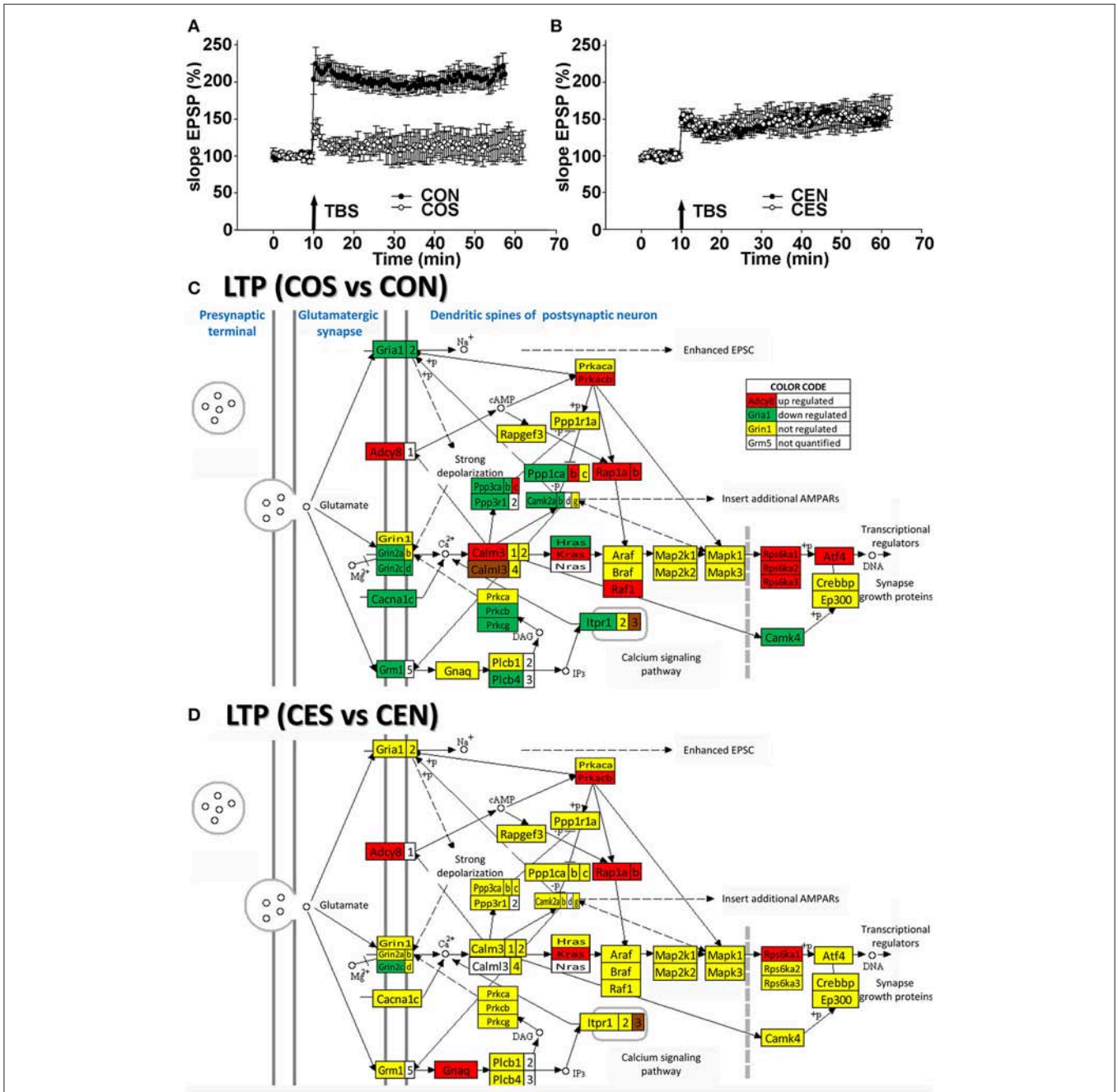


FIGURE 3 | Electrophysiology and transcriptomics of the long-term potentiation (LTP). **(A,B)** Medial perforant path LTP in the DG in OVX rats is lost following SE but preserved in E2-replaced animals. LTP induced by TBS following SE (filled circles) compared to rats with no seizures (open circles). An arrow marks a moment when the TBS was administered. In slices from oil-injected rats exposed to SE (COS; $n = 5$), TBS-induced LTP was severely reduced compared to LTP from rats with no SE (CON; $n = 10$). In contrast, the magnitude of TBS-induced LTP in slices from E2-replaced OVX rats following SE (CES; $n = 5$) was not different from LTP in slices from rats with no seizure experience (CEN; $n = 10$). **(C,D)** Dramatic regulation of LTP associated genes by SE in OVX rats compared to their non-SE counterparts (COS vs. CON) is in contrast to protection against the transcriptomic regulation of LTP associated genes in E2 replaced animals (CES vs. CEN). As specified in the "COLOR CODE" inset table, red/green/yellow background of the gene symbol indicates up-/down-/no regulation, while the white background indicates that expression of that gene was not quantified. Note the significantly lower number of regulated genes in rats with E2 replacement.

In addition to the percentages of up-/down-regulated genes, we have also computed the Weighted Pathway Regulation (WPR) and Pathway Protection (PPR, similar to Pathway Restoration Efficiency) that we have recently introduced (Iacobas et al., 2017). WPR and PPR are free of the arbitrary FC and *p*-value cut-offs to consider a gene as significantly regulated and take also into account the magnitude and significance of each gene regulation. Thus, a gene with 2 × fold-change at *p*-val = 0.01 contributes to WPR more than twice than a gene with 1.5 × fold-change at *p*-val = 0.05. This approach is different from the percentage of regulated genes that not only depends on the arbitrarily introduced cut-offs but treats all

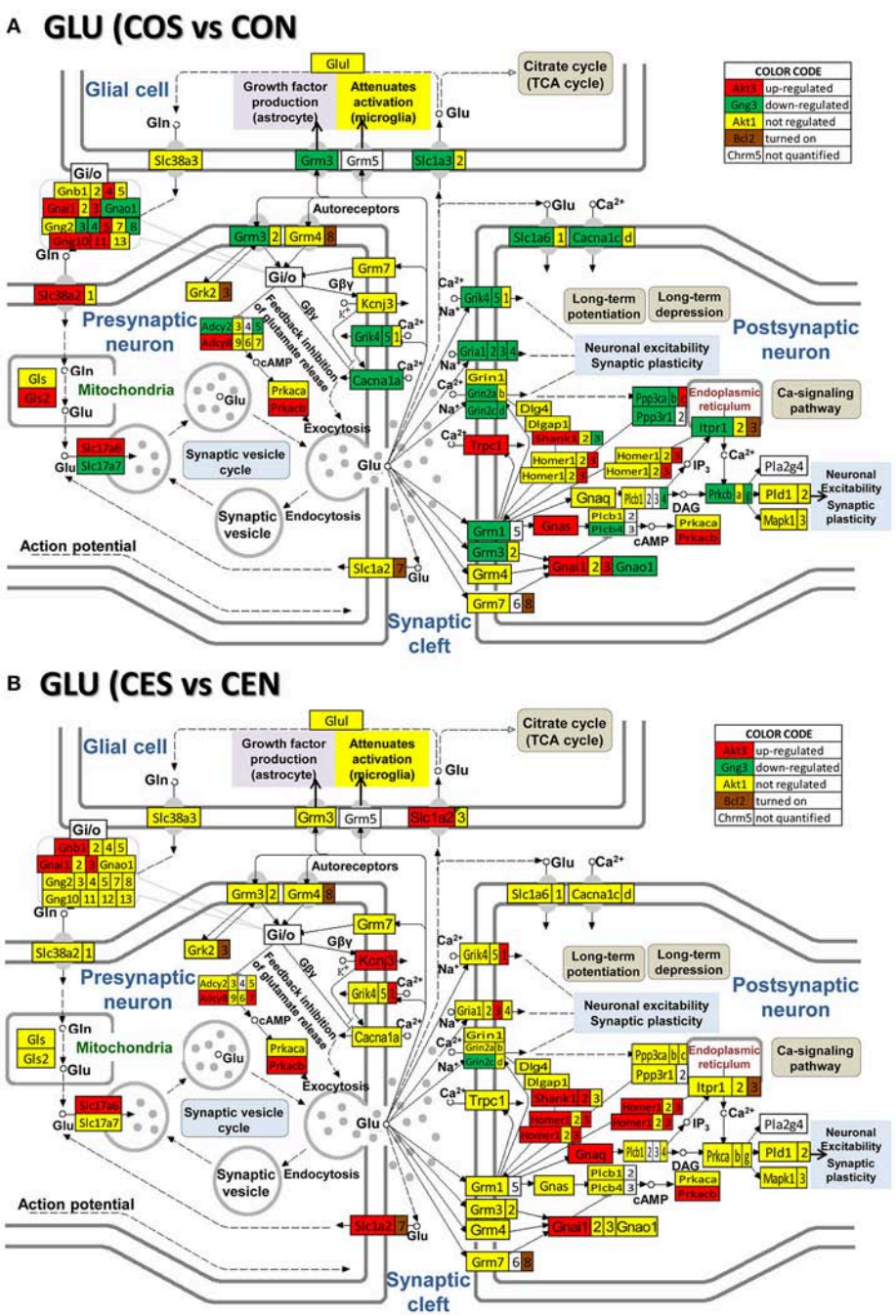


FIGURE 4 | Regulation of the glutamatergic synapse pathway by SE in OVX rats with (CES)/without (COS) E2-replacement with respect to their non-SE counterparts (CEN and CON). Note the large number of down-regulated genes by SE in oil injected rats [e.g., metabotropic glutamate receptor 3, *Grm3* in **(A)**] whose expression was not affected by SE in rats with E2 replacement **(B)**.

regulated genes as equal contributors. We have used also the Transcriptomic Protection (TPR), similar to the Transcriptomic Recovery Efficacy (Lachtermacher et al., 2011; Soares et al., 2011), representing the reduction of the percentage of regulated genes while considering also the side effects (i.e., the percentage of the regulated genes by the E2-replacement alone).

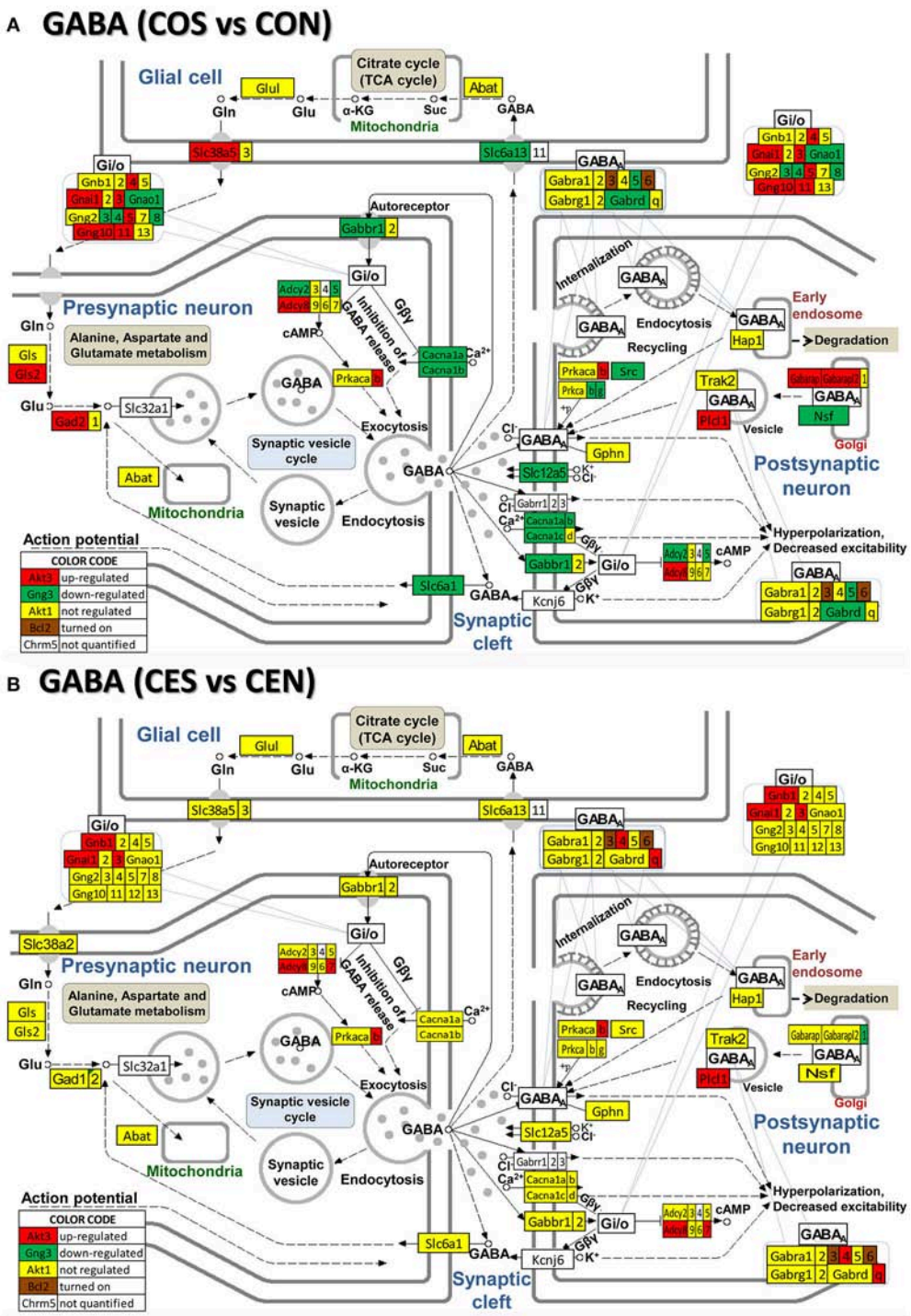


FIGURE 5 | Regulation of the GABAergic synapse pathway by SE in OVX rats with (CES)/without (COS) E2-replacement with respect to their non-SE counterparts (CEN and CON). Note large number of genes down-regulated by SE in oil-injected animals [e.g., GABA B receptor 1, *Gabbr1* in **(A)**] whose expression was not affected by SE in rats with E2 replacement **(B)**.

Transcriptomic Networks

For each condition (CON, COS, CEN, CES) we determined how much expression of each gene is coordinated with expression of each other gene by computing Pearson correlation coefficient between the expression levels of the two genes among biological replicates. Expression coordination of two genes may result from several molecular mechanisms that may include (but it is not restricted to) the regulation of the expressions of both genes by a common transcription factor. We used coordination analysis (Iacobas et al., 2007a; Iacobas and Iacobas, 2010) to determine the transcriptomic networks by which genes in the estrogen signaling pathway controls the genes involved in all

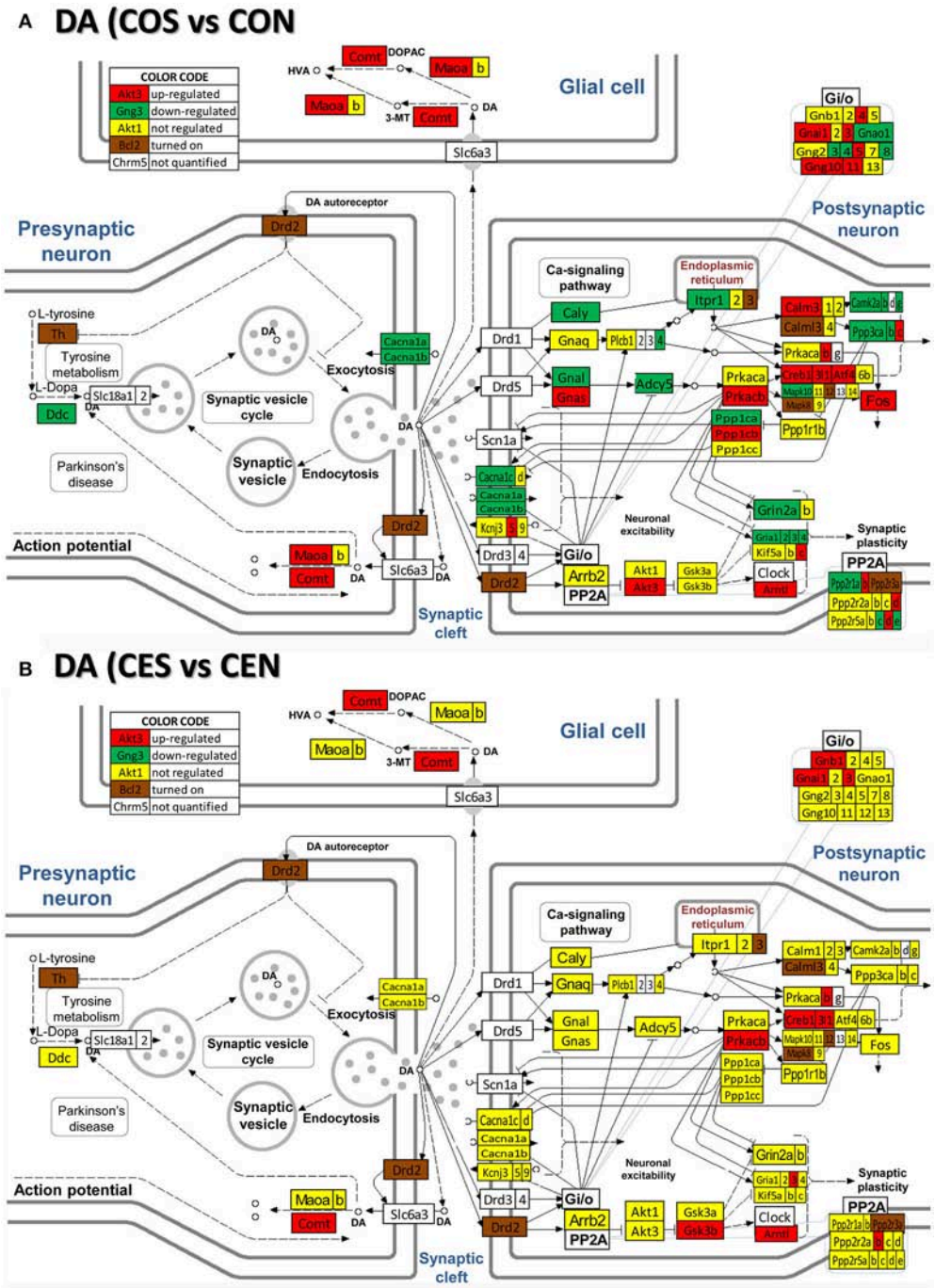


FIGURE 6 | Regulation of the dopaminergic synapse pathway by SE in OVX rats with (CES)/without (COS) E2-replacement with respect to their non-SE counterparts (CEN and CON). Note the number of genes down-regulated by SE in oil-injected animals [e.g., DOPA decarboxylase, *Ddc* in **(A)**] whose expression was not affected by SE in rats with E2 replacement **(B)**.

types of neurotransmission in the female DG. The analysis is based on our principle of "transcriptomic stoichiometry" (Iacobas et al., 2007b; Iacobas, 2016) stating that genes whose encoded proteins are working together in functional pathways should be coordinately expressed to produce the pathway players in the right proportions. In a previous paper (Iacobas et al., 2007c), we have shown that the transcriptomic networks do not stop at the cellular border. Instead, they may cross it to link pathways from (phenotypically similar or distinct) neighboring cells via intercellular signaling (Iacobas et al., 2006d).

RESULTS

We quantified expressions of 12,710 distinct genes in all 16 samples and computed 80,765,695 Pearson correlation coefficients among these genes for each of the four conditions. Detailed experimental procedure and raw and normalized gene

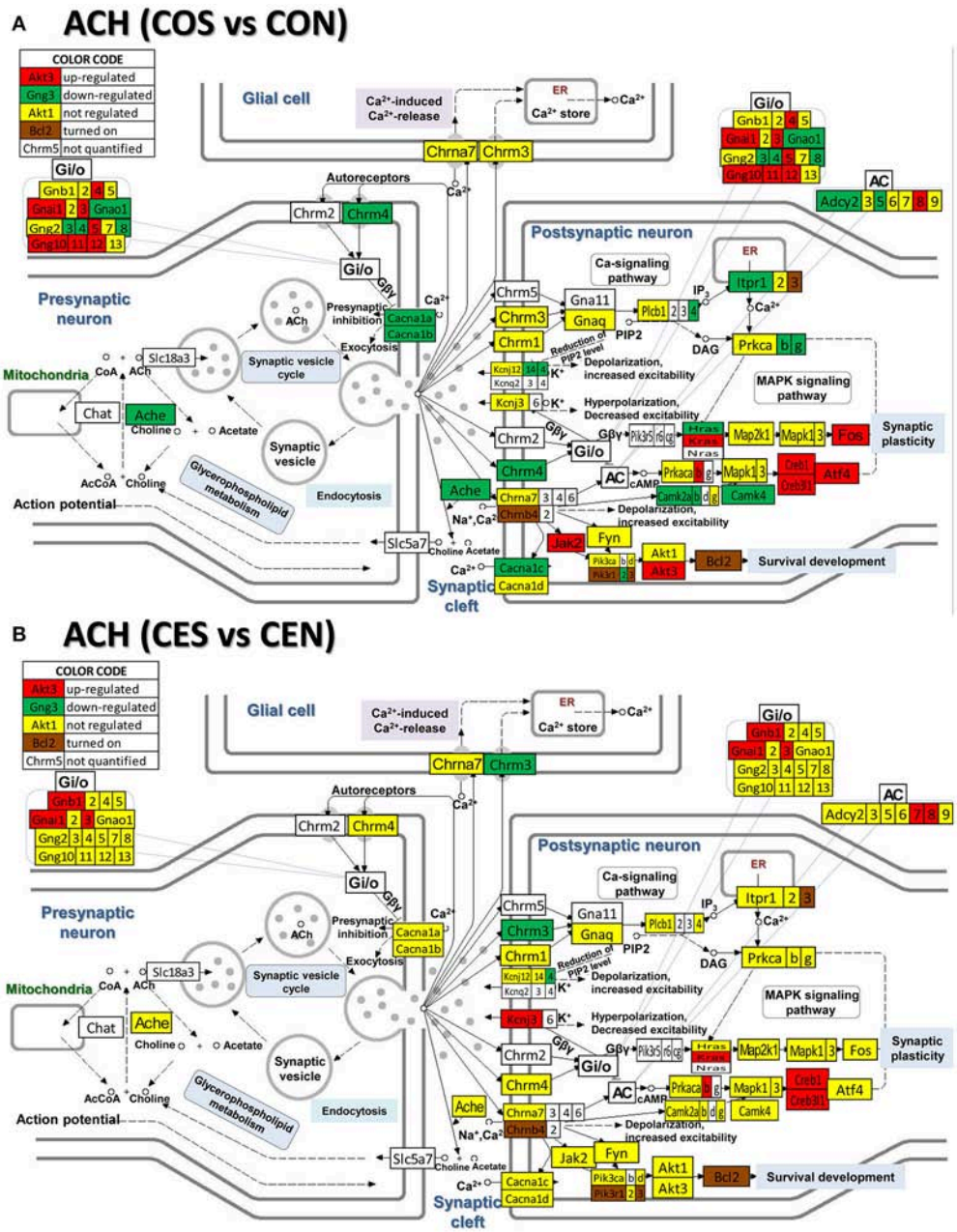


FIGURE 7 | Regulation of the cholinergic synapse pathway by SE in OVX rats with (CES)/without (COS) E2-replacement with respect to their non-SE counterparts (CEN and CON). Again, note the large number of genes down-regulated by SE in oil-injected animals [e.g., acetylcholinesterase, *Ache* in **(A)**] whose expression was not affected by SE in rats with E2 replacement **(B)**.

expression data are publically available at http://www.ncbi.nlm.nih.gov/geo as GSE60013 and GSE107725.

Gene Expression Regulation by SE and Protection by E2 Replacement

Figures 1B,E present the percentage of the SE-related regulated genes from all analyzed pathways in COS and CES groups with respect to their corresponding controls (CON and CEN groups). Of note is the substantial reduction of the percentage of regulated genes in OVX rats with EB replacement compared to the animals that received only oil. Note that larger percentages of genes were altered synaptic pathways than the overall alteration percentage in the entire transcriptome. **Figures 1C,F** present the WPR scores for selected pathways, confirming the

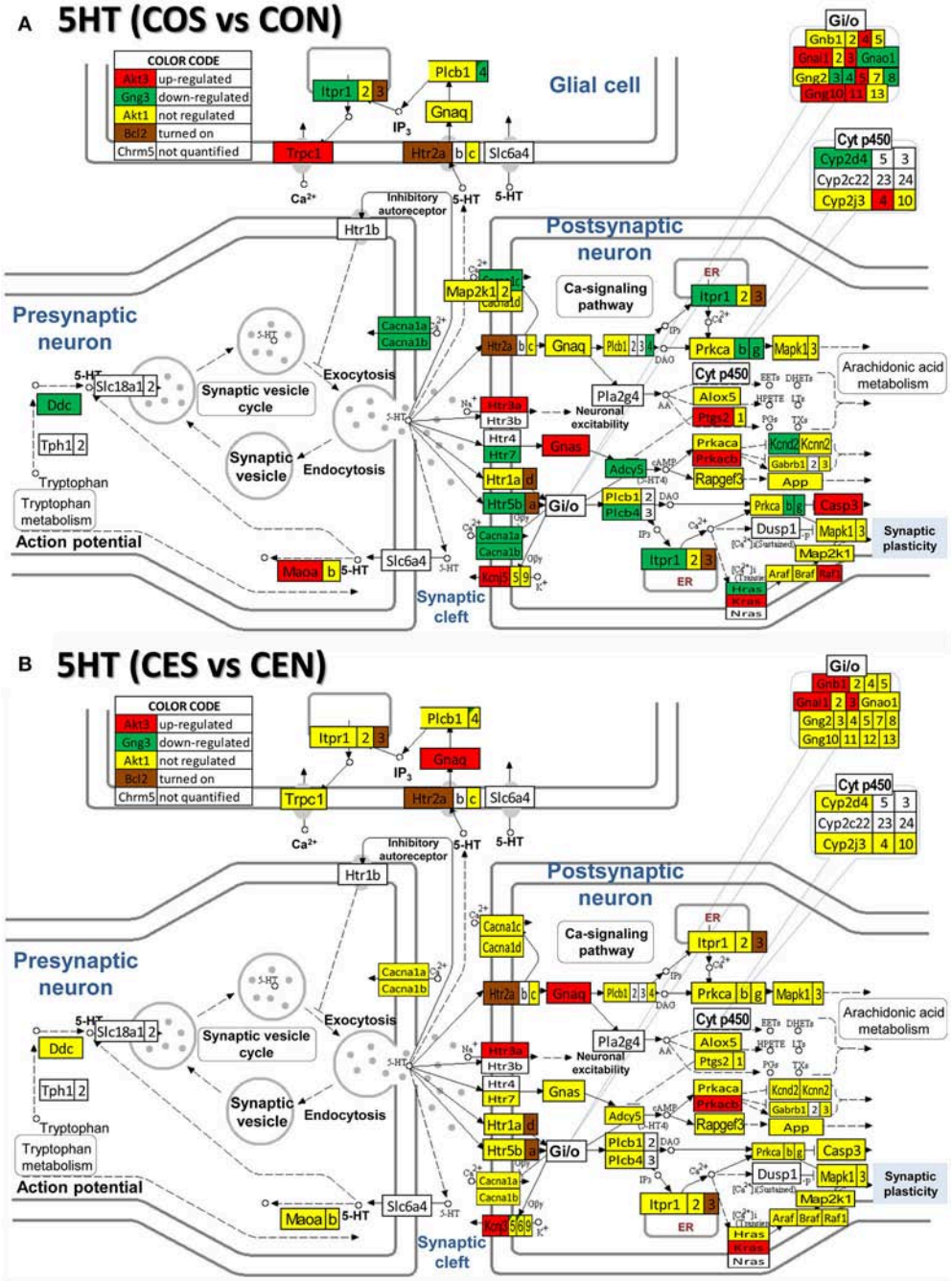


FIGURE 8 | Regulation of the serotonergic synapse pathway by SE in OVX with (CES)/without (COS) E2-replacement with respect to their non-SE counterparts (CEN and CON). Note the number of down-regulated genes by SE in oil-injected animals [e.g. serotonin receptor 5B, *Htr5b* in **(A)**] whose expression was not affected by SE in rats with E2 replacement **(B)**.

substantially lower alteration of these pathways in rats with E2 replacement. **Figures 1D,G** present the protection against SE alteration in E2 replaced animals as quantified by both TPR and PPR.

The expression ratios (x, negative for down-regulation), the absolute fold-change cut-offs (CUT) and the *p*-values for all regulated genes in the analyzed pathways are listed in the **Supplementary Table S1**. In order to illustrate how our method of flexible FC cut-off works, we present in **Figure 2** the distributions of the FC cut-offs and the list of "false negatives" (e.g., *Abl1* with $1.5 > \text{COS} = 1.477 > \text{CUT} = 1.298$) and "false positives" (e.g., *Chrna7* with $1.5 < \text{COS} = 2.226 < \text{CUT} = 2.297$) selected from the **Supplementary Table S1**. Of note from **Figure 1C** is that the FC ranges from 1.104 (for the "false negative" *Cdc16* in COS) to 2.821 (for *Raf1* in CES).

In addition to regulate the expression of thousands genes, both SE and E2 replacement separately or together turned ON/OFF many other genes. **Table 1** lists the turned on/off genes by E2 replacement alone in non-SE rats and the genes whose turning on by SE was prevented by E2 replacement.

Effects of SE on the LTP in the Hippocampal DG of OVX Oil-Injected and E2 Replaced Female Rats

To confirm the functional protection provided by the E2 replacement against SE, we compared the TBS-induced LTP in the hippocampal DG of animals with no SE experience to LTP from animals following kainic acid-induced SE. In oil-injected OVX females (no E2 replacement), we found that the TBS-induced LTP was lost following SE (**Figure 3A**) while in OVX

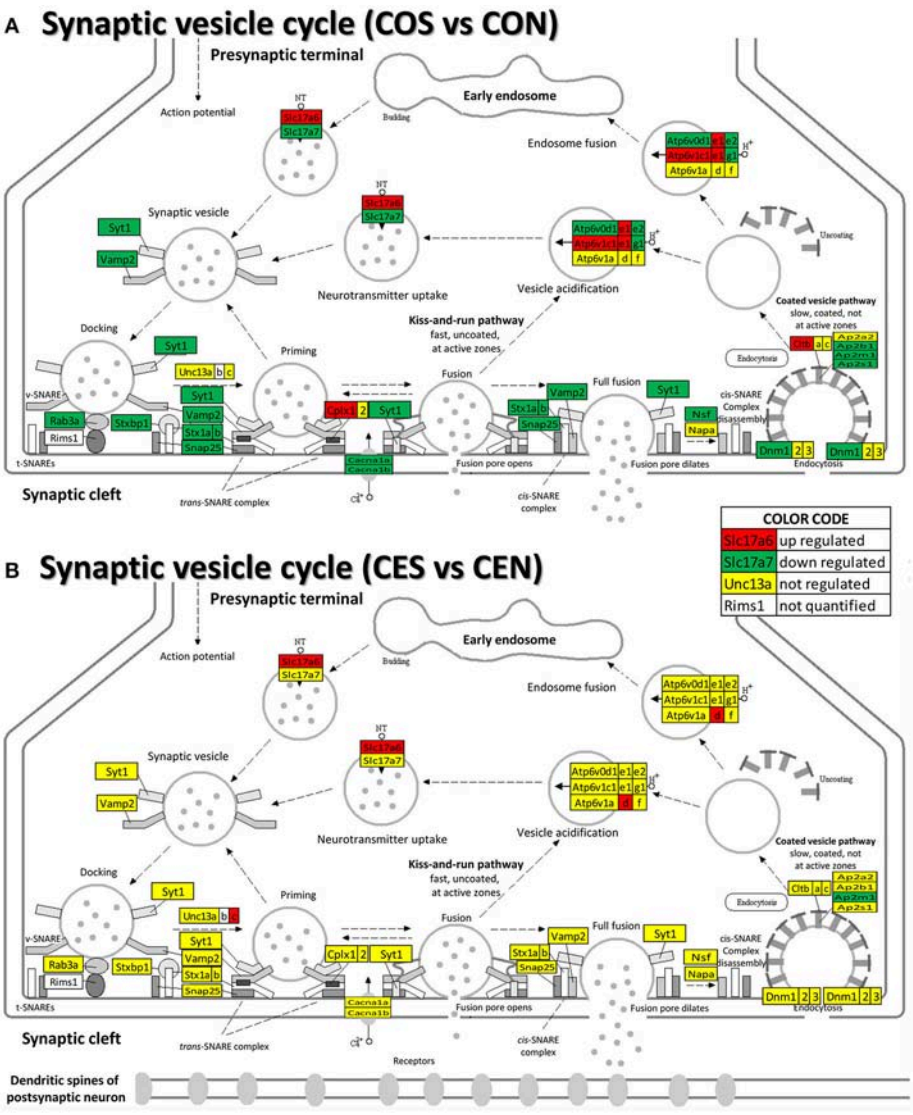


FIGURE 9 | Regulation of the synaptic vesicle cycle pathway by SE in OVX with (CES)/without (COS) E2-replacement with respect to their non-SE counterparts (CEN and CON). Note that in oil-injected animals **(A)** the vesicle cycle was altered by SE in all phases but only few genes were affected in animals with E2 replacement **(B)**.

rats with E2 replacement, the magnitude of the TBS-induced LTP in the DG was not affected by SE (**Figure 3B**). These data are in accord with the transcriptomic findings (**Figures 3C,D**). Thus, the LTP data clearly demonstrate that the DG plasticity was functionally severely compromised and associated with dramatic gene regulation in the LTP pathway following SE in OVX animals. In contrast, the SE did not disturb the LTP in animals with E2 replacement.

Regulation of Neurotransmission Pathways by SE w/o E2 Replacement

Figures 4–8 present the regulation of GLU, GABA, DA, ACH, and 5HT pathways by SE in OVX rats w/o E2-replaced with respect to their non-SE counterparts. Of note is the large number (58!) of down-regulated genes in oil-injected animals whose expression was preserved in E2-replaced rats. The protected genes include: adenylate cyclases (*Adcy2, Adcy5*), calcium (*Cacna1a, Cacna1b, Cacna1c*), potassium (*Kcnd2, Kcnj14*), and solute carrier (*Slc12a5, Slc17a7, Slc1a3, Slc1a6, Slc6a1, Slc6a13*) channels and kinases (*Camk2a, Camk2b, Camk4, Mapk10, Pik33r2, Prkcb, Prkcg*). E2 replacement preserved also expression of several receptor genes: *Chrm4, Gabr1, Gabr5, Gabrd, Gnal, Gnao1, Gng3, Gng4, Gria1, Gria2, Gria4, Grik4, Grik5, Grin2a, Grin2d, Grm1, Grm3, Htr5b, Htr7, Itpr1*). Moreover, the down-regulation of *Gria3* in oil-treated animals was switched to up-regulation in E2-replaced. E2 replacement also protected some binding proteins (*Gnb4, Gng10, Gng11, Gng12, Gng5*) and oncogenes (*Akt3, Fos, Jun, Raf1*) against the SE-related upregulation. However, other genes, not affected by SE in oil-injected animals were regulated in E2-replaced ones (*Adcy7, Chrm3, Gabarapl1, Gabra4, Gabrq, Gnb1, Grik1, Gsk3b, Homer1, Kcnj3, Ppp2r2b, Shank2, Slc1a2, Sos2*). The protection against SE-related down-regulation of *Itpr1* and calcium channels is particularly important for the role these genes play in the intercellular calcium signaling (Iacobas et al., 2006d).

Our data show (**Figure 9**) that also the synaptic vesicle cycle (SVC) was massively altered by SE in oil-injected females and on much smaller scale in rats with E2 replacement.

SE Remodels the Transcriptomic Networks by Which the ESG Pathway Controls Neurotransmission

We found that SE not only regulates the ESG pathway (**Figure 10**) but also profoundly remodeled the transcriptomic networks by which ESG regulates neurotransmission. Interestingly, in animals with E2 replacement, SE turned

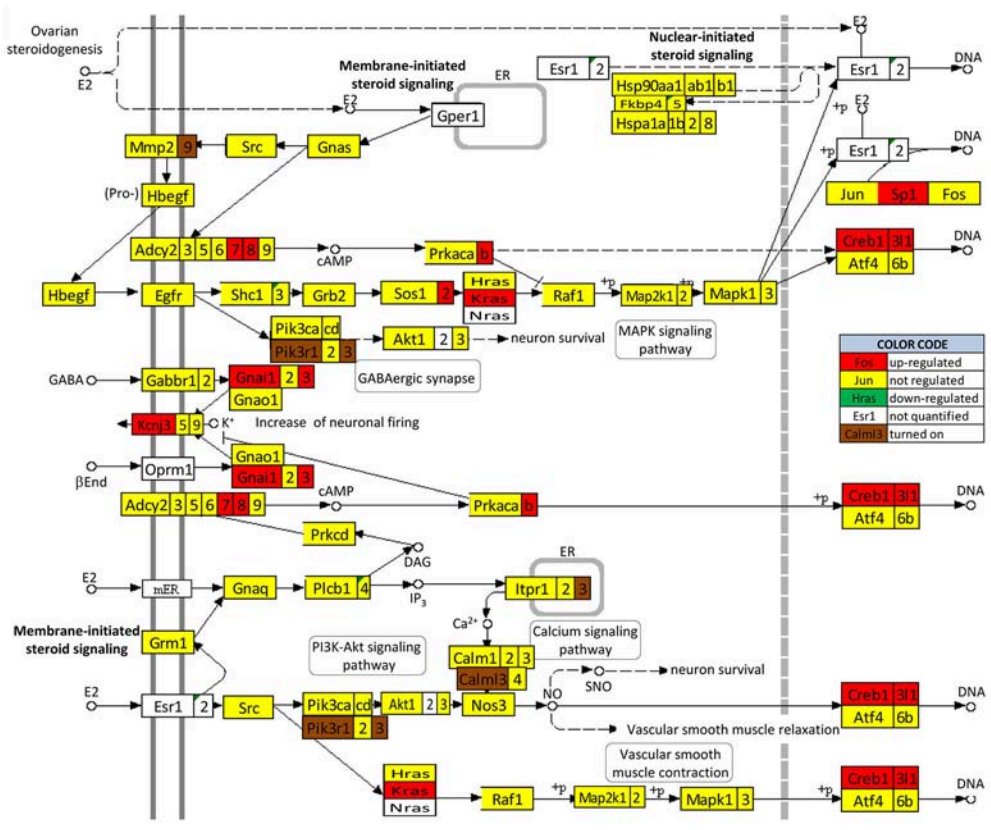


FIGURE 10 | Regulation of the estrogen signaling pathway by SE in OVX females with E2 replacement. Note that, in addition to up-regulating *Adcy7, Adcy8, Creb1, Creb3l1, Gnai1, Gnai3, Kcnj3, Kras, Prkacb,* and *Sp1,* SE turned on *Calml3, Itpr3, Mmp9, Pik3r1,* and *Pik3r3.* No gene of this pathway was found as down-regulated or turned off by SE.

on the very important *Pik3r1* and *Pik3r3* [phosphoinositide-3-kinase regulatory subunits 1 (alpha) and 3 (gamma)] that control numerous major pathways via the PI3K-AKT signaling.

Figures 11A and **12A** present the transcriptomic networks that interlink ESG pathway genes with glutamatergic and cholinergic transmission via common genes in OVX animals with E2 replacement. **Figures 11B**, **12B** show how SE altered these networks. The analysis revealed high degree (15%) of synergistic expression between genes involved in ESG pathway and all types of neurotransmission. This synergism means that when the ESG diminishes, the expression of neurotransmission genes decreases as well. This may explain why decline in E2 levels is associated with increased incidence of neurodegenerative diseases.

We also noticed that in animals with E2 replacement the synergistic expression of 131 ESG-neurotransmission gene pairs was switched by SE into antagonistic expression (albeit 25 pairs were switched from antagonistically to synergistically expressed). Thus, when expression of the ESG gene partner decreases, the expression of the paired neurotransmission gene goes up, likely compensating for the decrease of other synapse molecular factors. **Figure 13** presents the ESG-neurotransmission gene pairs that switched their expression coordination from synergistic to antagonistic or *vice versa* in E2-replaced animals following SE. For instance, the transcription factor *Jun*, known for its role in synaptic plasticity and term memory formation (Alberini, 2009) had 13 synergistic-to-antagonistic switch(with: *Arrb2, Ddc, Dlg4, Gabra1, Gabra2, Gabr4, Gabrg2, Gng2, Gria2, Gria3, Grik5,*

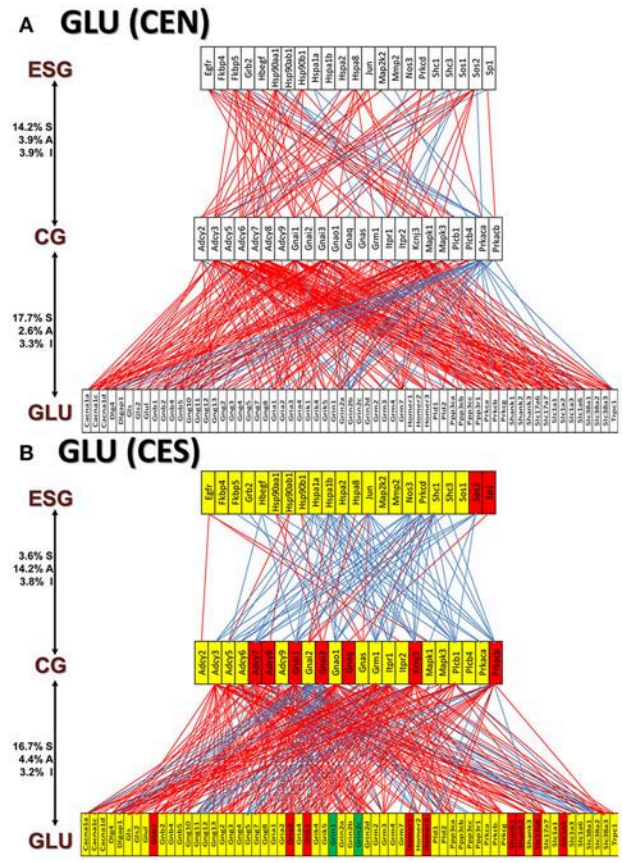


FIGURE 11 | The transcriptomic networks by which the estrogen-signaling (ESG) pathway genes regulate the glutamatergic synapse (GLU) genes through expression coordination with the common genes (CG) between the two pathways in OVX rats with E2-replacement. **(A)** Networks in the DG of non-SE animals. A red/blue line indicates that the linked genes are significantly (p-value < 0.05) synergistically/antagonistically expressed, respectively. Numbers on the left side represent the percentages of the synergistically (S)/antagonistically (A)/independently (I) expressed pairs that can be formed between the two groups of genes. **(B)** Networks in the DG of SE animals. Red/green/yellow background of the gene symbol indicates that that gene was up-/down-/not regulated by SE. Note that there is a substantial shift from synergistic to antagonistic expression between ESG and CG groups in SE animals.

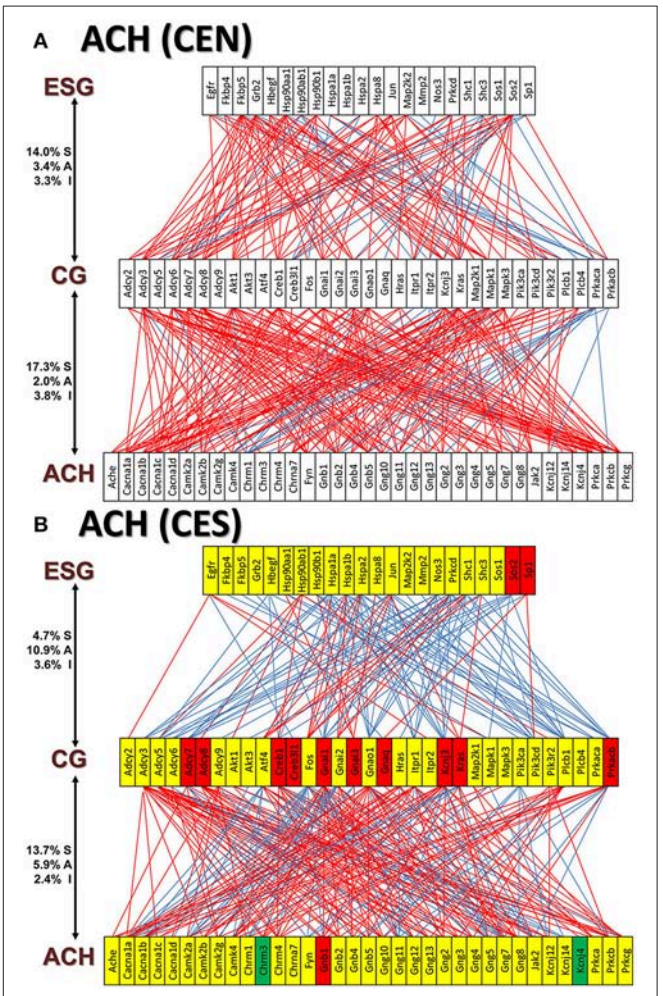


FIGURE 12 | The transcriptomic networks by which the estrogen-signaling (ESG) pathway genes regulate the cholinergic synapse (ACH) genes through expression coordination with the common genes (CG) between the two pathways in OVX rats with E2-replacement. **(A)** Networks in the DG of non-SE animals. **(B)** Networks in the DG of SE animals. Note the substantial shift from synergistic to antagonistic expression between ESG and CG groups in SE animals.

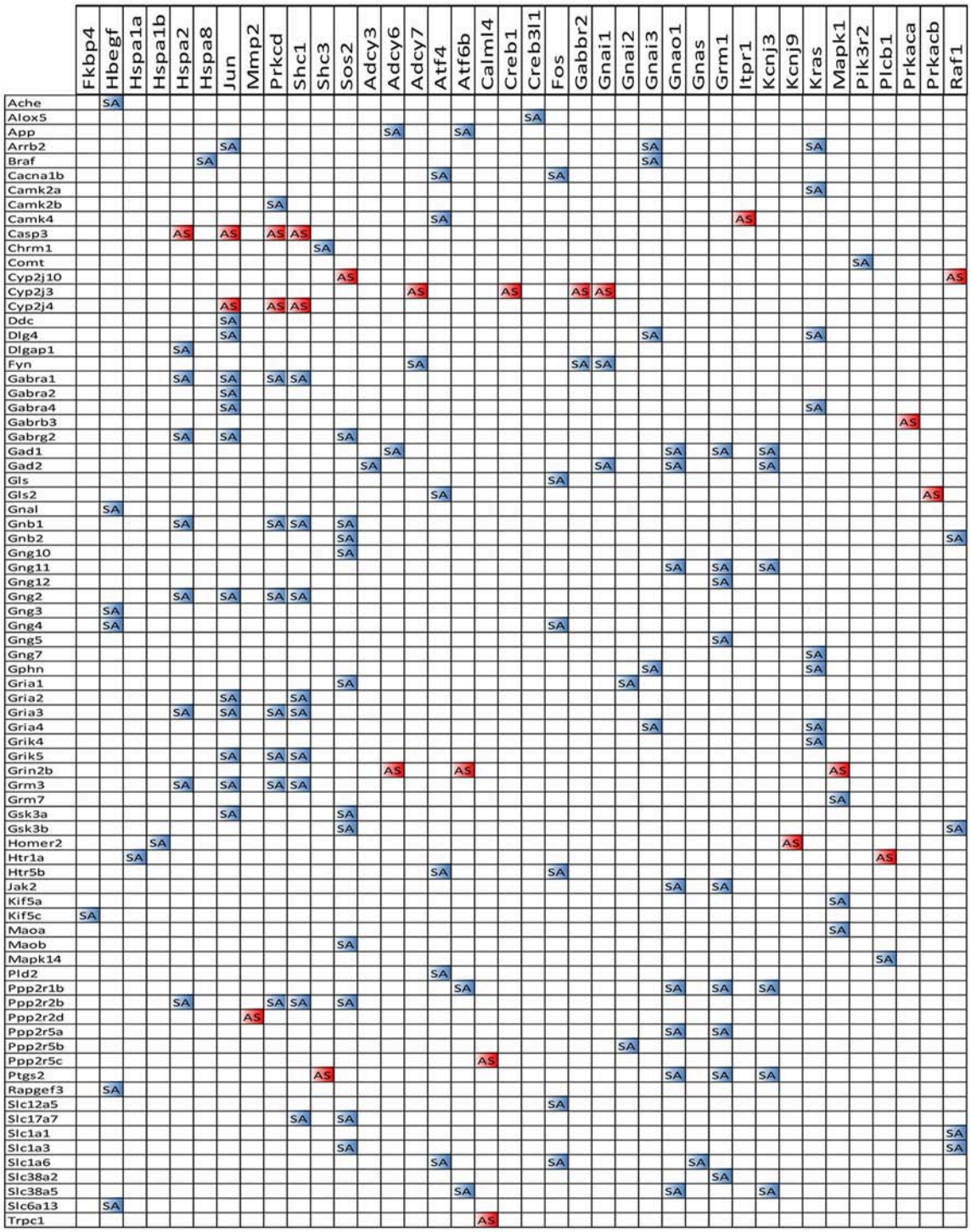


FIGURE 13 | ES-neurotransmission gene pairs that switched their expression coordination from synergistic to antagonistic (white to blue bullets) or from antagonistic to synergistic (white to red bullets) following SE.

Grm3, Gsk3a) and 2 antagonistic-to synergistic ones (*Casp3, Cyp2j3*).

It is interesting how the expression coordination of *Jun* with major synapse membrane receptors depends on the E2 presence and changes following SE. As illustrated in **Figure 14**, the E2-replacement doubled the number of synergistically expressed partners for *Jun* among the synaptic receptors and turned *Gabra1* from an independently to a synergistically expressed

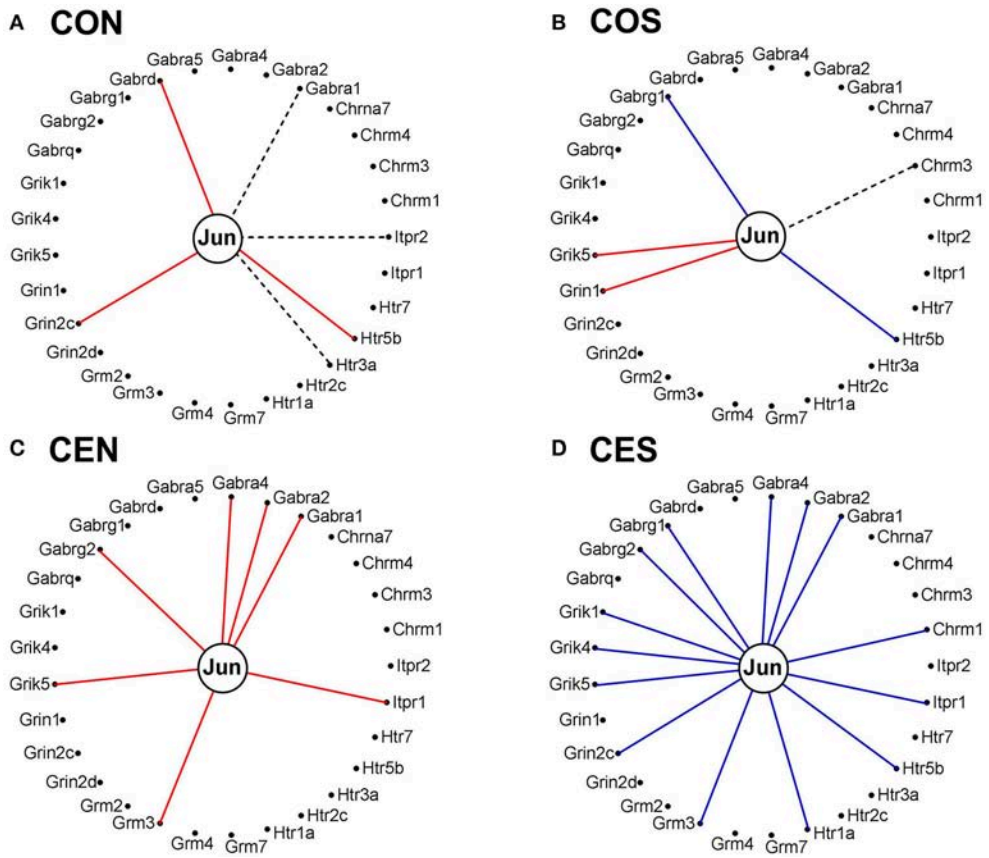


FIGURE 14 | Expression correlation of Jun oncogene with some synapse membrane receptors in the DG of OVX rat female rats. **(A)** Oil-injected, non-SE rats; **(B)** Oil-injected SE rats; **(C)** E2-replaced, non-SE rats; **(D)** E2-replaced, SE rats. A continuous red/blue line indicates significant (P-value < 0.05) expression synergism/antagonism of *Jun* with the linked gene, an interrupted black line indicates significant (p-value < 0.05) expression independence, while missing lines mean that we have not enough evidence to categorize the expression coordination of *Jun* with that gene.

gene. In both oil-injected and E2-replaced animals, SE induced antagonistic expression of *Jun* with several synaptic receptors.

DISCUSSION

We show that the well-known neuroprotective effects of E2, in this case against SE-induced hippocampal damage, involve complex, multifactorial changes including alteration of neurotransmission pathways. Our study stresses the importance of considering global genomic changes when studying effects of steroid hormones. The neuroprotective effects of E2 against SE-induced damage involve widespread regulation of neurotransmitter systems. Our data further shows that loss of estrogen leads to a state of transcriptomic instability, which may significantly contribute to neuronal vulnerability and, consequently, may render the system more susceptible to neurodegenerative processes. We identified a large number of down-regulated genes in the neurotransmission pathways induced by SE in oil-injected females and interpreted this finding as the major culprit for most of SE features. Indeed, E2 replacement led to restoration of the expression of many of these genes and we consider this as a good indication of the E2 protective role of brain circuitry.

The overall technical noise of the arrays hybridized with samples from oil-injected animals is practically equal to that of the samples from E2-replaced animals as indicated by comparing the control spots. Hence, the lower average (and median) of the FC distribution in the COS vs CON results from the reduction of expression variability in the compared conditions. This result is consistent with our robust finding in hundreds of transcriptomic experiments that the expression variability is larger in tissues of animals closer to their normal condition than in disease conditions. As presented in a previous publication (Iacobas, 2016) CES and CEN are closer to the conditions of intact females with/without SE than are COS and CEN.

The coordination analysis of the over 80 mil gene pairs in each condition, sorted in numerous interplays among the genomic fabrics of functional pathways provides an enormous amount of information. Complete presentation of this information is not possible to cover in a single report. Therefore, we have selected some results that may enhance our understanding of the complex molecular mechanisms triggered by the SE and the neuroprotective role of E2 replacement on brain circuitry.

Studies in patients with epilepsy suggest that particularly in the DG, seizure-induced hippocampal damage (sclerosis) is associated with memory deficits (Helmstaedter et al., 1997; Grunwald et al., 1999a,b). Recordings from resected human hippocampal specimens indicate that in patients with temporal lobe epilepsy (TLE) that was associated with hippocampal sclerosis, TBS-induced LTP in the DG is lost (Beck et al., 2000). Our present data show that following kainic acid-induced SE, a model of seizures originating in the temporal lobe (Velíšková et al., 2000; Velíšková and Velíšek, 2007), TBS-induced LTP is severely impaired in slices from oil-injected, but not E2-replaced OVX rats. Interestingly, an MRI study has demonstrated that young women are less likely to develop TLE-associated hippocampal damage suggesting possible protective effects of female ovarian hormones (Briellmann et al., 2000). Indeed, our previous studies showed that kainic-acid-induced SE was associated with severe neuronal loss in the vulnerable hilar region of the DG in OVX rats but the neuronal damage was significantly ameliorated in E2-replaced animals (Velíšková et al., 2000; Velíšková and Velíšek, 2007). We would like to emphasize that in OVX rats, the dose of E2 used in our studies produces circulating levels of E2 within the physiological range (Neal-Perry et al., 2005). This E2 dose is not only neuroprotective against the SE-induced hippocampal damage but it also has a mild anticonvulsant effect (Velíšková et al., 2000; Velíšková and Velíšek, 2007). Others have used various doses of E2 and showed consistent neuroprotective effects against seizure-induced hippocampal neuronal damage regardless the E2 dose but the supraphysiological E2 levels were associated with a proconvulsant effect (see Velíšková and DeSantis, 2013 for detailed review).

Very important findings of this study concern Jun oncogene (*Jun*) expression regulation and coordination with neurotransmission genes. As part of the activator protein-1 (AP-1), *Jun* is involved in major cellular processes including differentiation, proliferation and cell survival, frequently leading to cancerous processes, (Papoudou-Bai et al., 2017; Shrihari, 2017; Tewari et al., 2017). Significant up-regulation of *Jun* by SE in oil-injected rats (2.9x) can explain vast occurrence of SE-related brain damage through activation of pro-apoptotic genes (Kravchick et al., 2016). Importantly, expression of *Jun* was not altered by SE in E2-replaced animals, proving again the important protection E2 provides to the brain likely through its interference with apoptotic process here represented by *Jun*.

In a recent study (Iacobas et al., 2018) on a rat model of infantile spasms, we have analyzed how the epileptic spasms alter the neurotransmission in the hypothalamic arcuate nucleus and how the ACTH or PMX53 (a potent complement C5ar1 antagonist) treatment recovers most of these alterations. Thus, the question exists whether initiation of the E2 replacement in adult OVX females following the SE would also rescue the deteriorating effects of SE.

AUTHOR CONTRIBUTIONS

DI designed the microarray experiment, developed the analytical methods, and analyzed the data. SI performed the microarray experiment and the primary analysis. NN performed most of the animal work and analyzed the electrophysiology LTP data. LV contributed the biological interpretation of the experimental results. JV designed and supervised the entire project. All authors contributed to the manuscript writing.

ACKNOWLEDGMENTS

The studies were supported by the NIH grants NS-072966 (LV) and JV was partially supported by NS092786.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00332/full#supplementary-material

Supplementary Table S1 | Significantly regulated genes in the analyzed pathways. COS/CON, CES/CEN = expression ratios (negative for down-regulation) in the respective comparisons, CUT = fold-change cut-off computed for each gene in each comparison to be considered as regulated. Red/green background of the expression ratio indicates significant up/down regulation while yellow background indicates not significant regulation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 06 August 2018
doi: 10.3389/fendo.2018.00441

On the Role of Testosterone in Anxiety-Like Behavior Across Life in Experimental Rodents

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Testosterone affects brain functions and might explain some of the observed behavioral sex differences. Animal models may help in elucidating the possible involvement of sex hormones in these sex differences. The effects of testosterone have been intensively investigated, especially in anxiety models. Numerous experiments have brought inconsistent results with either anxiolytic or anxiogenic effects. Besides methodological variations, contradictory findings might be explained by the divergent metabolism of testosterone and its recognition by neurons during prenatal and postnatal development. Gonadectomy and subsequent supplementation have been used to study the role of sex hormones. However, the variable duration of hypogonadism might affect the outcomes and the effect of long-term androgen deficiency is understudied. Testosterone can be metabolized to dihydrotestosterone strengthening the androgen signaling, but also to estradiol converting the androgen to estrogen activity. Moreover, some metabolites of testosterone can modulate γ-aminobutyric acid and serotonergic neurotransmission. Here we review the currently available experimental data in experimental rodents on the effects of testosterone on anxiety during development. Based on the experimental results, females are generally less anxious than males from puberty to middle-age. The anxiety-like behavior of females and males is likely influenced by early organizational effects, but might be modified by activational effects of testosterone and its metabolites. The effects of sex hormones leading to anxiogenesis or anxiolysis depend on factors affecting hormonal status including age. The biological and several technical issues make the study of effects of testosterone on anxiety very complex and should be taken into account when interpreting experimental results.

Keywords: anxiety-like behavior, perinatal, adolescence, puberty, aging

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *31 January 2018*
Accepted: *17 July 2018*
Published: *06 August 2018*

Citation:
Domonkos E, Hodosy J, Ostatníková D and Celec P (2018) On the Role of Testosterone in Anxiety-Like Behavior Across Life in Experimental Rodents. Front. Endocrinol. 9:441. doi: 10.3389/fendo.2018.00441

INTRODUCTION

Besides depression, anxiety disorders, including generalized anxiety disorder, panic disorder, phobias, social anxiety disorder, separation anxiety disorder, obsessive-compulsive disorder and post-traumatic stress disorder, are the most common of all mental disorders (1). According to a survey performed in 2010, 14% of the population in Europe suffers from an anxiety disorder each year (2). The prevalence rate of anxiety disorders is influenced by various factors, including gender (3).

It is known that sex hormones affect brain morphology as well as brain functions. Based on the sex differences in prevalence of anxiety disorders in human, the role of sex hormones has been intensively investigated. A large body of animal experiments has brought contradictory results showing either anxiolytic or anxiogenic effects of testosterone (4, 5). These inconsistent findings might be due to differences in organizational and activational actions of testosterone affecting the neuronal circuits during prenatal and postnatal development. Therefore, in the present paper, the currently available experimental data on the sex differences in anxiety-like behavior and the potential role of testosterone affecting these differences across different period of life are reviewed.

SEX DIFFERENCES IN ANXIETY-LIKE BEHAVIOR IN RODENT MODELS

Anxiety is interconnected with behavioral and brain functions such as fear response. In clinical practice, anxiety is considered to be a consequence of overestimated response to threat in uncertain situations associated with prolonged hyper-vigilance and hyper-arousal (6). In neuropsychiatric studies, rodents are the most commonly used animal models (7). While in humans, anxiety might be assessed by self-report questionnaire, in rodents anxiety is evaluated based on temporary behavioral responses to various threatening stimuli. Therefore the translational value of such evaluation is disputable. On the other hand, animal experiments are crucial to elucidate causality behind observed associations. Although mice and rats may differ in some behavioral traits, the behavioral tests assessing anxiety in rodents are based on their common innate avoidance of certain conditions, such as bright illuminations (8) or open space (9–11). Therefore the most of the anxiety tests are suitable for both, mice and rats (7). In anxiety assessment it should be also considered that anxiety is associated with other behavioral phenotypes, such as aggression and violence (12–19). These exhibit sex differences and positive correlations with testosterone concentration (20, 21). Furthermore, anxiety-related behavior assessed in experimental rodents is often related to their cognitive performance, which might be affected by sex hormones (22, 23) and dependent on androgen signaling (24).

It should be noted that some behavioral traits, including anxiety, might be influenced by estrous cycle in females. It has been shown that female rat in proestrus display more explorative behavior in a novel environment and less freezing behavior following shock in defensive burying test, they spent more time in the open arm of the elevated plus maze and interact longer with an unfamiliar social partner in comparison to males but also to females during other phases of the estrous cycle (25). Although, to cover whole estrous cycle, females in the experiments are often selected for each stages and a heterogeneous group is used, but it leads to high inter-individual variability (26–28). Therefore, the assessment of sex differences in anxiety behavior is problematic.

Women are twice more likely to experience anxiety as men (26). However, it seems that sex differences in the behavioral stress responses mostly point to the opposite direction. Despite of the evidence suggesting the effect of hormonal fluctuation, the behavior of the females is often generalized without considering the estrous cycle, especially in the earlier experiments investigating sex differences. Such results show that adult female rats move in the open field more than males (29), while male rats display more freezing behavior and defecation, longer-lasting grooming reaction, less rearing as well as less time spent in the center zone of the arena (28). Females, mice as well as rats, make more entries into the open arms and spent greater time in open arms of the elevated plus maze, interact more with a same-sex social partner in a novel environment and bury fewer marbles than males (27, 28, 30, 31). On the contrary, in the Vogel conflict test, male rats seems to be less anxious than females indicated by more licking of the drinking spout during the punish period (30). In time spent in the bright chamber of the light-dark box, contradictory results have been found. In some experiments, females show higher anxiety than males (32), in other experiments, the opposite sex difference was proved (33), while the lack of sex differences is published rarely (27). It should be noted that sex differences in experimental animals in anxiety-like behavior might depend on many factors, such as strain (34) or breeding condition (32), and other sources of inter-individual variability are likely not identified yet (35). The sex differences in frequently used behavioral tests for anxiety changing with age, as well as the short- and long-term consequences of testosterone deprivation or exposure in males in different periods of life are illustrated in **Figure 1**.

THE ROLE OF TESTOSTERONE IN ANXIETY-LIKE BEHAVIOR OF RODENTS ACROSS DIFFERENT LIFE PERIODS

The sexually dimorphic behaviors related to anxiety can be influenced by both, organizational and activational effects of gonadal hormones. Organization of the neuronal circuits by testosterone and/or its metabolites predetermines the behavioral responses to the activational effects of sex hormones later in life. Besides the sensitive period of early—prenatal and perinatal development (15, 27), increasing amount of evidence supports the hypothesis that pubertal hormones (re)organize the brain and determine a variety of adult behaviors during adolescence (36, 37) or outside of the classic critical periods of neurodevelopment (38). An overview of the results of some experiments investigating sex differences and the role of testosterone in anxiety-like behavior, with particular regard to the age of animals with hormone manipulation and analysis of the outcomes, is provided in **Table 1**.

PRENATAL AND NEONATAL PERIOD

Early development is a critical period for brain formation and also for its modulation by sex hormones contributing to sexual dimorphism. Prenatal exposure to testosterone excess is associated with impaired neural development and mental

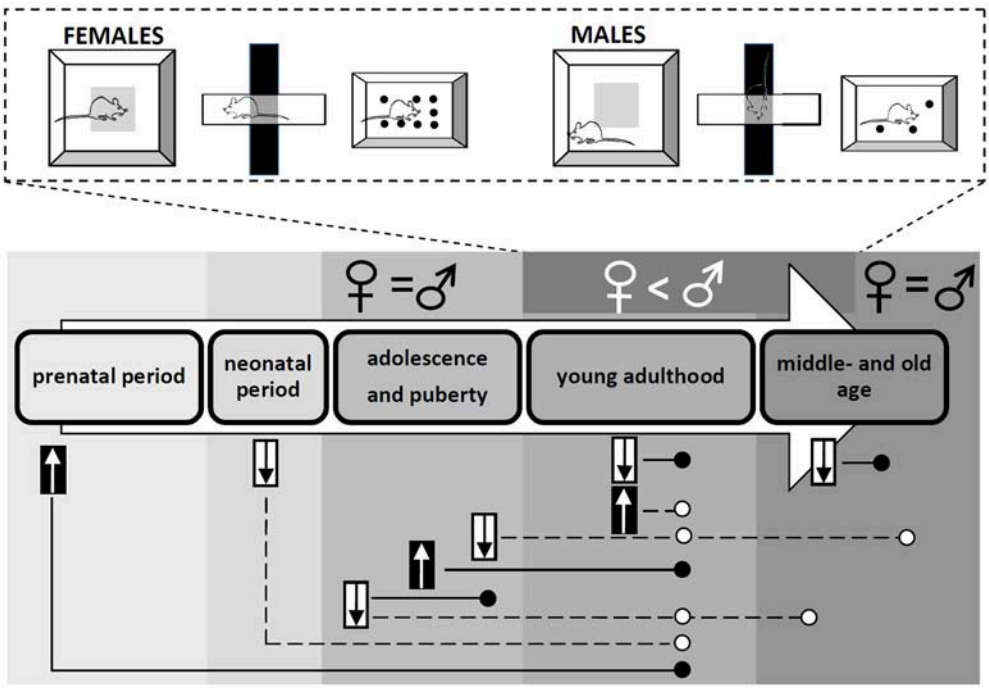

FIGURE 1 | Sex differences in anxiety-like behavior assessed using the open field, elevated plus maze and marble-burying tests. The consequences of testosterone exposure and deprivation in males are shown across the life. ↑ - testosterone exposure; ↓ - testosterone deprivation; ○ - anxiolytic effect; ● - anxiogenic effect.

functions (44, 45). Offspring of women with polycystic ovary syndrome is exposed to high concentration of androgens *in utero*. As shown in a rat model, maternal hypergonadism may result in increased risk for developing anxiety disorder in offspring, especially in female offspring (39). Inhibition of testosterone aromatization to estradiol in pregnant dams may induce increased defecation and freezing in the open field, as well as decreased number of hangings from and time spent on the open arms, pointing out an increased emotionality and anxiety in both, female and male offspring (28, 46). Accordingly, exposure to high concentration of testosterone during prenatal development leads to anxiogenesis in the adulthood, and females seems to be more sensitive to this effect of prenatal testosterone.

In addition to prenatal, also perinatal exposure to testosterone may predetermine anxiety-related behavior later during life. As found in adult rats and mice, testosterone injected on the day of birth or neonatal ovariectomy can masculinize anxiety-related behavior of females increasing the number of the marbles buried (27) and decreasing the time spent on the open arms (47). On contrary, adult male rats, castrated on the day of birth have a higher locomotor activity in the open arena, spent more time in the center zone, in the light chamber, in the open arms, and exhibit a higher number of novel object visits than their sham operated counterparts (40, 48). These results suggest that the absence of estrogens in females and testosterone in males during the perinatal period restrain the normal differentiation of gender-specific adult anxiety responses to the particular stimuli.

ADOLESCENCE

Puberty is a restricted period in adolescence characterized by a rapid elevation of circulating sex hormones accompanied by reproductive maturation. Adolescence, as the age between childhood and adulthood, represents a sensitive period in neurodevelopment as well. Numerous evidence proves that the remodeling of the adolescent brain occurs under the influence of sex hormones (36, 37). A large body of animal experiments has shown that sex differences in several anxiety tests arise from the organizational effects of testosterone at peri-pubertal age. In rats, male gonadectomy, either at puberty onset or shortly thereafter, results in lower anxiety in the open area, the behavioral response typical for females (29). Similar effects of pre-pubertal castration were found in the elevated plus maze and light-dark box, where adult male rats that had been gonadectomized prior to puberty exhibit a higher frequency of head-dipping and more time spent in the open arms or in the light part of the light-dark box (33). Environment-related social anxiety in males is organized also by pubertal sex hormones, where the aromatization of testosterone seems to be critical (49). In addition, middle- (12-months old) as well as old-aged male rats (30-months old) that have been castrated before puberty onset or at late adolescent age, respectively, display lower or similar anxiety in comparison to intact aged-matched females in a test-specific manner (41, 42). Pre-pubertal gonadectomy has anxiogenic consequences in male mice in the elevated plus maze, but anxiolytic effect in female mice in the marble-burying test (26). On the other side, testosterone exposure and social experience during adolescence

TABLE 1 | Overview of some experimental results on the effect of testosterone on anxiety-like behavior in regard to age at hormone manipulation.

References	Species/Gender	Age at intervention	Intervention	Age at anxiety examination	Anxiety examination	Outcome
PRE- UND PERINATAL PERIOD						
(39)	Rat (Wistar) Females and males	Prenatal period (GD 15–19)	Prenatal androgen exposure (PNA): pregnant dams administered with testosterone (0.5 mg/kg/d)	Young adulthood (PND 53-59)	Elevated plus maze	PNA offspring, particularly females, showed anxiety-like behavior
(28)	Rat (Wistar) Females and males	Prenatal period (GD 15–20)	Prenatal inhibition of testosterone aromatization: pregnant dams administered with 1,4,6-androstatriene-3.17-dione (ATD; aromatase inhibitor, 5 mg/rat)	Adolescence and young adulthood (1 and 3 months)	Open field, elevated cross maze	Gender differences: females were more active, less anxious and emotional than males at 3 months prenatal ATD resulted in increases in anxiety and emotionality, which in males depended on the age of examination
(27)	Mice (129:C57BL/6J) Females and males	Perinatal period (day of birth)	Testosterone injected in females (100 μg/pup)	Young adulthood (PND 67–78)	Marble-burying test, light-dark box	Gender differences: females were less anxious than males in marble-burying single injection of testosterone masculinized female anxiety-related behavior in marble-burying test
(40)	Rat (Long Evans) Males	Perinatal period (day of birth)	Gonadectomy	Young adulthood (ca. PND 120)	Open field, novel object exposure, light-dark box, elevated plus maze	Neonatal gonadectomy had anxiolytic effect in adult rats
PERIPUBERTAL AGE						
(26)	Mice (C57BL/6N) Females and males	Pre-pubertal age (PND 24-25)	Gonadectomy	Before puberty (PND 24) and late puberty (PND 40-47)	Elevated plus maze, open field, marble-burying test	Gender differences: in late adolescence males were less anxious than females, these gender differences were not found prior puberty or in young adulthood males as well as females showed an increase in anxiety-like behavior in marble-burying test from pre-pubertal to late-pubertal age; pre-pubertal gonadectomy had anxiogenic effect in males, but anxiolytic effect in females at late-pubertal age
(27)	Mice (129:C57BL/6J) Females and males	Pre-pubertal age (PND 28)	Ovariectomy + testosterone capsules implanted in females (3 mm/mouse)	Young adulthood (PND 67-78)	Marble-burying test, light-dark box	Testosterone treatment masculinized female anxiety-related behavior in marble-burying test
(41)	Rats (Lewis) Females and males	Pre-pubertal age (PND 29-31)	Gonadectomy in males	Middle-age (from 12 months)	Open field, light-dark box, elevated plus maze, PhenoTyper cage	Gender differences: females were less anxious than males in most of the conducted tests long-term androgen deficiency decreased sex differences (anxiolytic effect in males);
(33)	Rat (Lister hooded) Females and males	pre-pubertal age (PND 33-34) and post-pubertal age (PND 58-59)	Gonadectomy in males	Young adulthood (PND 101–110)	Elevated plus maze, light-dark box, open field	Gender differences: at PND 95–96, intact males were more anxious than intact females in light-dark box pre-pubertally castrated males displayed less anxious behavior than post-pubertally castrated males
(42)	Rats (Wistar) Females and males	Post-pubertal age (PND 47)	Gonadectomy in males	Old-age (30 months)	Open field, light-dark box, elevated plus maze, PhenoTyper cage	No gender differences were found in anxiety-related behavior at old age except the light-dark box (anxiolytic effects) gonadectomy had no effect on anxiety in males

(Continued)

TABLE 1 | Continued

References	Species/Gender	Age at intervention	Intervention	Age at anxiety examination	Anxiety examination	Outcome
MIDDLE- AND OLD AGE						
(22)	Rats (Fisher) Males	Young adulthood (4 months), middle-age (13 months) and old-age (24 months)	Gonadectomy + testosterone capsules implanted (10 mm/rat) in gonadally intact male rats: testosterone (1 mg/kg) 3α-diol (1 mg/kg)	Young adulthood (4 months), middle-age (13 months) and old-age (24 months)	Defensive freezing	13- and 24-months old rats displayed more freezing than 4-months old rats; gonadectomy in 4-months old rats, but not in 13- and 24-months old rats, increased freezing, which was reversed by chronic testosterone supplementation; a single injection of 3α-diol, but not testosterone, had anxiolytic effect regardless of the age
(41)	Rats (Lewis) Males	Middle-age (from 12 months)	In pre-pubertally gonadectomized rats: single injection of testosterone (1 mg/kg) single injection of estradiol (100 μg/kg) 2-week estradiol treatment (10μg/kg)	Middle-age (from 12 months)	Elevated plus maze	None of the treatment affected anxiety-related behavior in gonadectomized male rats
(23)	Mice (C57/B6) Males	Old-age (24 months)	Testosterone, 3α-diol or estradiol injected in aged intact mice (1 mg/kg)	Old-age (24 months)	Open field, light-dark box, elevated plus maze, zero maze, mirror chamber, Vogel conflict test	In gonadally intact aged testosterone and 3α-diol had anxiolytic effects in all of the conducted tests, while estradiol had anxiolytic effects in open field, light-dark box and mirror chamber only
(43)	Rats (Fisher) Females and males	Old-age (20 months)	Exposure to chronic restraint stress for 21 days	Old-age (21.5 months)	Open field, novel object exposure	Gender differences: males were generally more anxious than females stress increased male and decreased female anxiety-related behaviors, and had no effect on testosterone concentration

equally increase anxiety-like behavior, as shown in adult male hamsters (50). In females rats, single testosterone administration at the day of birth (organizational effect) as well as continual testosterone exposure from the puberty onset (activational effect) increases activity in marble-burying test of anxiety-like behavior, abolishing sex differences in adulthood (27). Furthermore, while in 1-month-old intact rats no sex differences were found in anxiety-like behavior, testing at 3 months of age revealed clear sex differences in reaction to the environmental novelty, females being more active and less anxious than males in the open field and in the elevated plus maze (28). Interestingly, in late adolescence, male mice spent more time on open arms than females. However, this sex difference in open-arm time is not present prior to puberty onset and does not persist into young adulthood (26).

ADULTHOOD

Most published experiments examine the role of testosterone in anxiety-like behavior and the underlying mechanisms in young adult animals. Unlike the anxiolytic effect of peri-pubertal castration, castration of adult males is frequently associated with increased anxiety-like behavior in a battery of behavioral tests, such as open field, elevated plus maze, and defensive-burying test (51–58). However, in some experiments in rats, no effect of adult gonadectomy was found (29, 33). Presumably, the different time interval between hormone deprivation and behavioral examination among the studies may result in these differences. The short-term effect of gonadectomy has been investigated in a large number of experiments, while the effects of the long-term hypogonadism are understudied.

The anxiogenic effect of adult castration might be reversed by hormone supplementation (52, 54, 59–63). The anxiolytic-like effect of testosterone has been revealed in gonadally intact healthy animals, as well (64–66). However, testosterone can be either reduced by 5α-reductase to the more potent androgen dihydrotestosterone, or aromatized by aromatase to estradiol converting the androgen to estrogen activity. In brain, dihydrotestosterone can be further metabolized to 5α-androstane-3α,17β-diol (3α-diol) and to 5α-androstane-3β,17β-diol (3β-diol), neuroactive steroids possessing neuromodulatory activity (67). One of the most cited study investigating the rapid effects of testosterone on anxiety-like behavior in mice has suggested that the anxiolytic effect of testosterone is mediated by its 5α-reduced metabolites (64). Likewise, it has been proved that administration of 3α-diol decreases anxiety-like behavior in male rats (52, 59, 60, 63) as well as in female rats (68), while inhibition of testosterone metabolism to 3α-diol increases anxiety in male rats (62). Similar to effect of hormone administration, sexual experience and the exposure of intact male rats or mice to female subjects may decrease anxiety-like behavior associated with increased concentration of testosterone in plasma and hippocampus, as well as increased hypothalamic testosterone and 3α-diol concentration (64, 69). On the other hand, estradiol (61) and another metabolite of testosterone, androsterone (70), may cause anxiolysis, as well. Fernández-Guasti and Martínez-Mota (54) have shown that repeated administration of testosterone, but not a single injection of testosterone, nor treatment with 3α-diol or androsterone produced anxiolysis in male gonadectomized rats (54). According to published data, the manifestation of the anxiolytic-like effect of testosterone or its metabolites is highly dependent on dose and duration of treatment (54, 58, 64, 65, 71).

Due to its complex metabolism, the effects of testosterone might be mediated through different mechanisms of action. Testosterone and dihydrotestosterone are ligands of the androgen receptor. The latter binds with a greater affinity to the receptor and activates gene transcription resulting in an increased androgen activity (72). The role of androgen signaling in the regulation of anxiety-related behavior was demonstrated by administration of the androgen receptor antagonist flutamide (52, 54, 66), but also using the animal model of testicular feminization mutation (73–76) and androgen receptor knockout mice (77). In addition, it has been shown that some selective androgen receptor modulators may exert neuroprotective effects (78) and may affect some behavioral and brain functions (79–83). However, experimental studies examining their effects on anxiety important for the deeper understanding of the association between testosterone and anxiety, but potentially also for therapeutic applications are lacking. Testosterone can exert its effects also via estrogen receptors following aromatization of testosterone to estradiol, as well as by conversion of dihydrotestosterone to 3α-diol and 3β-diol (23, 71). Furthermore, it was shown that testosterone may modulate γ-aminobutyric acid (GABA)-stimulated chloride influx (84). This might be due to the fact that 3α-diol and androsterone can bind to $GABA_A$/benzodiazepine receptor. Unlike to $GABA_C$ receptor, which seems not to be involved in the regulation of anxiety by testosterone (85), inhibition of $GABA_A$ receptor by bicucculine or picrotoxin (64), as well as by flumazenil (70) diminishes the anxiolytic effect of testosterone metabolites. In females, inhibition of $GABA_A$ and ERβ receptor diminishes the anxiolytic effect of testosterone, but not when aromatase is inhibited. Thus, the anxiolytic effect of testosterone might be mediated by its reduced metabolites via $GABA_A$ with participation of ERβ (86). On the other side, using βER-knockout mice, it has been shown that the anxiolytic effect of testosterone metabolites mediated by the $GABA_A$ receptor may be not as robust as via activation of ERβ receptor (70). In addition, it was shown that testosterone can regulate serotonin neurotransmission. Gonadectomy results in decreased expression of 5-HT_{2A} serotonergic receptors in hippocampus, testosterone replacement enhances 5-HT_{2A} expression accompanied by reduced anxiety (58). On the contrary, inhibition of 5-HT_{1A} receptor increases efficacy of testosterone treatment leading to enhanced anxiolytic-like effect (53). As shown by observation of inter-individual differences, anxious animals display higher expression of tryptophan hydroxylase—an enzyme involved in serotonin synthesis, and higher concentration of serotonin in amygdala than their non-anxious counterparts (35). Interestingly, while in gonadally intact rats, anxiety-like behavior positively correlates with the expression of tryptophan hydroxylase, this association is abolished by gonadectomy. As serotonin depletion diminishes

anxiety level in anxious individuals, it also reverses the anxiogenic effect of gonadectomy (57).

AGING

Aging is associated with a progressive decline of circulating testosterone resulting from decreased function of the hypothalamo-pituitary-gonadal axis. Unlike menopause in women, andropuase in men is a long-lasting process. The age-related hypogonadism in men is considered to cause some of the senescence symptoms, including anxiety (87). This has been proved in rats. While gonadectomy in young adulthood and, partially, at middle-age induces anxiety, this effect is less apparent in aged rats (22). On the other hand, in a genetic mouse model of aging, no association was found between low testosterone and anxiety (88). It should be considered that andropause is via negative feedback associated with increased concentration of luteinizing hormone. In adult female rats, agonist of gonadotropin-releasing hormone triptorelin, which inhibits luteinizing hormone release, alone as well as in combination with estradiol treatment reduces gonadectomy-induced anxiety (89). Further experiments are needed to examine the impact of luteinizing hormone modulation on anxiety in aging males.

In our laboratory, sex differences in anxiety-like behavior of middle-aged (12-months old) rats have been shown. Aging females were less anxious than aging males in a battery of behavioral tests (41). Similarly, male rats at older age (21-months old) have proved to be generally more anxious than females. In addition, stress in males increased, while in females it decreased anxiety-like behavior (43). However, we have shown that at very old age (30-months old) female and male rats do not differ any more in anxiety-like behavior in elevated plus maze and light-dark box, or in open-field ambulation, except rearing behavior (42). These results suggest that the causal role of endogenous testosterone in anxiety-related behavior at is doubtful at least in aging animals. However, it should be considered that the anxiety-like behavior in these rats might be biased by age-related decline in locomotor activity (90). Presumably, the sex differences, observed in young and middle-aged animals, result from the organizational effect of testosterone occurring at early development and puberty. These differences, however, do not persist into the old age, which can be caused by age-related cognitive and affective decline.

There is some evidence suggesting the anti-anxiety properties of exogenous testosterone as well as its metabolites, 3α-diol and estradiol, in old gonadally intact rats (23, 91) or in rats gonadectomized at old age (22). On contrary, we have demonstrated that neither single administration of testosterone or estradiol, nor short-term treatment with estradiol affect anxiety-like behavior in middle-aged rats, when they suffer from long-term hypogonadism initiated before puberty onset (41). Therefore, it seems to be possible that the length of testosterone deficiency as well as the age of animals might determine the activational effect of supplemented hormones on anxiety-like behavior. Furthermore, the age, dose and duration of hormone replacement therapy as well as their interactions were shown to influence the action of testosterone leading to either anxiolysis or anxiogenesis (91).

CONCLUSION

Sex differences in anxiety-like behavior origin from organizational effect and might be modified by activational effect of sex hormones. There are critical periods across life that are crucial in organization of neuronal circuits and pre-programming the activational effect of testosterone on anxiety-like behavior. According to the current experimental studies, testosterone exposure during brain development predetermines higher anxiety in males. Furthermore, the absence of testosterone during the activation of neuronal circuits involved in anxiety results in anxiogenesis, as well. Although it seems that testosterone might have anxiolytic effect in adult males following short-term androgen deficiency, the impact of testosterone on anxiety-related behavior following long-term hypogonadal condition is understudied, mainly in aged animals, and needs further examinations. Based on the experimental results, age is a crucial factor that modulates the effect of both, endogenous and exogenous sex hormones. Thus, studying the expression of the various androgen receptors in different brain regions during development will be important to understand the contradictory findings from different experiments. Testosterone exposure or even deficiency in critical periods of development may have long-lasting consequences persisting into middle- or old age. This should be considered when interpreting experimental results and potentially hormonal treatments in human patients.

AUTHOR CONTRIBUTIONS

ED drafted the manuscript, prepared the table and the figure. JH corrected the draft. DO corrected the draft. PC designed the review and corrected the draft. All authors approved the final version of the manuscript.

FUNDING

The authors are supported by Slovak research and development agency (grant number APVV-15-0045).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

OPINION
published: 06 August 2018
doi: 10.3389/fnins.2018.00482

Biomarkers for PTSD at the Interface of the Endocannabinoid and Neurosteroid Axis

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Keywords: allopregnanolone, biomarkers, N-palmitoylethanolamine, PPAR-α, endocannabinoids, 5α-reductase, contextual fear responses, PTSD

INTRODUCTION

In the search for reliable and, possibly, specific biomarkers for neuropsychiatric disorders, growing evidence has demonstrated that biosynthesis of neuroactive steroids and the endocannabinoid system are involved in the neuropathology of post-traumatic stress disorder (PTSD) and major depressive disorder (Uzunova et al., 1998; Rasmusson et al., 2006; reviewed in Locci and Pinna, 2017a). Although, undisputable progress has been made to assess validity of biomarkers for psychiatric disorders, the topic still remains underdeveloped as compared to other fields of neuroscience (Fernandes et al., 2017). The diagnosis of psychiatric disorders still relies on subjective measures centered on the DSM-5 criteria which have several shortcomings (Brewin et al., 2017). Psychiatric conditions are poorly understood and there is a wide heterogeneity in how illness manifests in several individuals. Furthermore, self-assessment of one's own feelings can be biased, ill-defined, and difficult, making psychological diagnoses unreliable and may lead to treatment inefficacy. Biomarkers discovery would significantly improve treatment matching. Thus, searching for potential biomarkers to guide precision medicine in the treatment of PTSD, and to increase the success of clinical trials and prompt the development of novel and specific treatments, is required. To aid this search, more sophisticated methodological tools and validated animal models has also become essential to reliably correlate behavioral changes with neurochemical alterations (reviewed in Ngounou Wetie et al., 2013). The overlap of symptoms and the comorbidity with other psychiatric disorders such as major depressive disorder, anxiety spectrum disorders, and even suicidal ideation (Franklin et al., 2017), suggest a bio-signature for PTSD should include the relation of numerous biomarkers rather than having only a few (Locci and Pinna, 2017a). A refined approach to more specifically "bio-define" PTSD can be to establish a *biomarker axis* or in other words, to assess the relation of various biomarkers, which fluctuate in concert and correlate uniquely with PTSD behavioral modifications. Insofar, a *biomarker axis* may provide a higher accuracy in the diagnosis of the disorder with benefits for prediction in PTSD treatment response and relapse (Locci et al., 2018; Pinna and Izumi, 2018). As a matter of fact, the "gold standard" treatment for PTSD and depression, the selective serotonin reuptake inhibitors (SSRIs), improve only half of the treatment-seeking patients and they are associated with severe side-effects (Golden et al., 2002; Rush et al., 2006; Kemp et al., 2008; reviewed in Bernardy and Friedman, 2017). This also suggests these psychiatric disorders are complex, multifaceted diseases arising from multiple and diverse neurobiological backgrounds and therefore, symptoms may not always recapitulate to a serotonergic deficit and administering an SSRI may not always improve symptoms. Unveiling reliable biomarkers is also a necessity for patient stratification in treatment selection as well as for drug development through clinical trials. The development of state-of-the-art technologies

OPEN ACCESS

Edited by:
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University of Ottawa, Canada

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Christine A. Rabinak,
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience

Received: *28 April 2018*
Accepted: *26 June 2018*
Published: *06 August 2018*

Citation:
Pinna G (2018) Biomarkers for PTSD at the Interface of the Endocannabinoid and Neurosteroid Axis. Front. Neurosci. 12:482. doi: 10.3389/fnins.2018.00482

and methodological rigor are essential to allow for the discovery of more reliable biomarkers in psychiatry. Employing the gas chromatography-mass spectrometry (GC-MS) to achieve this goal is highly innovative and provides reliable information based on a powerful technology with high sensitivity and unsurpassed structure selectivity (Uzunov et al., 1996; Pinna et al., 2000). Hence, by applying the GC-MS measurements of neuroactive steroids in serum, plasma, CSF and post-mortem brain, in the past decade, we have shed light in the fundamental role of neuroactive steroids in patients with neuropsychiatric disorders (Rasmusson et al., 2006, 2017; Agis-Balboa et al., 2014; Pineles et al., 2018; reviewed in Locci and Pinna, 2017a).

The biosynthesis of allopregnanolone, a positive allosteric modulator of GABA's action at $GABA_A$ receptors has been found deficient in a number of neuropsychopathologies, including epilepsy (e.g., PHDH19), major depression, PTSD, perceived social isolation, post-partum depression, premenstrual syndrome, and anorexia nervosa or obesity complicated by anxiety and depression symptoms in women (Romeo et al., 1998; Uzunova et al., 1998; Rasmusson et al., 2006, 2018; Nemeroff, 2008; Lovick, 2013; Trivisano et al., 2017; Dichtel et al., 2018; Pineles et al., 2018). Therapeutic measures aimed at reinstating normal allopregnanolone levels in deficient-patients correlates with improved symptoms (Kanes et al., 2017). The question arises as to whether allopregnanolone biosynthesis *per se* is a reliable biomarker to predict, diagnose and instruct treatment selection of patients or whether its relation with neurotransmitter systems ($GABA_A$ and NMDA receptors), stimulation of neurotropic factors (e.g., BDNF), and/or crosstalk with the endocannabinoid system (e.g., PPAR-α) may provide a valuable *biomarker axis* with a higher disorder-selectivity. This analysis includes both neurosteroids that are positive allosteric modulators of $GABA_A$ receptors (Pinna et al., 2000; Belelli and Lambert, 2005), such as allopregnanolone and pregnanolone and their sulfated forms that are inhibitors of NMDA-mediated tonic neurotransmission, which results in neuroprotection (Vyklicky et al., 2016).

The novel discovery that the endocannabinoid system regulates the biosynthesis of neurosteroids, including allopregnanolone has recently opened the field for assessing valuable PTSD biomarkers at the interface of these neuronal systems. In recent years, cannabinoid-based agents have become an integral part of drug discovery for PTSD treatment (Ruehle et al., 2012; Neumeister et al., 2014). The impact of the endocannabinoid system is under-scored by the density of receptors in glutamatergic neurons of emotion-relevant areas, including the the amygdaloid complex, the hippocampus and the frontal cortex (Katona, 2009). Synthetic cannabinoid receptor antagonists or knockouts enhance fear acquisition and impair fear extinction, a core feature of PTSD (Reich et al., 2008; Papini et al., 2015). In addition to the well assessed role of the endocannabinoid, anandamide (AEA) or 2-arachidonoyl-glycerol (2-AG) both in neuropsychiatric disorders and animal models of stress (Chhatwal et al., 2005; Umathe et al., 2011; Dubreucq et al., 2012), compelling evidence indicates stimulation of the intracellular endocannabinoid target, peroxisome-proliferator activated receptor (PPAR)-α by its endogenous neuromodulator, N-palmitoylethanolamine (PEA) engages the biosynthesis of neurosteroids to modulate emotional behavior (Locci and Pinna, 2017b; Locci et al., 2018) (please see **Figure 1** for a graphic representation).

This unforeseen behavioral and neurosteroidogenic function of PPAR-α, formally known to regulate pathophysiological functions, including inflammation and oxidative stress, opens the field for potential novel biomarkers for PTSD.

This article will discuss whether new discoveries in the field support a biomarker role for allopregnanolone biosynthesis and the endocannabinoid system for stress-induced disorders with focus on PTSD. The strategy of assessing a *biomarker axis*, which indicates the relation of various inter-related neurobiological deficits for one disorder (**Figure 2**), may help for diagnosis accuracy and for designing successful individualized treatments.

NEUROSTEROID ACTION AT $GABA_A$ AND NMDA RECEPTORS

Sulfated or unconjugated neuroactive steroids modulate ionotropic amino acid neurotransmitter receptors, including $GABA_A$ and NMDA receptors. The $GABA_A$ receptor offers two binding residues that express affinity for allopregnanolone and unconjugated congeners (e.g., pregnanolone) that act as potent positive allosteric modulators of the action of GABA at $GABA_A$ receptors. One is located at the interface of the α/β subunits, and the other is within the cavity of α subunits (Hosie et al., 2006). The α,β,γ $GABA_A$ receptor subtype is the most frequent synaptic configuration and is highly sensitive to benzodiazepines but shows lower sensitivity to GABA and neurosteroids (Nusser and Mody, 2002). The α,β,δ $GABA_A$ receptor subtype expressed in the extrasynaptic region is benzodiazepine-insensitive, show low efficacy for GABA, but neurosteroids increase its agonist efficacy (Stell et al., 2003; Shu et al., 2012). This receptor combination shows high efficacy for neurosteroids (Brown et al., 2002; Nusser and Mody, 2002; Wohlfarth et al., 2002; **Figure 1**). Sulfated neurosteroids such as pregnenolone sulfate, dehydroepiandrosterone sulfate, pregnanolone sulfate, and allopregnanolone sulfate may function as endogenous neuromodulators by inhibiting $GABA_A$ receptors, or depending on the receptor conformation and the sulfated neuroactive steroid examined, by activating or inhibiting NMDA-mediated neurotransmission (Park-Chung et al., 1999). Sulfation at C3 is essential to reverse the direction of modulation from positive to negative in $GABA_A$ receptors. Steroid negative and positive modulators act through distinct sites, which implies that steroid negative and positive modulators can act independently or coordinately to modulate the flavor of GABAergic-mediated inhibitory neurotransmission (reviewed in Smith et al., 2014). While, micromolar concentrations of pregnenolone sulfate negatively modulate $GABA_A$ receptors, pregnenolone sulfate can negatively or positively modulate NMDA receptors, depending on the receptor subunits expressed (Malayev et al., 2002; Smith et al., 2014). For instance, pregnenolone sulfate potentiates NMDA receptors that contain NR2A and NR2B subunits, but negatively modulates NR2C and NR2D-containing receptors (Malayev et al., 2002). Recent studies showed that pregnanolone

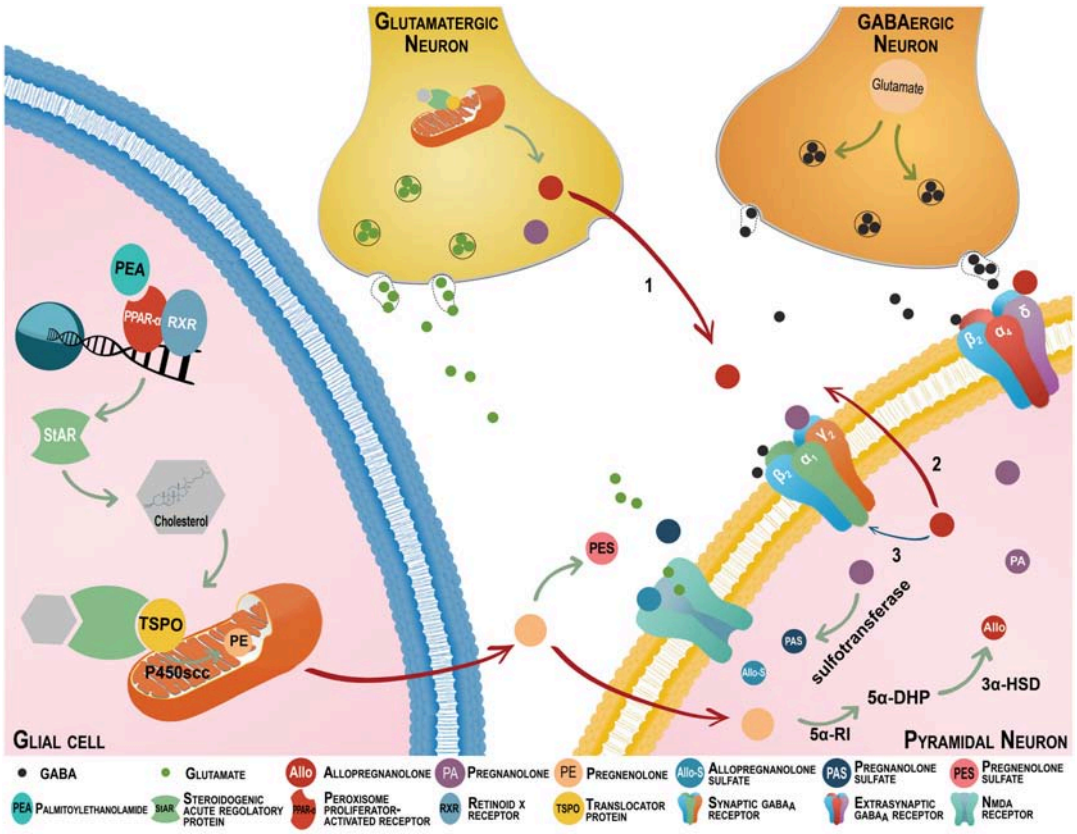

FIGURE 1 | The endocannabinoid and neurosteroid systems cross-talk regulates emotional behavior. The neurosteroids, allopregnanolone (Allo) and its equipotent isomer pregnanolone (PA) are primarily synthesized in glutamatergic corticolimbic neurons and upon secretion; they may act at $GABA_A$ receptors located on cell bodies or dendrites of distal pyramidal neurons (Arrow 1). They may also act at $GABA_A$ receptors located on glutamatergic neurons' dendrites or cell bodies by an autocrine mechanism (Arrow 2), or may access and act at the intracellular sites of $GABA_A$ receptors located in glutamatergic neurons that produced allopregnanolone itself (Arrow 3) (Agís-Balboa et al., 2006, 2007; Pinna et al., 2008). Allopregnanolone plays a central neuromodulatory role in facilitating the action of GABA at $GABA_A$ receptors (a primary target of anxiolytics) and in the fine-tuning of the receptor for agonists and GABAmimetic agents (Pinna et al., 2000). The finding that allopregnanolone facilitates the efficacy of $GABA_A$ receptor allosteric modulators substantiates its endogenous physiological relevance (Pinna et al., 2000, 2008; Guidotti et al., 2001). Importantly, $GABA_A$ receptors composed by α,β,γ subunits are the most common configuration in the synaptic membranes and they are responsible for the inhibitory *phasic* currents. These receptors are benzodiazepine-sensitive but show lower sensitivity to GABA and allopregnanolone (Nusser and Mody, 2002). The $GABA_A$ receptors including α,β,δ subtypes are mostly extrasynaptic and mediate inhibitory *tonic* currents. Of note, they are not sensitive to benzodiazepines and show low efficacy for GABA, however, allopregnanolone increase their efficacy (Stell et al., 2003; Shu et al., 2012). The efficacy of GABAergic neurosteroids is greatly enhanced for this receptor combination (Brown et al., 2002; Nusser and Mody, 2002; Wohlfarth et al., 2002). Remarkably, protracted stress favors a $GABA_A$ receptor composition with high sensitivity for allopregnanolone and its analogs (Locci and Pinna, 2017a). Following the action of sulphotransferase, allopregnanolone, and pregnanolone can be transformed into allopregnanolone sulfate (Allo-S) and pregnanolone sulfate (PAS). These sulfated steroids can be measured by gas chromatography-mass spectrometry in serum, CSF, and brain of patients or rodents in concentrations consistent with a physiological role in modulating neurotransmitter systems (Smith et al., 2014; Locci and Pinna, 2017b). Recently, pregnanolone sulfate has been shown to inhibit NMDA receptors. Pregnanolone sulfate can accumulate in plasma membranes and may accesses binding sites that are located at NMDA receptors (Borovska et al., 2012). Importantly, pregnanolone sulfate, and probably allopregnanolone sulfate, is highly potent at inhibiting tonic rather than synaptically mediated NMDA receptor neurotransmissions. While synaptic NMDA receptors play a pivotal role in synaptic plasticity, learning and memory, as well as in synaptogenesis, tonic-mediated NMDA receptor neurotransmission is mostly involved with excitotoxicity. Thus, the effects of pregnanolone sulfate negative modulation of tonic-mediated NMDA receptor neurotransmission have relevance for neuroprotection (Vyklicky et al., 2016). By this mechanism, these allopregnanolone and pregnanolone sulfated derivatives may play a role in the regulation of cognitive processes and of emotional behavior (reviewed in Locci and Pinna, 2017a). There is growing evidence that the intracellular peroxisome proliferator-activated receptor (PPAR-α) is also a cannabinoid target (depicted on the bottom right). PPAR-α heterodimerize with the retinoid X receptor (RXR) and binds to the consensus regions on the target gene promoters and initiates transcription (Neumeister, 2013). Given that endoannabinoids activate PPAR-α (Marsicano et al., 2002; Pistis and Melis, 2010), the activation of these nuclear receptors represents a novel mechanism by which cannabinoids may modulate behavior. The endocannabinoid congener, N-palmitoylethanolamine (PEA) is a PPAR-α endogenous agonist, which is decreased in PTSD patients (Wilker et al., 2016). Recent preclinical findings showed that supplementing PEA in rodent PTSD models improves emotional behavior by enhancing allopregnanolone biosynthesis in corticolimbic glutamatergic neurons. This effect is mimicked by PPAR-α agonists and prevented by allopregnanolone biosynthetic enzyme blockers and by deletion of the PPAR-α gene (Locci and Pinna, 2017b). Thus, anxiolytic, anti-aggressive and anti-fear effects of PEA and synthetic PPAR-α agonists may relate to an induction of corticolimbic allopregnanolone's biosynthetic enzymes. This may result in potentiation of $GABA_A$ receptor and, possibly, in an inhibition of tonic-mediated NMDA signal transduction associated with improved behavioral dysfunction. Stress effects on PEA levels and probably expression of PPAR-α may result in the downregulation of allopregnanolone's biosynthetic enzyme expression and allopregnanolone levels. The interface of the endocannabinoid and neurosteroid systems may provide an important *biomarker axis* to selectively predict, diagnose, and establish the best individualized treatment selection for PTSD patients.

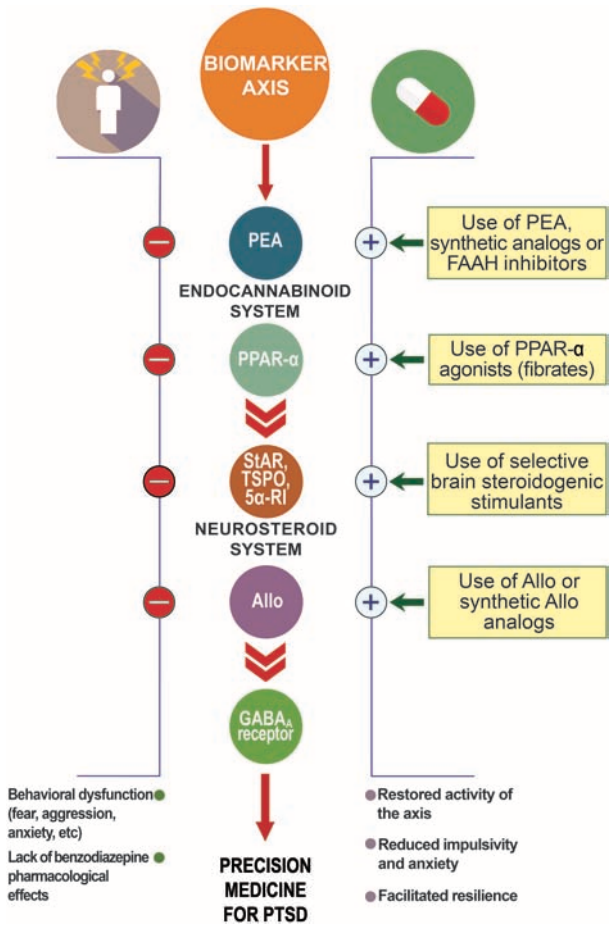

FIGURE 2 | Biomarker axis at the interface of the endocannabinoid and neurosteroid systems. In animal models of PTSD, protracted stress results in the downregulation of allopregnanolone biosynthetic enzymes (e.g., 5α-reductase type I, 5α-RI) and allopregnanolone concentrations in corticolimbic glutamatergic neurons of the frontal cortex, hippocampus, and basolateral amygdala. This allopregnanolone decrease correlates with behavioral dysfunction, such as increased aggression, enhanced contextual fear responses and anxiety-like behavior (Pinna et al., 2003; Pibiri et al., 2008). Supplying allopregnanolone or stimulating its biosynthesis decreases anxiety-like behavior, aggression and fear responses (Pinna, 2013; Pinna and Rasmusson, 2014). Stress may also result in changes in $GABA_A$ receptor subunit expression (Pinna et al., 2006; reviewed in Locci and Pinna, 2017a) with increased α4, α5, and δ subunits and decreased α1, α2, and γ2, which result in down-regulated benzodiazepine binding sites and inefficacy of benzodiazepine pharmacological action (Pinna et al., 2006; Nin et al., 2011b). Protracted stress results in increased $GABA_A$ receptor subunits, including α_{4-5},β,δ highly sensitivity for allopregnanolone (Locci and Pinna, 2017a). Both allopregnanolone biosynthesis downregulation and decreased benzodiazepine binding sites have been reported in PTSD patients (Rasmusson et al., 2006, 2018; Geuze et al., 2008). Thus, the combination of downregulation of allopregnanolone biosynthesis, changes in $GABA_A$ receptor subunit expression, and lack of benzodiazepine pharmacological action are peculiar changes observed in PTSD that may provide a selective *biomarker axis* for this disorder. Stress may affect PEA levels and expression of PPAR-α, which in turn may downregulate allopregnanolone concentrations. Thus, the PPAR-α-allopregnanolone axis may provide further biomarker candidates to support selection of the best individualized precision medicine for PTSD. Allo, allopregnanolone; GABA, γ-aminobutyric acid; PEA, N-palmitoylethanolamine; PPAR-α, peroxisome-proliferator activated receptor-α; StAR, steroidogenic acute regulatory protein; TSPO, 18 kDa translocator protein.

sulfate has a potent inhibitory action at tonic rather than synaptically-activated NMDA receptors, which provides neuroprotection and possibly improves emotional behavior and cognition (Vyklicky et al., 2016). This feature is relevant for developing a novel class of steroid-based NMDA-inhibitors devoid of the psychotomimetic effects that characterize classical NMDA receptor inhibitors, including ketamine. While $GABA_A$ receptor subunit expression during protracted stress has been previously investigated (discussed below), the role and action of sulfated pregnanolone, pregnenolone, allopregnanolone, and the expression of NMDA receptor subunits in PTSD patients and in rodent stress models, still warrants elucidation.

The Neurosteroid and Endocannabinoid Crosstalk

Intriguingly, studies conducted in cell cultures, brainstem and spinal cord showed the endocannabinoid, PEA binding at the ligand-activated nuclear receptor, PPAR-α stimulates allopregnanolone biosynthesis and potentiates pentobarbital-induced sedation (Sasso et al., 2010, 2012; Raso et al., 2011). These observations suggest that PPAR-α may play a role in the regulation of emotions by inducing neurosteroidogenesis in corticolimbic neurons following binding with its endogenous ligand, PEA, or synthetic agonists. Whereas the classic cannabinoid receptor type 1 (CB1) has been shown to regulate emotions and stress responses, PPAR-α's role on emotions remains poorly understood (Riebe and Wotjak, 2011; Häring et al., 2012). The relevance of the endocannabinoid system in behavior is highlighted by expression of CB1 and PPAR-α in glutamatergic neurons of emotion-relevant areas that have been identified by brain imaging to be critical in PTSD (amygdala, hippocampus, frontal cortex) (Moreno et al., 2004; Lo Verme et al., 2005; Shin et al., 2006; D'Agostino, 2007; D'Agostino et al., 2009; Katona, 2009; Petrosino and Di Marzo, 2017). Moreover, evidence suggests CB1 disruption leads to impaired fear extinction (Reich et al., 2008), depressive- and anxiety-like behavior, while agonists, like AEA, induce anxiolysis and improve fear responses (Hill and Patel, 2013). Current thought suggests that the effects of AEA at CB1 account for the majority of anti-fear effects of endocannabinoids (Marsicano et al., 2002; Viveros et al., 2005; Kamprath et al., 2006; Thiemann et al., 2008; Jacob et al., 2012), however this view seems no longer tenable (Pistis and Melis, 2010). In addition to these cell-surface cannabinoid receptors (O'Sullivan, 2007), there is growing evidence that PPAR-α's activation represents a novel mechanism by which cannabinoids modulate behavior. Stimulation of PPAR-α by PEA or synthetic PPAR-α agonists was recently shown to elevate corticolimbic allopregnanolone levels in hippocampus, amygdala, frontal cortex and in olfactory bulb, which correlated with improvement of PTSD-like behavior in socially isolated mice (Locci and Pinna, 2017a). PEA facilitates contextual fear extinction and fear extinction retention and induces anti-aggressive, anxiolytic, and antidepressant-like effects in socially isolated mice (Locci and Pinna, 2017b; Locci et al., 2017). PPAR-α synthetic agonists normalized allopregnanolone

levels and improved behavior, whereas antagonism at PPAR-α, inhibition of allopregnanolone biosynthetic enzymes, or PPAR-α KO mice prevented both PEA-induced behavior and its neurosteroidogenic effects (Locci and Pinna, 2017b). While the role of PPAR-α in neuropsychiatric disorders is just emerging, studies in the field suggest serum PEA and oleoylethanolamide (OEA) concentrations increase after acute social stressor (Dlugos, 2012) and decrease following recovery (Hill et al., 2009a). Stress evokes fast induction of fatty acid amide hydrolase (FAAH), which reduces PEA and AEA levels (Patel et al., 2005; Hill et al., 2009b). In PTSD patients, symptoms are inversely correlated with reduced hair levels of PEA, OEA and stearoylethanolamide (SEA) in both males and females (Wilker et al., 2016). PEA adjunctive therapy to citalopram improves symptoms in depressed patients (Ghazizadeh-Hashemi, 2018). Furthermore, intense workouts increase PEA and OEA levels and improve depression and PTSD (Heyman, 2012). In rodents, exposure to predator stressors reduces cardiac PEA and OEA levels (Holman et al., 2014), but, antidepressant-like effects are induced by increasing PEA and OEA (Adamczyk et al., 2008; Umathe et al., 2011; Melis et al., 2013). Collectively, the crosstalk between the endocannabinoid system and neurosteroid biosynthesis during stress may unveil *biomarker axis* uniquely altered in specific stress-induced mood disorders.

BIOMARKERS AND TREATMENT OPTIONS FOR PTSD AT THE INTERFACE OF THE ENDOCANNABINOID AND NEUROSTEROID AXIS

Psychiatric disorders, such as PTSD, are not currently amenable to objective neurobiological determinations as is routine practice in the diagnosis and treatment of other medical conditions. This is most likely due to the general complexity and multifactorial origins of these disorders and the difficulty to establish a consistent bio-signature. While no biomarkers for PTSD have to date been firmly assessed with diagnostic validity, a consistent progress in the field has been done. Biomarker candidates for PTSD have been proposed but often they share overlaps with other psychiatric disorders with similar symptoms and that are currently treated with same drugs. Indeed, the first-choice pharmacological treatments for PTSD, the SSRIs, act through multiple molecular mechanisms other than by inhibiting serotonin reuptake. These mechanisms include the stimulation of neurosteroid and endocannabinoid biosynthesis and neurotrophic factors, such as BDNF, which are found deficient in PTSD. Increasing allopregnanolone levels is also associated with increased BDNF expression (Nin et al., 2011a). Collectively, these findings have contributed to improve our understanding of the psychobiological abnormalities associated with PTSD and promote the development of novel targeted treatment options. For instance, the correlation between the impairment of neurosteroid biosynthesis and behavioral modifications in neuropsychiatric disorders has been the focus of several studies (van Broekhoven and Verkes, 2003; reviewed in Pinna, 2013; Agis-Balboa et al., 2014; Locci and Pinna, 2017a). A reduction in the content of the GABAergic modulator allopregnanolone and its equipotent isomer pregnanolone was reported in cerebrospinal fluid (CSF) and serum of major depression and PTSD patients (Romeo et al., 1998; Uzunova et al., 1998; Rasmusson et al., 2006, 2016; Pineles et al., 2018). A negative correlation between CSF allopregnanolone levels and PTSD symptoms was more recently confirmed in male patients (Rasmusson et al., 2018). Other clinical studies support the significance of allopregnanolone biosynthesis as a biomarker of mood disorders (Uzunova et al., 1998; Agis-Balboa et al., 2014; reviewed in Zorumski et al., 2013; Schüle et al., 2014; Locci and Pinna, 2017a) with finding showing decreased allopregnanolone levels in postpartum depression (Nemeroff, 2008), under treatment with finasteride, an allopregnanolone biosynthetic enzyme blocker (Altomare and Capella, 2002; Caruso, 2015; Welk et al., 2017), and with anorexia nervosa or obesity complicated by anxiety and depression (Dichtel et al., 2018). Intriguingly, SSRI treatments normalize plasma, CSF, and brain allopregnanolone content in association with improvement of symptoms in responders only (Romeo et al., 1998; Uzunova et al., 1998; Agis-Balboa et al., 2014). These findings are in support of the role of allopregnanolone in the mechanisms of SSRIs' anxiolytic effects (Pinna, 2015). The downregulation of neurosteroid levels found in PTSD and depressed patients can be modeled in rodents exposed to protracted stress, including the socially-isolated mouse. Allopregnanolone is produced in brain corticolimbic neurons (**Figure 1**) and a reduction of its levels by prolonged social isolation (Agís-Balboa et al., 2006, 2007) or exposure to single prolonged stressors, results in development of anxiety-like behavior, aggression and enhanced contextual fear conditioning responses associated with impairment of fear extinction and elevated spontaneous fear responses at recall (Pinna et al., 2003; Pibiri et al., 2008; Pinna and Rasmusson, 2014; Qiu et al., 2015). These preclinical studies further support allopregnanolone as a putative *biomarker* for stress-induced emotional modification, such as exaggerated fear responses and impaired fear extinction, a hallmark in PTSD (Pibiri et al., 2008; Pinna et al., 2008; Pinna and Rasmusson, 2012). This evidence also suggests that new therapeutic approaches should counteract the downregulation of neurosteroid biosynthesis to improve symptoms in PTSD patients. In recent phase 3 clinical trials, intravenous allopregnanolone (brexanolone or SAGE-547) or an oral analog (SAGE-217) showed a rapid and long-lasting remission of post-partum depression and major depressive disorder symptoms, conditions highly comorbid with PTSD (Kanes et al., 2017; http://investor.sagerx.com/news-releases/news-release-details/sage-announces-pivotal-phase-3-trial-status-sage-217-major). If successfully developed, SAGE-217 will be the first durable, rapid-acting, oral, short-course treatment for mood disorders with potential application for PTSD treatment. Stress tremendously affects the expression of $GABA_A$ receptor subunits (Pinna et al., 2006; reviewed in Locci and Pinna, 2017a). After social isolation, the α4, α5, and δ subunit expression was increased, and the α1, α2, and γ2 were significantly decreased in corticolimbic areas. These changes result in decreased benzodiazepine recognition sites and lower pharmacological response to benzodiazepines

(Pinna et al., 2006). Remarkably, protracted stress favors a $GABA_A$ receptor composition with high sensitivity for allopregnanolone and its analogs (Locci et al., 2017; reviewed in Locci and Pinna, 2017a). Clinical findings support lower benzodiazepine recognition site binding in brain of PTSD patients in association with benzodiazepine-insensitivity (Geuze et al., 2008). Altogether, these findings suggest that isolation stress results in: (i) changes in $GABA_A$ receptor subunit composition; (ii) downregulated neurosteroidogenesis; and (iii) lack of response to benzodiazepines, which may provide a unique *biomarker axis* for PTSD (**Figure 2**). Allopregnanolone, analogs or stimulation of allopregnanolone biosynthesis may be a valuable therapeutic strategy for stress-induced psychiatric disorders, characterized by benzodiazepine-inefficacy and poor response to SSRIs. The pharmacological profile of SSRIs on stimulation of neurotropic factors, including the brain-derived neurotrophic factor (BDNF), via stimulation of allopregnanolone biosynthesis is an additional important mechanism to consider when establishing biomarkers for PTSD. BDNF expression decrease in PTSD patients is associated with symptom severity. In the socially isolated mouse, fluoxetine improves behavior by elevating the corticolimbic levels of allopregnanolone and BDNF expression, independently from the action of these drugs on serotonin reuptake inhibition. This and other evidence suggest that neurosteroid biosynthesis and BDNF expression may be interrelated (Nin et al., 2011a; Frye et al., 2014), and this may provide further support for biomarker selection.

Hence, biomarkers that instruct which treatment would be most effective for a patient is expected to considerably reduce non-responders and non-completers rate. Discovering new targets and agents to stimulate allopregnanolone biosynthesis is pivotal in this process. While more research is required to elucidate the interaction between the endocannabinoid system and allopregnanolone biosynthesis and specifically, following activation of PPAR-α, undoubtedly their cross-talk offers a unique opportunity to assess a biomarker axis that encompasses these two systems (**Figure 2**). A better assessment can be done following clarification of how stress affects PPAR-α expression and function. The concentrations of its main endogenous modulators, PEA, OEA, and SEA, which were found decreased in hair of PTSD patients, should be confirmed systemically in serum and CSF of patients. Preclinical studies should also verify whether their biosynthesis is altered in brain of rodent models of stress-induced behavioral deficits. Furthermore, methods development to simultaneously determine both endocannabinoid (AEA), cannabinoid-like (PEA) and neurosteroid (allopregnanolone) concentrations in same samples will consistently enhance the understanding on how the two systems are coordinated in neuropsychiatric disorders.

Both endocannabinoids and neurosteroids can be measured by GC-MS, however, presently there is no method that can determine them simultaneously in the same samples. Our laboratory established GC-MS methods to determine neurosteroids and their sulfates in human and rodent samples and we are currently developing new methods to include the quantification of endocannabinoids with demonstrated involvement in the pathophysiology of PTSD. Hence, these studies will clarify whether allopregnanolone levels down-regulation is causally linked to a PPAR-α expression down-regulation and/or endocannabinoid concentrations. The goal is to provide a reliable bio-signature that may uniquely define neurobiological alteration in PTSD and shows diagnostic validity.

While many aspects that relate to the endocannabinoid and neurosteroid cross-talk remain presently obscure, our current findings highlight the potential for: (i) assessing novel biomarkers to predict, diagnose, and treat PTSD at the interface of the PPAR-α-allopregnanolone axis; and (ii) repurposing FDA-approved PPAR-α agonists for the treatment of PTSD after positive clinical trials. Very few drugs are direct agonists of PPAR-α, and none have been tested for their potential effects in fear responses. However, one class of drugs, the fibrates are fibric acid derivatives that are prescribed to lower plasma lipids and triglyceride levels and are synthetic PPAR-α agonists that may be exploited in rodent models of PTSD to improve behavioral deficits.

CONCLUSIONS

Progress in assessing biomarkers to predict PTSD and its treatment response will guide the future of novel PTSD medications that may be designed to improve neurotransmission (GABA, NMDA), and neuroendocrinologic (allopregnanolone biosynthesis) and anti-inflammatory (PPAR-α) responses. Research supports precision medicine for PTSD designed to stimulate neurosteroidogenesis after assessing in subpopulations of PTSD patients a downregulation of allopregnanolone biosynthesis. This can be achieved by acting at neurosteroidogenic targets or by mimicking allopregnanolone's function (e.g., analogs). Several neuronal targets to enhance steroidogenesis have recently been discovered and these include the endocannabinoid target PPAR-α. The crosstalk between the endocannabinoid system and the biosynthesis of neurosteroids, involving their targeted receptors or the biosynthetic enzymes promises to provide unique bio-signatures for stress-induced disorders.

Collectively, advances in the field suggest biomarker-based diagnosis and treatments for PTSD that encompass the neurosteroid and endocannabinoid systems may not be a far reach and these may provide a pivotal complement to the current practice of assessing the disorder based on self-reported symptoms and psychiatrist assessments.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

ACKNOWLEDGMENTS

The Author wishes to thank Mr. Dario Aspesi for graphic design. This study was supported by the United States Department of Defense Grant W81XWH-15-1-0521 and Veteran Affairs Grant VA241-15-D-0041 (to GP).

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 24 September 2018
doi: 10.3389/fendo.2018.00544

Luteinizing Hormone Involvement in Aging Female Cognition: Not All Is Estrogen Loss

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Pervasive age-related dysfunction in hypothalamic-pituitary-gonadal (HPG) axis is associated with cognitive impairments in aging as well as pathogenesis of age-related neurodegenerative diseases such as the Alzheimer's disease (AD). As a major regulator of the HPG axis, the steroid hormone estrogen has been widely studied for its role in regulation of memory. Although estrogen modulates both cognition as well as cognition associated morphological components in a healthy state, the benefits of estrogen replacement therapy on cognition and disease seem to diminish with advancing age. Emerging data suggests an important role for luteinizing hormone (LH) in CNS function, which is another component of the HPG axis that becomes dysregulated during aging, particularly in menopause. The goal of this review is to highlight the current existing literature on LH and provide new insights on possible mechanisms of its action.

Keywords: luteinizing hormone, luteinizing hormone receptor, cognition, menopause, estrogen, ovariectomy, inverse relationship

OPEN ACCESS

Edited by:
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University of Ottawa, Canada

Reviewed by:
Carol F. Elias,
University of Michigan, United States
Ben Nephew,
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *16 May 2018*
Accepted: *28 August 2018*
Published: *24 September 2018*

Citation:
Bhatta S, Blair JA and Casadesus G (2018) Luteinizing Hormone Involvement in Aging Female Cognition: Not All Is Estrogen Loss. Front. Endocrinol. 9:544. doi: 10.3389/fendo.2018.00544

INTRODUCTION

Our ability to successfully treat midlife diseases has increased human longevity as well as the prevalence of age-associated neurodegenerative diseases such as the Alzheimer's disease (AD). While a small percentage of AD is precipitated by dominant mutations, the majority is sporadic and is highly associated with environmental/life-style choices as well as the physiological aging process. In fact, aging is the strongest risk factor for development for AD, the most common form of dementia (1–3). As baby boomers age, millions of people are at risk for AD and other cognitive diseases. This is especially relevant as the number of AD diagnosis is expected to rise from current 5.7 million to 14 million by 2050, and the cost of disease is projected to rise to 1.1 trillion dollars creating a broad economic and social impact. Therefore, identifying therapeutic targets to delay, modify, or cure the disease is of paramount importance.

Menopause, a hallmark of aging in women, is associated with dysfunctions in the hypothalamic-pituitary-gonadal (HPG) axis and is implicated in the pathogenesis of AD. Age-related declines in reproductive hormones lead to a disarray of the HPG axis as it is tightly regulated by positive and negative feedback loops involving gonadotropin releasing hormone (GnRH), gonadal steroids, and gonadotropins. The loss of any component leads to significant dysregulation of the system and any type of hormonal imbalance likely affects homeostasis resulting in functional decline throughout

the body, including the brain (4–8). In fact, these declines in gonadal hormones, and associated increases in gonadotropins such as LH are implicated in cognitive dysfunction in aging as well as the pathogenesis of age-related disorders such as AD. This is particularly relevant for women as they have two-fold higher risk for development of AD after menopause compared to men.

HORMONE REPLACEMENT THERAPY (HRT) FOR COGNITION AND AD

Several studies attribute increased risk of AD to the menopausal loss of estrogen (9, 10) and considered estrogen replacement a promising therapeutic avenue to reduce the risk of AD (11–14). This was initially supported by early epidemiological studies identifying strong relationships between estrogen therapy in post-menopausal women and reductions in multiple types of memory decline, specifically, in menopausal women with and without AD (15, 16).

Clinically, estrogen replacement or HRT showed initial promise in smaller clinical retrospective (17–20) and prospective studies (21–23). However, a large scale, randomized, double-blind placebo-controlled trial, women's health initiative WIH found no overall cognitive benefits. While the use of estrogen immediately or after a smaller delay following onset of menopause was effective, HRT in women at least 15 years after onset of menopause slightly increased the risk of dementia instead (24–26). This discrepancy in actions of estrogen on memory and dementia risk lead several groups to hypothesize that a "critical period" may exist following onset of menopause where estrogen can confer benefits, beyond which it is ineffective and may instead exert harmful effects (27–31). These clinical observations were further supported by pre-clinical work in ovariectomized rodents (32) and primates (33) providing support for the existence of a critical window for the initiation of estrogen for optimal enhancement of cognitive function (34).

One mechanism purported to underlie the ineffectiveness of estrogen treatment beyond a critical period is the "*healthy cell bias*" wherein estrogen is beneficial to healthy cells, but detrimental to unhealthy ones (35, 36). This was further supported by the fact that critical plasticity and cognition related proteins such as BDNF (37) and the cholinergic system (38) differ in young and old rats. Based on the above theory, more recent clinical randomized trials addressed the potential benefit of estrogen therapy considering timing of replacement. Unfortunately, these studies have yielded little support for this hypothesis (39–42). Together, these data suggest that while estrogen is relevant and important for CNS function and structure during the reproductive periods, aspects beyond estrogen loss contribute to age-related menopausal dysfunction.

INVOLVEMENT OF LUTEINIZING HORMONE (LH) IN CNS FUNCTION AND AD

Accumulating evidence supports a role for LH in regulating cognition and AD-related parameters. LH, a gonadotropin hormone released from the anterior pituitary, functions to stimulate the production of sex steroids, which in turn negatively regulate hypothalamic GnRH release and further production of LH. In the absence of sex steroids providing negative feedback, LH levels drastically increase, as observed following menopause and andropause. Peripheral levels of LH increase three-fold in post-menopausal women (43) and two-fold in aging men (44). Importantly, these increases in peripheral LH levels correlate to cognitive deficits in aging men and women (45, 46) as well as AD patients (47–51). In fact, increased levels of peripheral LH in AD patients is associated with exacerbated pathology and cognitive deficits (50–52). Additionally, increased LH is correlated with increased plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in subjective memory complainers, suggesting LH may play a progressive role in early preclinical stages (51).

In rodents, earlier studies demonstrated that over-expression of LH in transgenic mice (53), or exogenous treatment with human LH (54) without any estrogen manipulations, impaired working memory. These studies are supported by work showing that chronic elevations in peripheral levels through exogenous application of the LH analog, human chorionic gonadotropin (hCG), results in similar attenuation of working memory as well as increases in total brain amyloid-β_{1-40} in a mouse model of AD (55, 56). Moreover, peripheral increase in hCG is capable of reversing estrogen associated benefits in spatial memory (55, 56).

Several studies involving pharmacological downregulation of peripheral LH after ovariectomy also support a role for LH in cognition and plasticity. To this end, work utilizing either GnRH super-agonist (57–60) or competitive antagonists (61–64) of the GnRH receptor reverse cognitive deficits (57–64) and neuronal plasticity loss (59, 60) in the absence of estrogen replacement. For example, several studies from our lab using GnRH super-agonist, leuprolide acetate, improved function in ovariectomized C57BL/6J mice (58, 60) as well as aged-ovariectomized 3xTg AD mice (59). Furthermore, these benefits were associated with activation of signaling cascades associated with long term potentiation (LTP), the cellular basis of learning and memory, Bryan et al. (58) in the absence of AD pathology (59), further supporting a direct role of LH-related mechanisms on cognition. More importantly, a key finding was that pharmacological reductions in peripheral levels of LH lead to cognitive improvements and rescue of dendritic spine density without any dependence on timing of treatment onset (60). This raises the exciting possibility that LH may provide an additional therapeutic target for rescuing cognitive and structural deficiencies associated with menopause, aging, and AD.

While there is no clinical data to support the benefits of peripheral downregulation of LH in menopausal women, clinical data in AD patients support its validity (65). A small, phase II, randomized, clinical trial, in female patients with moderate AD, showed that leuprolide acetate stabilized cognitive function in a subgroup treated with an acetylcholinesterase inhibitor, Aricept. Given Aricept is the primary treatment option for AD patients, these data are encouraging. However, while clinical and pre-clinical studies show promise for LH-related therapeutics, the

mechanisms underlying these effects remain unknown to date. In the next sections, we highlight a potential mechanism of action of these therapies in relation to signaling and function of the LH receptor.

LH SIGNALING AND COGNITION

LH as well its receptor LHR are expressed in the CNS (66–69). Importantly, LHR is present in regions associated with learning and memory (68–70). Similarly, LHR is also expressed in the choroid plexus, ependymal cells of the third, fourth and lateral ventricles as well as the area postrema (68), which are areas involved in production and circulation of cerebrospinal fluid CSF as well as blood brain barrier transport, suggesting a potential modulatory role of LHR in BBB permeability.

The LHR is a G-protein-coupled receptor involved in production of sex steroids in the gonads through Gs cAMP/PKA, ERK and Gq PLC pathways. G_s signaling results in phosphorylation of MAPK and CREB, while G_q induces intracellular Ca^{2+} release and activation of several protein kinases such as PKA and CAMKII (71–74). LHR activated cAMP/PKA signaling cascades in the immortalized cultures of hippocampal and hypothalamic neurons *in vitro* (75, 76). Interestingly, these signaling cascades downstream of LHR are associated with gene expression and structural changes that are the hallmark of synaptic plasticity and memory formation in the CNS (77–80) and induction of LTP (81–84). Furthermore, active kinases trigger activation of transcriptional factors such as MAPK and CREB leading to gene expression and structural remodeling of the synapse. This ultimately leads to enhanced synaptic transmission and BDNF production, as well as plasticity of dendritic spines (79, 85–87). However, as previously discussed above, downregulation of peripheral LH in the OVX model lead to CAMKII autophosphorylation at Thr 286 and CAMKII dependent GluR1 Ser 831 phosphorylation (58), inhibition of GSK3β and upregulation of β-catenin in 3X Tg mouse model of AD (59), and improved cognitive function. Based on the known canonical signaling cascades ascribed to the LHR, these data, at least indirectly, suggest activation rather than inhibition of the LHR, thus, in conflict with the prevalent idea that LH signaling in the CNS is detrimental.

The idea that LH signaling in the CNS is detrimental stems from the supposition that LH crosses the blood-brain-barrier (BBB); however, LH is a large glycoprotein that is unlikely to penetrate the BBB without a transporter molecule. Furthermore, both human and animal studies show contradictory evidence on whether hCG or LH can cross the BBB. One study showed some permeability of hCG into CNS when administered at supra-physiological levels (88) while another did not (89). Moreover, studies show hCG is not BBB permeable in human choriocarcinomas models until a high threshold is reached (90, 91), especially in patients with normal levels of plasma LH (92). These data suggest that hCG and LH may not be permeable in normal or OVX conditions, thus, the supposition that the LHR is detrimental is difficult to reconcile.

BRAIN-SPECIFIC LH AS MECHANISM FOR LHR-MEDIATED CNS FUNCTION

Several earlier studies have demonstrated the expression of LH in the brain of various species (59, 66, 67), as well as its ability to modulate synaptic function (93–95), neurogenesis (96), and behavior (59, 88, 97, 98). *In vitro*, administration of the LH analog hCG, which binds and activates the LHR similarly to LH (99, 100), stimulates neurite outgrowth in rat neuronal culture and differentiation in PC12 cells through activation of the erk/MAPK pathway (99, 101). Erk/MAPK is a pathway that is critical for memory function and plasticity (78). Similarly, LHR activation *in vitro* also leads to stem cell differentiation (102), and protection against excitotoxicity in primary neurons (103).

LHR transcripts as well as functional receptors have been found in neurons and glial cells *in vitro* (94, 95). LHR was functional *in vitro* in neurons cultured at embryonic day 19. Similarly, hCG treatment of mixed glial cultures, from either P0 or P1 neonates, for 3 days lead to a dose-dependent increase in anti-inflammatory prostaglandin (PG), PGD2 and decrease in proinflammatory PGE2. These actions of LHR may be important for neonatal brain development and function as PGE2 inhibits the proliferation of glial cells, while PGD2 may do the exact opposite. Therefore, LHR signaling may be promoting controlled proliferation of glial cells during brain development (94).

While the above cited works cannot conclusively identify the source of LH in the brain, these studies demonstrate the presence of LH in cognition associated areas and its ability to mediate signaling and behavior. Furthermore, the highlighted *in vitro* work validates CNS LHR functionality and signaling consistent with cognition and neuroplasticity, both through neurons and glial cells. This said, how the LHR is involved in menopause and ovariectomy-induced cognitive and neuroplasticity loss remains unclear but may be explained through alterations in brain and peripheral levels of LH, as highlighted in the section below.

INVERSE RELATIONSHIP BETWEEN PERIPHERY AND THE BRAIN

Accumulating research shows that LH levels in the brain may have an inverse relationship to the periphery. For example, in studies of cycling adult female rats, hypothalamic LH decreased during proestrus, when a drastic 10-fold increase in LH occurs in the pituitary and periphery (104). Similarly, LH treatment into the median eminence (ME) of the hypothalamus in intact or castrated males and females significantly decreased both LH levels in the pituitary as well as the periphery (105). In a similar experiment, hCG treatment into the median eminence of hypothalamus in ovariectomized as well as intact mature female rats decreased peripheral LH (106).

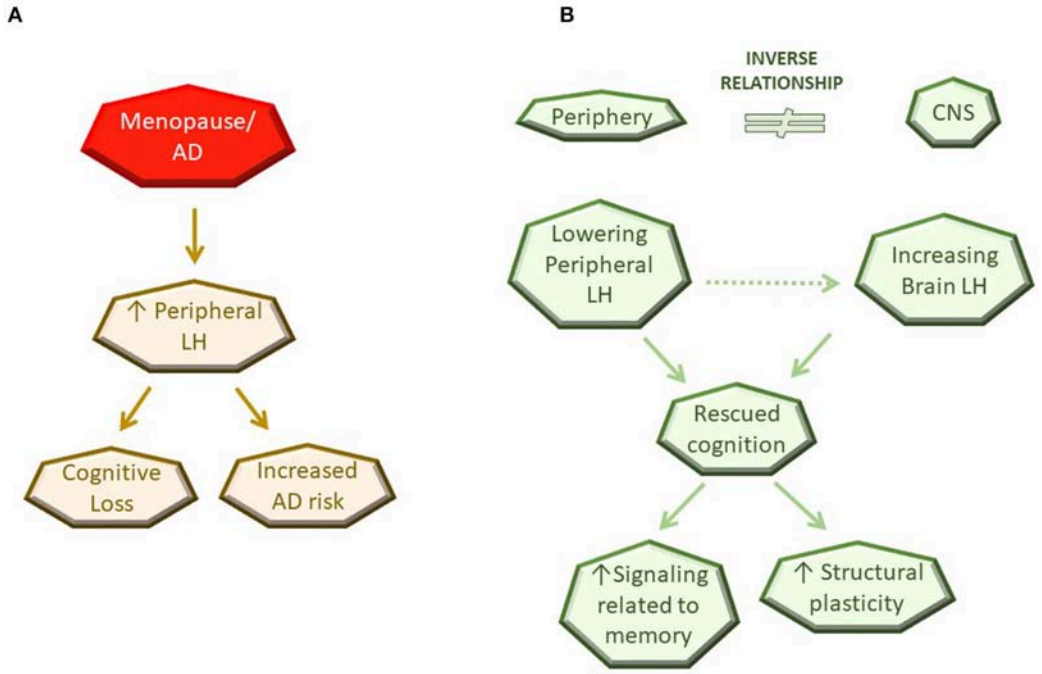

FIGURE 1 | Schematic diagram of current working model for LH action in the CNS. **(A)** Observation in the literature. **(B)** Potential mechanisms of LH action.

Additionally, LH mRNA levels are reduced in both the hippocampus and the cortex of human female AD brains (59), although they are increased in the periphery (47, 48). Interestingly, LH immunoreactivity is remarkably reversed when peripheral LH levels are downregulated and lead to improved cognition (59). These experiments, therefore, provide ample support to the idea of inverse relationship between peripheral LH and central LH, and further suggest that the LH and hCG in the periphery are capable of self-regulating their levels through short-loop feedback into the brain (105, 107, 108).

Interestingly, ovariectomized rats have no changes in hypothalamic LH, although the pituitary and serum LH levels are elevated eight-fold (104). Furthermore, the self-regulated decrease in peripheral LH in response to IV treatment of hCG and hLH seen in rabbits 2 weeks following castrations was absent 6 weeks following castrations, suggesting the sensitivity of the short-loop changes with time after castration (107). This begs the question of whether self-regulation of hypothalamic LH production is more pertinent than gonadal loss, and importantly, whether loss of this "short" feedback in menopause underlies the "critical period" sensitivity observed for estrogen. The fact that the LHR is rapidly internalized in the presence of its ligands (109–113) may provide a potential explanation for the loss of short-feedback loop, as impairments in LHR signaling due to ligand-induced internalization may reduce the ability to signal back to the hypothalamus. This may also explain the work postulating that activation of LHR is deleterious to cognition (52, 62, 88, 114) as the above studies used supraphysiological doses of exogenous hCG. In one of the studies above, deglycosylated hCG used as the LHR antagonist improved cognition; however, validation of the levels of LHR or verification of full deglycosylation of hCG was not performed (114). Therefore, further studies need to be carried out with careful consideration of LH dose and LHR internalization to clarify the role of LHR in the CNS.

An additional aspect that has confounded our ability to clearly dissect the function of the LHR in the CNS from that of loss of gonadal steroids is the fact that LHR has not been modulated in a brain-specific manner. For example, one article demonstrated that knocking out LHR in an AD mouse model attenuated the pathology and cognitive deficits (115). However, the LHR knockout model was global, not brain-specific, thus early developmental effects due to deficits in sex steroids, which are known in these animals, cannot be separated from the role of LHR loss.

CONCLUSIONS

Among a multitude of changes that occur with aging, menopause is a clear driver for development of AD in women. The role of steroid hormones in CNS function and structure, particularly during reproductive stages, is undisputed and here we provide evidence that LH and its receptor are important in CNS function as well as dysfunction as depicted in the working model of LH action in **Figure 1**. We also highlight a potential mechanism, specifically the inverse relationship of LH levels in the CNS vs. the periphery, to provide an explanation for the paradox observed between the ability of the LHR to activate cascades associated

with learning, memory and neuroplasticity, and the findings demonstrating benefits of peripheral LH downregulation on these aspects. Further investigation is necessary to understand how the inverse relationship of LH between the periphery and the CNS is maintained as well as to identify the molecular mechanisms underlying LH-related memory and plasticity benefits in a CNS-specific manner. Genetic approaches targeting the LHR specifically within the brain are likely to be useful models to conclusively address the role of the LHR in CNS function and structure.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

Our works cited in this manuscript have been funded by the NIH grant, RF1 AG054654-01.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 09 April 2019
doi: 10.3389/fendo.2019.00169

Neurosteroid Actions in Memory and Neurologic/Neuropsychiatric Disorders

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Memory dysfunction is a symptomatic feature of many neurologic and neuropsychiatric disorders; however, the basic underlying mechanisms of memory and altered states of circuitry function associated with disorders of memory remain a vast unexplored territory. The initial discovery of endogenous neurosteroids triggered a quest to elucidate their role as neuromodulators in normal and diseased brain function. In this review, based on the perspective of our own research, the advances leading to the discovery of positive and negative neurosteroid allosteric modulators of GABA type-A ($GABA_A$), NMDA, and non-NMDA type glutamate receptors are brought together in a historical and conceptual framework. We extend the analysis toward a state-of-the art view of how neurosteroid modulation of neural circuitry function may affect memory and memory deficits. By aggregating the results from multiple laboratories using both animal models for disease and human clinical research on neuropsychiatric and age-related neurodegenerative disorders, elements of a circuitry level view begins to emerge. Lastly, the effects of both endogenously active and exogenously administered neurosteroids on neural networks across the life span of women and men point to a possible underlying pharmacological connectome by which these neuromodulators might act to modulate memory across diverse altered states of mind.

Keywords: neurosteroid, memory, anxiety, depression, schizophrenia, allopregnanolone, pregnenolone sulfate, Alzheimer's disease

OPEN ACCESS

Edited by:
Vance L. Trudeau, University of Ottawa, Canada

Reviewed by:
David Vaudry, Institut National de la Santé et de la Recherche Médicale (INSERM), France
Xavier Xifró, University of Girona, Spain

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *24 August 2018*
Accepted: *28 February 2019*
Published: *09 April 2019*

Citation:
Ratner MH, Kumaresan V and Farb DH (2019) Neurosteroid Actions in Memory and Neurologic/Neuropsychiatric Disorders. Front. Endocrinol. 10:169. doi: 10.3389/fendo.2019.00169

INTRODUCTION

A major question in neuroscience since the initial discovery that somatically released gut peptides could alter central nervous system (CNS) function relates to whether and how the body can influence or modulate brain function. The science of neuroendocrinology was advanced conceptually 50 years ago by the independent discoveries of Schally, Leeman and Reichlin, demonstrating that the gut peptides thyrotropin-releasing hormone (TRH) (1, 2) and substance P (3, 4) were synthesized, stored, and released in the hypothalamus as endogenous neuromodulators. The demonstration of local synthesis of neuropeptides within the CNS presented a non-canonical mechanism for gut peptides to act as chemical neurotransmitters at synapses, without transport via the systemic circulation and transport across the blood-brain barrier (BBB).

The BBB does not impair access of sex steroids to the CNS to the same extent as gut peptides. Lipophilic steroid hormones, such as progesterone, estradiol and testosterone cross the BBB and readily gain access to the CNS (5) where they can serve as agonists of steroid hormone receptors that

in turn act at genomic response elements. In the early 1980s, several lines of evidence from Etienne, Baulieu, and Robel (6–9) challenged the central dogma that neuroactive steroids were exclusively synthesized peripherally, demonstrating for the first time that steroids could be synthesized from cholesterol within the CNS.

Such steroids were called *neurosteroids* and an intensive search began to identify which steroids belonged to this group and to define their function. An early clue came from the research of Selye (10) showing that steroids could have anesthetic effects. Four decades later, in 1983, radiolabeling studies by Sapolsky, McEwen, and Rainbow revealed uptake of corticosterone in the stratum oriens and apical dendrite regions of the hippocampus, suggesting that GABAergic interneurons in these regions might possess corticosterone receptors (11). Corticosterone treatment had been shown to affect GABA uptake in the hippocampus, possibly suggesting a mechanism for hormonal modulation of memory. In a seemingly unrelated study, while investigating the pharmacological mechanism of action of the synthetic steroid anesthetic alphaxalone, Harrison and Simmonds (12) demonstrated that alphaxalone and barbiturates shared a common mechanism of action via augmenting $GABA_AR$ action. Subsequent research by multiple investigators demonstrated that several reduced metabolites of progesterone and deoxycorticosterone act as positive allosteric modulators of $GABA_ARs$ (13–17), much like benzodiazepines (18, 19). Other research (20, 21) also suggested that neurosteroids might be capable of modulating inhibitory GABAergic neurotransmission.

As new ideas emerged from clinical studies by Andrew Herzog in the mid 1980s concerning the possible role of estrogen and progesterone in catamenial epilepsy (22), we hypothesized that progesterone might act as a positive allosteric modulator of the $GABA_AR$. This led to the early work of Fong-sen Wu and Terrell Gibbs in my lab (23) showing that progesterone did in fact modulate $GABA_A$ and glycine receptors. Unexpectedly, we also found that pregnenolone sulfate (PregS), a novel negatively charged steroid derived from the sulfation of pregnenolone (PREG), potentiated N-methyl-D-aspartate receptor (NMDAR) function (24) (**Figure 1** and **Table 1**).

Over the ensuing 25 years, endogenous neurosteroids have been implicated in learning and memory function, hippocampal information processes, and synaptic plasticity (28, 29, 48, 59–63). Neurosteroids have also been implicated in the etiology and treatment of learning and memory disturbances associated with certain neuropsychiatric disorders, including schizophrenia, depression, and anxiety (50, 64–66) (**Table 2**).

Memory dysfunction is frequently comorbid with age-related neurodegenerative diseases, such as Alzheimer's disease (AD) (84). From a therapeutic standpoint, the lack of an effective treatment for memory disorders extends beyond neurodegeneration to a wide range of neuropsychiatric disorders, such as depression and schizoprhenia.

Memory dysfunction seriously impacts performance of routine tasks necessary for a productive and healthy life, including the ability to maintain gainful employment and compliance with treatment plans (85). This review summarizes the field from the perspective of our own research, which has spanned the past three decades, and attempts to bring together state-of-the-art findings related to the role of neurosteroids in memory dysfunction, as seen in patients with schizophrenia, depression, and anxiety disorders. We believe that a greater understanding of how steroids modulate neural network activity will help lay the foundation for a unifying theory of neurosteroid action in the brain centered on a systems level "pharmacological connectome."

SYNTHESIS, STRUCTURE, TRANSPORT AND CELLULAR TARGETS OF NEUROSTEROIDS

Synthesis and Translocation

Neurosteroid synthesis involves translocation of cholesterol across the mitochondrial membrane by transport proteins, such as the steroidogenic acute regulatory protein (StAR protein), the translocator protein (TSPO), voltage-dependent anion channel (VDAC) protein and the adenine nucleotide transporter (ANT) protein (86–89). The conversion of cholesterol to PREG is catalyzed by the enzyme cytochrome P450 side chain cleavage (P450 scc) located on chromosome 15 in humans (90). Other enzymes that play a role in the biosynthesis of neurosteroids include 5α-reductase and 3α-hydroxysteroid dehydrogenase (91–93). These two enzymes are involved in the biosynthesis of allopregnanolone (ALLO) and tetrahydrodeoxycorticosterone (THDOC); the identification of neurons that express these enzymes in the rodent cerebral cortex, hippocampus, olfactory bulb, amygdala, and thalamus suggests that ALLO and THDOC can be synthesized locally from precursors within the CNS (94).

The sulfation and desulfation of neurosteroids further alters both the pharmacokinetic and pharmacodynamic properties of these endogenous neuromodulators (95). In humans, sulfation of PREG to PregS is catalyzed by SULT2B1a, whereas SULT2B1b

Abbreviations: ACTH, adrenocorticotropic hormone; AD, Alzheimer's disease; ALLO, allopregnanolone; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANT, adenine nucleotide transporter protein; BBB, blood-brain barrier; BNST, bed nucleus of the stria terminalis; CB1, cannabinoid receptor 1; CICR, Ca^{2+}-induced Ca^{2+} release; CNS, central nervous system; DA, dopamine; DAT, dopamine transporter; DAT KO, dopamine transporter knockout; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; GABA, gamma-aminobutyric acid; GAD, generalized anxiety disorder; GLIC, Gloeobacter ligand-gated ion channels; GnRH, gonadotrophin-releasing hormone; HPA axis, hypothalamic-pituitary-adrenal axis; HRSA, Hamilton Rating Scale for Anxiety; IL-6, interleukin-6; L-Type VGCC, L-type voltage-gated Ca^{2+} channel; LTD, long-term depression; LTP, long-term potentiation; MCI, mild cognitive impairment; NMDA, N-methyl-D-aspartate; OATPs, organic anion transporting peptides; ORAI1, Ca^{2+} release-activated Ca^{2+} channel protein 1; PAHS, pregnanolone hemisuccinate; pCREB, phosphorylated CREB; PET, positron emission tomography; PCOS, polycystic ovary syndrome; PREG, pregnenolone; PregS, pregnenolone sulfate; PTSD, post-traumatic stress disorder; PV, parvalbumin; PXR, pregnane xenobiotic receptor; P450 scc, cytochrome P450 side chain cleavage; sEPSC, spontaneous excitatory post-synaptic currents; 5-HT, serotonin; SSRIs, selective serotonin reuptake inhibitors; StAR protein, steroidogenic acute regulatory protein; STIM1, stromal interaction molecule 1; THDOC, tetrahydrodeoxycorticosterone; TRH, thyrotropin-releasing hormone; TRP channels, transient receptor potential channels; TSPO, translocator protein; VDAC, voltage-dependent anion channel.

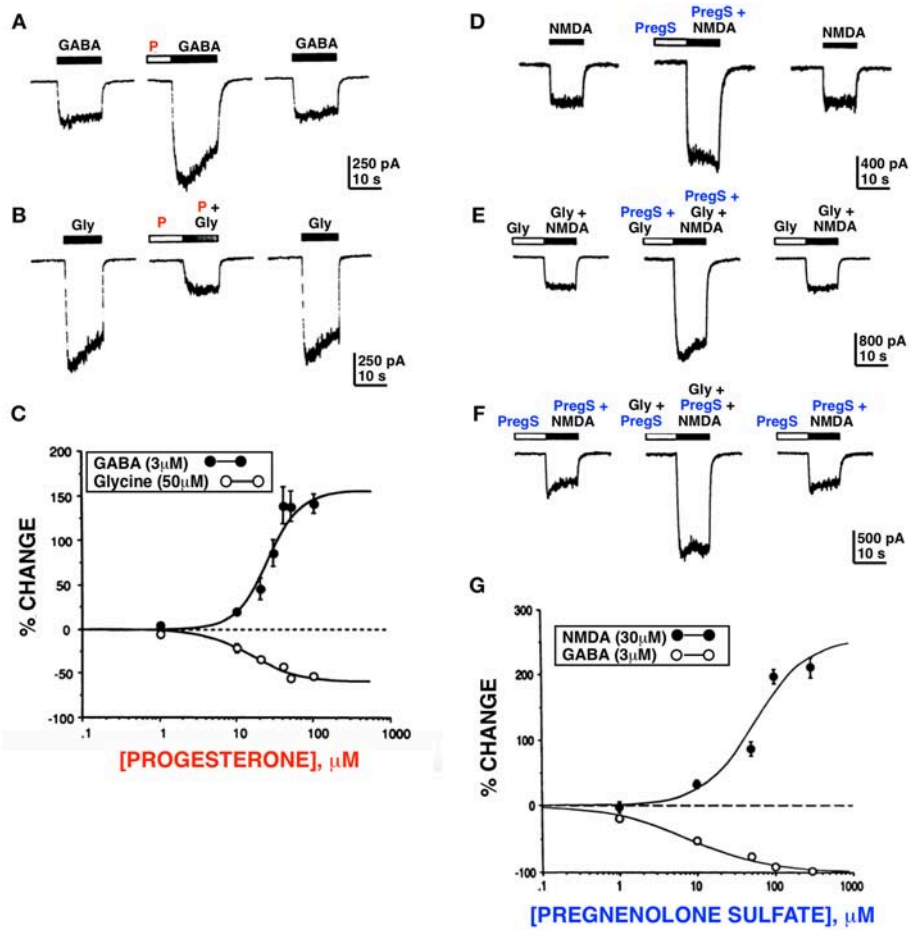

FIGURE 1 | Progesterone and PregS differentially modulate whole cell currents induced by GABA, glycine and NMDA. Progesterone (P) (100 μM) potentiates the GABA response **(A)** and inhibits the glycine **(B)** response. **(C)** Dose response curves for progesterone modulation of GABA and glycine currents; enhancement of the GABA response by progesterone occurs over the same concentration range as inhibition of the glycine response. **(D)** PregS (100 μM) potentiates the NMDA response (normal media [Gly]). **(E)** PregS and glycine potentiate NMDA response by different mechanisms. **(F)** In the presence of the maximal concentration (10 μM) of glycine, PregS (100 μM) enhances (179 ± 17.1%; $n = 4$) the response induced by 30 μM of NMDA; **(F)** In the presence of near maximal concentration of PregS (100 μM), glycine (10 μM) reversibly potentiates (210 ± 36.5%; $n = 4$) the NMDA response. **(G)** Dose response curves for PregS modulation of NMDA and GABA currents. Enhancement of the NMDA response by PregS occurs over the same concentration range as inhibition of the GABA response (*Horizontal bar above each trace represents period of drug application*) [Modified from Wu et al. (23, 24) with Permission].

preferentially catalyzes the sulfation of 3beta-hydroxysteroids. Non-human primate studies suggest that age-dependent changes in the expression of these enzymes could play a role in age-related changes in cognitive function (96, 97).

Neurosteroids and their sulfated conjugates can be characterized based on their core backbone structures as pregnanes, pregnenes, androstanes, progesterones, and deoxycorticosterones. Neurosteroids in these respective subcategories include: pregnanolone and pregnanolone sulfate; PREG and PregS; dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS); progesterone and ALLO; and, deoxycorticosterone and THDOC (98). While delineating the neurological function of sulfated neurosteroids remains a frontier in neuroendocrinology, some fundamental progress has been made these past few decades.

Physiological Actions

The physiologic effects of neurosteroids are mediated through direct interactions with neurotransmitter receptors and transporters, and indirectly via promotion of second-messenger signaling cascades (47, 48, 99–103). Their rapid non-genomic effects are exerted via the allosteric modulation of inhibitory and excitatory receptors located in the surface membrane. In some cases, neurosteroids exert genomic effects, at least in part, by activation of intracellular steroid receptors (104). The degree to which neurosteroids produce genomic and non-genomic effects depends on the extent to which they are metabolized (e.g., PREG to progesterone), and the extent to which the parent molecule and its neuroactive metabolites modulate extra- and intracellular receptors (104).

The modulation of GABAergic neurotransmission by neurosteroids is mediated by interactions with allosteric sites

TABLE 1 | Historical discoveries in pregnene series neurosteroids.

References	System and methodology	Key findings and novel outcomes
Majewska and Schwartz (21); Majewska, (25)	Measurement of inhibition of GABA-mediated uptake of labeled chloride in rat brain synaptosome preparations	Demonstration of a possible receptor target for PregS as an inhibitor of $GABA_A$ receptors
Wu et al. (24); Farb et al. (26)	Whole cell patch clamp of NMDARs currents in cultured chick spinal cord neurons	PregS and related sulfated neurosteroids of the pregnene series potentiate NMDARs acting as functional neuromodulators in glutamatergic synaptic transmission
Irwin et al. (27)	Micro-spectrofluorimetric measurement of intracellular calcium in primary neuronal cultures of rat hippocampus	Neurosteroids, such as PregS modulate excitation-inhibition balance in the CNS
Flood et al. (28–30); Plescia et al. (31); Plescia et al. (32); Abdel-Hafiz, (33)	Behavioral assays in rodent models	Pregnene group neurosteroid-mediated enhancement of cognitive function. Subsequent studies provide further demonstration that PREG and its metabolite, PregS, enhances memory.
Park-Chung et al. (34)	Whole cell patch clamp in cultured chick spinal cord neurons	Elucidation of subunit-specific effects of PregS and demonstration that pregnene neurosteroids modulate excitatory ionotropic GluRs.
Park-Chung et al. (35, 36)	Whole cell recordings in cultured chick spinal cord neurons. Structure-activity studies using recordings from recombinant NMDAR expressed in *Xenopus* oocytes	Identification of PregS binding site. First demonstration that steroids function by binding to an extracellular site on NMDAR.
Yaghoubi et al. (37); Malayev et al. (38); Cameron et al. (39)	Voltage clamp recordings of recombinant NMDAR in *Xenopus* oocytes. Bacterial cultures. Intrinsic fluorescence spectroscopy.	PregS positively modulates GluN2A- and GluN2B-containing NMDARs. PregS inhibits GluN2C- and GluN2D-containing NMDARs and AMPA/kainate receptors.
Partridge and Valenzuela, (40); Sliwinski et al. (41); Sabeti et al. (42)	Measurement of long-term potentiation using hippocampal slice electrophysiology	PregS modulates synaptic strength critical for learning and memory. nM PregS: modulates LTP via NMDARs; modulates presynaptic release of glutamate; voltage-gated Ca^{2+} channel induced LTP potentiation.
Jang et al. (43); Horak et al. (44); Kostakis et al. (45)	Electrophysiology; molecular modeling; recombinant chimeric NMDARs, with altered residues by means of site directed mutagenesis expressed in *Xenopus* oocytes.	PregS exhibits a rich modulatory repertoire enabled by the structural diversity of NMDARs. The extracellular steroid-modulatory site (SMD1) contains the J/K helices and contiguous TMD4. Extracellular loop between TMD3 and 4 mediates both excitatory and inhibitory effects.
Petrovic et al. (46)	Voltage-clamp studies in HEK293 cells expressing NR1/NR2B NMDARs and cultured rat hippocampal neurons.	PregS influences NMDAR-dependent responses via a phosphorylation dependent mechanism.
Kostakis et al. (47); Smith et al. (48)	Whole cell recordings of recombinant receptors expressed in oocytes and $[Ca^{2+}]i$ imaging using and primary neuronal cultures of embryonic cortical neurons	First demonstration that physiologically relevant concentrations of PregS modulate synaptic plasticity *in vitro*. Picomolar concentrations are sufficient to increase intracellular Ca^{2+}. Increased intracellular Ca^{2+} increases surface GluN1-NMDARs and CREB activation. PregS mediated modulation of NMDARs results in delayed onset potentiation occurs via a non-canonical G-protein and Ca^{2+} dependent manner. This potentiation is absent when the J/K helices and TMD4 of GluN2B are replaced with that of GluN2D further establishing the subunit-dependent action of PregS and importantly the extracellular binding site of PregS.
Smith et al. (48); Adamusová et al. (49)	$[Ca^{2+}]i$ imaging studies using primary rat hippocampal neuronal cultures and HEK293 cells	Picomolar to femtomolar concentrations of PregS increases intracellular $Ca2^{+}$
Marx et al. (50–52); Ritsner et al. (53)	Human subjects for clinical effects	Adjunctive treatment with PREG in schizophrenia and schizoaffective disorders shown to reduce negative symptoms and improve positive symptoms of verbal memory and attention. Post-treatment elevation of ALLO and PregS correlate with enhancement of cognitive function. Metabolism of PREG to PregS likely ameliorating NMDAR hypofunction implicated in schizophrenia.
Wilding et al. (54)	Whole cell recordings and molecular modeling using recombinant chimeric GluN and GluK2 receptor constructs in HEK 293 cells	Confirmation of binding sites and relationship to specific receptor domains elucidated. Confirmed extracellularly directed binding site for PregS. Requirement of TMD likely for pore formation.
Paul et al. (55); Linsenbardt et al. (56)	Investigations of synthetic PregS analogs and oxysterols as therapeutics using *in vitro* and *ex vivo* electrophysiological methods	The major brain-derived cholesterol metabolite, 24(S)-hydroxy cholesterol modulates NMDARs by binding to an intracellular site. This intracellular oxysterol binding site is distinct from the extracellular site that bind PregS.
Vyklicky et al. (57)	Electrophysiological investigations of *de novo* missense mutations of the hGluN2B expressed in HEK cells.	Missense mutations of the hGluN2B subunit located in membrane domains lead to multiple defects that manifest by the NMDAR loss of function that can be rectified by steroids.
Chisari et al. (58)	Hippocampal slice electrophysiology, *in vitro* electrophysiological recordings from cultured hippocampal neurons and *Xenopus* oocytes. expressing recombinant NMDARs.	Analogs of PregS and oxysterols, such as KK169 shown to exhibit properties of PregS.

TABLE 2 | Neurosteroids in human neurologic and neuropsychiatric disorders.

Disorder	Neurosteroid(s)	Clinical response	Memory
ALZHEIMER'S DISEASE			
Temporal cortex: Naylor et al. (67)	Increased DHEA and PREG decrease ALLO levels	ALLO levels inversely correlate with Braak and Braak neuropathological stage	NR
Striatum and cerebellum: Hypothalamus: Weill-Engerer et al. (60)	Low PregS and DHEAS Low DHEAS	Negative correlation between cortical β-amyloid and PregS in striatum and cerebellum Negative correlation between levels of pTau and DHEAS	NR
DHEAS: Carlson et al. (68)	Increases in plasma	AD risk not linked with DHEAS	Increased memory performance
Cortisol: Csernansky et al. (69)	Increases in plasma	More rapid disease progression	Increased memory performance
Cortisol: Carlson et al. (68)	Decreases in plasma	No relationship to AD risk	Increased Delayed Route Recall
DHEA in women: Rasmuson et al. (70)	Increases in serum	Associated with AD risk	NR
DHEA and DHEAS in men: Aldred and Mecocci (71)	Decreases in plasma	Associated with AD risk	NR
Cortisol in men: Rasmuson et al. (70)	Increases in serum	Associated with AD risk	NR
aMCI in men: Cherrier et al. (72)	Testosterone treatment	Reduced depression	Improved verbal memory
MOOD DISORDERS			
GAD in elderly: Mantella et al. (73)	Increased saliva cortisol	Positive correlation between symptoms and saliva cortisol	NR
Generalized social phobia in men: Heydari and Le Mellédo (74)	Decreased plasma PregS	PregS levels lower in generalized social phobia subjects	NR
PTSD in women: Rasmusson et al. (75)	Decreased CSF ALLO	ALLO/DHEA correlates negatively with PTSD and Profile of Mood States depression dejection scores	NR
PTSD in men: Rasmusson et al. (76)	ALLO and pregnanolone CSF	Negative correlation between ALLO + pregnanolone and symptoms severity	NR
Acute stress: Droogleever Fortuyn et al. (77)	Increased plasma ALLO	Peripheral benzodiazepine receptor density increased in blood platelets	NR
Acute psychosocial stress in elderly: Wolf et al. (78)	DHEA at 50 mg/kg/day for 2 weeks	DHEAS lower than in young adults. DHEA replacement increases DHEAS	Enhanced attention; Impaired declarative memory and recall, but not spatial memory.
Dysphoria: Premenstrual Girdler et al. (79)	Increased plasma ALLO/progesterone	Greater levels of premenstrual anxiety	NR
Post-partum depression: Kanes et al. (80, 81)	ALLO	Reduction in hamilton depression rating scale scores.	NR
SCHIZOPHRENIA			
Marx et al. (50)	Adjunctive PREG	Improves negative symptoms and ameliorates cognitive deficits	NR
Marx et al. (51)	Treatment with PREG for 8 weeks	Increases serum PREG and its metabolites ALLO and PregS	Increased serum PREG aligns with BACS score
CATAMENIAL EPILEPSY			
Herzog (22) Herzog and Frye (82)	Progesterone ALLO	Associated with progesterone No association between serum ALLO and seizure frequencies in women treated with progesterone stratified by catamenial vs. non-catamenial epilepsy Serum ALLO correlated with seizure reduction in progesterone-treated women who reported a 3-fold or greater perimenstrual increase in average daily seizure frequency	NR
Partial intractable epilepsy: Valencia-Sanchez et al. (83)	Adjunctive progesterone	No effect on catamenial or non-catamenial seizures	NR

NR, not reported.

on $GABA_AR$s (105–109), and neurosteroids appear to play a role in regulating the expression of specific $GABA_AR$ subunits (63). Classical uncharged neurosteroids modulate inhibitory GABA receptors and neurotransmission. Neurosteroids that are known to be relatively potent positive modulators of GABAergic neurotransmission include ALLO, pregnanolone, and TDHOC.

PregS is a relatively potent positive allosteric modulator of NMDAR-mediated synaptic transmission, while pregnanolone sulfate is a relatively potent negative allosteric modulator of NMDAR-mediated glutamatergic neurotransmission (24, 35, 110).

PregS is the most widely studied neurosteroid that potentiates NMDARs (111, 112). 17-hydroxy-PREG is metabolized to DHEA by cytochrome P450 17α-hydroxylase/17,20-lyase. The sulfated form of DHEA, like the sulfated form of PREG (i.e., PregS), is also an NMDAR potentiator. Electrophysiology studies of recombinant NMDARs expressed in *Xenopus* oocytes have established that the effects of PregS are dependent on NMDAR subunit composition. PregS potentiates GluN2A- and GluN2B-NMDARs, whereas it negatively modulates GluN2C- and GluN2D-NMDARs (38). Long known to be critical for learning and memory, transient activation of NMDARs is required for induction of long-term potentiation (LTP) or strengthening of synaptic transmission, as well as long-term depression (LTD) or weakening of synaptic transmission (113). Activation of NMDARs is crucial for many forms of activity-dependent plasticity responsible for learning and memory in the hippocampus and other brain nuclei (114–118).

PregS also acts as a negative allosteric modulator of GABA, glycine, kainate, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (34). The synthetic analog of PregS, PREG hemisuccinate, and other related PREG derivatives bearing a negative charge, potentiate the NMDA response (119). This observation suggests that positive modulation of NMDARs is not mediated by the sulfate group *per se*. Additional studies using other synthetic analogs of PregS revealed that a negatively charged moiety at the C3 position is, however, essential for positive modulation of NMDARs (35). PregS may also influence NMDAR-dependent responses via a phosphorylation-dependent mechanism (46). Low nanomolar concentrations of PregS induce a delayed onset increase of the neuronal response to NMDA and trafficking of NMDAR to the cell surface through an intracellular Ca^{2+} ($[Ca^{2+}]i$)-dependent and non-canonical mechanism involving G-proteins (47) (**Figure 2**). Moreover, low picomolar PregS concentrations appear to be sufficient to increase $[Ca^{2+}]i$ and CREB phosphorylation (48).

Effects of PregS on NMDARs are diverse (15). NMDARs possess at least two distinct modulatory sites (38). PregS increases the frequency and duration of NMDA-mediated channel opening while it inhibits AMPA and $GABA_AR$s (24, 110). PregS effects are dependent on the subunit composition of NMDARs (45, 120). PregS potentiates recombinant NMDARs with GluN1-1a/GluN2B through a steroid modulating domain in GluN2B that also modulates tonic proton inhibition and is pH independent. PregS-mediated potentiation of GluN-2C-NMDARs is similarly pH-dependent. On the other hand, PregS-mediated potentiation of GluN2A and 2D-NMDARs is enhanced at reduced pH. The presence of GluN1-1b subunit with an N-terminal exon-5 splicing insert modulates the extent of proton-dependent PregS potentiation (43, 45). The differential pH sensitivity of the NMDAR isoforms to modulation by PregS is likely to be critical in view of the importance of proton sensors in CNS health and disease (45). PregS acts at a site distinct from the PregS site and inhibits NMDARs irrespective of subunit composition (35, 38, 46).

PregS increases spontaneous excitatory post-synaptic currents (sEPSC) frequency but not amplitude. This demonstrates PregS-mediated presynaptic regulation of spontaneous glutamate release and points to a potential significant impact of PregS on hippocampal function. Presynaptic transient receptor potential channel (TRP channel) receptor activation by PregS modulates glutamate release and increases sEPSC in acutely isolated hilar neurons of the dentate gyrus, an increase that is blocked by TRP channel antagonists (121). Dong et al. (122) had previously demonstrated presynaptic effects of PregS. Lee et al. (121) identified a role for PregS in eliciting presynaptic plasticity by altering intracellular Ca^{2+} via Ca^{2+}-induced Ca^{2+} release (CICR). Moreover, PregS modulates CICR, which is a key mediator of neuronal plasticity. PregS may affect CICR indirectly by activation of NMDARs or L-type voltage-gated Ca^{2+} channels (L-Type VGCCs) and not by direct activation of the Ca^{2+} release-activated Ca^{2+} channel protein 1 (ORAI1) or stromal interaction molecule 1 (STIM1).

DHEA, which is structurally similar to PREG, is the most abundantly expressed neurosteroid in the human body. This neurosteroid is synthesized in the brain, and higher concentrations are found in the brain than in plasma (123). DHEA and DHEAS are neuroprotective, acting via NMDA and AMPA receptors (124, 125). DHEA also appears to play a role in neuronal cell differentiation and programmed cell death via interactions with neurotrophic tyrosine kinase receptors (126, 127). DHEAS, which is structurally similar to PregS except for substitution of carbonyl oxygen for the acetyl group at C17 on the steroid D ring, potentiates NMDA-mediated Ca^{2+} currents and inhibits $GABA_AR$-mediated chloride currents (124, 128–131). Neurosteroids are neuroprotective and reduce neuroinflammation (124, 125, 132, 133).

The Modulatory Recognition Sites

There is a paucity of information on the direct binding of neurosteroids to receptors and of the mechanisms underpinning neuromodulation (39, 134). The ability of neurosteroids to bind to and activate specific categories and subtypes of neuronal receptors is influenced by: (1) conjugation of the parent molecule with a sulfate group; (2) geometry (planar vs. bent); and (3) charge (38, 119). The complexity of neurosteroid-mediated effects, for instance gating of $GABA_AR$ (109, 135–139) and subunit-specific modulation of glycine and NMDARs (140), suggest the likelihood of multiple binding sites that contribute to potentiating and inhibitory effects (39). The effects of neurosteroids on $GABA_AR$s also appears to involve modulation

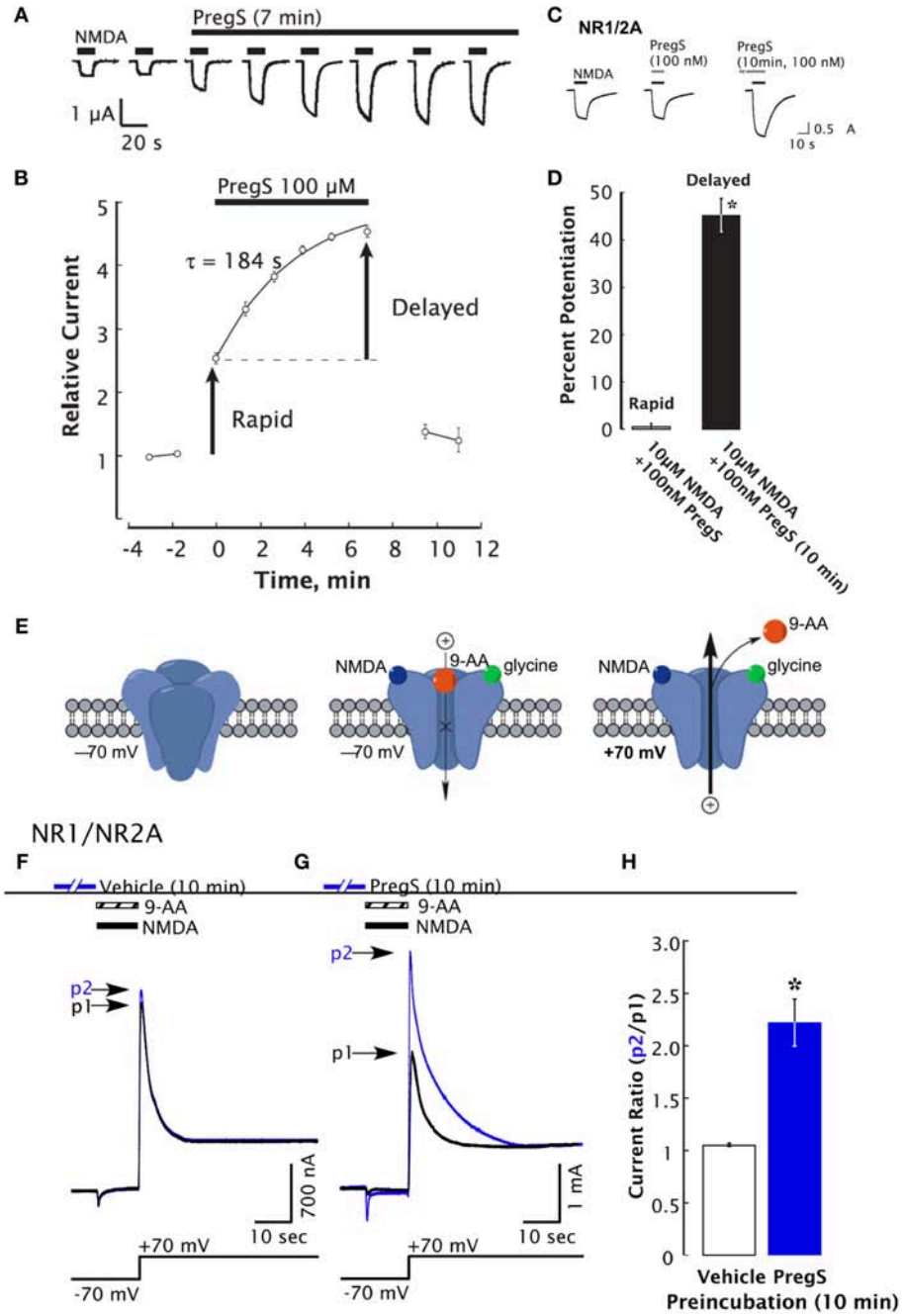

FIGURE 2 | Delayed-onset potentiation of NMDARs is induced by PregS. Two-electrode whole-cell voltage clamp recording from *Xenopus oocytes* expressing NR1/2A subunits following application of PregS (100 μM) and NMDA (300 μM). **(A)** Potentiation of the NMDA response by PregS. Black bars indicate successive applications of NMDA. **(B)** Peak NMDA-induced currents determined as in **(A)** are normalized to the average response before application of PregS beginning at $t = 0$. Smooth curve reflects an exponential fit ($t = 184$ s). Error bars indicate S.E.M. ($n = 3$). Arrows indicate rapid and delayed components of potentiation. **(D)** In oocytes expressing NR1/2A receptors, delayed potentiation but not rapid potentiation is induced by 100 nM PregS. **(D)** Averaged values of normalized peak current responses for rapid and delayed increase. When added simultaneously with NMDA, PregS produces a negligible potentiation of the NMDA response (1 ± 2%), whereas after 10 min pre-incubation with 100 nM PregS, the response to NMDA was enhanced by 45 ± 3%. Error bars represent S.E.M ($n = 8$–10). *Indicates a significant difference between rapid and delayed potentiation ($P < 0.0005$). **(E)** Cartoon shows NMDA and 9-aminoacridine (9-AA) (100 μM) co-applied to *Xenopus oocytes* expressing NR1/2A receptors results in a transient inward current as NMDA-activated channels are blocked by 9-AA (a voltage-dependent open-channel blocker). As the holding potential is switched from −70 to +70 mV, an outward tail current reflecting 9-AA unblock of NMDAR channels ensues (black traces). Cells were then exposed to vehicle (Ba-Ringer) **(F)** or PregS **(G)** for 10 min, and the 9-AA block and unblock sequences were repeated (blue traces). Peak tail currents after baseline subtraction are expressed relative to the control current (black trace) from the same cell (p2/p1). **(H)** The peak current ratio p2/p1 for PregS-treated oocytes (blue bar; $n = 8$) is significantly higher than for vehicle-treated oocytes (white bar; $n = 6$). *Relative current $P < 0.00001$, unpaired 2-tail t-test [From Kostakis et al. (47) with Permission].

of δ subunit-containing receptors which play a role in tonic inhibition (141–145).

The receptor transmembrane domain plays a role in neurosteroid-mediated modulation of NMDARs (43) (**Figure 3**) and $GABA_AR$s (109, 146, 147). Residues in the α1-subunit M1 and/or M2 membrane domains of the $GABA_AR$s are critical for neurosteroid action (109) (**Figure 4**) Recent studies using Gloeobacter ligand-gated ion channels (GLIC), a prototypic pentameric ligand-gated ion channel that is a homolog to the nicotinic acetylcholine receptor, have identified putative intersubunit and intrasubunit neurosteroid binding sites for ALLO within the transmembrane domain (134) (**Figure 5**). Using this innovative approach, Cheng et al. (134) found that substitutions at the 12 and 15 positions on the neurosteroid backbone altered modulation of GLIC channel activity, demonstrating the functional role of both sites. The interaction of neurosteroids with $GABA_AR$ is stereoselective, suggesting that the binding sites for these compounds are of a specific dimension and shape (12, 34, 148).

The results suggest that interactions between the extracellular domain and transmembrane domains play an essential role in the positive and negative modulatory actions of neurosteroids (149). Another important factor determining neurosteroid-dependent modulation of cell function is localization of the neurosteroid. Recent studies have started investigating this aspect using both endogenous neurosteroids and their synthetic

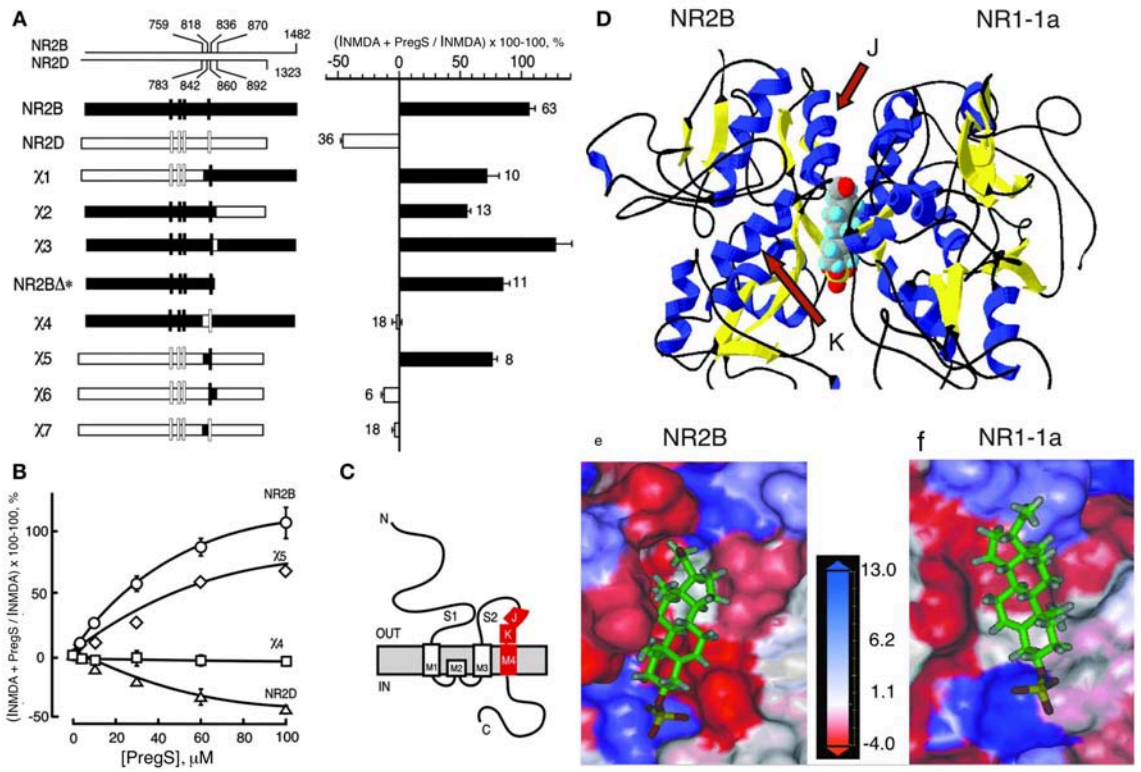

FIGURE 3 | Steroid modulatory domain of NMDARs. **(A)** (Left) Schematic representation of wildtype NR2B, NR2D, and the NR2B, NR2D chimeras. The contribution of NR2B and NR2D to chimeras is depicted in black and white, respectively. The scales at the top indicate the residue numbers in the wildtype subunits at junctions. Vertical bars represent the four hydrophobic membrane domains. (Right) Percent increase in the NMDA glycine response (elicited by 300 μM NMDA and 50 μM glycine in oocytes expressing NR1-1a and NR2 subunits) in the presence of 100 μM PS is indicated. Error bars are SEMs. Numbers adjacent to the error bars indicate the number of oocytes used in the study. **(B)** Concentration–response curves of PregS modulation for receptors containing NR2B (◯), NR2D (△), χ4 (), and χ5 (◇), were determined in the presence of saturating concentrations of NMDA (300 μM) and glycine (50 μM). The EC50 (NMDA) for NR1-1a χ4 and NR1-1a_NR2B are both 22 ± 1 μM, and EC50 (glycine) is 0.30 ± 0.02 and 0.10 ± 0.02 μM, respectively. **(C)** The topological representation of the NR2B subunit and the location of the identified segment are depicted in red. Membrane domains are denoted as M1–M4. The amino terminus (N) is located on the extracellular side and the carboxyl terminus **(C)** on the intracellular side of the plasma membrane. **(D)** Molecular modeling of potential binding pocket for PregS. The dimer comprising the S1/S2 domains of NR2B and NR1-1a is depicted in a 3D ribbon structure with helices colored in blue and sheets colored in yellow with PregS docked at the interface between the two subunits. Our finding that both J and K helices (see arrows) and M4 of the NR2B subunit are required to confer PS potentiation indicates that M4 is also critical in coupling allosteric modulation from extracellular binding regions to the gating mechanism. **(E)** Detailed view of the potential binding pocket for PregS on NR2B. **(F)** Detailed view of the potential binding pocket for PregS on NR1-1a. NR1-1a or NR2B have been removed from the models to show the hydrophobic pocket on NR2B **(E)** or NR1-1a **(F)**, respectively. The receptor surface is colored according to a hydrophobicity scale with hydrophobic residues in red and charged residues in blue. PregS is depicted in a stick configuration and colored by the atom type with hydrogen in white, carbon in green, oxygen in red, and sulfur in yellow. Our finding that both J and K helices (see arrows) and M4 of the NR2B subunit are required to confer PS potentiation indicates that M4 is also critical in coupling allosteric modulation from extracellular binding regions to the gating mechanism [From Jang et al. (43) with Permission].

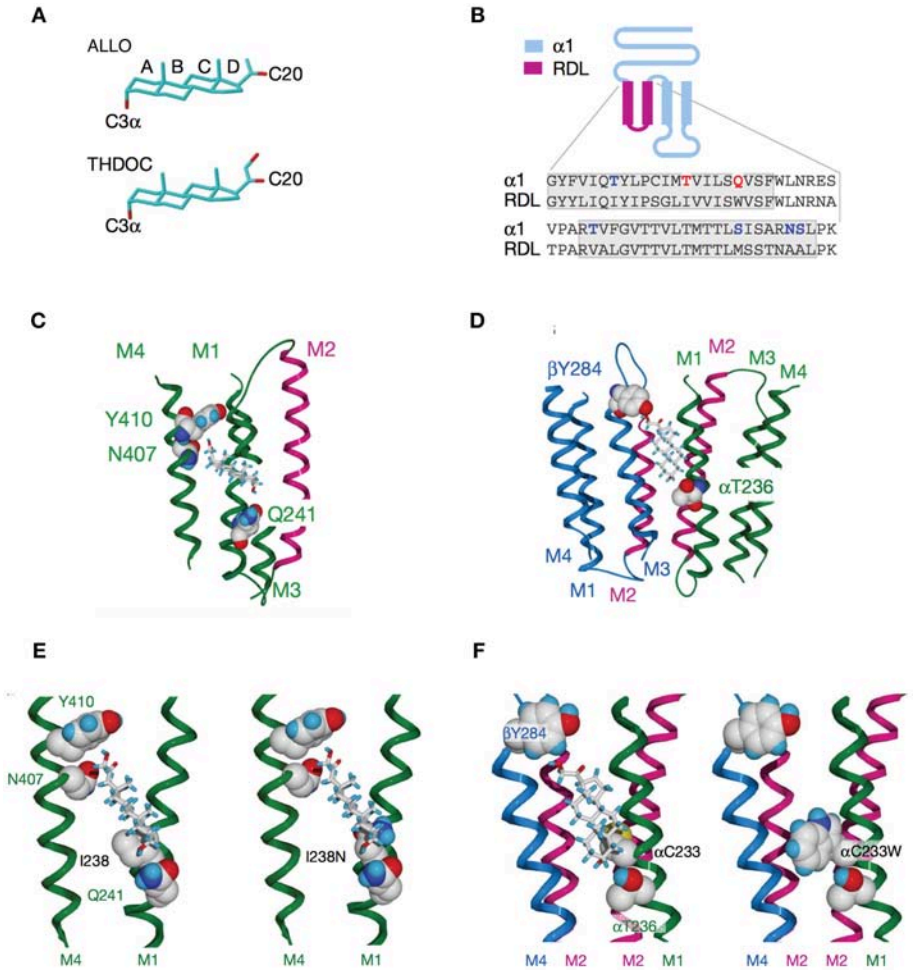

FIGURE 4 | Activation of $GABA_AR$s by neurosteroids, such as ALLO and THDOC depends on occupancy of both the activation and potentiation sites on the transmembrane domain. **(A)** Regulation of $GABA_AR$, is dependent on the C3a hydroxyl group on the A-ring and the C20 ketone on the D-ring shown here in structures of ALLO and THDOC. **(B)** *Neurosteroid activity is determined by α-subunit M1 domain residues.* Replacement of membrane domains M1 through to the end of M2 in the murine α1 and β2 subunits with the corresponding sequence from the RDL subunit, forming the chimeras αR and βR, respectively, was the first modification used to established their $GABA_AR$ pharmacology. Potentiation and direct activation of $GABA_AR$s by THDOC and ALLO was abolished in chimeric receptors incorporating αR; receptors containing βR were indistinguishable from the wild type. Polar residues in α1 (blue) are in bold, with Thr 236 and Gln 241 (red) highlighted. The transmembrane domains are boxed. **(C)** *Neurosteroid potentiation requires α-subunit M1 and M4 membrane domains.* Ribbon structure of α subunit viewed from the lipid bilayer showing αGln 241, αAsn 407, and αTyr 410 docking with a THDOC molecule. The channel lining the M2 membrane domain is shown in purple (a section of M3 domain is omitted for clarity). **(D)** *Neurosteroid activation binding site spans the β/α-subunit interface.* View of transmembrane region (extracellular and cytoplasmic domains removed) with a bound THDOC molecule. Replacing αThr 236 with non-hydrogen-bonding isoleucine or valine reduces the agonist potency of ALLO and THDOC. **(E)** *Neurosteroid potentiation requires α-subunit M1 and M4 membrane domains.* Homology model of THDOC bound to the potentiation site between M1 and M4 membrane domains of the [David: should the following show alpha or beta symbol?] α subunit (M3 membrane domain removed from figure for clarity). Ile 238 is predicted to lie close to the A-ring of THDOC (left). Introduction of a similar-sized but polar side chain at residue 238, such as replacement with asparagine repels the steroid (right). **(F)** Replacement of Cys 233 (left) with tryptophan (right) increases the steric hindrance for THDOC binding to βTyr 284 and αThr 236 [From Figures 1, 3, 4 of Hosie et al. (109)].

analogs. Neurosteroids that can permeate cell membranes can be localized within different intracellular compartments. This compartmentalization is likely to be of importance to the therapeutic function of the neurosteroid (150).

Other known molecular targets of neurosteroids include various TRP channels (151, 152), serotonin receptors and L-Type VGCCs (42). Subtypes of the TRP channels expressed in mammals include TRPC (canonical), TRPV (vallinoid), TRPM (melastatin), and TRPA (ankyrin) channels. PregS modulates Ca^{2+} influx via TRPM3 channels in pancreatic beta cells (151). In addition, TRPM3 is also activated by related substances PREG, DHEA and DHEA sulfate in these studies. Although previous studies by Chen and Wu (153) suggest that PregS activates TRPV1, also known as the capsaicin receptor, other members of the TRPV and TRPM subfamily tested by Wagner and colleagues were not activated by PregS.

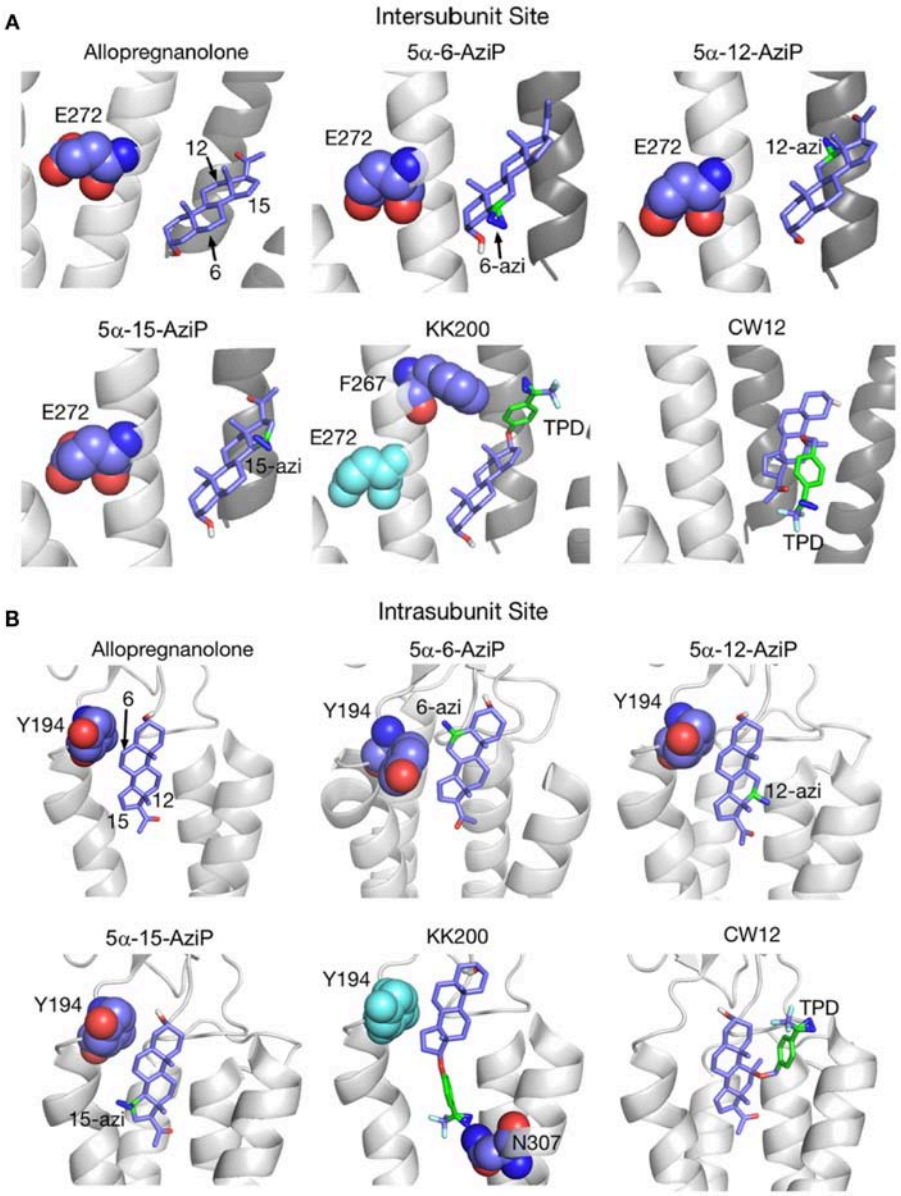

FIGURE 5 | Docking positions of ALLO and photolabeling reagents in the intersubunit and intrasubunit sites. **(A)** Intersubunit docking sites; the photolabeled residues for Glu-272 (5α-6-AziP, 5α-12-AziP, 5α-15-AziP) and Phe-267 (KK200), are shown as purple spheres. **(B)** The intrasubunit docking sites; the photolabeled residues, Tyr-194 (5α-6-AziP) and Asn-307 (KK200), are shown as purple spheres [From Cheng et al. (134), with Permission].

TRPM3 channels are expressed at glutamatergic synapses in neonatal Purkinje cells (154). The effect of PregS on AMPA receptor-mediated miniature excitatory post-synaptic current (mEPSC) frequency is blocked by lanthanide^{3+}, a non-selective TRP channel blocker (155), providing support for TRPM3 as a target for PregS modulation of glutamate release. Valenzuela et al. (156) demonstrated that PregS activates silent synapses by promoting Ca^{2+} influx in a TRP channel-dependent manner, increasing presynaptic glutamate release, and insertion of AMPARs into the post-synaptic membrane (**Figure 6**). Based on these results, Valenzuela et al. developed a working model to explain the actions of PregS at glutamatergic synapses (**Figure 7**). This model is consistent with the recent discovery of delayed onset potentiation of the NMDA response reported by Kostakis et al. (47). We subsequently demonstrated that the phenomenon of delayed onset potentiation of the NMDAR response induced by PregS occurs at physiologically relevant picomolar concentrations and is coupled to a downstream signal transduction pathway associated with learning and memory function (**Figures 8**, **9**).

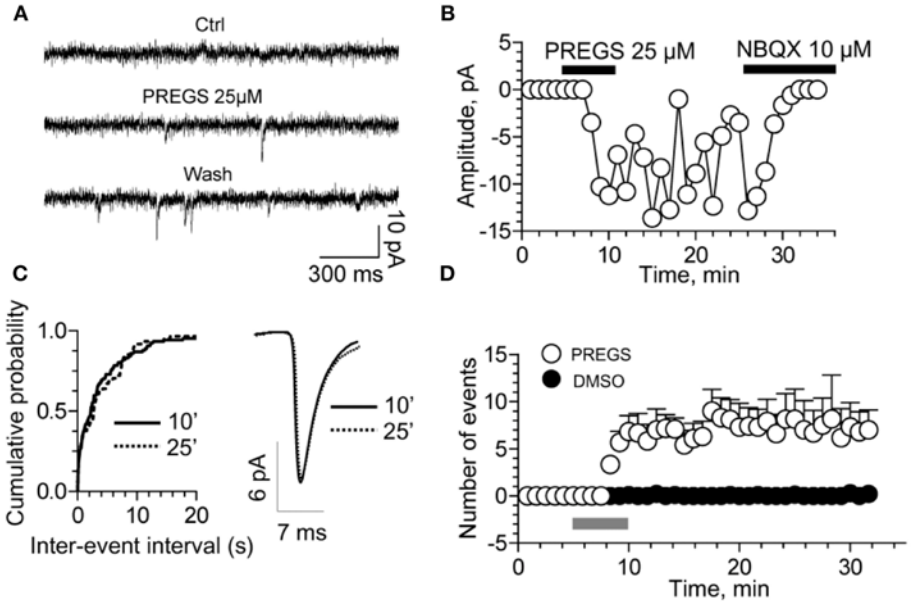

FIGURE 6 | PregS activates silent synapses. **(A)** Sample traces of whole-cell patch-clamp recording from CA1 pyramidal neurons in hippocampal slices from a P3 rat reveal mEPSCs after 25 μM PregS exposure. This effect, which is absent under control conditions, is not reversed by washout. **(B)** Depiction of the time course for the recordings shown in figure **(A)**. Note that NBQX blocks this effect, indicating it is mediated by AMPA receptors. **(C)** Cumulative probability plot. The distribution of the mEPSC inter-event intervals at 10 vs. 25 min is not influenced by PregS exposure. Average mEPSC traces illustrating the lack of an effect of PregS on mEPSC amplitude at these time points. **(D)** Summary figure showing effects of vehicle (DMSO) and PregS (application represented by the gray bar) on the number of events as a function of time (n = 8–12) [From Valenzuela et al. (156) with Permission].

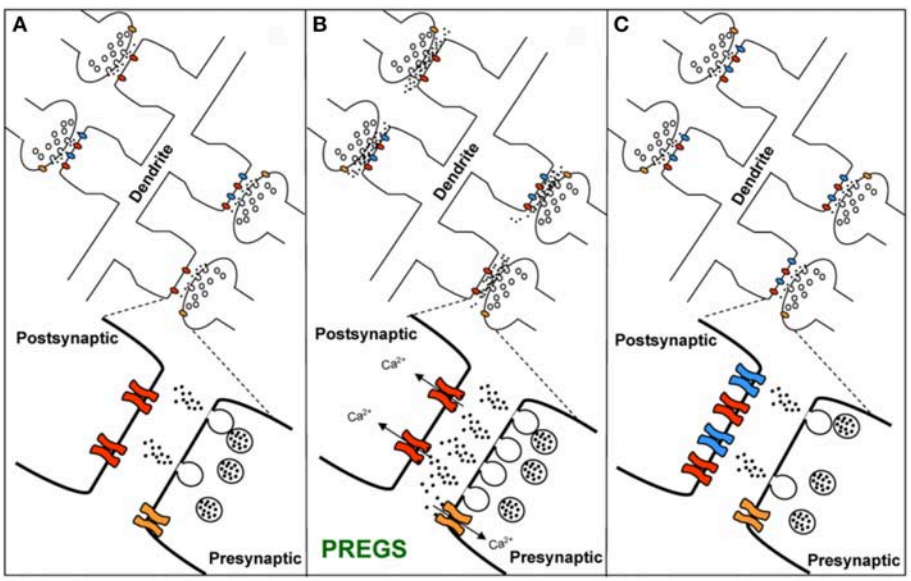

FIGURE 7 | Working model for the action of PregS and related neurosteroids at glutamatergic synapses in immature CA1 hippocampal pyramidal cells. **(A)** Under control conditions, neonatal CA1 hippocampal pyramidal neurons could have a mixture of glutamatergic synapses containing both post-synaptic AMPARs (blue) and NMDARs (red) (active synapses) or only post-synaptic NMDARs (silent synapses at rest). A "*silent synapse*" is enlarged. Presynaptic NMDARs are shown in orange. **(B)** PregS increases Ca^{2+} influx through presynaptic NMDARs, leading to an increase in the probability of glutamate release and activation of post-synaptic NMDARs. Pre- and post-synaptic NMDARs at these synapses are proposed to contain NR2D subunits, making them less sensitive to Mg^{2+} block. **(C)** Glutamate release probability returns to baseline levels after PregS washout. The post-synaptic increase in [Ca^{2+}] shown in panel B elicits delayed insertion of AMPARs only in "*silent synapses*" [From Valenzuela et al. (156) with Permission].

Modulation of TRP channels has been proposed as a therapeutic target for age-related neurologic disorders, such as AD (157). TRPA1 channels regulate astrocyte resting Ca^{2+} and inhibitory synapse efficacy (158). The TRPA1 channel has also been implicated in astrocytic hyperactivity and synaptic dysfunction mediated by Aβ in mouse models of AD (159). Recognized differences in TRPA1 channels across species have been cited as confounding the translational value of results from preclinical rodent models (160). For example, human TRPA1 activity is suppressed by caffeine but mouse TRPA1 channels are activated (161). More work is needed to fully elucidate the role of neurosteroids as functional modulators of the different subtypes of TRPs expressed in the brain.

Transport of Negatively Charged Steroids Across Cell Membranes

Sulfated steroids, namely PregS and DHEAS, are unique in that they are highly negatively charged and thus do not pass across cell membranes without specific transporters. It therefore seems unlikely, although not impossible, that PregS or DHEAS could rapidly associate with a receptor present within the cell without a specific membrane transporter. Our results show that PregS acts extracellularly (35) and that PREG is inactive as a modulator (36–38, 162). For these reasons, PregS more closely resembles the characteristics of classical neurotransmitters, such as acetylcholine, in which the parent molecule bears a full positive charge and is active only at an extracellularly directed recognition site while the immediate breakdown products (choline + acetic acid) are inactive. However, with respect to the neuronal membrane, PregS exhibits full effect even when applied extracellularly to cultured cortical and hippocampal neurons (27, 48) internally dialyzed with the same concentration of PregS via the whole cell patch clamp recording configuration (47).

The recognition site for steroids, such as estrogen (ER), progesterone and its metabolite ALLO is now relatively well-described. For instance, membrane-bound ER receptors, such as ERα localize at the cell surface where they regulate cell signaling mediated by ER produced in the CNS; this function is in addition to their well-recognized classical intracellular localizations where these receptors function to modulate transcription (163–166).

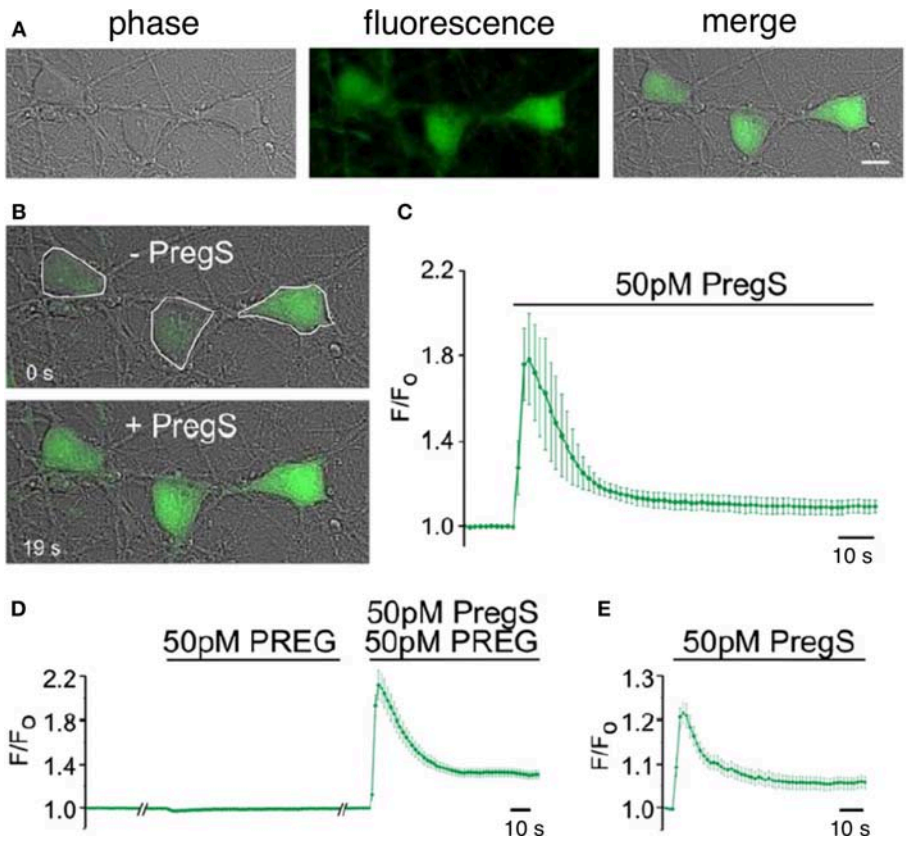

FIGURE 8 | Picomolar concentrations of PregS increase $[Ca^{2+}]_i$ in primary cultured cortical neurons. **(A)** Phase-contrast (left), fluorescence (middle), and merged (right) image of a field of cortical neurons. **(B)** Before (top, 0 s) and after (bottom, 19 s) application of 50 pM PregS. Regions of interest defined morphologically as neuronal cell bodies are outlined in white. **(C)** Mean 6 S.E.M. fluorescence intensity normalized to average initial intensity of the same cell (F/Fo) for 3 neurons in **(B)**. **(D)** Baseline Ca^{2+} fluorescence is shown in the (left) trace. PREG (50 pM) does not increase $[Ca^{2+}]$I (middle trace), whereas a subsequent application of 50 pM PregS in the presence of 50 pM PREG increases $[Ca^{2+}]$i. (right trace) (mean 6 S.E.M., 30 neurons). **(E)** 50 pM PregS increases $[Ca^{2+}]$i in primary cultured hippocampal neurons. Scale bar for **(A,B)**: 10 mm. [From Smith et al. (48) with Permission].

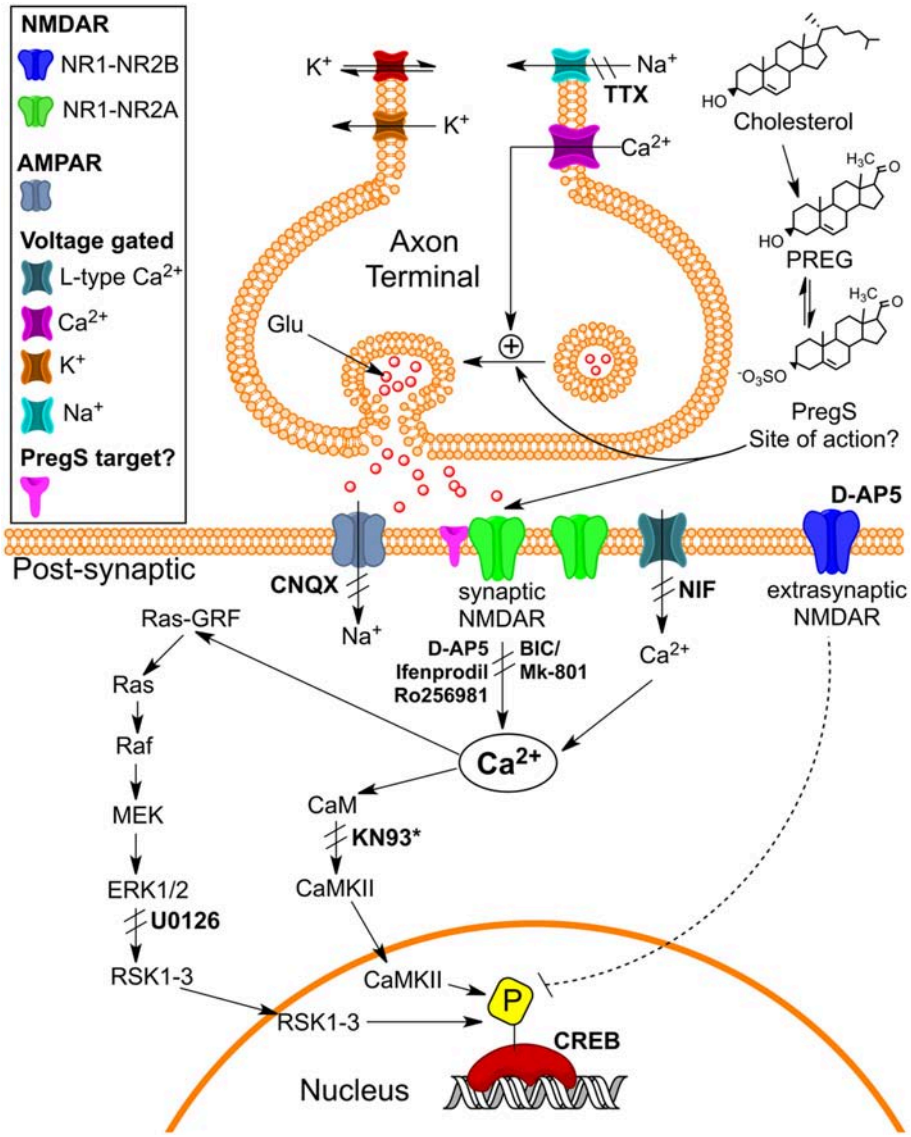

FIGURE 9 | Schematic illustrating pathways that may underlie pM PregS-induced [Ca^{2+}]i and pCREB increases. Diagram illustrates 50 pM PregS-stimulated increase in [Ca^{2+}]i via voltage-gated Na+ channels, NMDARs, and Ca^{2+}L and 50 pM PregS-induced pCREB increases via synaptic NMDAR and ERK activation. Inhibitors used in the study are in bold. *KN93 did not inhibit PregS-induced pCREB increases. [From Smith et al. (48) with Permission].

Interestingly, activation of these membrane receptors leads to rapid, non-genomic effects and regulates neuronal plasticity in the CNS (166–171).

An extracellular site of activation for PregS on endogenous NMDARs in primary rat hippocampal and chick spinal cord neurons in culture, as well as receptors expressed in *Xenopus* oocytes, was demonstrated by Farb et al. (26). Extracellular application of PregS activates NMDARs, whereas intracellular dialysis with PregS fails to elicit a neuronal response or to inhibit PregS applied extracellularly (35). An extracellularly directed PregS -binding domain and obligatory transmembrane domain TM4 participate in the positive allosteric modulation of NMDAR activation by neurotransmitter co-agonists glutamate and glycine [(43, 44) and reviewed by (62, 172)].

These findings were recently confirmed and extended by Wilding et al. (54), who generated chimeric receptors by replacing specific domains of the NMDAR with homologous domains from kainate receptors expressed in non-neuronal HEK293 cells to elucidate the contribution of specific domains to pore formation and allosteric modulation of the NMDARs. By contrast, potentiation by the dihydroxysterol does not require the ligand-binding domain but instead requires a membrane proximal portion of the carboxy terminal domain of the

NMDAR, consistent with an intracellularly directed site for receptor activation. Recently, Chisari et al. (58) have described the characterization of compound analogs of PregS, such as KK169. Like PregS, KK169 has the ability to potentiate NMDAR function, and possesses several characteristics that are consistent with an action for PregS and related sulfated steroids at a cell surface-oriented activation domain (35, 43). Interestingly, KK169 does not inhibit oxysterol potentiation of the NMDAR, consistent with its action at an extracellularly directed binding site (similar to that for PregS). However, some sequestration of KK169 was observed in cultured hippocampal neurons, revealing a possible mechanism for membrane transport and accumulation.

These considerations also relate to question of whether PregS might be able to cross the BBB, with the clear expectation that a specific transmembrane transporter would be needed. In fact, such a transporter has been observed in non-fenestrated intracerebral capillaries (173–177) and could well be present in glia and/or neurons, providing a possible pathway toward sequestration.

NEUROSTEROIDS AND MEMORY FUNCTION

PREG was initially thought of only as a precursor for other steroids and not as an active modulator. Fluorescence spectroscopy studies of the binding of PREG and the related sulfated neurosteroid 3α-hydroxy-5β-pregnan-20-one sulfate, which differentially modulate NMDA and AMPA receptors, suggest that the differential effects of these sulfated neurosteroids on current flow may be related to their binding at the SIS2 and amino terminal domains of these receptors (39). Cannabinoid receptor 1 (CB1) has been identified as a molecular target for PREG (178). Tetrahydrocannabinol or THC, the active ingredient in cannabis, induces PREG synthesis in a CB1 receptor-dependent manner. PREG then acts as an allosteric negative modulator of CB1 receptor in an autocrine-paracrine loop in the brain acting to ameliorate cannabis intoxication. CB1 receptor activation is well-known to modulate learning and memory function by depressing neurotransmitter release. Interestingly, two cannabinoid receptor-mediated signaling cascades have been identified: one is PREG sensitive and targets the vesicular protein Munc-18-1, thereby depressing transmitter release; the other is PREG-insensitive and involves the lateral perforant path of the hippocampus (179). When given as an adjunctive treatment to patients diagnosed with schizophrenia, PREG both improves negative symptoms and ameliorates cognitive deficits (50).

Although controversy still remains with respect to the ability of systemically administered sulfated neurosteroids to cross the BBB, acute treatment with PregS, which is well-recognized for its actions as a positive allosteric modulator of NMDARs, has been associated with an improvement in learning and memory function (21, 24, 31, 34, 35, 162, 180–185). The influx of the sulfated compounds is dependent upon transporters, such as organic anion transporting peptides (OATPs) situated in the BBB and choroid plexus (173, 177, 186) (**Figure 10**).

The role of these transporters in the efflux of negatively charged sulfate steroids across the BBB from the CNS into the systemic circulation is fairly well-established; however, the specific mechanisms associated with brain influx have not been fully elucidated, despite the finding that systemic administration of sulfated steroids produces effects on cognition including improved learning and memory function (31, 173, 176). It had been suggested, based on studies looking at the expression of 17α-Hydroxylase/C17-20-lyase and hydroxysteroid sulfotransferase, that sulfated steroids, such as DHEAS are unlikely to be synthesized *de novo* within the human hippocampus (186). This work has led to the suggestion that sulfated neurosteroids must be transported from the periphery into the CNS, but this hypothesis has not been validated (186). Using *in situ* rat brain perfusion, Qaiser et al. (176) found that PregS enters the brain more rapidly than DHEAS and that both sulfated steroids undergo extensive desulfation mediated by sulfatase located in the capillary fraction of the BBB. While more work is clearly needed to parse out these complex relationships (95), systemic administration of conjugated neurosteroids can nevertheless result in increased CNS levels of the unsulfated neurosteroids (176).

Whether or not the cognitive enhancing effects are due to PregS or PREG remains to be determined. However, the cognitive enhancing effects of PregS in healthy subjects are unlikely to be due to the PREG metabolite ALLO since acute administration of this and other GABAergic modulating neurosteroids to healthy subjects inhibits learning and memory function in a manner similar to benzodiazepines (187–191). On the contrary, ALLO may play a role in the effects of PREG seen in patients with schizophrenia and other neuropsychiatric and neurologic disorders in which neural network activity is dysregulated (51).

Unlike synthetic pharmaceuticals, the literature reporting on the role of neurosteroids in learning and memory function includes some studies looking at the role of endogenous levels and other studies looking at the effects of systemically administered neurosteroids. Because of this, there are several important factors that must be considered when interpreting the results of these studies, including: (1) metabolism of the parent neurosteroid molecule and active metabolites may have a different mechanisms of action; (2) dosing schedules can be acute or chronic; and (3) pharmacokinetic and pharmacodynamic interactions between the neurosteroids and endogenous levels of circulating steroid hormones can fluctuate over time.

Performance on tests of memory function also depends on when neurosteroids are administered in relationship to training or testing. For example, administration of neurosteroids before training can influence both acquisition and consolidation of new information, whereas administration after training is expected to influence consolidation and recall but not acquisition. The type of memory function being assessed (e.g., working memory vs. long-term memory) may also be

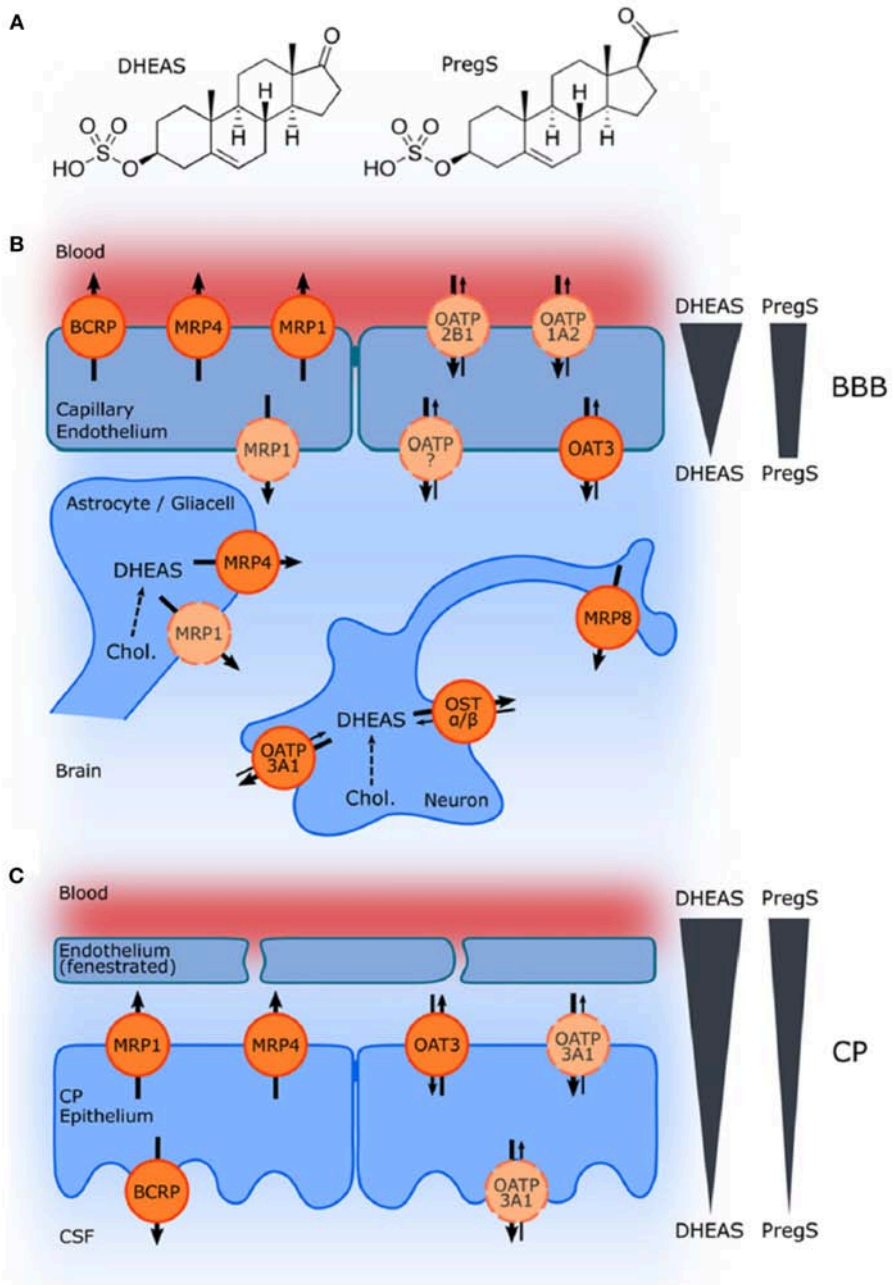

FIGURE 10 | Schematic illustration of ABC and SLC transporters putatively involved in DHEAS and PregS transport and their proposed localization. **(A)** Structures of DHEAS and PregS. **(B,C)** The ATP-binding cassette (ABC) and solute carrier (SLC)-type membrane proteins facilitate transport of neurosteroids at the BBB and in the choroid plexus (CP) The ABC transporters BCRP (ABCG2), MRP1, MRP4, and MRP8 (ABCC1, ABCC4, and ABCC11) facilitate efflux of conjugated steroids. The solute carriers OAT3 (SLC22A3), OATP1A2 (SLCO1A2), OATP2B1 (SLCO2B1), and OSTa/ß (SLC51A/B) are implicated in secretion of sulfated steroids from neurons and glial cells and in their transport across the BBB as well as blood–CSF barrier in the CP**.** Arrows indicate the directions of substrate transport. Proteins for which there is little or controversial evidence for expression and localization in the basal or apical membrane are indicated in light orange and by a dashed line. The DHEAS and PregS concentration gradients across the BBB and in the CP are depicted on the right [From Grube et al. (177) with Permission].

more or less sensitive to the effects of neurosteroids. Finally, the age and cognitive status of the study subjects must be considered. Each of these factors is explored in greater detail below.

Mechanism of Action and Metabolism of Parent Molecule to Active Metabolites

Neurosteroids are metabolized to other neurosteroids that can also influence neurologic function. For example, PREG is a

lipophilic precursor of ALLO, which is also a metabolite of progesterone. Administration of a loading dose of PREG is associated with an increase in serum ALLO levels (192). Although PREG is preferentially metabolized to ALLO, it is also metabolized to progesterone and DHEA (51, 52, 104). The extent to which a neurosteroid, such as PREG is metabolized to progesterone and vice versa determines whether the dose administered will modulate extra- or intracellular receptors to produce non-genomic vs. genomic effects (104).

Administration of 5α-reductase inhibitors, such as finasteride appears to prevent the metabolism of progesterone to ALLO and may thereby influence the neuromodulatory effects of endogenous as well as exogenous sources of these neurosteroids (193–195). Studies looking at the direct infusion of PregS into the nucleus basalis show that this neurosteroid improves spatial memory function in rats and increases acetylcholine release in the basolateral amygdala and frontoparietal cortex (196). Despite these promnestic effect, the metabolism of sulfated neurosteroids, such as PregS by sulfatases, coupled with limited transport across the BBB, has impeded the development of this compound as a novel therapeutic. This limitation can be overcome by using synthetic analogs (132).

Acute vs. Chronic Dosing Schedules

Acute administration of ALLO improves memory function in one mouse model of AD (197), but other studies using rodent models suggest that chronic exposure to ALLO may actually cause memory deficits and exacerbate disease-related functional impairments (198–200). The observation that ALLO transiently increases CREB phosphorylation and increases indicators of neurogenesis in wildtype rats (201) suggests that the acute and chronic effects may not be the same. The translational relevance of these observations to human subjects is unclear since, although men show an age-related decrease in serum ALLO, a similar age-dependent reduction in circulating levels of this neurosteroid is not seen in women (202). On the contrary, women show changes in circulating levels of ALLO that correlate with transient changes in circulating levels of steroid hormones (202). Interestingly, acute systemic administration of ALLO appears to interfere with episodic memory function in healthy women without impairing semantic or working memory function (203), suggesting that brain regions involved in these distinct memory processes, such as the frontal lobes and hippocampus are differentially modulated by this neurosteroid.

Studies looking at brain region-specific effects of neurosteroids on memory function have served to further elucidate the role these neuromodulators play in the acquisition, consolidation and retrieval of information. Systemic administration of the $GABA_AR$ positive modulator ALLO to mice interferes with acquisition and consolidation on a hippocampal-dependent novel object recognition task (204, 205). ALLO impairs memory acquisition/encoding in rodent models when it is injected into the nucleus basalis magnocellularis *before* an acquisition trial, implicating inhibition of cholinergic neurotransmission in the effects of ALLO on memory acquisition (181). Intrahippocampal administration of ALLO into the CA1 subregion *after* the acquisition phase of a passive avoidance paradigm has no measurable effect on retention (206).

Age-Dependent Effects of Neurosteroids

Neuroactive steroid hormones levels change during development, with aging and across the estrous cycle (207, 208). Age-dependent effects of neurosteroids on memory function are seen in rodents and humans. For example, neonatal exposure of female rats to estradiol has been associated with reduced brain levels of ALLO and improved learning and memory function in adulthood (209). It has been suggested, based largely on work in animal models, that enduring changes in $GABA_ARs$ expression induced by developmental exposure to steroid hormones, such as progesterone and its metabolites play a role in hippocampal neuronal excitability and in the etiology of sex-dependent neuropsychiatric disorders and memory deficits later in life (209–211). Administration of the 5α-reductase inhibitor, finasteride to pregnant rat dams late in gestation impairs cognitive and neuroendocrine function in their juvenile offspring (210). Treatment of neonatal female rats with estradiol increases the expression of extrasynaptic α4/δ subunit-containing $GABA_ARs$ and improves performance in the Morris water maze during adulthood (209).

In humans, serum levels of ALLO are normally stable during the first 2 years of life (212). Obese children have been reported to have elevated circulating levels of ALLO, but how this influences their learning and memory function later in life has not been elucidated (213). In post-menopausal women, the effects of ALLO on mood appears to be dose dependent and to follow an inverted U-shaped cure (214). The major contributor to endogenous levels of ALLO is the corpus luteum of the ovary and, not too surprisingly, serum levels of ALLO increase during puberty and with polycystic ovary syndrome (PCOS) (202, 212, 215–218). Functional imaging studies indicate that activity within the right superior and inferior parietal lobes is increased during performance of working memory tasks in untreated women with PCOS. The observed increase in neuronal activity is attenuated by antiandrogen therapy (219).

During the menstrual cycle in humans, ALLO and progesterone levels rise together, with the highest concentrations reached in the luteal phase (202, 220–223). Encoding of emotional memories appears to be better in the luteal phase than in follicular phase (207). It has been suggested that cyclical fluctuations in circulating steroid hormone levels can influence encoding and recall of emotional stimuli, and therefore may play a role in the expression of post-traumatic stress disorder (PTSD) symptomology (224, 225).

In rodents, the onset of puberty is associated with an increase in the expression α4βδ $GABA_ARs$ on dendrites of CA1 hippocampal pyramidal cells (226–228). There is also evidence to suggest that synaptic pruning is influenced by neurosteroids during puberty. Optimal spine density may depend in part on expression and modulation of α4βδ $GABA_ARs$ during puberty, which in turn appears necessary for optimal learning and memory function in adulthood (229). The estrus cycle influences memory function in female rodents (230). Spatial learning

and memory deficits observed on the morning of proestrus phase in rodent are associated with an increased expression of α4βδ $GABA_A$Rs on CA1 pyramidal cell dendrites (230). There is a reversible decrease in the expression of δ-$GABA_A$Rs in parvalbumin containing interneurons in the CA3 hippocampal subregion during pregnancy in rodents, and this change is associated with increased levels of ALLO (231). Reduced expression of δ-$GABA_A$Rs and increased ALLO may serve to counterbalance each other during pregnancy so that tonic inhibition is maintained. It has been suggested that dysregulation of this delicate balance alters cortical activity in the γ frequency range and that measuring drug-induced changes in cortical γ activity could serve as a non-invasive objective biomarker for predicting the efficacy of pharmacological interventions (231). A non-invasive objective biomarker of this type could prove to be very useful in open label studies.

Timing of Neurosteroid Administration in Relationship to Effect on Memory Function

Learning and memory function can be broken down into three phases: (1) acquisition; (encoding); (2) consolidation; and (3) retrieval (recall). As a result, in addition to their unique and sometimes complex mechanisms of action, the timing of neurosteroid administration can also influence performance on specific tests sensitive to the different aspects of learning and memory function. For example, post-acquisition administration of PregS is associated with improved retention in rodents on a passive avoidance paradigm, indicating that this positive modulator of glutamatergic neurotransmission facilitates consolidation (206). Systemic administration of PregS enhances acquisition (31), while direct infusion of this neurosteroid into the lateral septum 30 min before training appears to interfere with acquisition (185). This seeming discrepancy between the two studies can likely be explained by the complexity of the septo-hippocampal interaction and Pregs-induced increase in excitation and/or decreased excitation within the septum (232).

Specific Type of Memory Function Being Assessed

But what about the specific type of memory function being assessed with a specific task; how might this affect interpretation of behavioral response to neurosteroids? Different types of memories require activation of different brain regions. Regional changes in activation are also associated with specific neuropsychiatric and neurologic disorders. Problems with attention, concentration, and motivation can interfere with encoding of new information and, thus, with performance on tests of memory function (233). While the hippocampus is involved in most aspects of learning and memory function, certain types of memory appear to depend less on activation of this brain region than on other regions (234).

The role of neurosteroids in semantic memory function can only be effectively studied in humans who have the inherent ability to verbally express what they know about the world (235). Humans are also capable of using non-episodic strategies on tests looking at the temporal aspects of an episodic memory (236). This distinction can be very important when parsing out the effects of neurosteroid on episodic vs. semantic memory function. For example, systemic administration of ALLO impairs episodic memory but not working or semantic memory function in healthy adult women (203). The effects of neurosteroids on semantic memory function in schizophrenia has not been elucidated; however, PREG is metabolized to ALLO and PregS, both of which appear to improve working memory function in this patient population (51, 52, 65, 237, 238). Whether this effect on performance is mediated by PREG, ALLO, or PregS alone or via a combination of all three neurosteroids has not been determined.

The effects of neurosteroids on learning and memory for fear-inducing stimuli appears to be different in males and females, who show different brain levels of ALLO at baseline as well. Augmenting levels of ALLO in the bed nucleus of the stria terminalis (BNST) of males, who have lower levels of ALLO at baseline, promotes contextual freezing. By contrast, reducing intra-BNST levels of ALLO in females, who have higher brain ALLO levels at baseline, enhances the expression of contextual freezing (239). In addition to differences in responding due to sex-related baseline levels of neurosteroids, different aspects of memory function depend on recruitment of different brain regions, as well as on activation of different subregions within the hippocampus (e.g., dorsal vs. ventral hippocampus) (240).

Learning and memory of coordinated motor activities involves activation of the cerebellum where neurosteroids are synthesized *de novo* by Purkinje cells (241–243). It has recently been suggested that 17β-estradiol may also play a role in cerebellar motor memory formation in male rats (244). Animal studies also suggest that the glutamate-nitric oxide-cyclic GMP pathway is impaired in the cerebellum and cortex of subjects with hyperammonemia due to hepatic failure. Ammonia-induced disruption of this pathway has been implicated in impaired performance on certain types of procedural tasks that depend in part on optimization of motor performance. Restoring the pathway and cyclic GMP levels in the brain restores learning ability. The role of neurosteroids in cerebellar function and the acquisition of motor skills has not been fully elucidated. Elevated levels of GABAergic neurosteroids have been associated with hepatic failure, and it has been suggested that PregS may be of clinical benefit in the treatment of deficits in motor coordination and memory disturbances associated with hyperammonia seen in this patient population (245).

Where within the pharmacological connectome do neurosteroids act to modulate different types of learning and memory function *in vivo*? Not too surprisingly, direct infusion of agonists and antagonists into the brain regions implicated in these different types of memory can either augment or inhibit learning and memory function. For example, the effects of the neurosteroid ALLO on acquisition and extinction of memories associated with exposure to powerful emotion-evoking or fear-inducing stimuli are state independent when it is injected into the amygdala and hippocampus, but state-dependent when injected into the BNST (246). Due to the high concentration of $GABA_A$ receptors in the basolateral amygdala, infusion of

GABAergic neurosteroids, such as ALLO into this brain region interferes with the acquisition and expression of the contextual and auditory cue-induced freezing responses in male rats (246).

Age and Cognitive Status of Subjects at Time of Testing

Neurosteroids are implicated in age- and disease state-dependent impairments in learning and memory function (247–249). Because aging subjects can be comorbid for neuropsychiatric disorders, such as depression and age-related neurodegenerative diseases that both affect memory function (250), understanding the role of neurosteroids in age-related changes in memory function can shed light on potential therapeutic targets suitable for this unique patient population.

Low brain levels of ALLO have been associated with memory impairments in aged rats as well as in human subjects diagnosed with AD (67, 248). In addition, post-mortem studies of human subjects with AD reveal increased brain levels of DHEA and PREG (67). Autopsy studies in humans indicate that brain testosterone levels are lower in patients with AD in comparison to normal men (251) and free testosterone has been implicated as a risk factor for probable AD based on clinical diagnostic criteria (252). However, although low testosterone has been implicated in cognitive deficits in healthy men, testosterone replacement therapy does not appear to significantly improve memory function in cognitively impaired older men with low testosterone (253). It is important to point out that although *in vivo* biomarkers are expected to enhance the pathophysiological specificity of the diagnosis of AD dementia in future studies, most published studies to date looking at the role of neurosteroids on memory function have used core clinical criteria for the diagnosis of possible/probable AD type dementia. Interpretation of such results is hampered in human studies as inclusion of subjects who will ultimately not meet the definitive diagnostic criteria for AD at autopsy is a possible confounding factor.

Decreased plasma levels of DHEA and DHEAS have also been reported in humans with AD (71). It has been suggested that patients meeting clinical criteria for probable AD who have higher baseline levels of DHEAS may perform better on memory tasks than those with lower levels, while by contrast patients with lower circulating levels of cortisol may perform better than those with higher plasma cortisol (68). Men with probable AD based on clinical features of the disease show diurnal changes in cortisol levels characterized by a significant increases at 03 h 00 not seen in healthy elderly men. This increase in cortisol levels occurs despite a slight decrease in levels of adrenocorticotropic hormone (70). Increased levels of DHEA, adrenocorticotropic hormone (ACTH), and interleukin-6 (IL-6) are seen in the morning in women diagnosed with clinical symptoms consistent with AD (70). Cortisol levels are important to the different sleep stages.

Although sleep disturbances are implicated in AD, it is not entirely clear how the aforementioned diurnal changes in cortisol influence sleep and memory consolidation. Sleep plays a role in memory consolidation, and neurosteroids, such as PregS and ALLO can modulate activity within brain regions, such as the pedunculopontine tegmentum nucleus implicated in regulation of sleep (248, 254, 255). Neurosteroids, such as PregS have been implicated in disturbances of sleep and cognitive dysfunction in AD rodent models (247, 256). It has been suggested that the promnestic effects of PregS are mediated by an increase in paradoxical (a.k.a., rapid eye movement; REM) sleep (257, 258), but whether or not the effects of PregS on memory function in subjects with neuropsychiatric disorders and age-related neurodegenerative disease are mediated by enhancement of REM and/or non-REM sleep has not been fully elucidated (259, 260).

Positron emission tomography (PET) imaging studies using the high-affinity sigma-1 (σ-1) receptor selective PET tracer [18F]1-(3-Fluoropropyl)-4-[4-cyanophenoxy) methyl]piperidine ([18F]FPS) as a radioligand suggest that neurosteroids including DHEA bind to σ-1 receptors *in vivo* (261). Sigma-1 receptors modulate NMDA-mediated responses (262, 263). Agonists of the σ-1 receptor, which acts as a molecular chaperone on mitochondria-associated endoplasmic reticulum membranes, appear to provide neuroprotection in rodent models of AD (264).

PET scans using the radiotracer [carbonyl-(11)C]WAY-100635 indicate that progesterone and DHEAS modulate serotonin 1A (5-HT1A) receptor binding *in vivo* (265, 266). While there is evidence of disrupted serotonergic neurotransmission in AD (267), the role of neurosteroids, such as DHEAS in the progression of this age-related neurodegenerative disease has not been fully elucidated.

Hyperactivity of entorhinal cortical and hippocampal circuits are also thought to underlie neurodegenerative disorders, such as AD and mild cognitive impairment (MCI) (268–272). Early synaptic dysfunction or "synaptopathy" at the level of inhibitory interneurons within the entorhinal cortex and CA3 hippocampal subregion leads to hyperactivity of pyramidal cells, which appears to play a role in the progression of AD neuropathology (e.g., tauopathy) (271, 272). This hyperactivity may be due in part to reduced responsiveness of pyramidal neurons to GABAergic inhibitory inputs (272). Other studies implicate a loss of GABAergic interneurons in aging rat models of MCI, suggesting that multiple mechanisms may play a role and/or may interact to contribute to hyperactivity (273, 274). Studies using human tissue from patients with late stage AD reveal hippocampal subregion- and strata specific changes in receptor subunit expression that could potentially influence memory function (275). For example, α5 subunit expression, which is implicated in learning and memory function, is increased in the pyramidal layer and oriens of the CA1 subregion. In addition, expression of the α1 subunit, which is implicated in sedation, is increased in all strata of the CA3 subregion and in the granule cell layer and hilus of the dentate gyrus. An increase in α2 subunit expression, which has been implicated in anxiety, is seen in the oriens and radiatum in the CA3 subregion. Expression of α2 is increased in the oriens of the CA1 subregion, but is decreased in the pyramidal layer. There is a decrease in β3 subunit expression in the granular and molecular layers of the dentate gyrus, while expression of α3 and β1 subunits remain unchanged.

ALLO may be useful in attenuating the hyperactivity implicated in early stage AD (201, 276). In mouse models, ALLO appears to increase neurogenesis, reduce amyloid deposition and

improve performance on learning and memory tests, suggesting that it may serve as a regenerative therapeutic (197, 277–279). A clinical trial for ALLO is ongoing. Other investigations indicate that chronic treatment with riluzole, which includes a reduction in glutamatergic neurotransmission among its mechanisms of action, attenuates the spread of tauopathy in rodent models (280). Is it possible that neurosteroids, such as pregnanolone sulfate and its synthetic analog pregnanolone hemisuccinate, which negatively modulate glutamatergic neurotransmission, may also have potential therapeutic applications in AD and age-related MCI.

Upregulation of TSPO expression has been implicated in neuropsychiatric disorders and neurodegenerative disease (281–283). Oxidative stress is associated with induction of neurosteroid biosynthesis in the human brain (284), and TSPO ligands (*N*,*N*-dialkyl-2-phenylindol-3-ylglyoxylamides) that also show anxiolytic activity promote a reduction in oxidative stress and pro-inflammatory enzymes in glial cells via promotion of neurosteroid synthesis (133). The role of this pathway in the progression of neurodegenerative diseases associated with memory impairments, such as AD has not been fully elucidated. However, increasing oxidative stress by treating oligodendrocytes with beta-amyloid is associated with an increase in the synthesis of DHEA (284), which provides some neuroprotection in a rodent model of AD (285).

Radioligands are being used to measure TSPO expression *in vivo*. PET scans using 11C-PBR28 as a radiolabeled tracer indicate that binding to TSPO is greater in patients with early-onset AD than in those with late-onset disease. Binding to TSPO is also inversely correlated with gray matter volume and performance on measures of cognitive function in all patients with AD. Early-onset patients have greater 11C-PBR28 binding than late-onset patients. Additionally, an increase in TSPO binding is not seen in patients with age-related MCI, even though these patients show increased amyloid pathology as assessed by Pittsburgh Compound B PET scans and hippocampal atrophy as assessed by volumetric analysis of MRI scans (282). The largest differences in TSPO binding in these groups are seen in the temporal and parietal cortices.

These findings collectively point to a potential therapeutic use for neurosteroids in age-related neurodegenerative diseases. The unique modulatory properties of neurosteroids make these particularly well-suited for targeting comorbid anxiety and depression as well as memory deficits in these patient populations.

THE ROLE OF NEUROSTEROIDS IN MEMORY DEFICITS ASSOCIATED WITH STRESS AND ANXIETY DISORDERS

Three large scale major neural networks—the default mode network, central executive network, and salience network—contribute to cognitive processing within the human brain and function together to facilitate adaptive responses of the CNS (286). The *default mode network* plays a role in episodic memory function and self-related cognitive activities including autobiographical memories. Key functional nodes within the *default mode network,* which includes the hippocampus, amygdala, and medial prefrontal cortex, have been implicated in AD and epilepsy (287, 288). The frontal parietal connections of the *central executive network* control attention, working memory, and executive function.

Connections within the central executive network have been implicated in schizophrenia (289). Responses to emotional changes and reward stimuli are dependent on intact function of the *salience network*, within which the anterior insula, anterior cingulate cortex and amygdala, ventral tegmental area, and thalamus function together to segregate the most relevant and rewarding among internal and extra-personal stimuli in order to guide behavior. Stronger connectivity within the salience network has been associated with increased anxiety (290, 291). Pathological activation of the salience and default networks can interfere with the process of switching between these two networks, which would have a differential impact on mood and memory functionality. Neurosteroids can therefore differentially modulate these three major neural networks central to memory processing.

Neurosteroids play a role in anxiety and in the learning and memory deficits associated with certain anxiety disorders. Treatment of subjects under acute psychosocial stress with DHEA (50 mg/day) both improves attention but also impairs declarative memory function (78), suggesting that the benefits in one cognitive domain may be offset by deficits in another at this dose. Neurosteroid modulation of GABAergic neurotransmission in the central amygdala has been implicated in anxiety (131) and the effects of ALLO on anxiety appear to be mediated in part via modulation of activity within the amygdala, which in turn influences neural activity in brain regions involved in learning and memory function (64, 192, 292) (**Figures 11**, **12**). Although the role of 5α-reductase inhibition in the memory deficits associated with anxiety disorders has not been fully elucidated, it has nevertheless been suggested that serum and brain levels of ALLO are increased by 5α-reductase inhibition.

The BNST, which is also referred to as the extended amygdala, receives input from the hippocampus and shares reciprocal projections with the paraventricular nucleus of the thalamus (246, 293–296). This brain regions play a role in adaptive responses of the hypothalamic-pituitary-adrenal axis (HPA axis) to fear-inducing stimuli (239, 246, 296). The effects of ALLO on acquisition and extinction of hippocampal-dependent memories associated with fear-inducing stimuli appear to be state-dependent following direct injections of this neurosteroid into the BNST. Direct injections of ALLO into the BNST during conditioning or testing suppressed contextual fear, but this effect was not seen when the neurosteroid was injected into the BNST during both procedures (246).

Selective serotonin reuptake inhibitors (SSRIs) increase brain levels of ALLO in humans and animals, suggesting that the anxiolytic effects of SSRIs may be related in part to their effect on CNS levels of this endogenous GABAergic modulator (93, 297–301). Patients with anxiety disorders often self-medicate with ethanol. Acute exposure to ethanol also increases brain levels of ALLO (302). By contrast, preclinical studies in a rat

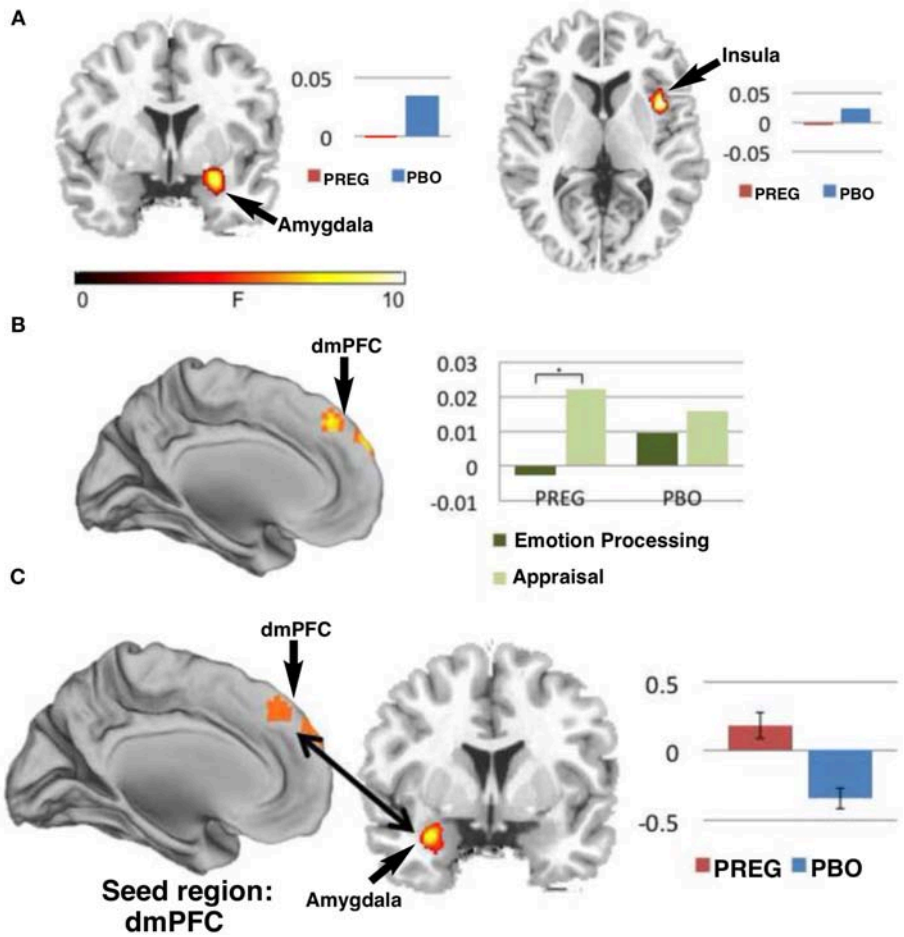

FIGURE 11 | Functional MRI studies show that treatment with PREG enhances activation of neural circuitry involved in emotional regulation as measured by a Shifted-Attention Emotion Appraisal (SEAT) paradigm. The SEAT task is designed to probe multiple aspects of emotional regulation in response to presentation of images depicting neutral, angry, or fearful expressions superimposed on pictures of indoor or outdoor scenes. Sixteen subjects were treated with PREG and 15 were treated with placebo (PBO). Subjects viewed the stimuli through MR-compatible goggles and responded using an MRI-compatible button box. Maps of cerebral activation in each condition indicated that: **(A)** There was a significant ($p < 0.05$) main effect of drug in the amygdala [Montreal Institute (MNI) coronal (y) plane coordinate = 2] and right insula [MNI axial (z) plane coordinate = −6] such that, treatment with PREG decreased activity in these two regions across all conditions and face types. **(B)** Treatment with PREG increased activation in dorsal medial prefrontal cortex (dmPFC) [MNI sagittal (x) plane coordinate = 0] during appraisal. **(C)** PREG treatment also significantly ($p < 0.001$) increased functional connectivity between the dmPFC and left amygdala during appraisal. Self-reported anxiety was inversely correlated ($r = -0.52$, $p = 0.046$) with functional connectivity between the dmPFC and amygdala in the PREG group. Percent signal change is displayed next to each figure [Modified from Sripada et al. (192) with Permission].

model suggest that chronic intermittent exposure to ethanol is associated with decreased ALLO levels in the hippocampus. Although protein levels were not reported, in this study, mRNA levels for 5alpha-reductase and 3alpha-HSD were noted to be reduced in the hippocampus of these rats, which also had impaired performance on a hippocampal-dependent memory test and increased sensitivity to the anxiolytic effects of alphaxalone (303). Could targeting neurosteroidogenesis (304) and/or steroid metabolism be a viable strategy for the treatment of patients presenting with comorbid anxiety and alcohol use disorders?

When compared with normal subjects and patients with major depression, elderly patients with generalized anxiety disorder (GAD) show more deficits on tests of short-term memory function (305). The severity of GAD has been found to positively correlate with cortisol levels in saliva of older adults (73). Elevated salivary cortisol in older adults is associated with impaired performance on tests of memory function (306). Treatment of older adults presenting with GAD and elevated baseline cortisol with SSRIs is associated with a reduction in salivary cortisol that correlates with reductions in anxiety (307). SSRIs increase levels of ALLO (299). and ALLO restores hippocampal-dependent learning and memory function in a rodent model of AD (308). AD is associated with non-cognitive behavioral and psychological symptoms, which can include paranoia and anxiety; therefore, ALLO may be well-suited for targeting these as well as the cognitive deficits associated with this form of dementia (309).

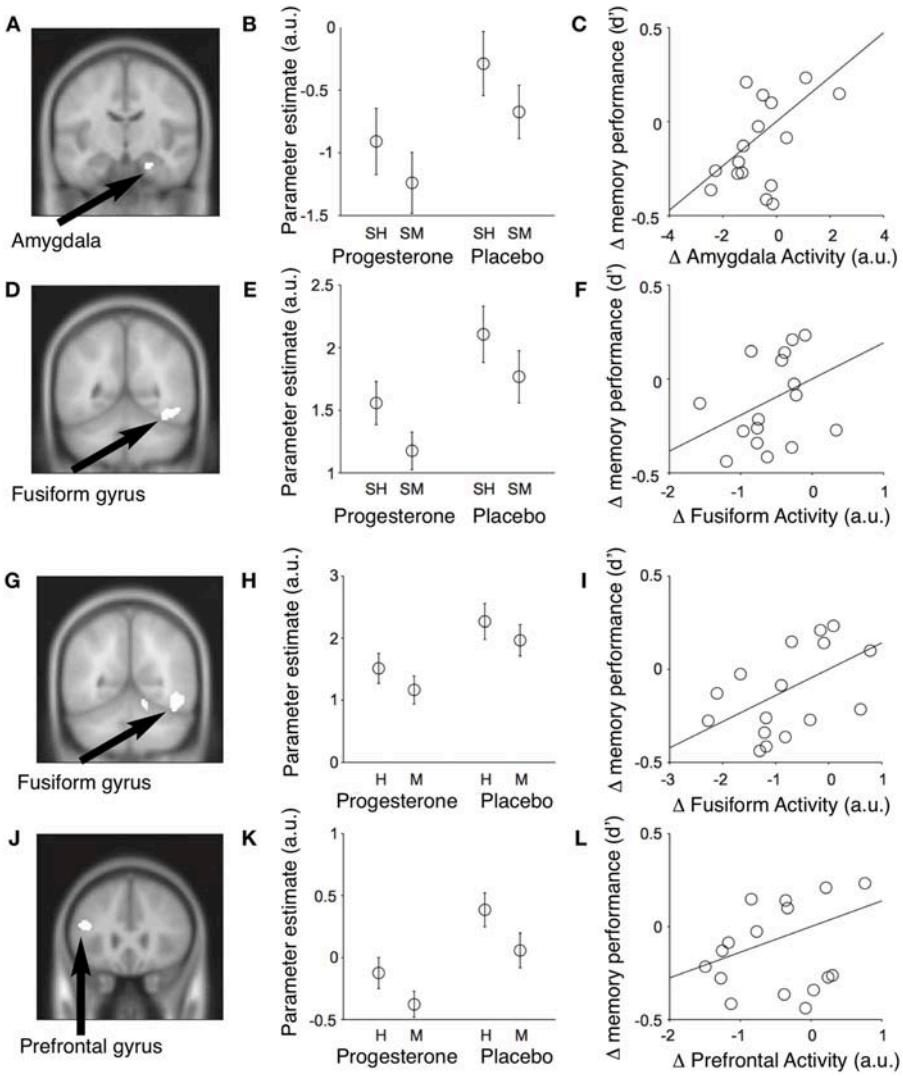

FIGURE 12 | Event-related fMRI studies in female subjects showing effects of progesterone on brain regions involved in the encoding and retrieval of memories of faces. Results of conjunction analyses for the relationships between neural activity in different brain regions and memory performance across subjects. **(A)** During encoding of memories for faces, progesterone administration was associated with reduced activity in the right amygdala (MNI coronal (y) plane coordinate = −12; $p < 0.001$, uncorrected) and fusiform gyrus [MNI coronal (y) plane coordinate = −52] which predicted a decrease in memory performance across subjects. **(B)** Parameter estimates for mean activity within each significant conjunction cluster (SH = subsequent hits; SM = subsequent misses; a.u. = arbitrary units; mean ± SEM). **(C)** The progesterone-induced decrease in amygdalar activity predicted the decrease in memory performance across subjects [recognition memory accuracy = d′]. **(D–F)** Identical figures for the significant conjunction of main effects in the right fusiform gyrus and memory performance. Progesterone reduces activity in the fusiform and inferior frontal gyri and impairs performance during retrieval of memories for faces**. (G)** Significant conjunction of main effect of memory (i.e., hits > misses) and main effect of drug [i.e., placebo (faces > null events) > progesterone (faces > null events)] in the right fusiform gyrus [MNI coronal (y) plane coordinate = −56; $p < 0.001$, uncorrected]. **(H)** Parameter estimates of the conjunction cluster (arbitrary units; mean ± SEM). **(I)** Progesterone-induced reduction in fusiform gyrus activity predicted the decrease in memory performance across subjects [recognition memory accuracy (d′)]. **(J–L)** Identical figures for the significant conjunction of main effects in the left inferior frontal gyrus [MNI coronal (y) plane coordinate = 26]. H, Hits; M, misses; a.u., arbitrary units. H, Hits; M, misses; a.u., arbitrary units [Modified from van Wingen et al. (64) with Permission].

Cortisol seems to enhance treatment outcomes in a small group (males and females) treated with exposure-based group therapy (310). In addition, high endogenous estradiol vs. low estradiol correlates with better treatment outcomes in exposure therapy (311) in female patients with spider phobias. Investigations of neural network activity using intracranial electroencephalography in human subjects indicate that β-frequency coherence (13–30 Hz) between the amygdala and hippocampus encodes variations in mood (312). Stress may mediate changes in mood and cognition in early adolescence and may play a role in the expression of psychopathologies in adulthood. During stress, corticotropin-releasing hormone promotes an increase in adrenocorticotropic hormone release. This promotes an increase in the concentration of cholesterol

at the inner mitochondria membrane of the adrenal cortical cell via activation of the steroidogenic acute regulatory protein. Cholesterol is then converted to PREG and its steroid metabolites, including PregS, cortisol, corticosterone, DHEA, and ALLO (77, 87, 313, 314).

DHEA and DHEAS appear to counteract the negative effects of increased cortisol on working memory function in women and men, respectively (315, 316). A dose-dependent inverted U-shaped response to DHEAS is observed on tests of learning and memory function in male mice (30). By contrast, DHEA is effective in a wider range of doses, suggesting it would be a better choice as a therapeutic (30). Although a positive correlation between DHEAS and cognitive function has been observed in women and men, more research is needed to determine if the effects of DHEA and DHEAS on learning and memory function in humans are sex, dose, and disease state dependent (30, 317, 318).

The response to stress appears to be influenced in part by circulating steroid hormones. Stress increases associative learning and dendritic spine density in the hippocampus of male rats, but impairs associative learning and reduces spine density in females (319). The potential effects of neurosteroids on development of anxiety disorders can be observed during puberty, which is not only associated with increase in levels of reproductive hormones but also with the onset of many psychiatric disorders, including GAD, social anxiety, and panic attacks (320–326). Anxiety symptoms have been found to increase from middle to late adolescence (323), with a particularly high prevalence of all anxiety disorders reported among adolescent girls (324, 327, 328). Of particular interest in this setting are the neurosteroids ALLO (in human and rat) and pregnanolone (in human only), metabolites of the reproductive hormone progesterone that are also produced in the brain in response to stress (79, 329).

Animal studies suggest that developmental exposure to ALLO influences subsequent responsivity to anxiolytics in adulthood (206, 330–332). Acute stress is associated with an increase in plasma ALLO levels that correlates with an increase in expression of the TSPO, also known as the peripheral benzodiazepine receptor. Because the TSPO plays a role in steroidogenesis, it has been considered as a therapeutic target for treatment of anxiety disorders (77, 333). It is interesting to note, that ALLO levels have been found to be decreased relative to controls in the CSF of women with chronic stress disorders, such as PTSD (75). A subsequent study from this group observed a negative correlation between CSF levels of total GABAergic neurosteroids levels (ALLO plus pregnanolone) and PTSD symptoms in men (76). This study also found an association between PTSD and reduced 5α-reductase mediated biosynthesis of ALLO in men, which is in contrast to the block at 3α-HSD previously observed in women with PTSD. Other studies have found that administration of sodium lactate and cholecystokinin tetrapeptide to persons diagnosed with panic disorder decreases plasma concentrations of both pregnanolone and ALLO, and increases the concentration of the functional antagonistic isomer 3β,5α-tetrahydroprogesterone (334). These findings suggest that both acute stress and chronic stress give rise to unique effects on ALLO levels in men and women. Although studies in post-menopausal women did not reveal any benefit on tests of short-term memory function, it has nevertheless been suggested that replenishing this neurosteroid may have beneficial effects on memory function in certain populations with age-related memory deficits (335, 336).

Restoration of altered endogenous neurosteroid levels via modulation of steroidogenesis mediated by pregnane xenobiotic receptors (PXR) and the endocannabinoid system has been suggested as an alternative to direct administration of neurosteroids (89, 178, 337). Because PREG is a precursor for all steroid hormones, it seems plausible that promotion of cortisol synthesis during stress may attenuate synthesis of other steroid hormones, but this suggestion has not been substantiated.

These findings suggest that changes in endogenous brain levels of neurosteroids associated with age, sex, stress, and administration of SSRIs may play a key role in the onset and clinical response to pharmacologic interventions in certain anxiety disorders. Supporting this hypothesis are findings related to the effects of ALLO on gonadotrophin-releasing hormone (GnRH), the primary chemical messenger implicated in the onset of puberty and sexual maturation (338). Central precocious puberty is associated with ALLO, which has been found to suppress the release of hypothalamic GnRH via allosteric modulation of $GABA_A$Rs (339, 340). Although inhibition of GnRH release is increased by ALLO administration before puberty and in adulthood, it is paradoxically reduced during puberty (212).

The reduction in GnRH release during puberty is also associated with increased excitability of pyramidal cells in hippocampal region CA1. This effect appears to be due in part to inhibition of α4-containing $GABA_A$Rs, which are expressed at higher levels than normal in the CA1 region of the hippocampus during puberty. $GABA_A$Rs of the α4β2δ subtype, which have a δ subunit instead of a γ subunit, play a role in tonic inhibition in areas, such as the dentate gyrus and cortex (341). $GABA_A$R-mediated conductance is normally inhibitory; however, the reversal potential of $GABA_A$R-mediated post-synaptic current in dentate gyrus granule cells is "positive" to the resting membrane potential, making membrane hyperpolarization of $GABA_A$Rs unlikely in this region. Inhibition of shunting appears to play a role in overcoming this process, such that non-hyperpolarizing inhibitory conductance reduces the depolarizing effect of post-synaptic potentials by decreasing proximal membrane resistance (342). In the dentate gyrus and cortex, the GABAergic current is inward (i.e., chloride flux is outward) (342, 343), and thus inhibition in these areas is enhanced by ALLO. However, in the CA1 hippocampal subregion the current is normally outward (344), and thus increased expression of α4β2δ GABA receptors paradoxically results in ALLO attenuating rather than enhancing inhibition. The reduction in currents generated by ALLO at α4β2δ $GABA_A$R is dependent upon the presence of arginine 353 in the intracellular loop of α4, where it may serve as a chloride modulatory site (345). This polarity-dependent decrease in

inhibition mediated by ALLO may have important implications for how we approach the memory deficits associated with anxiety disorders, which may prove to be amenable to therapeutic strategies targeting the expression and/or activity of $GABA_AR$s of the α4β2δ subtype (346).

THE ROLE OF NEUROSTEROIDS IN MEMORY DEFICITS ASSOCIATED WITH DEPRESSION

Changes in neurosteroid levels have been implicated in onset of depression and in the actions of medications used to treat depression. Animal studies using a synthetic analog of ALLO suggest that fluctuations in neurosteroids levels may also influence motivation to learn via modulation of dopaminergic pathways (347). The effects of ALLO on mood in women appear to follow an inverted U-shaped curve (214). SSRI treatment has been associated with increased brain levels of ALLO, suggesting that the memory enhancing effects of SSRIs in this patient population may be related in part to the actions of this neurosteroid (93, 297, 348, 349). Preclinical animal studies suggest that modulation of GABAergic neurotransmission by DHEA may also have therapeutic potential in the treatment of memory deficits associated with depression (350).

It is not entirely clear how ALLO, which is a positive allosteric modulator of GABAergic neurotransmission, acts to improve memory function in this patient population, but it has been suggested that stress-induced changes in GABA receptor expression levels are likely to play a role in the etiology of depression. This hypothesis is supported by preclinical studies showing that early life traumatic stress is associated with chronic anxiety, spatial memory deficits and reduced expression of $GABA_AR$ subunits in the adult rat brains (351).

It has been suggested that PREG and its metabolites may be efficacious in the treatment of depressive disorders. Treatment of depression poses several challenges and this is especially true in the treatment of depression in bipolar disorder (BPD). In a study conducted by Brown et al. (352), 80 adults with BPD and depressive mood state were treated with PREG or placebo as add-on therapy for 12 weeks. Outcome measures included the 17-item Hamilton Scale for Depression, Hamilton Rating Scale for Anxiety (HRSA) and Young Mania Rating Scale. Assessment of serum neurosteroid levels at baseline and treatment completion (week 12) revealed large baseline-to exit changes in neurosteroids in the PREG treatment group. In the PREG group, unlike the placebo group, HRSA changes negatively correlated with ALLO and PREG levels, indicative of reduced anxiety. The results of this small study should be interpreted with caution because subjects were taking a wide variety of medications including lithium, antidepressants (unspecified), sedative hypnotics/anxiolytics (unspecified), antipsychotics (unspecified), and stimulants (unspecified) and they were not stratified based on the drugs they were taking for their depression.

THE ROLE OF NEUROSTEROIDS IN MEMORY DEFICITS ASSOCIATED WITH SCHIZOPHRENIA

The cognitive deficits associated with schizophrenia are caused by multiple factors, and elucidating a single causative part of the cognitive component would provide some hope for understanding memory and a basis for therapeutic discovery. Neural circuitry-based studies highlight cortical disinhibition as a critical factor in schizophrenia affecting GABAergic interneurons (parvalbumin, SOM/NPY/CCK/expressing interneurons). GAD67 deficiencies together with changes in $GABA_AR$ expression, in particular the α2 subunit, are known (353–356). Hypofunction of NMDARs, particularly in inhibitory parvalbumin (PV) interneurons (357), may initiate the disease process, leading to decreased inhibition of pyramidal neurons. NMDAR hypofunction in inhibitory interneurons is implicated in the observed GABAergic deficits (358–364). Whatever the specific path for initiating dynamical imbalance or dysregulation of the circuitry, the resultant hyperactivity of downstream pyramidal neurons ultimately leads to dysregulated neural network activity, excitotoxicity, and eventually frank neuronal loss. Note that hypofunction of NMDARs expressed in pyramidal neurons themselves is also likely (365). Disruptions of neural network activity during sleep, including reduced sleep spindle activity, has been associated with impaired sleep dependent memory consolidation in schizophrenia (260).

Synchronous activity of PV interneurons generates gamma oscillations which are observed during performance of cognitive tasks. Cognitive deficits are thought to arise from a disturbance of these high frequency oscillations in the gamma range due to reduced excitatory drive to cortical PV interneurons (366, 367) with resultant network hypersynchrony (368, 369). Manipulations of various receptors such as α5 type $GABA_ARs$ and sodium channels have been shown to enhance cognition (356). The glutamate hypothesis of schizophrenia underscores the importance of NMDARs in neuropsychiatric disorders. NMDARs are critical for the generation of gamma oscillations, and dysfunctional NMDARs result in psychosis and deficits in specific cognitive domains. Genetically engineered mice lacking NMDARs show deficits in habituation, working memory and associative learning (370).

Enhancing NMDAR function is therefore vital for enhancing cognition in healthy individuals and those with neuropsychiatric disorders (111). However, attempts to enhance NMDAR neurotransmission thus far have not been successful (371). A recent meta-analysis of currently available NMDAR positive allosteric modulators reveals that these are ineffective in alleviating cognitive impairments (371). Maintaining a critical balance in NMDAR activation is optimal for avoiding the negative consequences of NMDAR stimulation a, making PregS, DHEAS and their analogs highly suitable candidates. Subunit selective modulation, combined with its ability to potentiate receptor function without overstimulation, renders

PregS a likely and highly suitable positive modulator of NMDARs (111, 371).

PREG and ALLO have been investigated as potential therapeutics for treating memory deficits associated with schizophrenia (50, 65, 238). Acute administration of either clozapine or olanzapine increases brain and plasma levels of PREG, which is a precursor of ALLO and PregS (372). Adrenalectomy prevents clozapine-induced increase in hippocampal PREG. By contrast, significant increases in PREG levels in rat hippocampus are not observed following acute administration of aripiprazole, quetiapine, or ziprasidone administration. This suggests that the effects of second-generation antipsychotics on memory function may be due in part to increased brain levels of PREG and/or PregS (28, 48, 51, 62, 373).

We have previously demonstrated that low picomolar concentrations of PregS are sufficient to increase $[Ca^{2+}]i$ and CREB phosphorylation (48). There is some evidence that sulfotransferase 4A1 haplotype 1 (SULT4A1-1)-positive subjects show a better response to olanzapine than do SULT4A1-1 negative subjects. However, the exact role this sulfotransferase isozyme plays in the formation of PregS from PREG and the amelioration of learning and memory deficits in humans with schizophrenia treated with second-generation antipsychotics has not yet been established (374). ALLO-mediated modulation of GABAergic neurotransmission has also been implicated in the antipsychotic effects of clozapine and olanzapine in rodent models (375–377).

Multiple clinical and preclinical studies have examined the effects of neurosteroids, PREG, PregS and DHEA on memory and other cognitive attributes. Cognitive deficits are a core feature of schizophrenia (378). Recent clinical trials in patients with schizophrenia suggest that treatment with PREG as add-on therapy alleviates cognitive deficits (50). In patients receiving PREG, plasma levels of its immediate metabolite, PregS (positive modulator of NMDARs) and ALLO (positive modulator of $GABA_A$Rs) are elevated, suggesting a possible role for PregS in improvement of learning and memory function (50). Earlier studies (28) had also described the cognition enhancing effects of PregS (29).

Neurosteroids, Dopamine Knockout Mouse Model and Schizophrenia

Dopamine (DA) is a neuromodulator and a key player in several neural disorders in which cognitive deficits are characteristic, such as schizophrenia, attention deficit hyperactivity disorder, and depression (379–381). The dopamine transporter (DAT) mediates re-uptake of extracellular DA, thus terminating DA receptor activation. DAT, a member of the Na^+/Cl^--dependent family of neurotransmitter transporters (382) is therefore a major therapeutic target for schizophrenia and other disorders (383–385). Several transgenic models have been generated with changes in DAT expression or function or mutations in the DAT gene (386). These models have proven to be crucial in elucidating neurotransmitter and neuromodulator function (or mechanistic underpinnings) of neuropsychiatric disorders (381, 387).

Wong et al. (373) have used a DAT knockout (DAT KO) mouse model exhibiting symptoms characteristic of schizophrenia to investigate the effects of PregS treatment on learning and memory function. Systemic administration of PregS, which is able to cross the BBB, alleviates positive and negative symptoms as well as cognitive deficits in the DAT KO mouse. Long-term systemic treatment with PregS rescues impaired episodic memory and poor discriminative abilities in the DAT KO mice without adverse effects. Consistent with observed reductions in cognitive deficits, long-term PregS

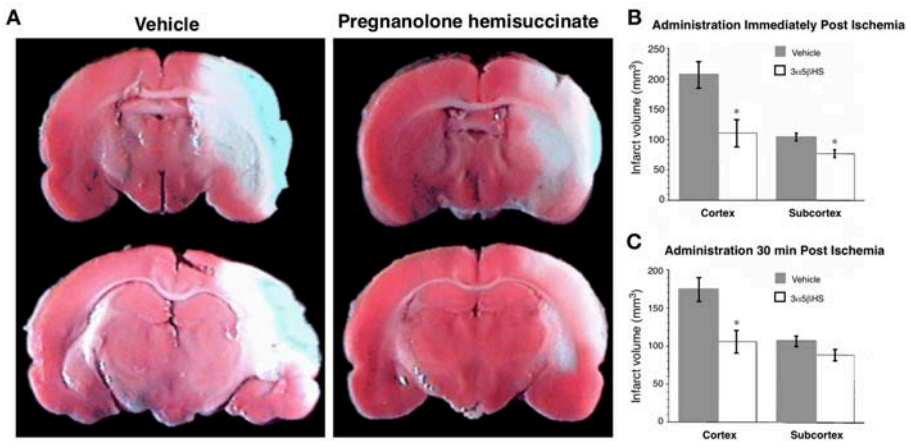

FIGURE 13 | Pregnanolone hemisuccinate (3α5βHS) is neuroprotective in an *in vivo* model of stroke. Rats were infused with either vehicle or 3α5βHS (6.9 mg/kg/h; 6.9 mg/kg loading dose), beginning immediately or 30 min after initiation of medial cerebral artery occlusion. Infusion of 3α5βHS was continued for an additional 22 h, at which time the rats were euthanized and their brains harvested and stained with 2,3,5-triphenyl tetrazolium Cl. **(A)** Representative coronal sections from animals receiving vehicle or 3α5βHS infusions immediately after initiation of ischemia. Infarct area appears pale. **(B)** Administration of 3α5βHS immediately after the onset of ischemia significantly reduced the volume of the cortical infarct from 206 ± 22 to 110 ± 21 mm^3 ($P < 0.005$) and subcortical infarct was reduced from 103 ± 6 to 76 ± 6 mm^3 (*$P < 0.005$) (vehicle, $n = 9$; 3α5βHS, $n = 10$). **(C)** When 3α5βHS was administered 30 min after the onset of ischemia the volume of cortical infarct was reduced from 173 ± 15 to 106 ± 15 mm^3 (*$P < 0.005$; $n = 13$), with no reduction apparent in the subcortical region [From Weaver et al. (132) with Permission].

treatment increases expression of the obligatory NMDAR subunit GluN1 in the hippocampus. Earlier, our laboratory characterized some of the mechanisms involved in increased expression of NMDAR subunits and demonstrated that PregS increases surface GluN1 in oocytes expressing recombinant NMDAR subunits in a non-canonical GPCR- and Ca^{2+}-dependent manner (47) (**Figure 2**).

SYNTHETIC NEUROSTEROIDS AS POTENTIAL COGNITIVE ENHANCERS

The findings reviewed herein suggest that the memory deficits seen in patients with schizophrenia, depression and anxiety disorders are influenced by changes in endogenous neurosteroid levels. Because not all systemically administered endogenously occurring neurosteroids readily cross the BBB and because these compounds can be metabolized to hormonally active steroids with different mechanisms of action, the therapeutic potential of these compounds is limited. For this reason, synthetic analogs of neurosteroids, which are more resistant to metabolism and better able to cross the BBB, are under investigation for use as anxiolytics, antidepressants, cognitive enhancers, anesthetics, and anticonvulsants (132, 388, 389). For example, synthetic neuroactive steroids bearing a hemisuccinate group are more resistant to hydrolysis than the corresponding sulfate esters and are partly unionized at physiological pH, allowing increased passage across the BBB. One such synthetic

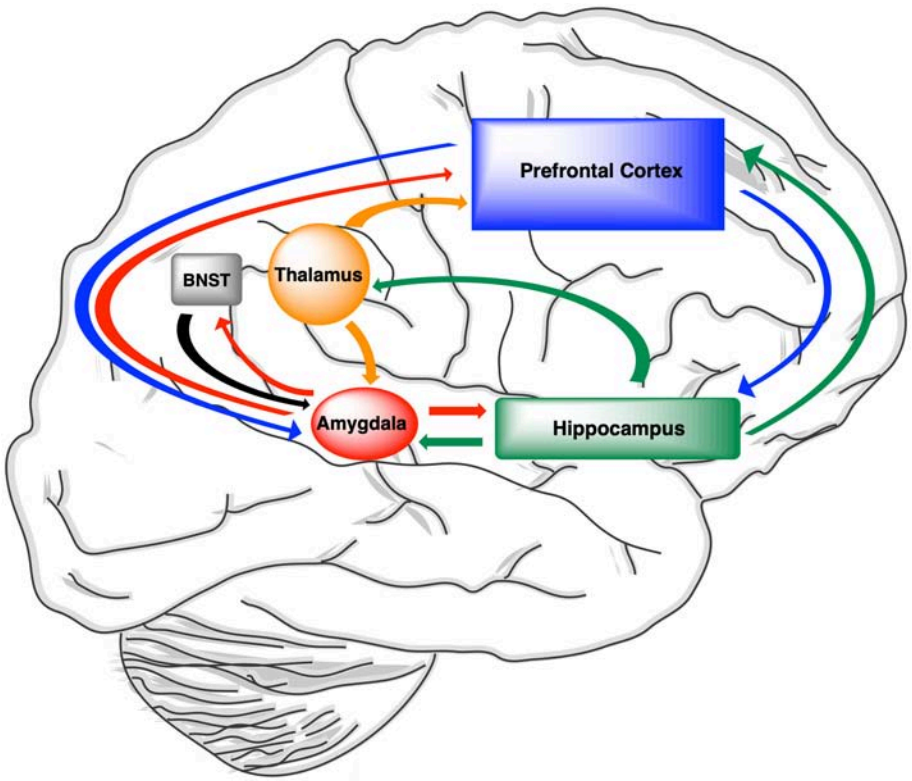

FIGURE 14 | Pharmacologic connectivity pathways implicated in neurosteroidal modulation of memory function. Neural networks that project to and/or share reciprocal connections with the hippocampus are modulated by neurosteroids as well as neurotransmitters. As a result, learning and memory deficits are associated with many of neurologic and neuropsychiatric disorders in which neurosteroids are implicated. The amygdala (shown in red), which is implicated in anxiety disorders, shares reciprocal connections with the hippocampus (shown in green). Progesterone, which is metabolized to ALLO, modulates emotional memory function by influencing amygdalar activity (64). The effects of neurosteroids on learning and memory for fear-inducing stimuli appears to be different in males and females which show different brain levels of ALLO at baseline as well (395). Neuroactive steroids, such as ALLO that enhance inhibitory neurotransmission can provide symptomatic relief from anxiety by reducing intra-network connectivity in the salience network and the amygdala (75, 192, 290, 291). ALLO induces an increase in functional connectivity between the amygdala and prefrontal cortex (shown in blue), which is involved in processing of complex social and non-social stimuli. However, the increased inhibition associated higher levels of ALLO can also interfere with episodic memory function which depends on intact functional connectivity between the hippocampus and the pre-cuneus (not shown) (203). Interestingly, both PREG, which is metabolized to ALLO, and PregS, which is a positive modulator of NMDARs, improve working memory function in patients with schizophrenia in which cortical disinhibition due to hypofunction of excitatory NMDARs on PV interneurons in the prefrontal cortex has been implicated (51, 52, 65, 237, 238). Parahippocampal and hippocampal structures including, the trisynaptic circuit receives sensory and emotional inputs from sensory modalities via the thalamus (shown in orange) and the amygdala, respectively (396–398). The impact of powerful emotion-evoking stimuli are state independent when ALLO is injected into the amygdala and hippocampus, but state-dependent when it is injected into the BNST (shown in gray) (246). The response of the hippocampal trisynaptic circuit to neurosteroids also depends on disease state-dependent changes in neurosteroid biosynthesis and receptor expression (399–401), as well as age-related changes in steroid hormone levels which converge to influence the responsivity of this circuit to endogenous and exogenous sources of neurosteroids and their synthetic analogs (229, 401–403).

neuroactive steroid, pregnanolone hemisuccinate, produces sedation and neuroprotection in mice and rats (132). Weaver et al. (132) demonstrated that pregnanolone hemisuccinate (PAHS) inhibits NMDA-induced currents and cell death in primary cultures of hippocampal neurons. Additionally, administration of a non-sedating dose of PAHS to rats following focal cerebral ischemia reduces cortical and subcortical infarct size (**Figure 13**).

Ganaxolone (3-hydroxy-3-methyl-5-pregnane-20-one), an orally bioavailable synthetic analog of ALLO, is a positive allosteric modulator of $GABA_AR$ with promising basic and early-stage clinical outcomes as a potential novel treatment for PTSD (388, 390–392). Based on the mechanism of action of ganaxolone and work in preclinical animal models suggesting improved spatial memory function in an animal model of Angelman Syndrome (393), future investigation looking at the clinical value of this agent in anxiety disorders appears warranted. Another synthetic analog of ALLO, 3β-ethenyl-3α-hydroxy-5α-pregnan-20-one (Co 3-0593), has been found to have anxiolytic effects comparable to benzodiazepines after both subcutaneous and oral administration in rodents. An absence of tolerance to this synthetic neuroactive steroid is suggested by the observation that its effects were maintained with chronic administration (394).

Current research into the function of PregS is impeded by the lack of selective/specific antagonists and by a lack of knowledge of validated binding site(s). These drawbacks are further exacerbated by the susceptibility of the essential 3-hydroxysulfate to hydrolysis by sulfatases. To overcome these limitations, we and others are using PregS analogs to decipher mechanistic aspects of PregS-mediated effects on learning and memory function. We recently reported that low nanomolar concentration of PregS induce a delayed-onset increase of the neuronal response to NMDA and trafficking of NMDAR to the cell surface through an intracellular ($[Ca^{2+}]i$)-dependent mechanism (47) (**Figure 2**). Moreover, we have demonstrated that low picomolar PregS increases $[Ca^{2+}]i$ and CREB phosphorylation and the frequency of spontaneous excitatory post-synaptic currents (48) (**Figures 8**, **9**). More work is needed to determine if the synthetic analog of PregS, PREG hemisuccinate, has the potential to overcome the limitations associated with systemic administration of PregS.

CONCLUSIONS

The effects of neurosteroids in memory function in neuropsychiatric and neurologic disorders reflect their modulatory interactions exerted via selective binding at the amino and transmembrane domains of specific subunits comprising GABA and glutamate receptors, among others. Age- and disease state-dependent changes in endogenous levels of neurosteroids appear to play a role in the emergence of the unique functional imbalances implicated in specific neuropsychiatric disorders and the associated memory deficits, which are mediated in part by changes in neural network activity within specific brain regions implicated in the encoding, consolidation, and retrieval of memories. It may be helpful to think of these findings in terms of a *pharmacological connectome* that reflects the interactions of neurosteroids with various neural networks involved with the encoding and recall of memories. We believe that a neural circuitry framework will help to guide future investigations into the potential role of neurosteroids and their synthetic analogs as neurotherapeutics for memory dysfunction (**Figure 14**).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

NIH/NIA Grant Number: AG056947.

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