RECENT PROGRESS AND PERSPECTIVES IN NEUROSTEROID RESEARCH

EDITED BY: Hubert Vaudry, Kiran K. Soma, Takayoshi Ubuka and Kazuyoshi Tsutsui

PUBLISHED IN: Frontiers in Endocrinology and Frontiers in Neuroscience

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-627-7
DOI 10.3389/978-2-88976-627-7

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

RECENT PROGRESS AND PERSPECTIVES IN NEUROSTEROID RESEARCH

Topic Editors:
Hubert Vaudry, Université de Rouen, France
Kiran K. Soma, University of British Columbia, Canada
Takayoshi Ubuka, Cancer medical service, Japan
Kazuyoshi Tsutsui, Hiroshima University, Japan

Citation: Vaudry, H., Soma, K. K., Ubuka, T., Tsutsui, K., eds. (2022). Recent Progress and Perspectives in Neurosteroid Research. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88976-627-7

Table of Contents

frontiers | Frontiers in Endocrinology

EDITORIAL
published: 27 July 2022
doi: 10.3389/fendo.2022.951990

Editorial: Recent Progress and Perspectives in Neurosteroid Research

Hubert Vaudry[1]*, *Takayoshi Ubuka*[2], *Kiran K. Soma*[3] and *Kazuyoshi Tsutsui*[4]

[1] Université de Rouen Normandie, Mont-Saint-Aignan, France, [2] International Cancer Laboratory, Tokyo, Japan, [3] University of British Columbia, Vancouver, BC, Canada, [4] Hiroshima University, Hiroshima, Japan

Keywords: neurosteroids, neurohormones, neuromodulators, neurotransmitters, neurotrophic factors

Editorial on the Research Topic

Recent Progress and Perspectives in Neurosteroid Research

The term "Neurosteroids" designates pregnenolone-derived bioactive compounds that are synthesized or catalyzed by neural cells and act locally on the central or peripheral nervous systems. At the molecular level, the actions of neurosteroids are mainly mediated through membrane receptors i.e. via their cognate receptors and/or via allosteric modulation of other receptors. At the cellular level, neurosteroids act as neurohormones, neuromodulators, neurotransmitters and/or neurotrophic factors. At the organismal level, neurosteroids regulate several physiological processes including arousal, sleep, learning, social and sexual behaviors. Thus, neurosteroids are now considered an important class of intercellular/intracellular signaling molecules in the nervous system, in very much the same as neurotransmitters, neuropeptides and growth factors. Not surprisingly, neurosteroids appear to be implicated in a number of pathophysiological conditions such as pain, neurodegenerative diseases, autism, stress, anxiety, depression, etc. Therefore, drugs targeting neurosteroid biosynthetic enzymes or neurosteroid receptors have strong potential for the development of novel therapeutic approaches.

This Research Topic compiles a series of review and original articles that provide a broad view of the current knowledge on the biosynthesis, functional roles and pathophysiological implications of neurosteroids, and highlights new concepts in this field.

We want to dedicate this Research Topic to the memory of our colleague and dear friend Kazuyoshi Tsutsui, an undisputed leader in neurosteroid research who, very sadly, passed away on September 16, 2021.

Progesterone and allopregnanolone are two neuroactive steroids that act primarily through membrane receptors (1). Progesterone can activate five metabotropic membrane receptors belonging to the progestin and adipoQ receptor (PAQR) family that are distinct from the GPCR family (2). Allopregnanolone and its 3β-methylated synthetic analog ganaxolone act as positive allosteric modulators of $GABA_A$ receptors (Pinna). The review by Thomas and Pang summarizes the current knowledge on the neuroprotective actions of allopregnanolone and ganaxolone.

Concurrently, Pinna describes the long journey between the discovery of allopregnanolone in the adrenal cortex (3) and the characterization of its anxiolytic and antidepressant properties (4, 5). The recent validation of allopregnanolone-based treatment of postpartum depression (6) opens new avenues for the development of other neurosteroid-derived drugs in neuropsychiatry.

Apolipoprotein A1 regulatory protein-1 (ARP-1), a member of the steroid receptor superfamily whose ligand is unknown (orphan receptor), regulates transcriptional activity of numerous genes

Edited and reviewed by:
Jeff M. P. Holly,
University of Bristol, United Kingdom

***Correspondence:**
Hubert Vaudry
hubert.vaudry@univ-rouen.fr

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

Received: *24 May 2022*
Accepted: *27 May 2022*
Published: *27 July 2022*

Citation:
Vaudry H, Ubuka T, Soma KK and Tsutsui K (2022) Editorial: Recent Progress and Perspectives in Neurosteroid Research. Front. Endocrinol. 13:951990. doi: 10.3389/fendo.2022.951990

including apolipoprotein-encoding genes (7). Using a yeast one-hybrid screening approach, Honda and Harada identified ARP-1 as a transcription factor that binds to a *cis*-element, aro-AII, responsible for the brain-specific expression of the mouse aromatase gene.

The recent years have seen substantial progress in the understanding of the bidirectional interactions between the hypothalamo-pituitary-adrenal (HPA) and the hypothalamo-pituitary-gonadal (HPG) axes (8). Hamidovic et al. have conducted a meta-analysis of data spanning over half a century of research on plasma cortisol levels in healthy women during the follicular *vs.* luteal phases of the menstrual cycle. Their study reveals that circulating cortisol levels are higher in the follicular phase. One possible explanation relies on the changes of $GABA_A$ receptor-modulating neurosteroids, including allopregnanolone, during the menstrual cycle (9).

GABAergic anesthetic agents such as isoflurane can exert adverse neuroendocrine effects, notably during the neonatal period (10). Li et al. have investigated the effects of sevoflurane on testosterone (T) and its derivative 17β-estradiol (E2) levels in 5-day old rats. They found that sevoflurane causes an increase in T levels in male rats only and an increase in E2 levels in both male and female rats. These data indicate that the adverse effects of general anesthesia at the beginning of the lifespan might be ascribed, at least in part, to a $GABA_A$ receptor-mediated increase of plasma sex steroids.

Biosynthesis of estrogens is catalyzed by the enzyme cytochrome P450 aromatase, also called estrogen synthase, that converts T into E2 (11). A recent study has revealed that administration of aromatase inhibitors may impair cognitive functions (12). In this context, Alia-Klein et al. have taken advantage of the availability of positron emission tomography (PET) aromatase radiotracers to correlate cognitive performance with aromatase levels in the human amygdala and thalamus.

During the perinatal period, neuroestrogens play a pivotal role in the sexual differentiation of the brain (13, 14). The review by Tsukahara and Morishita discusses the role of neuroestrogens of testicular origin on two sexually dimorphic brain regions, the preoptic area (POA) and the bed nucleus of the stria terminalis (BNST). Surprisingly, during the peripubertal period, testicular androgens, without aromatization, also contribute to sexual differentiation of the POA and BNST.

There is now strong evidence that estrogens synthesized within the CNS exert a neuroprotective action (15). Reciprocally, traumatic brain injury (TBI) can affect both estrogen biosynthesis and estrogen inactivation in the central nervous system (CNS) (16). Indeed, TBI can modulate various estrogen metabolizing enzymes including aromatase, steroid sulfatase, estrogen sulfotransferase and 17β-hydroxysteroid dehydrogenases.

Neuroactive steroids, like steroid hormones, can act through intracellular receptors (genomic actions) and/or plasma membrane receptors (non-genomic actions) (17). Neurosteroids are not only synthesized in the CNS but also in the peripheral nervous system (PNS) (18). Colciago et al. provide a comprehensive review of the various aspects of neurosteroid actions in the PNS through intracellular receptors, metabotropic receptors (*i.e.* G protein-coupled receptors) and ionotropic receptors (mainly $GABA_A$ receptors).

Various neurosteroids can negatively or positively modulate $GABA_A$ receptor activity and can thus act as proconvulsant or anticonvulsant agents (19). In their systematic review, Miziak et al. summarize the diverse effects of endogenous and exogenous neurosteroids on seizure activity in animal models and epileptic patients.

Astrocytes express both cytochrome P450scc and 3β-hydroxysteroid dehydrogenase, the two enzymes that are required for the biosynthesis of progesterone (20). Although estradiol initiates the luteinizing hormone (LH) surge that triggers ovulation and reproduction, estrogens do not act directly on gonadotropin-releasing hormone (GnRH) neurons (21). Sinchak et al. review the evidence that neuroprogesterone synthesized in hypothalamic astrocytes is involved in the estradiol-induced LH surge.

Fish, which exhibit intense aromatase activity in their CNS (22), represent attractive models to investigate the effect of neuroactive steroids on behavior. Silva et al. reviews the contribution of a weakly electric teleost fish, *Gymnotus omarorum*, to the understanding of the neuroendocrine mechanisms underlying non-breeding aggressive behavior. The data strongly support the involvement of brain-born estrogens in year-long territorial behavior.

In birds, the biosynthesis of various neurosteroids is higher in the pineal gland than in any other brain region. In particular, 7α-hydroxypregnenolone (23) and allopregnanolone (24) are actively produced in the chicken pineal gland. Haraguchi and Tsutsui review the physiological roles played, respectively, by 7α-hydroxypregnenolone and allopregnanolone in the control of locomotor activity and in Purkinje cell survival during development.

In human as in other vertebrates, sex steroids affect multiple neural and behavioral functions (25). Since the menopause transition is associated with a drop in estrogen levels, He et al. have performed functional MRI scan on premenopausal and perimenopausal women to investigate spontaneous brain activity. The results reveal altered brain functions in brain regions implicated in cognition and working memory in perimenopausal women.

The post-menopausal syndrome includes various neuropsychological disorders including depression, anxiety and dementia (26). In order to investigate the role of estrogens in these disorders, Renczès et al. have compared the effects of surgical (ovariectomy) and pharmacological (aromatase inhibitor) treatment on anxiety-like behavior and memory.

Neuroactive estrogenic and androgenic neurosteroids enhance hippocampal memory tasks (27). In their mini-review, Tozzi et al. recapitulate the evidence supporting the involvement of E2 and T in the induction of long-term potentiation (LTP) and long-term depression (LTD), and on dendritic spine formation in different brain areas. The data indicate that, while estrogens induce LTP and androgens induce LTD, both neurosteroids enhance dendritic spine formation.

Do transient changes in hormonal state during the menstrual cycle affect human behaviors? To answer this question, two types of experimental designs can be set up: within-subject designs where the same women are tested during different phases of their cycle or between-subject designs where two groups of women are tested at different cycle phases. Here, Diekhof et al. have performed a between-subject study to explore the effect of hormonal changes

during the late follicular phase and the mid luteal phase on avoidance learning capacity, and they have concurrently conducted a meta-analysis of previously reported within-subject studies. Both approaches concur to demonstrate a decline in avoidance learning during the follicular phase compared to the luteal phase.

In conclusion, the contributions gathered in this Research Topic give an overview on recent advances in our understanding of the physiological roles and potential therapeutic implications of neurosteroids. They also highlight the challenges that remain to be addressed for the next decade. It is our hope that the readers will enjoy reading these articles, and that this Research Topic will become a major set of references for all researchers involved in this rapidly expanding field.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

We want to thank all the authors of this Research Topic for their excellent contributions, and the dedicated reviewers for their insightful comments that helped maintain the articles at the highest standards. We also gratefully acknowledge the excellent secretarial assistance of Mrs. Catherine Beau and the invaluable support of Emilie Schrepfer, Margaret Georgevits and the whole Frontiers staff.

REFERENCES

1. Guennoun R, Labombarda F, Gonzalez Deniselle MC, Liere P, De Nicola AF, Schumacher M. Progesterone and Allopregnanolone in the Central Nervous System: Response to Injury and Implication for Neuroprotection. *J Steroid Biochem Mol Biol* (2015) 146:48–61. doi: 10.1016/j.jsbmb.2014.09.001
2. Thomas P. Characteristics of Membrane Progestin Receptor Alpha (mPRalpha) and Progesterone Membrane Receptor Component 1 (PGMRC1) and Their roles in Mediating Rapid Progestin Actions. *Front Neuroendocrinol* (2008) 29:292–312. doi: 10.1016/j.yfrne.2008.01.001
3. Beall D, Reichstein T. Isolation of Progesterone and Allopregnanolone From the Adrenal. *Nature* (1938) 142:479.
4. Meltzer-Brody S, Kanes SJ. Allopregnanolone in Postpartum Depression: Role in Pathophysiology and Treatment. *Neurobiol Stress* (2020) 12:100212. doi: 10.1016/j.ynstr.2020.100212
5. Walton N, Maguire J. Allopregnanolone-Based Treatments for Postpartum Depression: Why/How Do They Work?. *Neurobiol Stress* (2019) 11:100198. doi: 10.1016/j.ynstr.2019.100198
6. Meltzer-Brody S, Colquhoun H, Riesenberg R, Epperson CN, Deligiannidis KM, Rubinow DR, et al. Brexanolone Injection in Post-Partum Depression: two Multicentre, Double-Blind, Randomised, Placebo-Controlled, Phase 3 Trials. *Lancet* (2018) 392:1058–70. doi: 10.1016/S0140-6736(18)31551-4
7. Ladias JA, Karathanasis SK. Regulation of the Apolipoprotein AI Gene by ARP-1, A Novel Member of the Steroid Receptor Superfamily. *Science* (1991) 251:561–5. doi: 10.1126/science.1899293
8. Acevedo-Rodriguez A, Kauffman AS, Cherrington BD, Borges CS, Roepke TA, Laconi M. Emerging Insights Into Hypothalamic-Pituitary-Gonadal Axis Regulation and Interaction With Stress Signalling. *J Neuroendocrinol.* (2018) 30:e12590. doi: 10.1111/jne.12590
9. Gunn BG, Cunningham L, Mitchell SG, Swinny JD, Lambert JJ, Belelli D. GABAA Receptor-Acting Neurosteroids: a Role in the Development and Regulation of the Stress Response. *Front Neuroendocrinol.* (2015) 36:28–48. doi: 10.1016/j.yfrne.2014.06.001
10. Vutskits L, Xie Z. Lasting Impact of General Anaesthesia on the Brain: Mechanisms and Relevance. *Nat Rev Neurosci* (2016) 17:705–17. doi: 10.1038/nrn.2016.128
11. Simpson ER, Clyne C, Rubin G, Boon WC, Robertson K, Britt K, et al. Aromatase–A Brief Overview. *Annu Rev Physiol* (2002) 64:93–127. doi: 10.1146/annurev.physiol.64.081601.142703
12. Rosenfeld CS, Shay DA, Vieira-Potter VJ. Cognitive Effects of Aromatase and Possible Role in Memory Disorders. *Front Endocrinol (Lausanne)* (2018) 9:610. doi: 10.3389/fendo.2018.00610
13. Amateau SK, McCarthy MM. Sexual differentiation of astrocyte morphology in the Developing Rat Preoptic Area. *J Neuroendocrinol.* (2002) 14:904–10. doi: 10.1046/j.1365-2826.2002.00858.x
14. Sano K, Nakata M, Musatov S, Morishita M, Sakamoto T, Tsukahara S, et al. Pubertal Activation of Estrogen Receptor α in the Medial Amygdala Is Essential for the Full Expression of Male Social Behavior in Mice. *Proc Natl Acad Sci USA* (2016) 113:7632–7. doi: 10.1073/pnas.1524907113
15. Pedersen AL, Nelson LH, Saldanha CJ. Pubertal Activation of Estrogen Receptor α in the Medial Amygdala Is Essential for the Full Expression of Male Social Behavior in Mice. *Endocrinology* (2016) 157:2041–51. doi: 10.1210/en.2015-1991
16. Duncan KA. Estrogen Formation and Inactivation Following TBI: What We Know and Where We Could Go. *Front Endocrinol (Lausanne)* (2020) 11:345. doi: 10.3389/fendo.2020.00345
17. Do Rego JL, Seong JY, Burel D, Leprince J, Vaudry D, Luu-The V, et al. Regulation of Neurosteroid Biosynthesis by Neurotransmitters and Neuropeptides. *Front Endocrinol (Lausanne)* (2012) 3:4. doi: 10.3389/fendo.2012.00004
18. Melcangi RC, Magnaghi V, Galbiati M, Martini L. Formation and Effects of Neuroactive Steroids in the Central and Peripheral Nervous System. *Int Rev Neurobiol* (2001) 46:145–76. doi: 10.1016/s0074-7742(01)46062-4
19. Herd MB, Belelli D, Lambert JJ. Neurosteroid Modulation of Synaptic and Extrasynaptic GABA(A) Receptors. *Pharmacol Ther* (2007) 116:20–34. doi: 10.1016/j.pharmthera.2007.03.007
20. Do Rego JL, Seong JY, Burel D, Leprince J, Luu-The V, Tsutsui K, et al. Neurosteroid Biosynthesis: Enzymatic Pathways and Neuroendocrine Regulation by Neurotransmitters and Neuropeptides. *Front Neuroendocrinol.* (2009) 30:259–301. doi: 10.1016/j.yfrne.2009.05.006
21. Wintermantel TM, Campbell RE, Porteous R, Bock D, Gröne HJ, Todman MG, et al. Definition of Estrogen Receptor Pathway Critical for Estrogen Positive Feedback to Gonadotropin-Releasing Hormone Neurons and Fertility. *Neuron* (2006) 52:271–80. doi: 10.1016/j.neuron.2006.07.023
22. Diotel N, Do Rego JL, Anglade I, Vaillant C, Pellegrini E, Vaudry H, et al. The Brain of Teleost Fish, a Source, and a Target of Sexual Steroids. *Front Neurosci* (2011) 5:137. doi: 10.3389/fnins.2011.00137
23. Hatori M, Hirota T, Iitsuka M, Kurabayashi N, Haraguchi S, Kokame K, et al. Light-Dependent and Circadian Clock-Regulated Activation of Sterol Regulatory Element-Binding Protein, X-Box-Binding Protein 1, and Heat Shock Factor Pathways. *Proc Natl Acad Sci USA* (2011) 108:4864–9. doi: 10.1073/pnas.1015959108
24. Haraguchi S, Hara S, Ubuka T, Mita M, Tsutsui K. Possible Role of Pineal Allopregnanolone in Purkinje Cell Survival. *Proc Natl Acad Sci USA* (2012) 109:21110–5. doi: 10.1073/pnas.1210804109
25. McEwen BS, Milner TA. Understanding the Broad Influence of Sex Hormones and Sex Differences in the Brain. *J Neurosci Res* (2017) 95:24–39. doi: 10.1002/jnr.23809
26. Dotlic J, Radovanovic S, Rancic B, Milosevic B, Nicevic S, Kurtagic I, et al. Mental Health Aspect of Quality of Life in the Menopausal Transition. *J Psychosom. Obstet. Gynaecol.* (2021) 42:40–9. doi: 10.1080/0167482X.2020.1734789
27. Jacome LF, Barateli K, Buitrago D, Lema F, Frankfurt M and Luine VN. Gonadal Hormones Rapidly Enhance Spatial Memory and Increase Hippocampal Spine Density in Male Rats. *Endocrinology* (2016) 157:1357–62. doi: 10.1210/en.2015-1959

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.

this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 24 April 2020
doi: 10.3389/fendo.2020.00231

Avoidance Learning Across the Menstrual Cycle: A Conceptual Replication

Esther K. Diekhof, Sina Korf, Franziska Ott, Carolin Schädlich and Sarah K. C. Holtfrerich*

Faculty of Mathematics, Informatics and Natural Sciences, Department of Biology, Institute of Zoology, Neuroendocrinology and Human Biology Unit, Universität Hamburg, Hamburg, Germany

Hormonal transitions across the menstrual cycle may modulate human reward processing and reinforcement learning, but previous results were contradictory. Studies assessed relatively small samples ($n < 30$) and exclusively used within-subject designs to compare women in hormonally distinct menstrual cycle phases. This increased the risk of sporadic findings and results may have been disproportionally affected by expectancy effects. Also, replication studies are widely missing, which currently precludes any reliable inferences. The present study was intended as a conceptual replication of a previous study [(1), Neuropsychologia 84; $n = 15$]. There, we had observed a reduction in avoidance learning capacity when women were in the high estradiol state of the late follicular phase as compared to the mid luteal phase with enhanced progesterone influence. These results conformed to the idea that estradiol and progesterone may antagonistically modulate dopaminergic transmission as a dopamine agonist and antagonist, respectively. Heightened progesterone in the luteal phase thereby supported the ability to learn from the negative outcomes of one's actions, while the follicular rise in estradiol interfered with this capacity. Here, we re-examined the above described within-subject difference between the follicular and the luteal phase in a between-subjects design. Seventy-five women were tested once with a probabilistic feedback learning task, while being either in the follicular (36 women) or luteal phase (39 women), and were compared for phase-related differences in behavior. Secondly, we combined the new data with data from three previous studies from our laboratory that used the same task and menstrual cycle phases. This meta-analysis included only data from the first test day, free of any biasing expectancy effects. Both analyses demonstrated the consistency of the decline in avoidance learning in the follicular relative to the luteal phase. We also showed that this decline reliably occurred in all of the included samples. Altogether, these results provide evidence for the consistency of a behavioral difference and its apparent association with a transient change in hormonal state that occurs in the natural menstrual cycle. Our findings may also open new avenues for the development of reliable between-subjects test protocols in menstrual cycle research.

Keywords: estrogen (17β-estradiol), menstrual cycle, replication crisis in psychology, reinforcement learning (RL), meta-analysis, progesterone and estradiol, reward processing

OPEN ACCESS

Edited by:
Kiran K. Soma,
University of British Columbia, Canada

Reviewed by:
Rebecca Todd,
University of Toronto, Canada
Kim Wallen,
Emory University, United States

****Correspondence:***
Esther K. Diekhof
esther.diekhof@uni-hamburg.de

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

Received: *23 November 2019*
Accepted: *31 March 2020*
Published: *24 April 2020*

Citation:
Diekhof EK, Korf S, Ott F, Schädlich C and Holtfrerich SKC (2020) Avoidance Learning Across the Menstrual Cycle: A Conceptual Replication. Front. Endocrinol. 11:231. doi: 10.3389/fendo.2020.00231

INTRODUCTION

There is an ongoing debate about whether menstrual cycle phase related differences in the concentrations of estradiol and progesterone significantly affect human reinforcement learning and reward seeking behavior as well as the associated neural processes. Although previous studies demonstrated a relationship between these hormones and different aspects of human reward processing (2), results showed a high variability and were not always consistent (3). Moreover, most studies in this domain were largely underpowered [$n < 30$; average sample size $= 17$ women; (3)] and replication studies are currently lacking. It is therefore unclear whether previous observations in humans were a product of the prevailing publication bias in the cognitive sciences or whether they indeed reflected the relatively strong effects of estradiol and progesterone in the mesocorticolimbic dopamine system that are suggested by animal studies.

In female rodents estradiol acts as natural dopamine agonist, which promotes the sensitivity for reward and interferes with the ability to avoid actions that lead to an undesired outcome (4, 5). In contrast, progesterone may partly inhibit dopaminergic transmission, and there is evidence that it can antagonize estradiol's action in the dopamine system (6–8). Progesterone should thus in turn support the ability to avoid actions that lead to a negative outcome (9). In line with the rodent evidence, the neuroimaging study by Diekhof and Ratnayake (1) found that women showed a reduced ability to learn from negative feedback in the high estradiol state of the late follicular phase compared to the mid luteal phase, in which progesterone reached its cyclic maximum. However, their results were based on the data of 15 young women, who were repeatedly tested. A repeated test protocol is not unproblematic, as the task repetition can lead to expectancy or carry-over effects, which may contaminate the experimental effect, one is actually interested in (10–12). Further, such a small sample may preclude the generalizability of the results to a larger population or could even reflect a false positive, sporadic finding. The present study was therefore intended as a conceptual replication of results of Diekhof and Ratnayake (1). The term "conceptual replication," as we use it here, refers to the repetition of a test of a hypothesis or a result of earlier research work with a different method. This means that the immaterial information focus (i.e., the experimental task performed by the participants) remained the same between studies. In contrast, the material realization of this information differed in line with the experimental idea (i.e., here we intended to replicate a within-subject effect related to changes in hormonal state with a between-subjects design). In contrast, the term "direct replication" refers to the exact repetition of the experimental procedure of a previous study. This can be accomplished by testing a larger repetition sample with the same experimental setup as used in the first study [in our example this would have been equivalent to a within-subject design simply applied to a bigger sample; see Schmidt (13) for discussion and overview].

Here, we assessed the above described difference in avoidance learning capacity between the late follicular and the mid luteal phase (1) in a larger sample and with a between-subjects design, a procedure through which we avoided contamination by repeated testing. For further confirmation of the observed effect, we combined the newly collected behavioral data with those collected in three previous studies from our laboratory that employed the same probabilistic feedback learning task and tested women in comparable menstrual cycle phases. All data for this meta-analysis came from independent observations collected during the first, naïve test day. The meta-analytic data were examined for between-subjects effects associated with menstrual cycle phase. In that way we wanted to evaluate the consistency of the behavioral findings across studies and also intended to compare the results derived from the between-subjects design with the outcome of the commonly used within-subject approach in menstrual cycle research.

MATERIALS AND METHODS

Participants

In total, 93 healthy young women [age [mean ± sem] = 25.2 ± 0.4 years] were tested for this study. They had no current or previous psychiatric or neurological diagnosis, reported to have no history of drug abuse, and did not have any chronic disorder related to the hormone system (e.g., Diabetes, Hashimoto-Thyroiditis, PCO). All reported to have regular menstrual cycles (cycle length between 21 and 35 days), were fluent in German, and had corrected-to-normal vision. Thirty-six women had never used hormonal contraception. The remaining 57 women had taken hormonal contraceptives in the past and reported the month of the last intake. None of them had used hormonal contraceptives within the month preceding the study [average distance between test and last intake [mean ± sem] = 27.7 ± 4.3 months; median distance = 15 months]. Three women had stopped oral contraction <3 months before the actual test, two of them were in the follicular phase on the test day. 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*).

Power Analysis for Determination of Optimal Sample Size

Here, we opted for testing a group of sufficient size to reach an acceptable level of statistical power (≥80%). The sample size for the given project was determined based on the results of Diekhof and Ratnayake (1). There, women were better at avoiding the least rewarded option "B" in the mid luteal phase [mean avoidance frequency ± SD = 77.0 ± 21.1%] compared to the late follicular phase [mean avoidance frequency ± SD = 62.5 ± 16.1%; correlation between paired values = 0.136; Cohen's d = −0.52]. Assuming a power of at least 80%, this behavioral difference translated to an effect size of $d_z = 0.59$ in G*Power [(14); please note that in G*Power $d_z = |\mu_{x-y}| / \sigma_{x-y,}$ which is somewhat different from Cohen's $d = [\mu\text{-}c]/\sigma$]. In order to achieve the same effect size and a power of 80% at $p < 0.05$ in a between-subjects design, G*Power indicated an optimal sample size of 37 women for each test group in the direct comparison of the two cycle phases. For the assessment of the interaction between "*learning preference*" in the reinforcement learning task

and "*cycle phase*" a slightly higher number of participants per group was indicated ($n = 42$).

Post-test Exclusion Criteria

Menstrual cycles are highly variable and thus crucial events, like ovulation, are to a certain extent unpredictable. Cycle phase was therefore determined by a two-step procedure. The test appointment was made based on the onset of menstruation in the given cycle and the expected cycle length based on retrospective information on average cycle length provided by the participant. After the behavioral test took place and the given cycle ended with the onset of the next menstruation, we adjusted the test day to the actual cycle length (actual test day). The actual test day was then standardized to a 28-day cycle for all participants (see Experimental procedure below for standardization formula).

Further, to make our test procedure most similar to the procedures commonly used in within-subject designs, we also decided to exclude participants whose standardized cycle day indicated that they had been tested too late in the follicular phase, when hormone level could have been highly unstable (on the 3 days during and around ovulation). We also excluded participants who were tested when estradiol and progesterone were at their nadir (near the onset or offset the menstrual cycle). These post-test exclusion criteria applied to 16 of the already tested women. Eight of these women were sampled directly before, during or after ovulation, namely at standardized cycle days 13, 14, and 15 of the standard 28-day cycle. Another two women had a positive ovulation test at the time of testing. While the remaining 6 women were either tested at cycle onset (before standardized cycle day 2; $n = 4$) or near its offset (after standardized day 27; $n = 2$). Please note, that based on the pre-definition of time bin, not all of the subjects, who were actually tested on standardized cycle days 13–15 underwent an ovulation test, as this applied only for the predefined time bins 4–6 (see **Supplementary Table 1**). Another two women did not report the onset of the next cycle and did not reply to our further email inquiries, which also led to their exclusion. Thus, altogether 18 of the 93 women had to be excluded after the behavioral test was completed.

Experimental Procedure

We planned to test 100 women over the course of 6 months (November 2017–May 2018). Tests were performed under supervision of two female experimenters. Altogether, we tested 93 women during this period, of whom 75 were included in the final analysis after application of the post-test exclusion criteria (see above). Each subject was tested once within one of ten pre-determined time bins that comprised two to four cycle days (**Supplementary Table 1**). The pre-definition of time-bins was used to schedule the tests of a sufficient number of subjects in an equal distribution across the two cycle phases of interest and to balance testing between the two female experimenters in charge of data collection. The pre-defined test day of a given woman, i.e., the prospective test day, was based on the onset of her menstruation in the present menstrual cycle and the expected cycle length, which was determined from the average length of two previous menstrual cycles. Following the behavioral test and the onset of the next menstruation this information was then adjusted to the actual cycle length of the given test cycle and standardized to a cycle length of 28 days [standardized cycle day = actual test day/ actual cycle length * length of standard 28-day cycle]. Based on this calculation, we determined that 36 of the 75 women were in fact tested during the follicular phase (standardized cycle days 2–12), while 39 women performed the test in the luteal phase (standardized cycle days 16–26), approximating the optimal sample size as determined by G*Power. The result of this calculation was thereby comparable to other counting methods previously used to determine cycle phase, such as the "reverse counting method" [e.g., Puts (15)]. The reverse counting method uses the participant's date of the onset of menstruation of the next cycle and counts back from that date by 14–15 days to retrospectively approximate the date of ovulation. Relative to this ovulation date the current cycle position of the test day is then determined. We also applied this method to our data and found that 35 of the 36 women were classified by the reverse counting method as being in the follicular phase, while the reverse counting method assigned one of our follicular phase women (actual test day = 11; cycle length of given cycle = 25; standardized cycle day = 12) to the ovulation day (ovulation day according to reverse counting method = 11). Since this woman did not have a positive ovulation test before or at the test day and following our counting method was not tested between standardized days 13–15, we kept her original assignment to the follicular phase. Further, 38 luteal phase tests overlapped between our and the reverse counting method. One woman, who had a relatively long test cycle of 40 days, despite reporting regular cycles earlier, was placed in the luteal phase by our counting method (actual test day = 24; cycle length of given cycle = 40; standardized cycle day = 17), while the reverse counting method indicated that she might have performed the test during the late follicular phase (ovulation day according to reverse counting method = 25). Since she was tested in bin 7 (see **Supplementary Table 1**), she did not perform an ovulation test prior to the test. However, if we excluded her case from the subsequent analyses below, for example in the ANOVA the interaction between "learning outcome" and "cycle phase" remained significant nevertheless ($p = 0.048$) and the effect size remained identical (partial eta$^2 = 0.05$). Since otherwise there was no indication that the reverse counting method was somehow superior to our standardization procedure, we kept the cycle phase determined by our method for all women.

Finally, in order to match the two groups of women for various characteristics, they completed a battery of neuropsychological questionnaires and behavioral tests to assess relevant personality characteristics, cognitive capacity as well as mood and premenstrual symptoms. Working memory capacity was measured with the Digit span test and the combined score of forward and backward span. Impulsiveness was measured with the Barratt Impulsiveness Scale (BIS) (16), and color vision discrimination—as an index of dopamine functioning—was measured with the Lanthony Desaturate Panel D−15 [see Colzato et al. (17)]. The Lanthony score was thereby determined according to Geller (18). Current mood was examined with the Multidimensional Mood Questionnaire

[MDBF; (19)] and premenstrual symptoms were determined with the Premenstrual Symptoms Questionnaire by Ditzen et al. (20). Altogether, the women in the two cycle phase groups did not differ in age, education level, working memory capacity (Digit span), impulsiveness (BIS score), dopaminergic capacity (Lanthony score), mood (MDBF score), and premenstrual symptoms (PMS score) during the test day.

Task Description

We used the probabilistic feedback learning task already employed by Diekhof and Ratnayake (1). The task entailed a learning phase, in which participants learned to associate certain stimuli with probabilistic feedback that varied between the stimuli (**Figure 1A**). During the learning phase (session 1) participants were required to choose the better option from three fixed stimulus pairs (so called pairs "AB," "CD," and "EF") to maximize the incidence of positive feedback (smiley face). The stimuli denoted here as A, B, C, D, E, and F were different hiragana and kanji symbols. Once participants selected one of the symbols from a given stimulus pair, they received direct probabilistic feedback to enforce the different stimulus-feedback contingencies. Pair AB had the highest discriminatory power. Selection of symbol A was "rewarded" with a positive feedback (smiley face) in 80% of selections, while a grumpy face was shown as negative feedback in 20% of selections. In contrast, selection of symbol B yielded a grumpy face in 80% of selections, while only 20% of selections were followed by the positive smiley feedback. The pairs CD and EF yielded a positive feedback in 70:30 and 60:40, respectively. This made symbol A the most often "rewarded" option (best option), while B was the least "rewarded" option (worst option) in the task. Within the other pairs, C and E were the relatively better choices to be made. Before starting the learning phase, participants were instructed to collect as many smiley faces as possible and to avoid the negative grumpy feedback. Unbeknownst to the participants, the combination of the two symbols in the three stimulus pairs was fixed during the 360 trials of session 1. The actual screen location of the two stimuli (left or right) from each pair was pseudorandomly varied as was the sequence of the three stimulus pairs. At the end of the learning phase participants were expected to choose the relatively better options of the three pairs more often than their worse counterparts.

The learning session was followed by a transfer phase (session 2) that also included novel stimulus pairings (e.g., AC, BD, CF). But this time participants did not receive informative performance feedback after their decision [**Figure 1B**; see also Diekhof and Ratnayake (1)]. Participants were informed about the absence of feedback before session 2 was started, and were instructed to continue with their choices like in session 1, nevertheless. They were not informed about the changes in stimulus pairs. Novel pairs that contained either the symbol A or B allowed us to examine whether subjects rather learned through a preference for the best option A or through avoidance of the worst option B. Preferentially choosing A, the option associated with the highest probability of positive feedback, above all other stimuli in novel pairs is considered as an indicator of the ability to learn from the positive outcome of one's actions. In contrast, an increased avoidance of option B, that was associated with the highest probability for negative feedback, in novel pairs is believed to reflect avoidance learning capacity (21). In all, old and novel pairs were shown 12 times each in a pseudorandomized sequence of pairs and individual screen locations within pairs. The timing of the task remained the same as in the neuroimaging study by Diekhof and Ratnayake (1) (see also **Figure 1**). The task included the emulation of an fMRI-trigger signal with the repetition time of 2,000 ms as well as a temporal jitter at trial onset.

The transfer phase allowed us to disentangle the ability to learn from the positive outcome of one's actions, here the positive feedback, from the capacity to successfully avoid a negative action outcome, here the negative feedback. It has been assumed that these two aspects of learning may rely on two anatomical routes in the basal ganglia that either promote or inhibit action selection depending on current dopaminergic state (9). These routes may also be subject to the modulation by estradiol vs. progesterone, which may act as a dopamine agonist vs. antagonist, respectively (3). Based on Diekhof and Ratnayake (1), we predicted to find a reduction in the ability to learn from the negative feedback in the high estradiol state of the follicular phase compared to the high progesterone state of the luteal phase. The ability to effectively learn from negative feedback was measured from the percentage of the correct avoidance of the worst option B ("Avoid B" performance) in the novel stimulus pairs of the transfer phase. This required the participant to choose the relatively better stimuli C, D, E, or F from the respective pairs with B, which had all led to a higher incidence of positive feedback than stimulus B in session 1. Conversely, the ability to identify A as the best option was measured by the percentage of selections of A from the novel pairs with A (i.e., AC, AD, AE, and AF) in the transfer phase ("Choose A" performance).

Collection and Analysis of Saliva Samples

Saliva sample collection followed the common procedure used by our laboratory (1). This included collection of five saliva samples in the morning of the test day. Participants started directly after waking up and provided saliva samples in regular intervals over the course of 2 h. For this they used five 2 ml polypropylene Eppendorf tubes. The samples were frozen at −20°C upon arrival at the laboratory. For the analysis, equal amounts from each of the five samples were combined in an aliquot that was refrozen and then evaluated with a 17beta-Estradiol Saliva ELISA und a Progesterone Saliva ELISA Kit from IBL International (Tecan Group) following the manual provided by the manufacturer. Optical densities were transferred to concentrations with the internet tool https://elisaanalysis.com. The lowest detection level of estradiol in saliva was 2.1 pg/ml, and 3.13 pg/ml for progesterone.

Description of the Comparison Samples for the Meta-Analysis

For the second analysis, we combined our new behavioral data with previous results from the first test day of Diekhof and Ratnayake (1). In addition, the data from two other unpublished within-subject studies from our laboratory were also included.

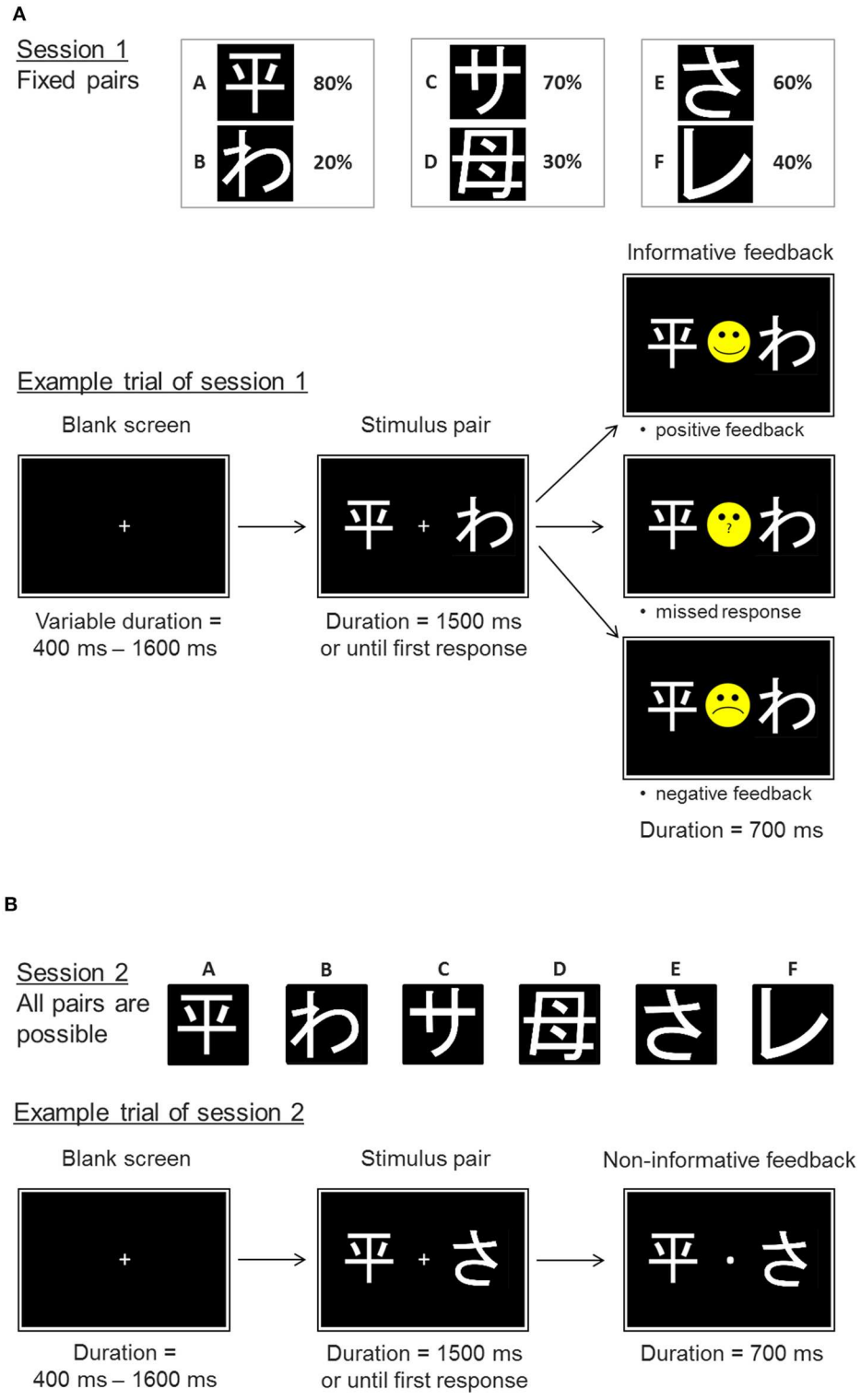

FIGURE 1 | Experimental paradigm. Example trials of the probabilistic feedback task. **(A)** Session 1 represents the learning phase with the fixed stimulus pairs AB, CD, and EF. The probabilities of positive feedback are displayed next to the respective symbols. Example trial: a trial starts with a blank screen delay of variable length. Then the stimulus pair is shown until a response is made or until 1,500 ms have passed without responding. Following the response or the 1,500 ms the participant receives an informative feedback, which either indicates a positive or negative performance outcome (smiley vs. grumpy face) or informs the participant that no response has been made. **(B)** In the transfer phase (session 2) the participants are not only confronted with the original pairs, but also face novel pairs. Responses are no longer followed by informative feedback, but participants just receive a dot indicating that a response has been made.

In all studies women were tested in the late follicular or the mid luteal phase at the first, naïve test day. All studies used the same version of the probabilistic feedback learning task described above, which was always performed first, before any other cognitive tasks included in the different studies. Other study specificities (like daytime or season) were not further considered here. All studies had in common that they assessed healthy young women with a comparable degree of education (on average undergraduate or graduate university education) and age (mean age ± sem = 24.27 ± 0.35 years; age range = 20–30 years), and the pre-test exclusion criteria were identical. All data included here were from the first, naïve test day.

The fMRI study by Diekhof and Ratnayake (1) contributed 15 cases of whom nine were tested in the late follicular phase and six in the mid luteal phase on test day one. The data for this fMRI study were collected by three female experimenters from August 2012 to March 2013. Session 1 of the probabilistic feedback learning task was performed in the MR-scanner, while the subsequent transfer task (session 2) was completed in a secluded test room directly after the participant left the MR-scanner.

The first unpublished study included in the meta-analysis was an EEG study. Originally this study tested women in three hormonally distinct cycle phases (menstruation, late follicular, and mid luteal phase), using a counterbalanced within-subject design. Three female experimenters collected the data between November 2012 and April 2013. Here, we used the data of the first naïve test day. Eight women started the EEG study in the late follicular phase and six were in the mid luteal phase at test day one. Women who started the test protocol during menstruation were not considered for the meta-analysis. Both sessions of the probabilistic feedback learning task were performed in a secluded test room, while the EEG was recorded.

The second unpublished study was designed to assess the influence of menstrual cycle phase on the human transcriptome in peripheral blood and assessed its relation to reinforcement learning. Women completed three repeated tests during menstruation, late follicular, and mid luteal phase in a counterbalanced within-subject design. Data for this transcriptome study were collected by two female experimenters from March to June 2017. Ten women were in the late follicular phase on the first test appointment, while eight started the study in the mid luteal phase. The data of the menstruation test were not included here. In the transcriptome study women arrived with an empty stomach at the test facility between 8 a.m. and 9 a.m., where a blood sample for transcriptome analysis was drawn. After that women had a small breakfast and commenced to the secluded test room, where they completed the probabilistic feedback learning task.

The data from the first test day of the three previous studies were combined with the present data to perform a meta-analysis of all data. Since the three previous studies specifically focused on the late follicular and the mid luteal phase, we decided to include only the data points from the late follicular ($n = 19$) and the mid luteal ($n = 22$) phase of the present study. We also standardized the cycle days from previous studies to a 28-day cycle (see procedure above). This resulted in the following distribution of standardized cycle days in the late follicular phase (mean ± sd): fMRI study = 12.3 ± 2.4; EEG study = 11.4 ± 2.4; Transcriptome study = 13.3 ± 2.3; Present study = 10.2 ± 1.5; All studies combined ($n = 45$) = 11.4 ± 2.3. The standardized cycle days of the mid luteal phase were: fMRI study = 22.5 ± 2.5; EEG study = 21.3 ± 4.8; Transcriptome study = 22.7 ± 2.0; Present study = 22.3 ± 1.8; All studies combined ($n = 40$) = 22.2 ± 2.5.

Statistical Analysis

The statistical analyses were performed with IBM SPSS Statistics (Version 22). We were primarily interested in the results of the transfer phase (session 2) that reflects overall learning outcome and the difference in punishment sensitivity (Avoid B performance), which had been observed when comparing the two cycle phases in the within-subject design of Diekhof and Ratnayake (1). For this we used a repeated measures two-way ANOVA with the within-subject factor "*learning outcome*" (Choose A and Avoid B performance) and the between-subjects factor "*cycle phase*" (follicular and luteal phase). *T*-tests were used for direct *post-hoc* comparisons. Statistical significance was assumed at $p < 0.05$, two-tailed.

RESULTS

Group comparisons showed that the two test groups were well-matched for the various characteristics of working memory, personality and mood (**Table 1**). Salivary hormone concentrations were measured in 28 women of the follicular and in 35 of the luteal phase. Similar to Diekhof and Ratnayake (1) the mean estradiol level did not differ between the follicular and the luteal phase ($p = 0.97$), but there was a significant difference in progesterone ($p < 0.001$) and in the estradiol to progesterone ratio ($p < 0.001$) (**Table 1**), suggesting different relative contributions of estradiol and progesterone in the two cycle phases (see also **Figure 2A** for a descriptive overview of the hormonal transitions over time bins).

Learning outcome of session 1 was comparable between the cycle phases. Accordingly, all participants learned to select the better option from the three pairs AB, CD, and EF with a higher frequency regardless of the cycle phase (all $p > 0.331$; see **Table 2**). This was similar to Diekhof and Ratnayake (1).

The data from the subsequent transfer phase were subjected to a repeated measures, two-way ANOVA with the within-subject factor "*learning outcome*" (Choose A and Avoid B performance) and the between-subjects factor "*cycle phase*" (follicular and luteal phase). Apart from a significant main effect of "*learning outcome*" [$F_{(1, 73)} = 17.3$, $p < 0.001$, partial eta$^2 = 0.19$], and the absence of a main effect of "*cycle phase*" [$F_{(1, 73)} = 1.6$, $p = 0.204$, partial eta$^2 = 0.02$], we observed a significant interaction between the between-subjects factor "*cycle phase*" and the within-subject factor "*learning preference*" [$F_{(1, 73)} = 4.0$, $p = 0.049$, partial eta$^2 = 0.05$]. This was the result of a significant decline of the ability to avoid the option that most often yielded negative feedback (Avoid B performance) in the follicular phase relative to the luteal phase [mean ± sem: follicular phase = 56.38 ± 3.48%; luteal phase = 66.81 ± 3.04%; $t_{(73)} = -2.27$, $p = 0.026$, Cohen's d = 0.52,

TABLE 1 | Demographic characteristics of the participants.

	Follicular phase Mean ± sem	Luteal phase Mean ± sem	*t*-value	*p*-value (2-tailed)	95% CI (lower, upper)
Age (years)	25.1 ± 0.5 [*n* = 36]	25.2 ± 0.6 [*n* = 38]	−0.16	0.873	−1.69, 1.44
Estradiol (pg/ml)	5.32 ± 0.62 [*n* = 28]	5.34 ± 0.42 [*n* = 35]	−0.04	0.971	−1.47, 1.42
Progesterone (pg/ml)*	**48.74 ± 3.32 [*n* = 28]**	**161.75 ± 14.28 [*n* = 35]**	**−7.71**	**<0.001**	**−142.70, −83.32**
Cycle length (days)	29.7 ± 0.5 [*n* = 36]	30.3 ± 0.6 [*n* = 39]	−0.69	0.494	−2.08, 1.01
Duration of menstrual cycle (days)	29.7 ± 0.5 [*n* = 36]	30.3 ± 0.6 [*n* = 39]	0.09	0.925	−0.47, 0.52
Duration of menstrual bleeding (days)	4.9 ± 0.2 [*n* = 36]	4.9 ± 0.2 [*n* = 39]	0.16	0.870	−0.63, 0.75
Standardized cycle day in which women were tested (comparison of all women)*	**7.3 ± 0.6 [*n* = 36]**	**20.9 ± 0.5 [*n* = 39]**	**−17.93**	**<0.001**	**−15.1, −12.1**
Standardized cycle day in the comparison of selective parts of the two cycle phases (part of the sample)*	**10.2 ± 0.3 [*n* = 19]**	**22.3 ± 0.4 [*n* = 22]**	**−23.52**	**<0.001**	**−13.1, −11.1**
BIS score	63.4 ± 1.7 [*n* = 36]	62.9 ± 1.6 [*n* = 38]	0.21	0.833	−4.2, 5.1
Lanthony score	60.8 ± 1.5 [*n* = 36]	68.5 ± 5.3 [*n* = 38]	−1.41	0.166	−18.74, 3.32
Mood score	90.2 ± 2.6 [*n* = 36]	87.7 ± 2.3 [*n* = 39]	0.745	0.459	−4.28, 9.40
Self-reported stress	2.4 ± 0.2 [*n* = 36]	2.5 ± 0.1 [*n* = 38]	−0.35	0.729	−0.57,0.40
PMS score	20.3 ± 2.5 [*n* = 36]	22.5 ± 2.1 [*n* = 39]	−0.67	0.507	−8.61, 4.29
Digit span (combined forward and backward span)	17.7 ± 0.5 [*n* = 36]	17.4 ± 0.5 [*n* = 39]	0.41	0.684	−1.1, 1.7

*Groups may slightly vary in size, as some data could not be acquired from all subjects. *Significant effects ($p < 0.05$) are plotted in bold and are marked with an asterisk.*

95% CI [lower, upper] = −19.60, −1.27]. In contrast Choose A performance remained unaffected by cycle phase [mean ± sem: follicular phase = 73.62 ± 3.12%; luteal phase = 72.86 ± 3.60%; $t_{(73)} = 0.16$, $p = 0.875$, 95% CI [lower, upper] = −8.81, 10.32] (**Figure 2B**).

A second two-way ANOVA with the same factors, which was however restricted to data points from the late follicular phase, near the pre-ovulatory estradiol peak (standardized cycle days 7–12, $n = 19$), and of the mid luteal phase when progesterone approached its maximum (cycle days 19–24, $n = 22$), confirmed the significant two-way interaction between "*cycle phase*" and "*learning preference*" [$F_{(1, 39)} = 4.50$, $p = 0.04$, partial eta^2 = 0.10]. The *post-hoc* test showed that avoidance learning was again significantly different between cycle phases [mean ± sem: follicular phase = 54.07 ± 4.65%; luteal phase = 68.48 ± 4.31%; $t_{(39)} = -2.28$, $p = 0.028$, Cohen's d = 0.71, 95% CI [lower, upper] = −27.23, −1.61]. The augmented effect size d however suggests that despite a reduction in sample size the assessment of the late follicular and the mid luteal phase, which should be most distinct in terms of their relative estradiol and progesterone influence, may even enhance discriminatory power in the between-subjects approach. Again, Choose A performance did not differ between cycle phases [mean ± sem: follicular phase = 73.35 ± 4.46%; luteal phase = 70.93 ± 5.04%; $t_{(39)} = 0.35$, $p = 0.726$, 95% CI [lower, upper] = −11.40, 16.23].

In a second step we performed the two-way ANOVA on the combined data of the transfer task from the three previous and the present study ($n = 88$; $n_{\text{late follicular phase}} = 49$). The associated data from session 1 can be found in **Table 3**, which shows the comparable learning outcome in both cycle phases. In the transfer phase, a significant interaction between "*cycle phase*" and "*learning outcome*" could be observed [$F_{(1, 86)} = 4.86$, $p = 0.030$, partial eta^2 = 0.05]. The main effect of "*learning outcome*" was also significant [$F_{(1, 86)} = 19.79$, $p < 0.001$, partial eta^2 = 0.19], while the main effect of "*cycle phase*" was not [$F_{(1, 86)} = 2.46$, $p = 0.120$, partial eta^2 = 0.03]. Women were better at avoiding the worst option B during the mid luteal compared to the late follicular phase [mean ± sem: follicular phase = 56.64 ± 2.79%; luteal phase = 67.79 ± 3.23%; $t_{(86)} = -2.62$, $p = 0.010$, Cohen's d = 0.56, 95% CI [lower, upper] = −19.60, −2.70], but demonstrated no difference in choosing stimulus A from novel pairs [mean ± sem: follicular phase = 74.41 ± 2.73%; luteal phase = 73.78 ± 3.39%; $t_{(86)} = 0.147$, $p = 0.884$, 95% CI [lower, upper] = −7.96, 9.23] (**Figure 2C**). As the sample size of the individual studies that contributed to the meta-data was very small, we only visually inspected the performance data of test day one from each of the four studies (**Figures 3A,B**). This showed that the difference in Avoid B performance when subtracting the percentage of the late follicular phase from that of the luteal phase (Δluteal-follicular phase) was always negative ($\text{mean}_{\Delta\text{luteal}-\text{follicular phase}}$: EEG study = −5.15%; fMRI study = −6.71%; Transcriptome study = −13.25%; Present study = −14.42%). In contrast, the delta of Choose A performance varied considerably between studies ($\text{mean}_{\Delta\text{luteal}-\text{follicular phase}}$: EEG study = −17.04%; fMRI study = 18.29%; Transcriptome study = −5.99%; Present study = 2.42%). This implied that only the ability to avoid a negative outcome appeared to be consistently reduced in the late follicular compared to the mid luteal phase across the four studies from our laboratory, even when comparing independent observations in the two critical menstrual cycle phases.

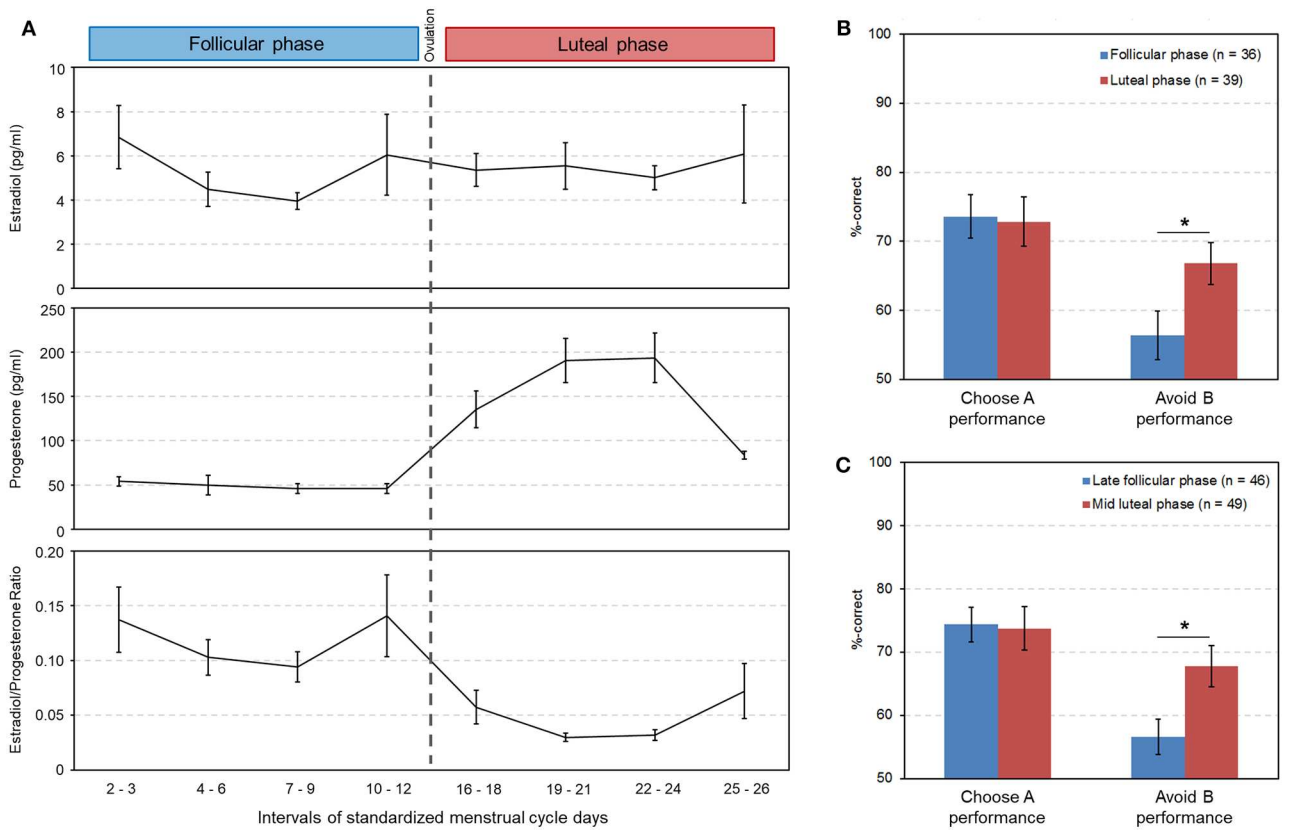

FIGURE 2 | Hormonal changes across the menstrual cycle and their association with reinforcement learning. **(A)** Changes in salivary estradiol, progesterone and in the estradiol/progesterone ratio across the menstrual cycle (data are from independent subjects; cycle days were standardized to a 28-day cycle). Days 13–15, during which ovulation most likely occurred, are not shown here. **(B)** Significantly lower punishment sensitivity in the follicular phase as opposed to the luteal phase in the between-subjects study [$t_{(73)} = -2.27$, $p = 0.026$, Cohen's d = 0.52, 95% CI [lower, upper] = −19.60, −1.27]. **(C)** Significantly lower punishment sensitivity in the late follicular phase compared to the mid luteal phase in the meta-analysis [$t_{(86)} = -2.62$, $p = 0.01$, Cohen's d = 0.56, 95% CI [lower, upper] = −19.60, −2.70]. Significant differences between cycle phases ($p < 0.05$) are indicated with an asterisk.

TABLE 2 | Learning performance did not differ between the follicular and luteal phase.

Stimulus pair	Follicular phase: selection of better option (%) ($n = 36$)	Luteal phase: selection of better option (%) ($n = 39$)	*t*-value (*p*-value)
Session 1: Pair AB	68.13 ± 3.12	71.36 ± 3.62	−0.67 (0.504)
Session 1: Pair CD	54.87 ± 2.83	59.15 ± 3.30	−0.98 (0.332)
Session 1: Pair EF	55.25 ± 2.38	51.61 ± 2.98	0.95 (0.348)
Session 2: Old Pair AB	75.87 ± 3.90	80.53 ± 4.12	−0.82 (0.416)

Mean percentage ± sem for the selection of the better option from the respective pairs. Data are from the present between-subjects design.

TABLE 3 | Learning performance did not differ between the late follicular and mid luteal phase in the meta-analysis based on the combined data from four independent studies.

Stimulus pair	Late follicular phase: selection of better option (%) ($n = 46$)	Mid luteal phase: selection of better option (%) ($n = 42$)	*t*-value (*p*-value)
Session 1: Pair AB	69.81 ± 2.69	72.98 ± 2.97	−0.80 (0.429)
Session 1: Pair CD	56.77 ± 2.65	61.10 ± 3.24	−1.04 (0.301)
Session 1: Pair EF	54.26 ± 2.13	55.82 ± 2.59	−0.47 (0.642)
Session 2: Old Pair AB	79.00 ± 3.64	83.31 ± 3.14	−0.89 (0.375)

Mean percentage ± sem for the selection of the better option from the respective pairs.

DISCUSSION

The present study collective was recruited as an independent replication sample, based on which we wanted to evaluate previous results from a study that used the common within-subject design (1). Our study thus constitutes the first attempt of a conceptual replication in the field of menstrual cycle research. Additionally, the present study was intended to demonstrate the feasibility of a between-subjects design in menstrual cycle

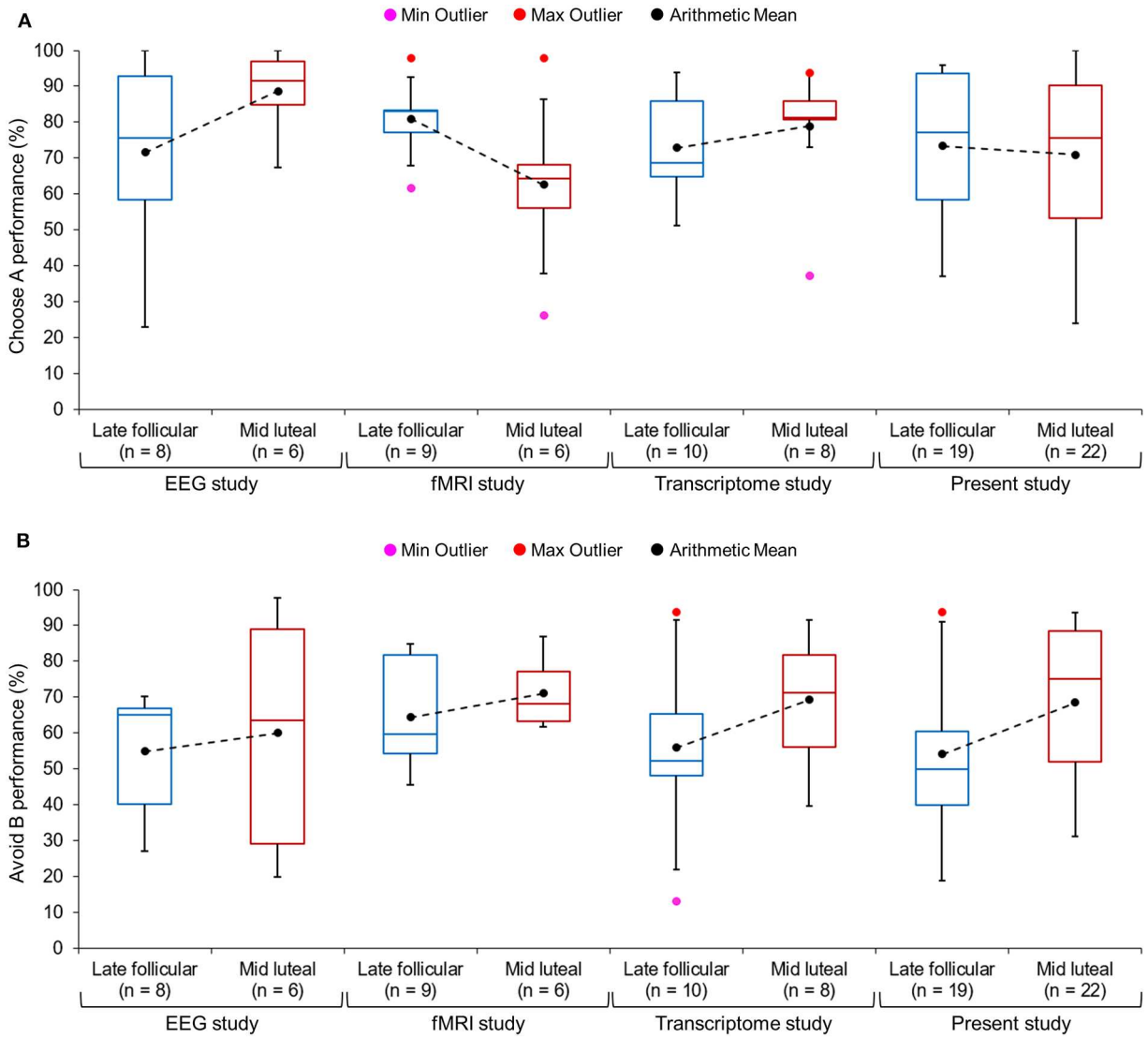

FIGURE 3 | Boxplots of the percentage of **(A)** correct choice of the best stimulus A, and of **(B)** correct avoidance of the worst stimulus B in novel stimulus pairs presented during the transfer phase. The ends of the whisker are set at 1.5*interquartile-range above the third quartile and 1.5*interquartile-range below the first quartile. Only the minimum (Min Outlier) and maximum (Max Outlier) outliers are shown here. We also added the arithmetic mean of the percentage of correct selection for each study and cycle phase to the box plot (see black dots; mean values from the same study are connected by a dashed line). The figure was created with the box plot template by Wittwer (22).

research. For this purpose, 75 women were tested once with a probabilistic feedback learning task in one of the designated cycle phases. The data from the follicular and the luteal phase were then compared. In a second step, we combined the newly acquired behavioral data with data from three previous studies of our laboratory. Notably, we included only data points from the first, naïve test day of these studies and compared behavior in the late follicular and mid luteal phase. Both analyses demonstrated the consistency of the phase-related difference in avoidance learning and the observed medium effect sizes were in the range of the previously documented within-subject effect. We also showed that, although the actual extent of the reduction in the ability to avoid negative feedback in the high estradiol phase relative to the high progesterone state varied between studies, it occurred in all four studies. Altogether, these results provide evidence for the consistency of a behavioral effect across studies and further underscore the assumption that it may indeed reflect a hormone-related variation in female reinforcement learning ability.

Replications are still rare in the cognitive sciences. Yet, they are necessary to evaluate the implications of fundamental findings for societal and health-related considerations, and to create a solid basis for innovative research (23). Behavioral changes across the menstrual cycle have been assumed to reflect

the degree to which estradiol and progesterone influence neural transmission in various cognitive networks, but previous findings were mixed (2, 3). Most results thereby came from within-subject designs that tested only small samples ($n < 30$) and replication studies are currently missing. Therefore, it is possible that many findings were in fact sporadic or might have been disproportionally inflated by involuntary data dredging (24). The present study was an attempt to account for previous shortcomings. We were able to replicate the cycle phase related differences in avoidance learning in a between-subjects design. In that way, our study represents the first conceptual replication of a behavioral within-subject variation (1), which suggests that the observed effect can probably be attributed to changes in hormonal state across the menstrual cycle.

Here, we observed the hypothesized alteration in avoidance learning in women, who were tested only once in a task-naïve state. The behavioral difference was thereby already visible when we compared data that covered the complete follicular and luteal phase (**Figure 2B**). Diekhof and Ratnayake (1) restricted the two tests to the hormonally more distinct phases of the late follicular and the mid luteal phase. If we did so in our sample, we found that the effect remained significant and the *post-hoc* comparison yielded a slightly higher effect size than the complete sample. This suggests that the finding of reduced avoidance learning in the state, in which the estradiol effect is unopposed by progesterone, i.e., the late follicular phase, compared to the state of increased progesterone influence, i.e., the mid luteal phased, is in fact reliable and can be replicated when comparing two groups of independent subjects in the respective phases of the menstrual cycle.

In addition to that, we combined the present data from the late follicular and the mid luteal phase with the data from the first test appointment of Diekhof and Ratnayake (1) and of two unpublished studies from our laboratory. This meta-analysis comprised 88 data points that were collected by nine different female experimenters between August 2012 and May 2018 and with different ramifications (e.g., in the early morning following a blood-draw for transcriptome analysis, while undergoing EEG-measurement, or in the fMRI scanner). Yet, all studies used the same probabilistic feedback learning task as the first experimental paradigm in the test procedure and assessed young healthy women of comparable age and education. Again, we found that the effect under research was reliable. First, the combined data confirmed the difference in the ability to avoid the least rewarded option between the luteal and follicular phases (**Figure 2C**), and second, in each of the included studies this difference was negative and thus conformed to the original direction of the within-subject finding (**Figure 3B**). This latter observation provides further evidence for the inferential reproducibility of the original finding. Amrhein et al. (24) argue that the faulty interpretation of a replication as being non-significant and therefore as representing a contradiction to the original results, only because the *p*-value exceeds 0.05, lets many researchers overlook the fact that *p*-values may rather reflect graded evidence against the null hypothesis. In that way, *p*-values cannot be considered as the main indicator for the reliability of research. This is because *p*-values and significance are hardly replicable, even if the alternative hypothesis is true. Amrhein et al. (24) provide several examples that show that even at a good statistical power of 80%, two studies can be "conflicting," in that one of the results will be significant and the other will not fulfill the statistical criterion, in one third of the cases, if there is a true effect. A replication can therefore not be interpreted as having failed only because it is non-significant (24). It is often neglected that the replication findings in fact point in the same direction as the original ones, even if the statistical criterion of $p < 0.05$ is not fulfilled. Since the individual samples of our previous studies were too small to statistically compare the data separately, the combined results (**Figure 2C**) as well as the descriptive finding of a negative Δluteal-follicular phase in all studies (**Figure 3A**) further support the reliability of the reduction of avoidance learning in the high estradiol state of the follicular phase relative to the luteal phase that was dominated by the effect of progesterone.

What are the implications of the present findings for future research? First, they demonstrate a reliable behavioral effect that is reproducible across different studies, and in a between-subjects comparison, given a matched sample of sufficient size. In that way the present data are consistent with rodent evidence that demonstrated the partly antagonistic effects of estradiol and progesterone on dopaminergic transmission [e.g., (5–8)]. Estradiol may thereby act as a dopamine agonist that promotes reward seeking behaviors, but inhibits the ability to adequately adapt to a punishing outcome. Conversely, progesterone may suppress dopaminergic responses and down-regulates tonic dopamine thus acting in the opposite direction of estradiol (3). Second, the present results may open new avenues for research protocols that examine menstrual cycle effects. By showing that a between-subjects approach may produce comparable results as the common within-subject design, could help to overcome two problems that always accompany within-subject designs: To begin with, the repeated testing of a typical menstrual cycle study can lead to expectancy effects that may contaminate the already small behavioral effects related to changes in hormonal state. Wallen and Rupp (10) showed that the menstrual cycle phase during first exposure to sexual stimuli predicted subsequent interest in sexual stimuli. If women started their test protocol in the high estradiol state of the late follicular phase they showed not only increased interest in the sexually explicit photos there, but this effect was also transferred to the other cycle phases. In contrast, no increased sexual interest in the late follicular phase could be demonstrated, if the women started in any of the remaining cycle phases (10). Leeners et al. (11) noted that "[…] *it is important to recognize that the specific timing of the first test application still introduces a major bias even in counterbalanced test-sequencing designs.*" This is because in one group the practice effect will most likely parallel the actual effect under research (e.g., the influence of estradiol on sexual interest), which could then disproportionally contribute to the expected outcome, or bias processing in the other cycle phases. Therefore, counterbalancing cannot completely wipe out practice effects [see also Leeners et al. (11) for discussion]. Only two studies that assessed changes in stress responsivity across the menstrual cycle intentionally used a between-subjects design and compared the follicular and luteal phase (25) or the early follicular phase and the period around ovulation (26). Maki et al.

(25) found that the cortisol stress response was significantly increased in women, who were tested during the follicular phase, and this was also related to the extent of emotional memory impairment women experienced during this phase. Albert et al. (26) reported a reduced distress experience during ovulation that was reflected in altered neural responses. Yet, while in stress-related research between-subjects designs are quite common and also mandatory, since expectations associated with the stress intervention could facilitate modulatory mechanisms like stress coping, to our knowledge the present study is the first between-subjects design employed in the context of reinforcement learning and reward processing.

Moreover, repeated tests at two or even three predetermined cycle phases constitute a logistic challenge. Menstrual cycles tend to show irregularities so that critical phases can be missed, and the daily obligations of a given subject often interfere with test schedules synchronized to individual cycles. Given the limited time frame of research grants this can also considerably limit the sample size. In fact, the average sample size of previous studies in the domain of reward processing is about 17 women [see Diekhof (3) for overview]. Apart from that, lengthy data collection periods may also incur the risk of contamination by other unwanted factors, like seasonal variations in the neuroendocrine response [e.g., Eisenberg et al. (27)]. With regard to the meta-data, we found the largest behavioral difference in the present study which also included the biggest sample. The second largest and numerically almost comparable difference between cycle phases was found in the Transcriptome study, for which the data were collected under the most controlled conditions (**Figure 3B**). In fact, to keep the blood transcriptome free from contaminating factors (e.g., food intake, circadian, or seasonal influences), women were always tested in the early morning and the study was completed within 3.5 months. In contrast, the two neurophysiological studies tested women whenever time slots were available on the desired test day. Also, data collection was not confined to a certain season. Still, even these latter studies identified the behavioral effect, although it was numerically smaller (**Figure 3B**). This suggests that a controlled test environment that also considers circadian and seasonal influences on the neuroendocrine response may further support data quality, which again underscores the fact that menstrual cycle research could benefit from test protocols that favor the collection of large samples over a short period of time. Nevertheless, it is also important to point out that between-subjects designs do not only offer advantages, but can produce a number of potential confounds caused by inter-subject variability. The present study tried to closely match participants with regard to education level, age, working memory capacity, impulsiveness, dopaminergic capacity, mood-state, and premenstrual symptoms. All subjects were healthy and did not report any previous or present psychiatric or neurological problems. Yet, it is still possible that the subjects from the two cycle phases differed in an aspect that was not matched here. For example, genetic variability in dopaminergic baseline capacity could represent a significant source of inter-subject variance (28), which was not controlled here. Since the results from the meta-analysis conformed to the present observation, and further replicated an already published within-subject finding, we think that it is very unlikely that an unknown inter-subject aspect determined the group-difference in avoidance learning.

CONCLUSION

Taken together, both the present study and the meta-analysis show that our previous within-subject finding can be replicated with a between-subjects design. This does not only support the reliability of the behavioral effect, but also opens new possibilities for future test protocols in menstrual cycle research. The present design circumvents the possible complications caused by repeated testing, since subjects are naïve to the test paradigm. Further, it also does not incur the logistic restrictions of within-subject designs and avoids lengthy periods of data collection. Given sufficient statistical power, i.e., a sufficient sample size that should be pre-determined by a power analysis, close matching of participants, and with careful control of the ramifications that accompany data collection (e.g., by restricting tests to a certain day time or season), future studies should achieve even more valuable contributions to menstrual cycle research by using comparable between-subjects approaches. Within this context, pre-registration of test protocols may further contribute to the reliability of future findings made by menstrual cycle research.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethikkommission der Ärztekammer Hamburg. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ED and SH have contributed in all steps of the research, including development of the experimental design, data collection, preprocessing, and analysis. ED has written the first draft of the paper. FO and CS have contributed in data collection, preprocessing, and analysis. SK has contributed in data analysis. SH, FO, CS, and SK have reviewed the first draft of the paper and provided valuable suggestions for its improvement.

FUNDING

This research was funded by resources provided by the Universität Hamburg (Germany).

ACKNOWLEDGMENTS

We would like to thank A. Kroll for her contribution in the analysis of salivary estradiol and progesterone, M. Langbehn for

programming the computer test and the preprocessing batches, and Y. Hartmann, M. Ratnayake, S. Weinkouff, S. Scholz, S. Bader, and F. Schuran for help with data acquisition. In addition, we would like to thank all the participants of this research project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00231/full#supplementary-material

REFERENCES

1. Diekhof EK, Ratnayake M. Menstrual cycle phase modulates reward sensitivity and performance monitoring in young women: preliminary FMRI evidence. *Neuropsychologia*. (2016) 84:70–80. doi: 10.1016/j.neuropsychologia.2015.10.016
2. Sakaki M, Mather M. How reward and emotional stimuli induce different reactions across the menstrual cycle guyer, Amandae. *Soc Personal Psychol Compass*. (2013) 6:1–17. doi: 10.1111/j.1751-9004.2011.00415.x
3. Diekhof EK. Estradiol and the reward system in humans. *Curr Opin Behav Sci*. (2018) 23:58–64. doi: 10.1016/j.cobeha.2018.03.010
4. Becker JB. Gender differences in dopaminergic function in striatum and nucleus accumbens. *Pharmacol Biochem Behav*. (1999) 64:803–12. doi: 10.1016/S0091-3057(99)00168-9
5. Becker JB. Sex differences in addiction. *Dialogues Clin Neurosci*. (2016) 18:395–402.
6. Luine VN, Rhodes JC. Gonadal hormone regulation of MAO and other enzymes in hypothalamic areas. *Neuroendocrinology*. (1983) 36:235–41. doi: 10.1159/000123461
7. Dluzen DE, Ramirez VD. Intermittent infusion of progesterone potentiates whereas continuous infusion reduces amphetamine-stimulated dopamine release from ovariectomized estrogen-primed rat striatal fragments superfused *in vitro*. *Brain Res*. (1987) 406:1–9. doi: 10.1016/0006-8993(87)90762-1
8. Motzo C, Porceddu ML, Maira G, Flore G, Concas A, Dazzi L, et al. Inhibition of basal and stress-induced dopamine release in the cerebral cortex and nucleus accumbens of freely moving rats by the neurosteroid allopregnanolone. *J Psychopharmacol*. (1996) 10:266–72. doi: 10.1177/026988119601000402
9. Maia TV, Frank MJ. From reinforcement learning models to psychiatric and neurological disorders. *Nat Neurosci*. (2011) 14:154–62. doi: 10.1038/nn.2723
10. Wallen K, Rupp HA. Women's interest in visual sexual stimuli varies with menstrual cycle phase at first exposure and predicts later interest. *Horm Behav*. (2010) 57:263–8. doi: 10.1016/j.yhbeh.2009.12.005
11. Leeners B, Kruger THC, Geraedts K, Tronci E, Spanaus K, Schippert C, et al. Lack of associations between female hormone levels and visuospatial working memory, divided attention and cognitive bias across two consecutive menstrual cycles. *Front Behav Neurosci*. (2017) 11:120. doi: 10.3389/fnbeh.2017.00120
12. Shirazi TN, Bossio JA, Puts DA, Chivers ML. Menstrual cycle phase predicts women's hormonal responses to sexual stimuli. *Horm Behav*. (2018) 103:45–53. doi: 10.1016/j.yhbeh.2018.05.023
13. Schmidt S. Shall we really do it again ? The powerful concept of replication is neglected in the social sciences. *Rev Gen Psychol*. (2019) 13:90–100. doi: 10.1037/a0015108
14. Faul F, Erdfelder E, Lang AG, Buchner A. G * Power 3 : a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods*. (2007) 39:175–91. doi: 10.3758/BF03193146
15. Puts DA. Mating context and menstrual phase affect women's preferences for male voice pitch. *Evol Hum Behav*. (2005) 26:388–97. doi: 10.1016/j.evolhumbehav.2005.03.001
16. Patton JH, Stanford MS, Barratt ES. Factor structure of the barratt impulsiveness scale. *J Clin Psychol*. (1995) 51:768–74. doi: 10.1002/1097-4679(199511)51:6<768::AID-JCLP2270510607>3.0.CO;2-1
17. Colzato LS, Sellaro R, Hulka LM, Quednow BB, Hommel B. Cognitive control predicted by color vision, and vice versa. *Neuropsychologia*. (2014) 62:55–9. doi: 10.1016/j.neuropsychologia.2014.07.010
18. Geller AM. A table of color distance scores for quantitative scoring of the lanthony desaturate color vision test. *Neurotoxicol Teratol*. (2001) 23:265–7. doi: 10.1016/S0892-0362(01)00139-8
19. Steyer R, Schwenkmezger P, Notz P, Eid M. *Der Mehrdimensionale Befindlichkeitsfragebogen (MDBF)*. Hogrefe: Handanweisung (1997). Available online at: http://136.199.85.91:80/pubpsych/Search.action?q=ID%3DPDID_srrf91en15&isFullView=true&stats=BMD&search=
20. Ditzen B, Nussbeck F, Drobnjak S, Spörri C, Wüest D, Ehlert U. Validierung eines deutschsprachigen DSM-IV-TR basierten fragebogens zum prämenstruellen syndrom. *Zeitschrift Klinische Psychol Psychother*. (2011) 40:149–59. doi: 10.1026/1616-3443/a000095
21. Frank MJ, Seeberger LC, O'Reilly RC. By carrot or by stick: cognitive reinforcement learning in parkinsonism. *Science*. (2004) 306:1940–3. doi: 10.1126/science.1102941
22. Wittwer JW. *Box Plot Template, Vertex42.com*. (2009). Available online at: https://www.vertex42.com/ExcelTemplates/box-whisker-plot.html (accessed November 11, 2019).
23. Open Science Collaboration. Estimating the reproducibility of psychological science. *Science*. (2015) 349:aac4716. doi: 10.1126/science.aac4716
24. Amrhein V, Korner-Nievergelt F, Roth T. The earth is flat ($p > 0:05$): significance thresholds and the crisis of unreplicable research. *PeerJ*. (2017) 5:e3544. doi: 10.7717/peerj.3544
25. Maki PM, Mordecai KL, Rubin LH, Sundermann E, Savarese A, Eatough E, et al. Menstrual cycle effects on cortisol responsivity and emotional retrieval following a psychosocial stressor. *Horm Behav*. (2015) 74:201–8. doi: 10.1016/j.yhbeh.2015.06.023
26. Albert K, Pruessner J, Newhouse P. Estradiol levels modulate brain activity and negative responses to psychosocial stress across the menstrual cycle. *Psychoneuroendocrinology*. (2015) 59:14–24. doi: 10.1016/j.psyneuen.2015.04.022
27. Eisenberg DP, Kohn PD, Baller EB, Bronstein JA, Masdeu JC, Berman KF. Seasonal effects on human striatal presynaptic dopamine synthesis. *J Neurosci*. (2010) 30:14691–4. doi: 10.1523/JNEUROSCI.1953-10.2010
28. Jakob K, Ehrentreich H, Holtfrerich SKC, Reimers L, Diekhof EK. DAT1-genotype and menstrual cycle, but not hormonal contraception, modulate reinforcement learning: preliminary evidence. *Front Endocrinol*. (2018) 9:60. doi: 10.3389/fendo.2018.00060

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.

frontiers in Endocrinology

OPINION
published: 14 May 2020
doi: 10.3389/fendo.2020.00236

Allopregnanolone, the Neuromodulator Turned Therapeutic Agent: Thank You, Next?

*Graziano Pinna**

Department of Psychiatry, The Psychiatric Institute, University of Illinois at Chicago, Chicago, IL, United States

Keywords: brexanolone, allopregnanolone (3α,5α-THP), postpartum depression, fast-acting antidepressant, $GABA_A$ receptor, 5α-reduced steroids, 5α-reductase, 3α-HSD

INTRODUCTION

Allopregnanolone, today best known as brexanolone and marketed as Zulresso™ for the treatment of postpartum depression is part of only two recently Food and Drug Administration (FDA)-approved fast-acting antidepressants, with esketamine nasal spray, an NMDA receptor antagonist used in treatment-resistant depression being the other (1).

The trajectory, lasting 80 years, that brought allopregnanolone from its discovery (2) in 1938 in the adrenal glands, to understanding its fast non-genomic mechanism in potentiating membrane neurotransmitter receptors, including $GABA_A$ receptors (3, 4), underlying its role in acute and chronic stress (5–7), discovering its powerful non-sedative pharmacological effects as anxiolytic and antidepressant agent in animal models and humans (8–10), to the design of the first clinical trials for postpartum depression (11), and finally to the shelves of the clinics in 2019, is regarded as one of the best examples of translational drug development in neuropsychopharmacology (12, 13).

This article redraws the most significant milestones in allopregnanolone discoveries and evaluates future perspective for a new generation of neurosteroid-based treatments in neuropsychiatry. The role of allopregnanolone as a potential biomarker for mood disorders and its pharmacological mechanism in improving behavioral deficits will be discussed.

ALLOPREGNANOLONE: 80 YEARS OF SCIENTIFIC DISCOVERIES

Following its discovery in the adrenal glands (2), Baulieu's laboratory observed (1981) that allopregnanolone can be produced in brain in a manner unrelated to peripheral renovation rates (14). This finding led to coin the term "*neurosteroid*" to define a chemically identical steroid specifically produced by the brain as opposed to "*neuroactive steroids*," coined by Paul and Purdy (15), which defines steroids produced peripherally that reach and act in the brain. However, it took 25 years to demonstrate that allopregnanolone and its biosynthetic enzymes, 5α-reductase type I (5α-RI) and 3α-hydroxysteroid dehydrogenase (3α-HSD), are expressed in glutamatergic neurons in cortex, hippocampus and basolateral amygdala, and in long-projecting GABAergic neurons in reticulus thalamic nucleus, striatum, central amygdala, and cerebellum but not in glial cells of rodent and human brain (16–20). In 1986, Paul's laboratory observed allopregnanolone is a potent positive allosteric modulator of GABA's action at synaptic and extrasynaptic $GABA_A$ receptors (3, 4, 21). Costa and Guidotti' laboratories later cloned and described the function of 18 kDa translocator (TSPO), involved in gating cholesterol entry into the inner mitochondrial membranes, where cholesterol is converted to pregnenolone, the precursors of all neurosteroids (22, 23). Acute stress in rodents fast induces allopregnanolone biosynthesis underlying its role in stress response and demonstrating allopregnanolone present in brain is synthesized independently from peripheral glands (24). However, prolonged stress in rodent models of behavioral dysfunction correlated with downregulated allopregnanolone biosynthesis in corticolimbic circuitry regulating fear responses,

OPEN ACCESS

Edited by:
Hubert Vaudry,
Université de Rouen, France

Reviewed by:
Jamie Maguire,
Tufts University School of Medicine,
United States

***Correspondence:**
Graziano Pinna
gpinna@uic.edu;
graziano_pinna@yahoo.com

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

Received: *19 February 2020*
Accepted: *31 March 2020*
Published: *14 May 2020*

Citation:
Pinna G (2020) Allopregnanolone, the Neuromodulator Turned Therapeutic Agent: Thank You, Next? Front. Endocrinol. 11:236. doi: 10.3389/fendo.2020.00236

anxiety-like, and depression-like phenotypes (7, 25, 26). The first evidence suggesting that allopregnanolone is involved in the etiopathology of depression originated by studies in rodents and depressed patients (27–29). Evidence showed that treatment with the SSRI, fluoxetine normalized the stress—induced decrease of allopregnanolone in rodent brain as well as its lower levels observed in CSF/serum of patients with depression (25, 28, 30). This finding was corroborated by observing SSRIs act as *selective brain steroidogenic stimulants* (SBSSs), increasing selectively allopregnanolone in a manner independent from SSRI mechanisms, underlying a novel mechanism of classical antidepressants (7, 29, 31). Endogenously-produced allopregnanolone in corticolimbic neurons modulates the fine-tuning of $GABA_A$ receptors for GABAmimetic, $GABA_A$ receptor agonists and positive allosteric modulators. This function underlies allopregnanolone's neurophysiological role. This finding also suggested that allopregnanolone, by this mechanism, may regulate emotional behavior in corticolimbic circuitry (32). Indeed, decreased allopregnanolone biosynthesis in these neurons occurred in association with behavioral dysfunction that are reminiscent of deficits observed in the spectrum of mood disorders (17, 19, 20). Independent laboratories, meanwhile, discovered that allopregnanolone enhances tonic inhibition in δ-containing $GABA_A$ receptors, that pregnancy reduces $GABA_A$ γ and δ-containing subunits, and that two membrane binding sites on $GABA_A$ receptors mediate activation and potentiation of neurosteroid signaling (21, 33–35). Preclinical studies of stress-induced allopregnanolone biosynthesis downregulation contributed to the discovery of several neurosteroidogenic targets through which, agents that increase allopregnanolone biosynthesis, are beneficial in improving behavioral deficits (36–39).

Collectively, these and many more observations in the field by many talented neurosteroid scientists, led to clinical trials that demonstrated the efficacy of intravenous allopregnanolone in postpartum depression (40, 41). Given the remarkable pharmacological efficacy of this novel therapeutic, on March 19th, 2019, the FDA approved intravenous allopregnanolone (i.e., brexanolone) as the first specific treatment for postpartum depression (**Figure 1**). Clinical studies are currently evaluating the pharmacological efficacy of an orally-active allopregnanolone called SAGE 217 for the treatment of major depressive disorders (47). A new era of fast-acting, short-course, long-lasting, neurosteroid-based treatments is born.

MECHANISMS LINKING BIOSYNTHESIS OF ALLOPREGNANOLONE TO MOOD DISORDERS

Allopregnanolone, a positive allosteric modulator of GABA's action at $GABA_A$ receptors (3, 4, 15, 48), is deficient in mood disorders (28, 30, 43, 45). Allopregnanolone and progesterone change significantly in pregnancy and after parturition (33, 49). The increase in plasma progesterone throughout pregnancy triggers upregulation of allopregnanolone levels, which reaches the highest blood concentrations during the third trimester (49, 50). Following childbirth, these neurohormones abruptly decrease (51, 52). Among the hypotheses linking allopregnanolone decrease and post-partum depression, the suggestion that allopregnanolone drops quicker and to lower levels than in mothers who fail to develop post-partum depression is particularly intriguing. This effect may be resulting from abnormal neurosteroid enzyme expression. Mechanistically, $GABA_A$ receptor function may fail to adapt to the rapid allopregnanolone level decline during the weeks following parturition (53). Studies conducted in estrous cycle in rats demonstrated that the drastic decrease of progesterone concentrations during diestrus is associated with overexpression of extrasynaptic α4β1δ-containing $GABA_A$ receptors in periaqueductal gray, which mediates anxiolytic and mood regulating effects of allopregnanolone in this estrous phase (54, 55). The expression of specific subunits of the $GABA_A$ receptor is coordinated with fluctuations in neurosteroid concentrations during menstrual/estrous cycle, pregnancy, and perinatally function (21, 33, 56). Pharmacological treatments, including finasteride and oral contraceptives, that inhibit 5α-RI, which results in a blood and brain allopregnanolone decrease also affect subunit expression of $GABA_A$ receptor and are associated with mood symptoms and suicide and are part of postfinasteride syndrome (57, 58). Post-finasteride syndrome, in addition to depression, anxiety and cognitive deficits also induces sexually-related side effects, such as loss of libido, erectile dysfunction, decreased arousal and difficulty in achieving an orgasm that persist despite drug withdrawal (58). Evidence suggests during pregnancy and across the estrous cycle a switch of extrasynaptic δ with synaptic γ2 subunits may be operative (33, 56). Rapid and dynamic changes among synaptic and extrasynaptic $GABA_A$ receptor conformation in areas that regulate cognitive functions and emotions, including the hippocampus have been reported (59).

Altogether, stressful condition, hormonal changes, pharmacological treatment (e.g., finasteride, oral contraceptives) may coordinately change $GABA_A$ receptor expression resulting in alterations in receptor function underlying mood disorders. They may alter $GABA_A$ receptor pharmacology in response to anxiolytics (42). Conversely, allopregnanolone, its analogs, and neurosteroidogenic agents may offer a therapeutic advantage for disorders that arise by these deficits.

ALLOPREGNANOLONE-BASED TREATMENTS

To contrast the rapid post-partum depletion of allopregnanolone and the rise of mood deficits, directly supplementing synthetic neuroactive steroids or their analogs, may offer a quick strategy in treating post-partum depression and other mood disorders linked with the drastic drop in endogenous allopregnanolone (53, 60). Following this concept, brexanolone, a β-cyclodextrin-based parenterally-administered soluble formulation of allopregnanolone, was developed and FDA-approved for treating post-partum depression. In an open-label study, a single brexanolone IV administration showed rapid and

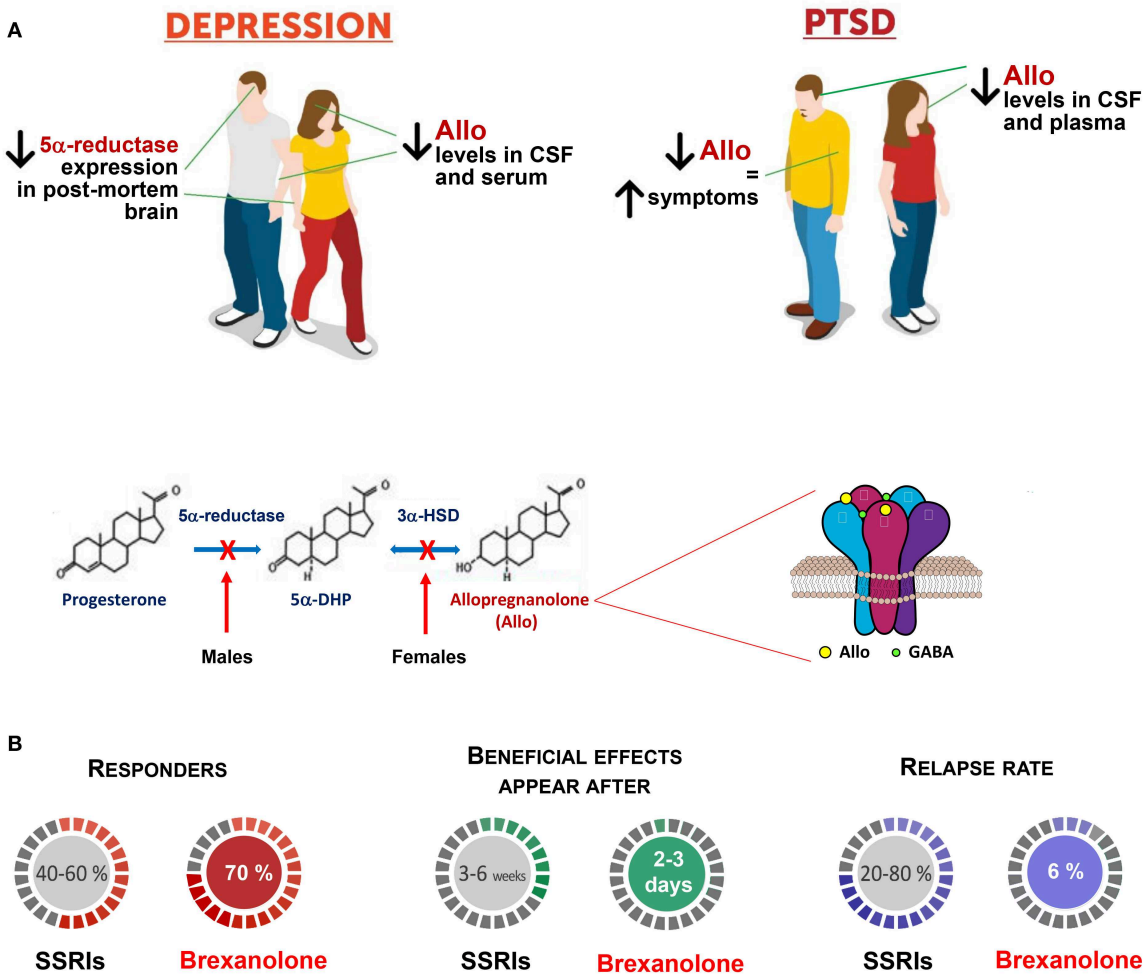

FIGURE 1 | Brexanolone is superior to traditional antidepressants in the treatment of mood disorders. **(A)** Patients with mood disorders, including major unipolar depression and PTSD, exhibit serum, plasma, CSF, and brain reduction of allopregnanolone levels and/or biosynthesis, which includes the enzymes, 5α-reductase type I (5α-RI), and 3α-hydroxysteroid dehydrogenase (3α-HSD) [(18, 28, 30); reviewed in (10, 42)]. In women with PTSD, progesterone, and the immediate allopregnanolone precursor, 5α-dihydroprogesterone (5α-DHP) levels fail to change and their ratios with allopregnanolone and pregnanolone (allopregnanolone's equipotent GABAergic isomer), concentration in the CSF and plasma points to a possible deficit at the 3α-HSD enzyme expression/function levels (43). Likewise, in PTSD male patients, the CSF allopregnanolone concentrations are decreased for an apparent deficit in 5α-RI expression/function, which negatively correlates with PTSD and depression symptoms (43–45). Thus, the concentration and the ratio of allopregnanolone with its parental neuroactive steroids can suggest deficits in their enzymatic pathway, which may unveil biomarkers of sex hormone-related mood disorders. Allopregnanolone's mechanism of action includes activation of mainly extrasynaptically-expressed $GABA_A$ receptors. $GABA_A$ receptor offers two residues for neurosteroid action; one is located between α and β subunits, and the second is a cavity on α subunits (34). The efficacy of neurosteroids at $GABA_A$ receptors is greatly enhanced by the αβδ-containing $GABA_A$ receptor subtype, which is characteristic of tonic inhibition mediated by extrasynaptic receptors (21). Allopregnanolone plays a pivotal neurophysiological role by modulating the fine-tuning and strength of $GABA_A$ receptors (32). By this mechanism, allopregnanolone appears to regulate emotional behavior and the pharmacological response of $GABA_A$ receptor. Altered $GABA_A$ receptor subunit composition has been observed in several pathophysiological conditions, including across the menstrual cycle, changes in hormonal shape during pregnancy, as well as during protracted stress (46). Stress, specifically, results in a $GABA_A$ receptor composition with increased sensitivity for neurosteroids and neurosteroid-like molecules (e.g., synthetic allopregnanolone analogs) [(29); reviewed in (42)]. These observations are in support of treatments that stimulate allopregnanolone biosynthesis for the therapeutic management of stress-induced psychiatric disorders, for which traditional anxiolytics or antidepressants are ineffective. **(B)** Brexanolone, a β-cyclodextrin-based parenterally administered soluble formulation of allopregnanolone is marketed as Zulresso™ and it is the first and only specific treatment for postpartum depression. Brexanolone is one of only two recently FDA-approved fast-acting antidepressants. In clinical trials, women with postpartum depression treated with brexanolone improved their symptoms compared with placebo in 2.5 days. Symptoms were measured before and after treatment. Follow-up studies showed that women receiving the treatment maintained the therapeutic gains for at least 30 days (41). Side effects include risk of sedation or loss of consciousness during treatment. For these reasons women who undergo treatment will be monitored by a healthcare professional in a healthcare setting. Other side effects may include sleepiness, dry mouth, flushing of the skin or face. A clinical trial using the orally-active allopregnanolone analog, SAGE 217 has recently failed for non-compliance issues that were noted with about 10% of patients presenting no blood drug levels. However, statistical significance was achieved at days 3, 8, 12, and 15 in patients with measurable drug concentration levels of SAGE-217. Hence, these allopregnanolone derivatives are highly promising in the treatment of mood disorders, from postpartum depression to major depression and, probably, in PTSD, which, as mentioned above, is characterized by low allopregnanolone levels (43, 45). Another approach is to use neurosteroidogenic drugs (38). These agents may selectively elevate allopregnanolone levels by stimulating enzyme activity/expression levels where a deficit emerges thereby improving mood symptoms avoiding a global expression of allopregnanolone levels.

long-lasting antidepressant effects in severe post-partum depression (40). Safety and efficacy was further confirmed in two double-blind, randomized clinical trials (41). Brexanolone presumably acts by reinstating normal allopregnanolone levels, and thereby tuning GABAergic neurotransmission function, promptly improved symptom severity in with post-partum depression patients (**Figure 1**). However, it still remains to be clarified the precise treatment targets, including levels of endogenous allopregnanolone, verify altered biosynthetic enzyme expression/function, and $GABA_A$ receptor assembly modifications pre, during, and post-brexanolone treatment. The elevation of brain derived neurotropic factor (BDNF) is also conceivable among allopregnanolone's mechanisms (61). Each of these factors may be critical for understanding why—and for whom—brexanolone is best indicated to improve mood symptoms. First, deficient allopregnanolone levels may be critical for predicting who may benefit from varying doses of direct neurosteroid replacement (via brexanolone or other allopregnanolone analogs). Second, baseline allopregnanolone concentrations are crucial to select the most effective brexanolone dose and avoid unwanted side-effects, including excessive sedation (62). Third, by directly affecting both the HPA and HPG axes, allopregnanolone may alter expression of key biosynthetic enzymes (e.g., 5α-RI and 3α-HSD) involved in neurosteroid synthesis. Indeed, the HPA axis is modulated by GABAergic neuron activation within the hypothalamus (63). Allopregnanolone potently inhibits HPA axis activity and repress stress elevation of ACTH and corticosterone (64, 65). This finding suggests that allopregnanolone administration may alter HPA axis responsiveness by affecting gonadal steroid concentrations (e.g., estradiol) with documented roles in maintaining expression/function of neurosteroidogenic enzymes (e.g., 3α-HSD) and sustainably change endogenous neurosteroids production (66).

Collectively, these reports suggest that more studies are needed to verify the diverse mechanisms involved in brexanolone treatment.

WHAT'S NEXT? ALLOPREGNANOLONE AS A BIOMARKER FOR MOOD DISORDERS

While converging evidence suggests a neurosteroid biosynthesis deficit involvement in the underlying neurobiology of mood disorders, the yet unanswered question is whether allopregnanolone biosynthesis (allopregnanolone levels and expression of rate-limiting biosynthetic enzymes) provide a reliable biomarker to prevent mood disorder, predict occurrence, diagnose, and indicate treatment selection. Another valid option suggests analyzing neurosteroid biosynthesis relative to $GABA_A$ receptor subunit dynamic changes. $GABA_A$ receptor expression and neurosteroid biosynthesis in post-partum depression and in general in mood disorders remains underinvestigated. Furthermore, analysis of neurosteroids that positively modulate $GABA_A$ receptors (allopregnanolone and pregnanolone), and of their sulfates (e.g., pregnanolone sulfate), that inhibit NMDA-mediated tonic neurotransmission, which results in neuroprotection and cognitive improvement (67), has been poorly investigated. Establishing predictive biomarkers of treatment response will enable follow-up analysis of neuroactive steroid biosynthesis and $GABA_A$ receptor composition that will help predict whether brexanolone pharmacological effects are associated with permanent neurobiological improvements or, alternatively, whether GABAergic functional deficits may anticipate relapses following drug discontinuation. Assessing a *biomarker axis*, indicating the dynamic changes of several inter-related neurobiological deficits will facilitate a more thorough diagnosis of mood disorders as well as predict which patients will likely respond to treatment. This will increase efficacy and limit occurrence of side-effects.

In neuropsychopharmacology establishing reliable biomarkers and efficient treatments is urgently needed. Currently, patients show large non-response and relapse-rate to traditional antidepressants and significant side-effects.

CONCLUSIONS

Eighty years of neurosteroid research originated from many talented neuroscientists around the world guided investigations that from the discovery of allopregnanolone led to its approval as a fast-acting agent to treat post-partum depression. One of the most significant achievements still remaining to be accomplished in neuropsychopharmacology and, in general in psychiatry, is the assessment of valid biomarkers to predict, diagnose, select, and treat patients more efficiently, avoiding drug non–responders and side-effects. Neurosteroidogenic targets have been recently suggested that may result in new drug development (38, 68). The opportunity of increasing allopregnanolone levels and improving deficits with functional foods (69) is an emerging novel approach to treat mood disorders in a more natural way without exposing pregnant women to drugs.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

This work was supported by the US Department of Defense Grant W81XWH-15-1-0521 to GP.

REFERENCES

1. Krystal JH, Abdallah CG, Duman RS. Ketamine: a paradigm shift for depression research and treatment. *Neuron.* (2019) 101:774–8. doi: 10.1016/j.neuron.2019.02.005
2. Beall D, Reichstein T. Isolation of progesterone and allopregnanolone from the adrenal. *Nature.* (1938) 142:479. doi: 10.1038/142479b0
3. Majewska MD, Harrison NL, Paul SM. Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science.* (1986) 232:1004–7. doi: 10.1126/science.2422758

4. Puia G, Santi MR, Vicini S, Seeburg PH, Costa E. Neurosteroids act on recombinant human GABAA receptors. *Neuron*. (1990) 4:759–65. doi: 10.1016/0896-6273(90)90202-Q
5. Purdy RH, Morrow AL, Moore PH Jr, Paul SM. Stress-induced elevations of gammaaminobutyric acid type A receptor-active steroids in the rat brain. *PNAS*. (1991) 88:4553–7. doi: 10.1073/pnas.88.10.4553
6. Matsumoto K, Puia G, Dong E, Pinna G. GABAA receptor neurotransmission dysfunction in a mouse model of social isolation-induced stress. *Stress.* (2007) 10:3–12. doi: 10.1080/10253890701200997
7. Pinna G, Dong E, Matsumoto K, Costa E, Guidotti A. In socially isolated mice, the reversal of brain allopregnanolone down-regulation mediates the anti-aggressive action of fluoxetine. *PNAS.* (2003) 100:2035–40. doi: 10.1073/pnas.0337642100
8. Crawley JN, Glowa JR, Majewska MD, Paul SM. Anxiolytic activity of an endogenous adrenal steroid. *Brain Res.* (1986) 398:382–5. doi: 10.1016/0006-8993(86)91500-3
9. Girdler SS, Straneva PA, Morrow AL. Allopregnanolone levels and reactivity to mental stress in premenstrual dysphoric disorder. *Biol Psychiatr.* (2001) 49:788–97. doi: 10.1016/S0006-3223(00)01044-1
10. Zorumski CF, Paul SM, Mennerick S. Neurosteroids, stress and depression: potential therapeutic opportunities. *Neurosci Biobehav Rev*. (2013) 37:109–22. doi: 10.1016/j.neubiorev.2012.10.005
11. Meltzer-Brody SE, Kanes SJ. Allopregnanolone in postpartum depression: Role in pathophysiology and treatment. *Neurobiol Stress.* (2020) 19:22. doi: 10.1016/j.ynstr.2020.100212
12. Walton N, Maguire J. Allopregnanolone-based treatments for postpartum depression: why/how do they work? *Neurobiol Stress*. (2019) 11:100198. doi: 10.1016/j.ynstr.2019.100198
13. Paul SM, Pinna G, Guidotti A. "NEUROSTEROIDS: from molecular pathophysiology to therapeutics - a brief historical perspective. *Neurobiol Stress.* (2020) 378:112309. doi: 10.1016/j.ynstr.2020.100215
14. Corpéchot C, Robel P, Baulieu EE. Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. *PNAS.* (1981) 78:4704–7. doi: 10.1073/pnas.78.8.4704
15. Paul SM, Purdy RH. Neuroactive steroids. *FASEB J.* (1992) 6:2311–22. doi: 10.1096/fasebj.6.6.1347506
16. Agis-Balboa RC, Pinna G, Costa E, Guidotti A. Location and expression of brain enzymes catalyzing neurosteroid biosynthesis. *PNAS.* (2006) 103:14602–7. doi: 10.1073/pnas.0606544103
17. Agís-Balboa RC, Pinna G, Costa E, Guidotti A. Downregulation of 5αreductase type I mRNA expression in cortico-limbic glutamatergic neurons in socially-isolated mice. *PNAS.* (2007) 104:18736–41. doi: 10.1073/pnas.0709419104
18. Agis-Balboa RC, Guidotti A, Pinna G. Allopregnanolone biosynthesis is downregulated in the prefrontal cortex/Brodmann's area 9 (BA9) of depressed patients. *Psychopharmacology*. (2014) 231:3569–80. doi: 10.1007/s00213-014-3567-5
19. Pinna G, Agis-Balboa R, Pibiri F, Nelson M, Guidotti A, Costa E. Neurosteroid biosynthesis regulates sexually dimorphic fear and aggressive behavior in mice. *Neurochem Res.* (2008) 33:1990–2007. doi: 10.1007/s11064-008-9718-5
20. Pibiri F, Nelson M, Costa E, Guidotti A, Pinna G. Decreased corticolimbic allopregnanolone expression during social isolation enhances contextual fear: a model relevant for posttraumatic stress disorder. *PNAS.* (2008) 105:5567–72. doi: 10.1073/pnas.0801853105
21. Maguire J, Mody I. GABAAR plasticity during pregnancy: relevance to postpartum depression. *Neuron.* (2008) 59:207–13. doi: 10.1016/j.neuron.2008.06.019
22. Sprengel R, Werner P, Guidotti A, Krueger KE. Molecular cloning and expression of cDNA encoding a peripheral-type benzodiazepine receptor. *J Biol Chem*. (1989) 264:20415–21.
23. Papadopoulos V, Baraldi M, Gavish M. Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor. *Trends Pharmacol Sci*. (2006) 27:402–9. doi: 10.1016/j.tips.2006.06.005
24. Purdy RH, Moore PHJr, Morrow AL, Paul SM. Neurosteroids and $GABA_A$ receptor function. *Adv Biochem Psychopharmacol.* (1992) 47:87–92.
25. Matsumoto K, Uzunova V, Pinna G, Guidotti A, Costa E. Permissive role of brain allopregnanolone content in the regulation of pentobarbital-induced righting reflex loss. *Neuropharmacology*. (1999) 38:955–63. doi: 10.1016/S0028-3908(99)00018-0
26. Guidotti A, Dong E, Matsumoto K, Pinna G, Rasmusson AM, Costa E. The socially-isolated -dihydroprogesterone in psychiatric disorders. *Brain Res Rev.* (2001) 37:110–5. doi: 10.1016/S0165-0173(01)00129-1
27. Uzunov DP, Cooper TB, Costa E, Guidotti A. Fluoxetine-elicited changes in brain neurosteroid content measured by negative ion mass fragmentography. *PNAS*. (1996) 93:12599–604. doi: 10.1073/pnas.93.22.12599
28. Uzunova V, Sheline Y, Davis JM, Rasmusson A, Uzunov DP, Costa E, et al. Increase in the cerebrospinal fluid content of neurosteroids in patients with unipolar major depression who are receiving fluoxetine or fluvoxamine. *PNAS.* (1998) 95:3239–44. doi: 10.1073/pnas.95.6.3239
29. Pinna G, Costa E, Guidotti. Fluoxetine and norfluoxetine stereospecifically and selectively increase brain neurosteroid content at doses inactive on 5-HT reuptake. *Psychopharmacology*. (2006) 186:362–72. doi: 10.1007/s00213-005-0213-2
30. Romeo E, Strohle A, Spalletta G, Holsboer F, Pasini A, Rupprecht R. Effects of antidepressant treatment on neuroactive steroids in major depression. *Am J Psychiatr.* (1998) 155:910–3. doi: 10.1176/ajp.155.7.910
31. Pinna G, Costa E, Guidotti A. Fluoxetine and norfluoxetine stereospecifically facilitate pentobarbital sedation by increasing neurosteroids. *Proc Natl Acad Sci USA*. (2004) 101:6222–5. doi: 10.1073/pnas.0401479101
32. Pinna G, Uzunova V, Matsumoto K, Puia G, Mienville JM, Costa E, et al. Brain allopregnanolone regulates the potency of the $GABA_A$ receptor agonist muscimol. *Neuropharmacology*. (2000) 39:440–8. doi: 10.1016/S0028-3908(99)00149-5
33. Concas A, Mostallino MC, Porcu P, Biggio G. Role of brain allopregnanolone in the plasticity of γ-aminobutyric acid type A receptor in rat brain during pregnancy and after delivery. *PNAS.* (1998) 95:13284–9. doi: 10.1073/pnas.95.22.13284
34. Hosie AM, Wilkins ME, da Silva HM, Smart TG. Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. *Nature.* (2006) 444:486–9. doi: 10.1038/nature05324
35. Brickley SG, Mody I. Extrasynaptic GABA(A) receptors: their function in the CNS and implications for disease. *Neuron.* (2012) 73:23–34. doi: 10.1016/j.neuron.2011.12.012
36. Rupprecht R, Papadopoulos V, Rammes G, Baghai TC, Fan J, Akula N et al. Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. *Nat Rev Drug Discov*. (2010) 9:971–88. doi: 10.1038/nrd3295
37. Rupprecht R, Rammes G, Kucher K. Translocator protein (18 kD) as target for anxiolytics without benzodiazepine-like side effects. *Science*. (2009) 325:490–3. doi: 10.1126/science.1175055
38. Raber J, Arzy S, Bertolus JB, Depue B, Haas HE, Hofmann SG, et al. Current understanding of fear learning and memory in humans and animal models and the value of a linguistic approach for analyzing fear learning and memory in humans. *Neurosci Biobehav Rev.* (2019) 105:136–77. doi: 10.1016/j.neubiorev.2019.03.015
39. Locci A, Pinna G. Stimulation of PPAR-α by N-palmitoylethanolamine engages allopregnanolone biosynthesis to modulate emotional behavior. *Biol Psychiatr.* (2019) 85:1036–45. doi: 10.1016/j.biopsych.2019.02.006
40. Kanes S, Colquhoun H, Gunduz-Bruce H, Epperson CN, Rubinow D, Paul S, et al. Brexanolone (SAGE-547 injection) in post-partum depression: a randomised controlled trial. *Lancet.* (2017) 390:480–9. doi: 10.1016/S0140-6736(17)31264-3
41. Meltzer-Brody S, Colquhoun H, Kanes S. Brexanolone injection in postpartum depression: two multicentre, double-blind, randomised, placebo-controlled, phase 3 trials. *Lancet*. (2018) 392:1058–70. doi: 10.1016/S0140-6736(18)31551-4
42. Locci A, Pinna G. Neurosteroid biosynthesis downregulation and changes in GABAA receptor subunit composition: A biomarker axis in stress-induced cognitive and emotional impairment. *Br J Pharmacol.* (2017) 174:3226–41. doi: 10.1111/bph.13843
43. Rasmusson AM, Pinna G, Krystal J, Guidotti A. Decreased cerebrospinal fluid allopregnanolone levels in women with posttraumatic stress disorder. *Biol Psychiatr.* (2006) 60:704–13. doi: 10.1016/j.biopsych.2006.03.026
44. Pineles SL, Nillni YI, Pinna G, Irvine J, Webb A, Arditte Hall KA, et al. PTSD in women is associated with a block in conversion

of progesterone to the GABAergic neurosteroids allopregnanolone and pregnanolone measured in plasma. *Psychoneuroendocrinology*. (2018) 93:133–41. doi: 10.1016/j.psyneuen.2018.04.024
45. Rasmusson AM, Nillni G, Anderson M, Pinna G. Relationships between cerebrospinal fluid GABAergic neurosteroid levels and symptom severity in men with PTSD. *Psychoneuroendocrinology*. (2018) 102:95–104. doi: 10.1016/j.psyneuen.2018.11.027
46. Pinna G, Agis Balboa RC, Zhubi A, Matsumoto K, Grayson DR, Costa E et al. Imidazenil and diazepam increase locomotor activity in mice exposed to protracted social isolation. *Proc Natl Acad Sci USA*. (2006) 103:4275–80.
47. Gunduz-Bruce H, Silber C, Paul SM, Kanes SJ. Trial of SAGE-217 in patients with major depressive disorder. *N Engl J Med*. (2019) 381:903–11. doi: 10.1056/NEJMoa1815981
48. Belelli D, Lambert JJ. Neurosteroids: endogenous regulators of the GABA(A) receptor. *Nat Rev Neurosci*. (2005) 6:565–75.
49. Luisi S, Petraglia F, Luisi M, Genazzani AR. Serum allopregnanolone levels in pregnant women: changes during pregnancy, at delivery, and in hypertensive patients. *J Clin Endocrinol Metab*. (2000) 85:2429–33. doi: 10.1210/jcem.85.7.6675
50. McEvoy K, Payne JL, Osborne LM. Neuroactive steroids and perinatal depression: a review of recent literature. *Curr Psychiatry Rep*. (2018) 20:78. doi: 10.1007/s11920-018-0937-4
51. Osborne LM, Betz JF, Payne JL. The role of allopregnanolone in pregnancy in predicting postpartum anxiety symptoms. *Front Psychol*. (2019) 10:1033. doi: 10.3389/fpsyg.2019.01033
52. Payne JL, Maguire J. Pathophysiological mechanisms implicated in postpartum depression. *Front Neuroendocrinol*. (2019) 52:165–80. doi: 10.1016/j.yfrne.2018.12.001
53. Mody I. GABAA R modulator for postpartum depression. *Cell*. (2019) 176:1. doi: 10.1016/j.cell.2018.12.016
54. Griffiths JL, Lovick TA. GABAergic neurones in the rat periaqueductal grey matter express alpha4, beta1 and delta GABAA receptor subunits: plasticity of expression during the estrous cycle. *Neuroscience*. (2005) 136:457–66. doi: 10.1016/j.neuroscience.2005.08.013
55. Lovick TA. Plasticity of GABAA receptor subunit expression during the oestrous cycle of the rat: implications for premenstrual syndrome in women. *Exp Physiol*. (2006) 91:655–60. doi: 10.1113/expphysiol.2005.032342
56. Maguire JL, Stell BM, Mody I. Ovarian cycle-linked changes in GABAA receptors mediating tonic inhibition alter seizure susceptibility and anxiety. *Nat Neurosci*. (2005) 8:797–804. doi: 10.1038/nn1469
57. Porcu P, Mostallino MC, Concas A. Long-term administration with levonorgestrel decreases allopregnanolone levels and anxiety-like behavior. *Pharmacol Biochem Behav*. (2012) 102:366–72. doi: 10.1016/j.pbb.2012.05.011
58. Diviccaro S, Melcangi RC, Giatti S. Post-finasteride syndrome: an emerging clinical problem. *Neurobiol Stress*. (2020) 12:100209. doi: 10.1016/j.ynstr.2019.100209
59. Jacob TC, Moss SJ, Jurd R. GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nat Rev Neurosci*. (2008) 9:331–43. doi: 10.1038/nrn2370
60. Pinna G. Targeting neurosteroidogenesis as therapy for PTSD. *Front Pharmacol*. (2014) 4:166. doi: 10.3389/fphar.2013.00166
61. Nin MS, Martinez LA, Pibiri F, Nelson M, Pinna G. Neurosteroids reduce social isolation-induced behavioral deficits: a proposed link with neurosteroid-mediated upregulation of BDNF expression. *Front Endocrinol (Lausanne)*. (2011) 2:73. doi: 10.3389/fendo.2011.00073
62. Leader LD, O'Connell M, VandenBerg A. Brexanolone for postpartum depression: clinical evidence and practical considerations. *Pharmacotherapy*. (2019) 39:1105–12. doi: 10.1002/phar.2331
63. Boudaba C, Szabo K, Tasker JG. Physiological mapping of local inhibitory inputs to the hypothalamic paraventricular nucleus. *J Neurosci*. (1996) 16:7151–60. doi: 10.1523/JNEUROSCI.16-22-07151.1996
64. Brunton PJ, McKay AJ, Russell JA. Central opioid inhibition of neuroendocrine stress responses in pregnancy in the rat is induced by the neurosteroid allopregnanolone. *J Neurosci*. (2009) 29:6449–60. doi: 10.1523/JNEUROSCI.0708-09.2009
65. Biggio G, Pisu MG, Biggio F, Serra M. Allopregnanolone modulation of HPA axis function in the adult rat. *Psychopharmacology (Berl)*. (2014) 231:3437–44. doi: 10.1007/s00213-014-3521-6
66. Mitev YA, Darwish M, Wolf SS, Holsboer F, Almeida OF, Patchev VK. Gender differences in the regulation of 3 alpha-hydroxysteroid dehydrogenase in rat brain and sensitivity to neurosteroid-mediated stress protection. *Neuroscience*. (2003) 120:541–9. doi: 10.1016/S0306-4522(03)00287-2
67. Vyklicky V, Borovska J, Krausova B, Balik A, Korinek M, Borovska J, et al. Preferential inhibition of tonically over phasically activated NMDA receptors by pregnane derivatives. *J Neurosci*. (2016) 36:2161–75. doi: 10.1523/JNEUROSCI.3181-15.2016
68. Pinna G. Animal models of PTSD: the socially isolated mouse and the biomarker role of allopregnanolone. *Front Behav Neurosci*. (2019) 13:114. doi: 10.3389/fnbeh.2019.00114
69. Matrisciano F, Pinna G. PPAR and functional foods: Rationale for natural neurosteroid-based interventions for postpartum depression. *Neurobio Stress*. (2020). doi: 10.1016/j.ynstr.2020.100222

Conflict of Interest: 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

CORRECTION
published: 30 July 2020
doi: 10.3389/fendo.2020.00507

Corrigendum: Allopregnanolone, the Neuromodulator Turned Therapeutic Agent: Thank You, Next?

*Graziano Pinna**

Department of Psychiatry, The Psychiatric Institute, University of Illinois at Chicago, Chicago, IL, United States

Keywords: brexanolone, allopregnanolone (3α,5α-THP), postpartum depression, fast-acting antidepressant, $GABA_A$ receptor, 5α-reduced steroids, 5α-reductase, 3α-HSD

OPEN ACCESS

Approved by:
Hubert Vaudry,
Université de Rouen, France

***Correspondence:**
Graziano Pinna
gpinna@uic.edu;
graziano_pinna@yahoo.com

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

Received: *11 June 2020*
Accepted: *24 June 2020*
Published: *30 July 2020*

Citation:
Pinna G (2020) Corrigendum: Allopregnanolone, the Neuromodulator Turned Therapeutic Agent: Thank You, Next? Front. Endocrinol. 11:507. doi: 10.3389/fendo.2020.00507

A Corrigendum on

Allopregnanolone, the Neuromodulator Turned Therapeutic Agent: Thank You, Next?
by Pinna, G. (2020). Front. Endocrinol. 11:236. doi: 10.3389/fendo.2020.00236

In the original article, there was an error. "Allopregnanolone, today best known as brexanolone and marketed as Zulresso™ for the treatment of postpartum depression is part of only two recently Food and Drug Administration (FDA)-approved fast-acting antidepressants, with esketamine nasal spray, an NMDA receptor antagonist used in treatment-resistant depression with suicidality being the other." **However, SPRAVATO® (esketamine) CIII Nasal Spray was approved by the FDA last year to treat treatment-resistant depression in adults when used along with an oral antidepressant, and is not indicated for the treatment of suicidality.**

A correction has been made to the **Introduction** section, First paragraph:

"Allopregnanolone, today best known as brexanolone and marketed as Zulresso™ for the treatment of postpartum depression is part of only two recently Food and Drug Administration (FDA)-approved fast-acting antidepressants, with esketamine nasal spray, an NMDA receptor antagonist used in treatment-resistant depression being the other (1)."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

frontiers in Endocrinology

PERSPECTIVE
published: 29 May 2020
doi: 10.3389/fendo.2020.00345

Estrogen Formation and Inactivation Following TBI: What we Know and Where we Could go

*Kelli A. Duncan**

Department of Biology and Program in Neuroscience and Behavior, Vassar College, Poughkeepsie, NY, United States

Traumatic brain injury (TBI) is responsible for various neuronal and cognitive deficits as well as psychosocial dysfunction. Characterized by damage inducing neuroinflammation, this response can cause an acute secondary injury that leads to widespread neurodegeneration and loss of neurological function. Estrogens decrease injury induced neuroinflammation and increase cell survival and neuroprotection and thus are a potential target for use following TBI. While much is known about the role of estrogens as a neuroprotective agent following TBI, less is known regarding their formation and inactivation following damage to the brain. Specifically, very little is known surrounding the majority of enzymes responsible for the production of estrogens. These estrogen metabolizing enzymes (EME) include aromatase, steroid sulfatase (STS), estrogen sulfotransferase (EST/SULT1E1), and some forms of 17β-hydroxysteroid dehydrogenase (HSD17B) and are involved in both the initial conversion and interconversion of estrogens from precursors. This article will review and offer new prospective and ideas on the expression of EMEs following TBI.

Keywords: TBI, estrogen, aromatase, androgen, sulfatase, HSD17B

OPEN ACCESS

Edited by:
Hubert Vaudry,
Université de Rouen, France

Reviewed by:
Barney A. Schlinger,
University of California, Los Angeles, United States
Benedetta Leuner,
The Ohio State University, United States

***Correspondence:**
Kelli A. Duncan
keduncan@vassar.edu

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

Received: *01 March 2020*
Accepted: *04 May 2020*
Published: *29 May 2020*

Citation:
Duncan KA (2020) Estrogen Formation and Inactivation Following TBI: What we Know and Where we Could go. Front. Endocrinol. 11:345. doi: 10.3389/fendo.2020.00345

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of human death and morbidity worldwide. TBI is a broad term to explain any injury or damage to the central nervous system, ranging from concussive, penetrating, to ischemic stroke. Over 70,000 cases are reported a year and it affects millions of people in the United States alone (1, 2). At-risk groups range from children, who frequently participate in contact sports to military personnel, and to an increasingly active elderly population (3, 4). Disturbingly high injury rates have made TBI not only an issue of social and economic concern, but also the target of nearly 50 years of dedicated clinical research (5–7). Despite research and improvements in effective patient care, there still remain very few recommended treatments which leads to poor patient outcomes (8).

Injury to the brain occurs in two phases, regardless of the cause. The irreversible primary phase is the injury itself. The majority of research around the primary phase focuses on decreasing the chance of injury (e.g., helmet design). The secondary and potentially reversible phase, which begins after the initial injury and continues for days to weeks afterword, is characterized by an induction of a neuroinflammatory response. This response is characterized by a permeation of inflammatory cells around the injury, followed by endothelial activation, and an accumulation of inflammatory cytokines (9–11). This secondary phase has some beneficial effects, but commonly results in an exacerbation of deleterious inflammatory effects, decreased cognitive ability, motor

loss, and increased risk of neurodegenerative diseases, such as multiple sclerosis (12–17). This secondary response to injury is well-characterized across vertebrates (rodents, birds, fish) (18–20). The majority of research has focused on preventing this secondary response (21, 22). One pharmacotherapeutic strategy for treatment of secondary injury has revolved around the role of sex steroids, specifically estrogens on the attenuation of the immune response (22–25). Briefly, we will review the effects of estrogens on cell survival and neuroinflammation following TBI.

Following damage to the brain, estrogens can promote neurogenesis and neural recovery by attenuating neural outgrowth and glial activity (25–29). Estrogens are also indicated to serve as an antioxidant against intrinsic free radical production following TBI (30, 31). The majority of these effects are through estrogen receptor (ER) signaling pathways. The genomic signal transduction pathway of estrogens involves the dimerization of ERs and transcriptional regulation of estrogen mediated target genes which in turn can provide neuroprotective effects, evidenced by decreased inflammation, reactive gliosis, and edema (30, 32–34). Furthermore, activation of ER alpha, the primary mediator of steroid induced neuroprotection, can also alter neurovascular function and promote myelin repair (24, 35). However, rapid non-genomic signaling has been shown to upregulate estrogen mediated neuroprotection via the pro-survival signaling pathway, phosphoinositide 3-kinase/Akt. Activation of this pathway results in increased cell survival, differentiation, and growth (36). Estrogens can also mediate cell death and apoptosis by inhibiting subcellular trafficking of p38α. Jun N-terminal kinase (JNK) and p38 are necessary for the activation of pro-apoptotic signaling pathways (34, 36, 37). Moreover, when estrogens are given or produced immediately after injury, they can decrease pro-inflammatory and increase anti-inflammatory cytokines and thus have strong antioxidant and anti-inflammatory effects. In terms of behavior and cognitive ability following TBI, very little is known about the role of estrogens in ameliorating these symptoms. However, estrogens can also enhance memory function and neurological outcome in male rodents following TBI (38–47). In addition to these effects on cell/neuronal survival, estrogens can also regulate neuroinflammation following injury.

Estrogen synthesis following TBI reduces the concentration of pro-inflammatory cytokines and thus decreases the secondary wave of degeneration observed following damage to the brain (30, 48–50). Through regulation of pro-inflammatory cytokines, estrogens subsequently reduce leukocyte recruitment, cerebral edema, apoptosis, and reactive astrogliosis, thusly improving outcomes following injury (51). Thus, estrogens are neuroprotective on many fronts following TBI (52, 53). While, I've only touched on a few of the pre-clinical studies, the majority of these studies suggest that estrogens are neuroprotective following TBI. However, clinical trials in humans involving estrogen administration have not shown as robust of a beneficial result, thus identifying how the natural/systemic productions of estrogens differs from that of exogenous/synthetic production is key. Aside from the neuroprotective effects of estrogens, they exert many biological effects as well. Estrogens are mostly widely known for their sex-specific effects related to sexual determination and differentiation (54). Both genomic and non-genomic signal transduction pathways of estrogens alter the regulation of the cell cycle, this includes, but is not limited to proliferation and differentiation, but specifically cyclines and cycline-2dependent kinases (55–57). As a result of this effect on cell growth and cell progression, estrogens have direct cancerogenic effects and damage DNA and cellular proteins via highly reactive oxygen reactivity (55, 58). Thus, understanding and parsing out the hormonal effects from neuroprotective actions of estrogens are key to their use as therapeutics.

FORMATION OF ESTROGENS

Four estrogens are produced naturally: estrone (E1), 17β-estradiol (estradiol or E2), estriol (E3), and estetrol (E4; **Figure 1**) The weakest form estrone is primarily found following menopause while estriol and estetrol are the predominant estrogens produced during pregnancy (30). Weak estrogens can bind to estrogen receptors, but generally lack a dramatic effect within tissues or cells. However, some weak estrogens such as estriol exhibit greater protection than estradiol in autoimmune disorders, such as multiple sclerosis (59). Estradiol is the most common and strongest of the estrogens and is thought to mediate the neuroprotection following various damage to the nervous system (30). Estradiol is produced during the menstrual cycle and also *de novo* in the brain (60–62).

Estrogens are formed following the enzymatic conversion and interconversion from cholesterol-based precursors via a subset of enzymes termed estrogen-metabolizing enzymes (EME). The most prevalent of these enzymes is aromatase or CYP19A1. The aromatase pathway forms estrone and estradiol from androgenic precursors androstenedione and testosterone, respectively (**Figure 1**) (63). In addition to this estrogen synthase activity, aromatase has been proposed to regulate estrogen-2-hydroxylase activity in placental tissue and in Japanese quail brains (64–66). This activity also paired with aromatase's interaction with TH and DA signaling suggest that aromatase plays a role in catecholaminergic transmission (67, 68). Thus, aromatase may be involved in both the production and inactivation of estrogens (68). Another EME, 17β-hydroxysteroid dehydrogenases 1 and 2 (HSD17B1, HSD17B2) is also necessary for the conversion of estrone to estradiol (61, 69, 70). Finally, estrogens can be made inactive by both degradation and sulfonation. In the sulfatase pathway, inactive estrogen sulfate is the source or precursor for the active estradiol and estrone. This is mediated via the enzymes steroid sulfatase (STS) and estrogen sulfotransferase (SULT1E1) (**Figure 1**)(71, 72). Below I will review what is known about these EMEs and their role following TBI.

EMES AND TBI

Aromatase

Among the EMEs, aromatase is the most prominent and widely studied. Across vertebrates aromatase expression is found in gonads, placenta, adipose tissue, bone, and other tissues including both male and female brains (73–75). Within the

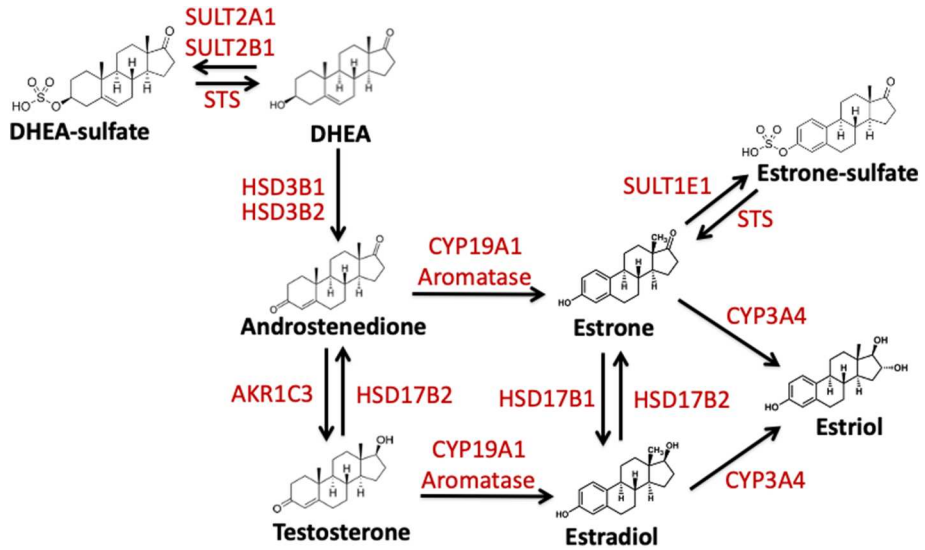

FIGURE 1 | Schematic representation of the enzymatic conversion and synthesis of biologically active estrogens. Estrogens are produced from C19 steroid precursors through several enzymatic conversions. DHEA, dehydroepiandrosterone. DHEA-S, dehydroepiandrosterone-sulfate; Aromatase/CYP19A1, estrogen synthase; HSD3B1, hydroxysteroid 3 beta-1; HSD3B2, hydroxysteroid 3 beta-2; HSD17B1, hydroxysteroid 17-beta dehydrogenase; HSD17B2, hydroxysteroid 17-beta dehydrogenase 2; STS, steroid sulfatase; SULT2A1, Sulfotransferase Family 2A Member 1; SULT2B1, Sulfotransferase Family 2B Member 1; SULT1E1, estrogen sulfotransferase; AKR1C3, Aldo-Keto Reductase Family 1 Member C3; CYP3A4, Cytochrome P450 3A4.

vertebrate brain, high concentrations of aromatase are expressed within the hypothalamus, amygdala, hippocampus, and cerebral cortex (76, 77). Aromatase is broadly expressed within neurons and not glial cells in the above listed brain areas of the uninjured brain (78–80). Aromatase is also present in pre-synaptic boutons, suggesting direct perisynaptic actions (81). Following injury or neuroinflammation, aromatase is also found in glial cells, specifically astrocytes (80, 82). In the songbird brain, females exhibit higher expression immediately after injury, but these differences disappear by 24 h post injury (83). This upregulated glial aromatase appears to affect neurodegenerative pathways by decreasing apoptosis (84, 85). In songbirds, as in the mammals (86), administration of fadrozole (aromatase inhibitor) dramatically increases the volume of damage induced by penetrating mechanical injury (84), sometimes in a sexually dimorphic manner (87). Replacing estradiol at the time of injury prevents this fadrozole-induced damage (88).

Cytokines increase aromatase expression without concurrent cell death or damage to neuronal tissues (25, 26, 89). Using TNF-α and IL-1β KO mice, we were able to determine that TNF-α, but not IL-1β signaling is necessary for the induction of aromatase following brain injury (25). Interestingly, while inflammation appears to regulate aromatase expression, increasing aromatase decreases expression of TNF-α and IL-1β following injury furthermore aromatase inhibition results in prolonged elevation of TNF-α and IL-1β (29, 89). Another mechanism by which estrogens may become inactive following TBI is through aromatase's estrogen-2-hydroxylase activity, that converts estrogens to catechol-estrogens (64–66). The role of this method of estrogen inactivation following TBI remains unknown. This cycle of both upregulation and inhibition of neuronal aromatase and cytokine expression may suggest a broadly conserved mechanism for protecting the CNS following detection of a threat (25).

Steroid Sulfatase

In addition to the aromatase pathway described previously, estrogens can also be formed from inactive precursors by the removal of sulfate groups (90–93). When sulfated, estrogens are unable to bind and dimerize to estrogen receptors. This protects cells and tissues from excess estrogen activity (55). Thus, sulfonation can potentially regulate active estrogen signaling and serve as a hormone "reservoir" for future use (91, 94, 95). Steroid sulfatase (STS) hydrolyzes the removal of sulfate groups from estrone sulfate (E1-S) to E1 and dehydroepiandrosterone sulfate (DHEA-S) to dehydroepiandrosterone (DHEA), also known as androstenolone (**Figure 1**) (96). STS is expressed broadly across vertebrates in both males and females with highest levels being found in the placenta, but low levels found across the majority of steroid sensitive tissues. *STS* expression and the mechanism that control its expression remain poorly understood (97). However, estrogen signaling pathways regulate expression of STS, and thus potentially create a positive feedback loop to increase estrogen production and signaling (71).

The majority of studies examining STS expression and TBI have focused more on the beneficial effects DHEA vs. E2. As STS alters both estrogens and DHEA at this time we are unable to separate out the neuroprotective effects of estrogens vs. those of DHEA. Both DHEA and DHEA-S are neuroprotective following damage to the CNS in rodents and birds. Specifically, DHEA and DHEA-S protect rodent neurons against various forms of toxicity including overactivation of N-methyl-D-aspartate

(NMDA) glutamate receptors (98, 99), amyloid beta expression (100), and oxygen-glucose deprivation (101). Importantly, DHEA-S prevents against rodent hippocampal neuronal cell loss and damage observed following ischemia and stroke (102). Additionally, the progesterone precursor pregnenolone and pregnenolone-sulfate also exhibit neuroprotective benefits (103–105). More work is necessary to discern estrogen mediated STS effects vs. DHEA mediated ones following TBI.

Like aromatase, we see a connection between STS expression and inflammatory signals. Interleukin (IL)-1β suppresses STS expression in endometrial stromal cells (106). However, in breast cancer cells, various other cytokines IL-1α, IL-6, TNFα, and enhanced or increased activity of STS (107). For both IL-6 and TNFα, STS mRNA expression was unchanged and the difference in activity was due to posttranslational modifications via STS glycosylation (72). Our studies examining the effects of a single penetrating injury in the adult zebra finch (*Taeniopygia guttata*) brain showed that STS expression was unchanged following TBI (**Figures 2A,B**). The zebra finch serves as a model organism for the study of steroid induced neuroprotection, because they rapidly and robustly respond to injury and express the full suite of steroidogenic enzymes (53, 82, 108). Furthermore, increased expression of pro-inflammatory cytokines is observed anywhere from 2 to 4 h following injury (83). Thus, the lack of a change in STS mRNA expression was surprising. As STS mRNA levels are unaffected in zebra finches, more research is necessary to determine if STS glycosylation or estrone bioavailability is increased following injury.

Estrogen Steroid Sulfotransferases (SULT1E1)

SULT1E1 (**Figure 1**) when compared to other members of the steroid sulfotransferase (SULT) family has greater affinity for estrogens than other steroids, and thus readily inactivates estrone and estradiol. *SULT1E1* is expressed in various tissues in both sexes (109) including the brain (110) and its expression is regulated by steroid hormones, specifically progesterone (96). Estrone-sulfate and its naturally occurring counterpart estradiol-sulfate (E2-SO_4) are biologically important as they regulate neuronal network formation, activity, and synaptogenesis (111–114) in vertebrates.

Very little is known about the expression of SULT1E1 following TBI, however there is some data on the role of estradiol-sulfate (E2-SO_4) in rodent models. In terms of expression, like STS, we did not see a significant increase or decrease in expression of SULT1E1 following TBI (**Figures 2C,D**), however the expression was far more variable than that of STS. Studies are on-going to determine if protein expression matched the mRNA expression. Sulfated estrogens reduce neurodegeneration by altering cell and tissue damage and oxidative stress in rodents (115, 116). This is accomplished by improving blood and water flow to damaged tissue and thus increasing cerebral perfusion pressure and decreasing cell loss (22). Furthermore, the solubility of E2-SO_4 as opposed to estradiol enables intravenous delivery of supraphysiological quantities and thus one could give much higher doses and have it be eliminated from the body much more quickly (117, 118). Sulfated estrogens provide a noteworthy avenue for both future research and future treatment options following TBI (118).

With respect to SULT1E1 and TBI induced inflammation, very little is known, but there appear to be a strong connection between SULT1E1 expression and release of cytokines. Knockdown of SULT1E1 increases pro-inflammatory cytokines and decreases anti-inflammatory signals (119). Furthermore, both edema and intercranial pressure are altered by SULT1E1 expression (115, 118). Understanding SULT1E1 expression and its role following TBI is key to understanding how sulfate estrogens can be used as a treatment. The time of SULT1E1 expression may also change the results. In the finch brain, we examined expression at 24 h, however the knockdown expression studies were 72 h. It would be interesting to extend the timepoints at which expression was examined.

HSD17B1 and HSD17B2

17β-hydroxysteroid dehydrogenases (HSD17B) are enzymes responsible for the synthesis and deactivation of estrogens and androgens, specifically the formation of testosterone and estradiol from precursors (61, 76, 108, 120, 121) (**Figure 1**). HSD17B1 primarily catalyzes the conversion of estrone to estradiol in various tissues including the brain. In addition to its role in estrogen synthesis, HSD17B1 facilitates the formation of various androgens as well (76, 122–124). Conversely, the enzyme HSD17B2 mediates the oxidation of estradiol back to estrone, and testosterone and androstendiol back to androstenedione dehydroepiandrosterone. Furthermore, HSD17B2, is responsible for the production of the active progestin, progesterone (76, 125). The expression of HSD17B2 has previously only been identified in non-neuronal tissues, however a few reports have identified transcript and protein in the brain (126).

Surprisingly, there remains a dearth of information and studies on the role or expression of either HSD17B1 or HSD17B2 following TBI. The majority of past studies have examined the role of HSD17Bs in steroid sensitive cancers (69). Specifically, in terms of brain damage, there has been some work showing a connection between HSD17B1 and risk of Alzheimer's disease in Down Syndrome patients (127). In our work, we did not find a significant change in expression of HSD17B1 following TBI, suggesting that conversion to estrone is not a probable path to increasing estrogen signaling following injury (**Figures 2E,F**). While estradiol is the predominate estrogen mediating neuroprotection, estrone, has been shown to be neuroprotective following various damage or insults to the brain (128–130). Estrone increases the signaling of neuroprotective pathways (ERK1/2 and BDNF) and decreases cell death and thus ischemic injury size (38). These results suggest that despite the lack of change in HSD17B1, vertebrates may still be getting the protection from higher levels of estrone (38). As estrone is the most abundant estrogen in menopausal women (131, 132), it is hypothesized to be extremely important in mediating neuroprotection in this population (38). Much more work is necessary to understand the levels of estrone relative to estradiol following TBI.

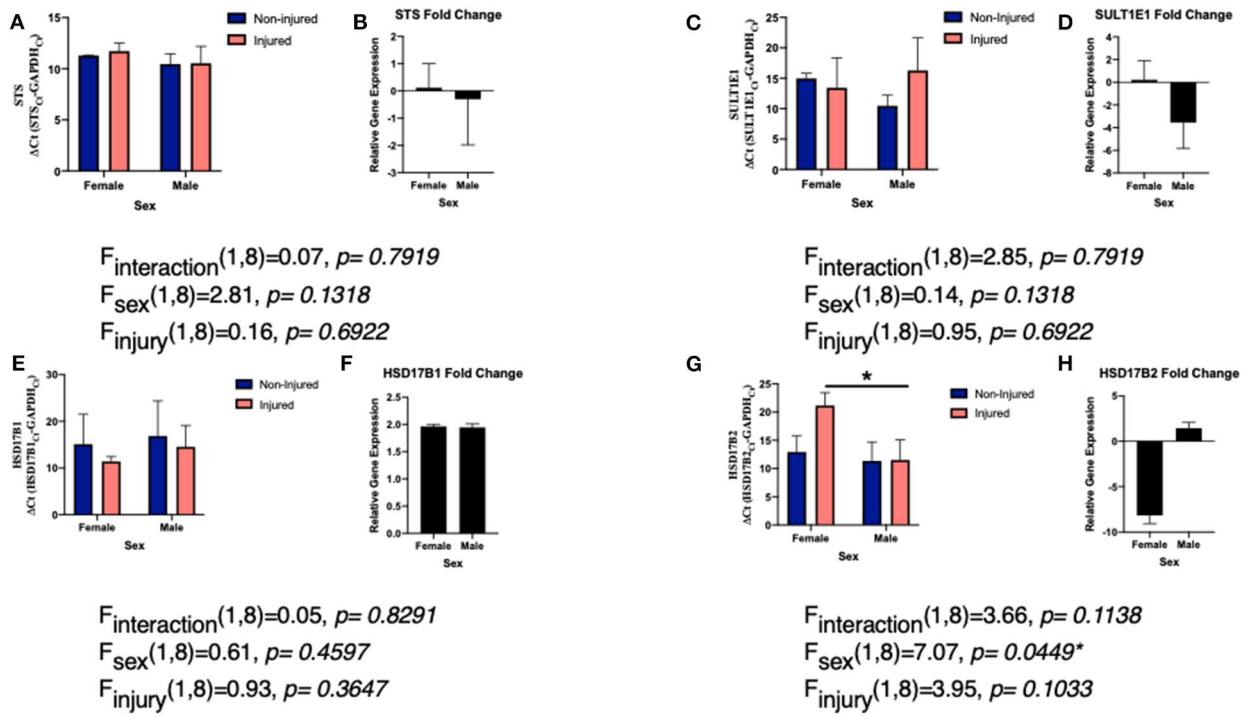

FIGURE 2 | Levels of EMEs mRNA relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; delta CT value and fold change) in both male and female zebra finches 24 h post-surgery. Adult male and female zebra finches were subjected to an unilateral penetrating injury directed toward the entopallium and collected 24 h later. Finches were sacrificed and total RNA was extracted from microdissections immediately adjacent to the cortical needle and the corresponding location on uninjured hemisphere. Expression of STS **(A)**, SULT1E1 **(C)**, HSD17B1 **(E)**, and HSD17B2 **(G)** relative to GAPDH was measured using quantitative PCR using primers specific for the ZF mRNA sequence. Mean ΔCT values were compared with a two-way ANOVA. Fold change in gene expression was also calculated STS **(B)**, SULT1E1 **(D)**, HSD17B1 **(F)**, and HSD17B2 **(H)**. Neither expression of STS, SULT1E1, or HSD17B1 were significantly changed following injury. However, HSD17B2 was significantly downregulated following injury. *denotes a significant difference at $P < 0.05$. All protocols were approved by the Vassar College Institutional Animal Care and Use Committee following National Institutes of Health Guidelines.

While HSD17B1 expression was not altered by TBI, HSD17B2 expression was downregulated in a sex specific manner following TBI in the finch brain (**Figures 2G,H**). Females decreased expression of HSD17B2 following injury while males did not. Again, much remains unknown surrounding the function of HSD17B2 in the brain, specifically because it is noticeably absent from human and rodent brains (133). Notably other HSDs are found throughout the avian brain and regulate conversion from cholesterol to the sex steroids (108, 120, 134). Further research is needed in order to conclude if the expression of HSD17B2 following injury is unique to songbird brains, or if this represents a more evolutionarily conserved pathway. Despite the abundant questions remaining, the results are promising. One could hypothesize that females are decreasing expression of HSD17B2 in order to keep more estradiol available for neuroprotection and repair, while not having to produce more estrogens. As males use estrogen to undergo sexual differentiation, having a local upregulation of estrogens in the female brain may not be beneficial long term. Furthermore, while HSD17B2 is the key HSD17B isozyme in androgen and estrogen inactivation, it also activates 20α-hydroxyprogesterone into progesterone (70, 135). Females may be using progesterone in combination with estradiol as neuroprotective steroids following injury. These results are compelling as we have previously found a role for progesterone in cell survival following TBI in the brain (105) and this supports a large body of research examining progesterone treatment following TBI (136, 137). Interestingly, like with estradiol despite positive pre-clinical studies, larger clinical, and human trials of progesterone use following TBI have been unsuccessful (138). Together, these data suggest that much more research is needed in order to understand the very complex steroidal milieu following TBI.

CONCLUSIONS

As has been discussed in the preceding sections, there is ample evidence to support that estrogens are neuroprotective following injury. Yet, when estrogen use has been used in clinical trials of TBI, the majority of evidence has not been positive and has led many to question its use as a treatment option (139, 140). Thus, more research on the formation of these estrogens and how they are inactivated is necessary in order to better develop treatment plans and options for mirroring estrogen's endogenous neuroprotective effects found preclinically without the undesirable hormonal ones observed.

ETHICS STATEMENT

The animal study was reviewed and approved by Vassar College IACUC.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

This work was supported by Vassar College Salmon Fund to KD.

ACKNOWLEDGMENTS

The author wishes to acknowledge Dr. Colin Saldanha, and the many undergraduates that contributed to the work presented here.

REFERENCES

1. Lenzlinger PM, Morganti-Kossmann MC, Laurer HL, McIntosh TK. The duality of the inflammatory response to traumatic brain injury. *Mol Neurobiol*. (2001) 24:169–81. doi: 10.1385/MN:24:1-3:169
2. Maas AI, Stocchetti N, Bullock R. Moderate and severe traumatic brain injury in adults. *Lancet Neurology*. (2008) 7:728–41. doi: 10.1016/S1474-4422(08)70164-9
3. Stein DG. Is progesterone a worthy candidate as a novel therapy for traumatic brain injury? *Dialogues Clin Neurosci*. (2011) 13:352–9.
4. Griesbach GS, Kreber LA, Harrington D, Ashley MJ. Post-acute traumatic brain injury rehabilitation: effects on outcome measures and life care costs. *J Neurotrauma*. (2015) 32:704–11. doi: 10.1089/neu.2014.3754
5. Bruns J, Hauser WA. The epidemiology of traumatic brain injury: a review. *Epilepsia*. (2003) 44:2–10. doi: 10.1046/j.1528-1157.44.s10.3.x
6. Corrigan JD, Selassie AW, Orman JA. The epidemiologyo of traumatic brain injury. *J Head Trauma Rehabil*. (2010) 25:72–80. doi: 10.1097/HTR.0b013e3181ccc8b4
7. Langlois JA, Rutland-Brown W, Wald MM. The epidemiology and impact of traumatic brain injury: a brief overview. *J Head Trauma Rehabil*. (2006) 21:375–8. doi: 10.1097/00001199-200609000-00001
8. Abou-Abbass H, Bahmad H, Ghandour H, Fares J, Wazzi-Mkahal R, Yacoub B, et al. Epidemiology and clinical characteristics of traumatic brain injury in Lebanon: a systematic review. *Medicine*. 95:e5342. doi: 10.1097/MD.0000000000005342
9. Morganti-Kossmann MC, Satgunaseelan L, Bye N, Kossmann T. Modulation of immune response by head injury. *Injury*. (2007) 38:1392–400. doi: 10.1016/j.injury.2007.10.005
10. Xu J, Fan G, Chen S, Wu Y, Xu XM, Hsu CY. Methylprednisolone inhibition of TNF-alpha expression and NF-kB activation after spinal cord injury in rats. *Brain Res Mol Brain Res*. (1998) 59:135–42. doi: 10.1016/S0169-328X(98)00142-9
11. Ziebell JM, Morganti-Kossmann MC. Involvement of pro- and anti-inflammatory cytokines and chemokines in the pathophysiology of traumatic brain injury. *Neurotherapeutics*. (2010) 7:22–30. doi: 10.1016/j.nurt.2009.10.016
12. Kang JH, Lin HC. Increased risk of multiple sclerosis after traumatic brain injury: a nationwide population-based study. *J Neurotrauma*. (2012) 29:90–5. doi: 10.1089/neu.2011.1936
13. Prins M, Greco T, Alexander D, Giza CC. The pathophysiology of traumatic brain injury at a glance. *DMM Dis Model Mech*. (2013) 6:1307–15. doi: 10.1242/dmm.011585
14. Lye TC, Shores EA. Traumatic brain injury as a risk factor for Alzheimer's disease: a review. *Neuropsychol Rev*. (2000) 10:115–29. doi: 10.1023/A:1009068804787
15. Sorby-Adams AJ, Marcoionni AM, Dempsey ER, Woenig JA, Turner RJ. The role of neurogenic inflammation in blood-brain barrier disruption and development of cerebral oedema following acute central nervous system (CNS) injury. *Int J Mol Sci*. (2017) 18:1788. doi: 10.3390/ijms18081788
16. Fitch MT, Doller C, Combs CK, Landreth GE, Silver J. Cellular and molecular mechanisms of glial scarring and progressive cavitation: *in vivo* and *in vitro* analysis of inflammation-induced secondary injury after CNS trauma. *J Neurosci*. (1999) 19:8182–98. doi: 10.1523/JNEUROSCI.19-19-08182.1999
17. Thelin EP, Hall CE, Gupta K, Carpenter KLH, Chandran S, Hutchinson PJ, et al. Elucidating pro-inflammatory cytokine responses after traumatic brain injury in a human stem cell model. *J Neurotrauma*. (2018) 35:341–52. doi: 10.1089/neu.2017.5155
18. Rothwell NJ, Strijbos PJ. Cytokines in neurodegeneration and repair. *Int J Dev Neurosci*. (1995) 13:179–85. doi: 10.1016/0736-5748(95)00018-C
19. Duncan KA, Walters BJ, Saldanha CJ. Traumatized and inflamed - but resilient: glial aromatization and the avian brain. *Horm Behav*. (2013) 63:208–15. doi: 10.1016/j.yhbeh.2012.02.026
20. McCutcheon V, Park E, Liu E, Sobhebidari P, Tavakkoli J, Wen XY, et al. A novel model of traumatic brain injury in adult zebrafish demonstrates response to injury and treatment comparable with mammalian models. *J Neurotrauma*. (2017) 34:1382–93. doi: 10.1089/neu.2016.4497
21. Bramlett HM, Dietrich WD. Progressive damage after brain and spinal cord injury: pathomechanisms and treatment strategies. *Prog Brain Res*. (2007) 161:125–41. doi: 10.1016/S0079-6123(06)61009-1
22. Day NL, Floyd CL, D'Alessandro TL, Hubbard WJ, Chaudry IH. 17β-estradiol confers protection after traumatic brain injury in the rat and involves activation of G protein-coupled estrogen receptor 1. *J Neurotrauma*. (2013) 30:1531–41. doi: 10.1089/neu.2013.2854
23. Herson PS, Koerner IP, Hurn PD. Sex, sex steroids, and brain injury. *Semin Reprod Med*. (2009) 27:229–39. doi: 10.1055/s-0029-1216276
24. Saldanha CJ, Duncan KA, Walters BJ. Neuroprotective actions of brain aromatase. *Front Neuroendocrinol*. (2009) 30:106–18. doi: 10.1016/j.yfrne.2009.04.016
25. Duncan KA, Saldanha CJ. Central aromatization: a dramatic and responsive defense against threat and trauma to the vertebrate brain. *Front Neuroendocrinol*. (2020) 56:100816. doi: 10.1016/j.yfrne.2019.100816
26. Duncan KA, Saldanha CJ. Neuroinflammation induces glial aromatase expression in the uninjured songbird brain. *J Neuroinflammation*. (2011) 8:81. doi: 10.1186/1742-2094-8-81
27. Stein DG. Brain damage, sex hormones and recovery: a new role for progesterone and estrogen? *Trends Neurosci*. (2001) 24:386–91. doi: 10.1016/S0166-2236(00)01821-X
28. Acaz-Fonseca E, Avila-Rodriguez M, Garcia-Segura LM, Barreto GE. Regulation of astroglia by gonadal steroid hormones under physiological and pathological conditions. *Prog Neurobiol*. (2016) 144:5–26. doi: 10.1016/j.pneurobio.2016.06.002
29. Pedersen AL, Nelson LH, Saldanha CJ. Centrally synthesized estradiol is a potent anti-inflammatory in the injured zebra finch brain. *Endocrinology*. (2016) 157:2041–51. doi: 10.1210/en.2015-1991
30. Raghava N, Das BC, Ray SK. Neuroprotective effects of estrogen in CNS injuries: insights from animal models. *Neurosci Neuroeconomics*. (2017) 6:15–29. doi: 10.2147/NAN.S105134
31. Culmsee C, Vedder H, Ravati A, Junker V, Otto D, Ahlemeyer B, et al. Neuroprotection by estrogens in a mouse model of focal cerebral ischemia and in cultured neurons: evidence for a receptor-independent antioxidative mechanism. *J Cereb Blood Flow Metab*. (1999) 19:1263–9. doi: 10.1097/00004647-199911000-00011
32. Lee JY, Choi HY, Na WH, Ju BG, Yune TY. 17β-estradiol inhibits MMP-9 and SUR1/TrpM4 expression and activation and thereby attenuates BSCB disruption/hemorrhage after spinal cord injury in male rats. *Endocrinology*. (2015) 156:1838–50. doi: 10.1210/en.2014-1832

33. Cox A, Varma A, Barry J, Vertegel A, Banik N. Nanoparticle estrogen in rat spinal cord injury elicits rapid anti-inflammatory effects in plasma, cerebrospinal fluid, and tissue. *J Neurotrauma*. (2015) 32:1413–21. doi: 10.1089/neu.2014.3730
34. Han D, Scott EL, Dong Y, Raz L, Wang R, Zhang Q. Attenuation of mitochondrial and nuclear p38α signaling: a novel mechanism of estrogen neuroprotection in cerebral ischemia. *Mol Cell Endocrinol*. (2015) 400:21–31. doi: 10.1016/j.mce.2014.11.010
35. Dubal DB, Zhu H, Yu J, Rau SW, Shughrue PJ, Merchenthaler I, et al. Estrogen receptor alpha, not beta, is a critical link in estradiol-mediated protection against brain injury. *Proc Natl Acad Sci USA*. (2001) 98:1952–7. doi: 10.1073/pnas.98.4.1952
36. Zhu C, Wang S, Wang B, Du F, Hu C, Li H, et al. 17β-estradiol up-regulates Nrf2 via PI3K/AKT and estrogen receptor signaling pathways to suppress light-induced degeneration in rat retina. *Neuroscience*. (2015) 304:328–39. doi: 10.1016/j.neuroscience.2015.07.057
37. Hill RA, Chua HK, Jones ME, Simpson ER, Boon WC. Estrogen deficiency results in apoptosis in the frontal cortex of adult female aromatase knockout mice. *Mol Cell Neurosci*. (2009) 41:1–7. doi: 10.1016/j.mcn.2008.12.009
38. Gatson JW, Liu MM, Abdelfattah K, Wigginton JG, Smith S, Wolf S, et al. Estrone is neuroprotective in rats after traumatic brain injury. *J Neurotrauma*. (2012) 29:2209–19. doi: 10.1089/neu.2011.2274
39. Simpkins JW, Rajakumar G, Zhang YQ, Simpkins CE, Greenwald D, Yu CJ, et al. Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat. *J Neurosurg*. (1997) 87:724–30. doi: 10.3171/jns.1997.87.5.0724
40. Kondo Y, Suzuki K, Sakuma Y. Estrogen alleviates cognitive dysfunction following transient brain ischemia in ovariectomized gerbils. *Neurosci Lett*. (1997) 238:45–8. doi: 10.1016/S0304-3940(97)00847-1
41. McCullough LD, Blizzard K, Simpson ER, Oz OK, Hurn PD. Aromatase cytochrome P450 and extragonadal estrogen play a role in ischemic neuroprotection. *J Neurosci*. (2003) 23:8701–5. doi: 10.1523/JNEUROSCI.23-25-08701.2003
42. Merchenthaler I, Dellovade TL, Shughrue PJ. Neuroprotection by estrogen in animal models of global and focal ischemia. *Ann. N. Y. Acad. Sci.* 1007:89–100. doi: 10.1196/annals.1286.009
43. Sudo S, Wen TC, Desaki J, Matsuda S, Tanaka J, Arai T, et al. Beta-estradiol protects hippocampal CA1 neurons against transient forebrain ischemia in gerbil. *Neurosci Res*. (1997) 29:345–54. doi: 10.1016/S0168-0102(97)00106-5
44. Suzuki S, Gerhold LM, Böttner M, Rau SW, Dela Cruz C, Yang E, et al. Estradiol enhances neurogenesis following ischemic stroke through estrogen receptors α and β. *J Comp Neurol*. (2007) 500:1064–75. doi: 10.1002/cne.21240
45. Yang SH, Shi J, Day AL, Simpkins JW. Estradiol exerts neuroprotective effects when administered after ischemic insult. *Stroke*. (2000) 31:745–50. doi: 10.1161/01.STR.31.3.745
46. Brotfain EE, Gruenbaum S, Boyko M, Kutz R, Zlotnik A, Klein M. Neuroprotection by estrogen and progesterone in traumatic brain injury and spinal cord injury. *Curr Neuropharmacol*. (2016) 14:641–53. doi: 10.2174/1570159X14666160309123554
47. Emerson CS, Headrick JP, Vink R. Estrogen improves biochemical and neurologic outcome following traumatic brain injury in male rats, but not in females. *Brain Res*. (1993) 608:95–100. doi: 10.1016/0006-8993(93)90778-L
48. Cook S, Hung V, Duncan KA. Crosstalk between estrogen withdrawal and NFκB signaling following penetrating brain injury. *Neuroimmunomodulation*. (2018) 25:193–200. doi: 10.1159/000493506
49. Pozzi S, Benedusi V, Maggi A, Vegeto E. Estrogen action in neuroprotection and brain inflammation. *Ann N Y Acad Sci*. (2006) 1089:302–23. doi: 10.1196/annals.1386.035
50. Wen Y, Yang S, Liu R, Perez E, Yi KD, Koulen P, et al. Estrogen attenuates nuclear factor-kappa B activation induced by transient cerebral ischemia. *Brain Res*. (2004) 1008:147–54. doi: 10.1016/j.brainres.2004.02.019
51. Pedersen AL, Saldanha CJ. Reciprocal interactions between prostaglandin E2- and estradiol-dependent signaling pathways in the injured zebra finch brain. *J Neuroinflammation*. (2017) 14:262. doi: 10.1186/s12974-017-1040-1
52. Walters BJ, Alexiades NG, Saldanha CJ. Intracerebral estrogen provision increases cytogenesis and neurogenesis in the injured zebra finch brain. *Dev Neurobiol*. (2011) 71:170–81. doi: 10.1002/dneu.20839
53. Duncan KA, Saldanha CJ. *Inducible Aromatase in Astroglia: Protection and Recovery from Neural Perturbation in Birds*. New York, NY: Oxford University Press (2013). doi: 10.1093/acprof:oso/9780199841196.003.0020
54. MacLusky N, Naftolin F. Sexual differentiation of the central nervous system. *Science*. (1981) 211:1294–302. doi: 10.1126/science.6163211
55. Secky L, Svoboda M, Klameth L, Bajna E, Hamilton G, Zeillinger R, et al. The sulfatase pathway for estrogen formation: targets for the treatment and diagnosis of hormone-associated tumors. *J Drug Deliv*. (2013) 2013:957605. doi: 10.1155/2013/957605
56. Leitman DC, Paruthiyil S, Vivar OI, Saunier EF, Herber CB, Cohen I, et al. Regulation of specific target genes and biological responses by estrogen receptor subtype agonists. *Curr Opin Pharmacol*. (2010) 10:629–36. doi: 10.1016/j.coph.2010.09.009
57. Koos RD. Minireview: putting physiology back into estrogens' mechanism of action. *Endocrinology*. (2011) 152:4481–8. doi: 10.1210/en.2011-1449
58. Bolton JL, Thatcher GRJ. Potential mechanisms of estrogen quinone carcinogenesis. *Chem Res Toxicol*. (2008) 21:93–101. doi: 10.1021/tx700191p
59. Voskuhl RR, Gold SM. Sex-related factors in multiple sclerosis susceptibility and progression. *Nat Rev Neurol*. (2012) 8:255–63. doi: 10.1038/nrneurol.2012.43
60. Arnold AP, Schlinger BA. Sexual differentiation of brain and behavior: the zebra finch is not just a flying rat. *Brain Behav Evol*. (1993) 42:231–41. doi: 10.1159/000114157
61. Tsutsui K. Neurosteroid biosynthesis and function in the brain of domestic birds. *Front Endocrinol*. (2011) 2:37. doi: 10.3389/fendo.2011.00037
62. Naftolin F. Brain aromatization of androgens. *J Reprod Med*. (1994) 39:257–61.
63. Labrie F. Intracrinology. *Mol Cell Endocrinol*. (1991) 78:C113–8. doi: 10.1016/0303-7207(91)90116-A
64. Balthazart J, Stoop R, Foidart A, Granneman JCM, Lambert JGD. Distribution and regulation of estrogen-2-hydroxylase in the quail brain. *Brain Res Bull*. (1994) 35:339–45. doi: 10.1016/0361-9230(94)90111-2
65. Almadhidi J, Moslemi S, Drosdowsky MA, Séralini GE. Equine cytochrome p450 aromatase exhibits an estrogen 2-hydroxylase activity *in vitro*. *J Steroid Biochem Mol Biol*. (1996) 59:55–61. doi: 10.1016/S0960-0760(96)00085-4
66. Osawa Y, Higashiyama T, Shimizu Y, Yarborough C. Multiple functions of aromatase and the active site structure; aromatase is the placental estrogen 2-hydroxylase. *J Steroid Biochem Mol Biol*. (1993) 44:469–80. doi: 10.1016/0960-0760(93)90252-R
67. Balthazart J, Ball GF. New insights into the regulation and function of brain estrogen synthase (aromatase). *Trends Neurosci*. (1998) 21:243–9. doi: 10.1016/S0166-2236(97)01221-6
68. Charlier TD, Cornil CA, Patte-Mensah C, Meyer L, Mensah-Nyagan AG, Balthazart J. Local modulation of steroid action: rapid control of enzymatic activity. *Front Neurosci*. (2015) 9:83. doi: 10.3389/fnins.2015.00083
69. Hilborn E, Stål O, Jansson A. Estrogen and androgen-converting enzymes 17β-hydroxysteroid dehydrogenase and their involvement in cancer: with a special focus on 17β-hydroxysteroid dehydrogenase type 1, 2, and breast cancer. *Oncotarget*. (2017) 8:30552–62. doi: 10.18632/oncotarget.15547
70. Moeller G, Adamski J. Multifunctionality of human 17β-hydroxysteroid dehydrogenases. *Mol. Cell. Endocrinol.* (2006) 248:47–55. doi: 10.1016/j.mce.2005.11.031
71. Zaichuk T, Ivancic D, Scholtens D, Schiller C, Khan SA. Tissue-specific transcripts of human steroid sulfatase are under control of estrogen signaling pathways in breast carcinoma. *J Steroid Biochem Mol Biol*. (2007) 105:76–84. doi: 10.1016/j.jsbmb.2006.12.101
72. Newman SP, Purohit A, Ghilchik MW, Potter B V, Reed MJ. Regulation of steroid sulphatase expression and activity in breast cancer. *J Steroid Biochem Mol Biol*. (2000) 75:259–64. doi: 10.1016/S0960-0760(00)00177-1
73. Stocco C. Tissue physiology and pathology of aromatase. *Steroids*. (2012) 77:27–35. doi: 10.1016/j.steroids.2011.10.013
74. Stocco C. Aromatase expression in the ovary: hormonal and molecular regulation. *Steroids*. (2008) 73:473–87. doi: 10.1016/j.steroids.2008.01.017
75. Naftolin F, Horvath TL, Jakab RL, Leranth C, Harada N, Balthazart J. Aromatase immunoreactivity in axon terminals of the vertebrate brain. an immunocytochemical study on quail, rat, monkey and human tissues. *Neuroendocrinology*. (1996) 63:149–55. doi: 10.1159/000126951

76. Stoffel-Wagner B, Watzka M, Schramm J, Bidlingmaier F, Klingmüller D. Expression of CYP19 (aromatase) mRNA in different areas of the human brain. *J Steroid Biochem Mol Biol*. 70:237–41. doi: 10.1016/S0960-0760(99)00114-4
77. Hojo Y, Hattori TA, Enami T, Furukawa A, Suzuki K, Ishii HT, et al. Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017α and P450 aromatase localized in neurons. *Proc Natl Acad Sci USA*. (2004) 101:865–70. doi: 10.1073/pnas.2630225100
78. Saldanha CJ, Remage-Healey L, Schlinger BA. Synaptocrine signaling: steroid synthesis and action at the synapse. *Endocr Rev*. (2011) 32:532–49. doi: 10.1210/er.2011-0004
79. Yague JG, Wang AC, Janssen WG, Hof PR, Garcia-Segura LM, Azcoitia I, et al. Aromatase distribution in the monkey temporal neocortex and hippocampus. *Brain Res*. (2008) 1209:115–27. doi: 10.1016/j.brainres.2008.02.061
80. Zhang QG, Wang R, Tang H, Dong Y, Chan A, Sareddy GR, et al. Brain-derived estrogen exerts anti-inflammatory and neuroprotective actions in the rat hippocampus. *Mol Cell Endocrinol*. (2014) 389:84–91. doi: 10.1016/j.mce.2013.12.019
81. Remage-Healey L, Saldanha CJ, Schlinger BA. Estradiol synthesis and action at the synapse: evidence for "synaptocrine" signaling. *Front Endocrinol*. (2011) 2:28. doi: 10.3389/fendo.2011.00028
82. Peterson RS, Saldanha CJ, Schlinger BA. Rapid upregulation of aromatase mRNA and protein following neural injury in the zebra finch (taeniopygia guttata). *J Neuroendocrinol*. (2001) 13:317–23. doi: 10.1046/j.1365-2826.2001.00647.x
83. Saldanha CJ, Burstein SR, Duncan KA. Induced synthesis of oestrogens by glia in the songbird brain. *J Neuroendocrinol*. (2013) 25:1032–8. doi: 10.1111/jne.12067
84. Wynne RD, Saldanha CJ. Glial aromatization decreases neural injury in the zebra finch (taeniopygia guttata): influence on apoptosis. *J Neuroendocrinol*. (2004) 16:676–83. doi: 10.1111/j.1365-2826.2004.01217.x
85. Wynne RD, Walters BJ, Bailey DJ, Saldanha CJ. Inhibition of injury-induced glial aromatase reveals a wave of secondary degeneration in the songbird brain. *Glia*. (2008) 56:97–105. doi: 10.1002/glia.20594
86. Azcoitia I, Sierra A, Veiga S, Honda S, Harada N, Garcia-Segura LM. Brain aromatase is neuroprotective. *J Neurobiol*. (2001) 47:318–29. doi: 10.1002/neu.1038
87. Gölz C, Kirchhoff FP, Westerhorstmann J, Schmidt M, Hirnet T, Rune GM, et al. Sex hormones modulate pathogenic processes in experimental traumatic brain injury. *J Neurochem*. (2019) 150:173–87. doi: 10.1111/jnc.14678
88. Saldanha CJ, Rohmann KN, Coomaralingam L, Wynne RD. Estrogen provision by reactive glia decreases apoptosis in the zebra finch (taeniopygia guttata). *J Neurobiol*. (2005) 64:192–201. doi: 10.1002/neu.20147
89. Pedersen AL, Brownrout JL, Saldanha CJ. Neuroinflammation and neurosteroidogenesis: reciprocal modulation during injury to the adult zebra finch brain. *Physiol Behav*. (2018) 187:51–6. doi: 10.1016/j.physbeh.2017.10.013
90. Purohit A, Potter B V, Parker MG, Reed MJ. Steroid sulphatase: expression, isolation and inhibition for active-site identification studies. *Chem Biol Interact*. (1998) 109:183–93. doi: 10.1016/S0009-2797(97)00132-4
91. Hobkirk R. Steroid sulfation current concepts. *Trends Endocrinol Metab*. (1993) 4:69–74. doi: 10.1016/S1043-2760(05)80018-9
92. Aksoy IA, Wood TC, Weinshilboum R. Human liver estrogen sulfotransferase: identification by cDNA cloning and expression. *Biochem Biophys Res Commun*. (1994) 200:1621–29. doi: 10.1006/bbrc.1994.1637
93. Falany CN, Vazquez ME, Kalb JM. Purification and characterization of human liver dehydroepiandrosterone sulphotransferase. *Biochem J*. (1989) 260:641–6. doi: 10.1042/bj2600641
94. Pasqualini JR, Chetrite G, Blacker C, Feinstein MC, Delalonde L, Talbi M, et al. Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. *J Clin Endocrinol Metab*. (1996) 81:1460–4. doi: 10.1210/jcem.81.4.8636351
95. Adjei AA, Thomae BA, Prondzinski JL, Eckloff BW, Wieben ED, Weinshilboum RM. Human estrogen sulfotransferase (SULT1E1) pharmacogenomics: gene resequencing and functional genomics. *Br J Pharmacol*. (2003) 139:1373–82. doi: 10.1038/sj.bjp.0705369
96. RiŽner TL. The important roles of steroid sulfatase and sulfotransferases in gynecological diseases. *Front Pharmacol*. (2016) 7:30. doi: 10.3389/fphar.2016.00030
97. Nardi A, Pomari E, Zambon D, Belvedere P, Colombo L, Dalla Valle L. Transcriptional control of human steroid sulfatase. *J Steroid Biochem Mol Biol*. (2009) 115:68–74. doi: 10.1016/j.jsbmb.2009.02.017
98. Mao X, Barger SW. Neuroprotection by dehydroepiandrosteronesulfate: role of an NF?B-like factor. *Neuroreport*. (1998) 9:759–63. doi: 10.1097/00001756-199803090-00036
99. Kimonides VG, Khatibi NH, Svendsen CN, Sofroniew M V., Herbert J. Dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS) protect hippocampal neurons against excitatory amino acid-induced neurotoxicity. *Proc Natl Acad Sci USA*. (1998) 95:1852–7. doi: 10.1073/pnas.95.4.1852
100. Cardounel A, Regelson W, Kalimi M. Dehydroepiandrosterone protects hippocampal neurons against neurotoxin-induced cell death: mechanism of action. *Proc Soc Exp Biol Med*. (1999) 222:145–9. doi: 10.1046/j.1525-1373.1999.d01-124.x
101. Kaasik A, Kalda A, Jaako K, Zharkovsky A. Dehydroepiandrosterone sulphate prevents oxygen-glucose deprivation-induced injury in cerebellar granule cell culture. *Neuroscience*. (2001) 102:427–32. doi: 10.1016/S0306-4522(00)00489-9
102. Li ZK, Feng JX, Zhao CY, Ke H, Shen L. Protection of androgen against hypoxic-ischemic brain damage in neonatal rats and possible mechanisms. *Zhongguo Dang Dai Er Ke Za Zhi*. (2006) 8:441–6.
103. Weaver CE, Marek P, Park-Chung M, Tam SW, Farb DH. Neuroprotective activity of a new class of steroidal inhibitors of the N-methyl-D-aspartate receptor. *Proc Natl Acad Sci USA*. (1997) 94:10450–4. doi: 10.1073/pnas.94.19.10450
104. Gursoy E, Cardounel A, Kalimi M. Pregnenolone protects mouse hippocampal (HT-22) cells against glutamate and amyloid beta protein toxicity. *Neurochem Res*. (2001) 26:15–21. doi: 10.1023/a:1007668213330
105. Blackshear K, Giessner S, Hayden JP, Duncan KA. Exogenous progesterone is neuroprotective following injury to the male zebra finch brain. *J Neurosci Res*. (2018) 96:545–555. doi: 10.1002/jnr.24060
106. Matsuoka R, Yanaihara A, Saito H, Furusawa Y, Toma Y, Shimizu Y, et al. Regulation of estrogen activity in human endometrium: effect of IL-1beta on steroid sulfatase activity in human endometrial stromal cells. *Steroids*. (2002) 67:655–9. doi: 10.1016/S0039-128X(02)00016-8
107. Ren X, Wu X, Hillier SG, Fegan KS, Critchley HOD, Mason JI, et al. Local estrogen metabolism in epithelial ovarian cancer suggests novel targets for therapy. *J Steroid Biochem Mol Biol*. (2015) 150:54–63. doi: 10.1016/j.jsbmb.2015.03.010
108. Mirzatoni A, Spence RD, Naranjo KC, Saldanha CJ, Schlinger BA. Injury-induced regulation of steroidogenic gene expression in the cerebellum. *J Neurotrauma*. (2010) 27:1875–82. doi: 10.1089/neu.2010.1330
109. Coughtrie MWH. Sulfation through the looking glass - recent advances in sulfotransferase research for the curious. *Pharmacogenomics J*. (2002) 2:297–308. doi: 10.1038/sj.tpj.6500117
110. Uhlén M, Björling E, Agaton C, Szigyarto CAK, Amini B, Andersen E, et al. A human protein atlas for normal and cancer tissues based on antibody proteomics. *Mol Cell Proteomics*. (2005) 4:1920–32. doi: 10.1074/mcp.M500279-MCP200
111. Garcia-Segura LM, Luquin S, Parducz A, Naftolin F. Gonadal hormone regulation of glial fibrillary acidic protein immunoreactivity and glial ultrastructure in the rat neuroendocrine hypothalamus. *Glia*. (1994) 10:59–69. doi: 10.1002/glia.440100108
112. Roof RL, Hall ED. Gender differences in acute CNS trauma and stroke: neuroprotective effects of estrogen and progesterone. *J Neurotrauma*. (2000) 17:367–88. doi: 10.1089/neu.2000.17.367
113. McEwen B. Estrogen actions throughout the brain. *Recent Prog Horm Res*. (2002) 57:357–84. doi: 10.1210/rp.57.1.357
114. Beyer C. Estrogen and the developing mammalian brain. *Anat Embryol*. (1999) 199:379–90. doi: 10.1007/s004290050236
115. Liu X, Huang J, Sun Y, Zhan K, Zhang Z, Hong M. Identification of multiple binding sites for substrate transport in bovine organic anion transporting polypeptide 1a2. *Drug Metab Dispos*. (2013) 41:602–7. doi: 10.1124/dmd.112.047910

116. Stein DG, Hoffman SW. Estrogen and progesterone as neuroprotective agents in the treatment of acute brain injuries. *Pediatr Rehabil*. (2003) 6:13–22. doi: 10.1080/1363849031000095279
117. Kim H, Yu T, Cam-Etoz B, Van Groen T, Hubbard WJ, Chaudry IH. Treatment of traumatic brain injury with 17α-ethinylestradiol-3-sulfate in a rat model. *J Neurosurg*. (2017) 127:23–31. doi: 10.3171/2016.7.JNS161263
118. Kim H, Cam-Etoz B, Zhai G, Hubbard WJ, Zinn KR, Chaudry IH. Salutary effects of estrogen sulfate for traumatic brain injury. *J Neurotrauma*. (2015) 32:1210–6. doi: 10.1089/neu.2014.3771
119. Xu Y, Yang X, Wang Z, Li M, Ning Y, Chen S, et al. Estrogen sulfotransferase (SULT1E1) regulates inflammatory response and lipid metabolism of human endothelial cells via PPARγ. *Mol Cell Endocrinol*. (2013) 369:140–9. doi: 10.1016/j.mce.2013.01.020
120. London SE, Itoh Y, Lance VA, Wise PM, Ekanayake PS, Oyama RK, et al. Neural expression and post-transcriptional dosage compensation of the steroid metabolic enzyme 17β-HSD type 4. *BMC Neurosci*. (2010) 11:47. doi: 10.1186/1471-2202-11-47
121. Tomaszycki ML, Peabody C, Replogle K, Clayton DF, Tempelman RJ, Wade J. Sexual differentiation of the zebra finch song system: potential roles for sex chromosome genes. *BMC Neurosci*. (2009) 10:24. doi: 10.1186/1471-2202-10-24
122. Peltoketo H, Isomaa V, Mäentausta O, Vihko R. Complete amino acid sequence of human placental 17 beta-hydroxysteroid dehydrogenase deduced from cDNA. *FEBS Lett*. (1988) 239:73–7. doi: 10.1016/0014-5793(88)80548-9
123. The VL, Labrie C, Zhao HF, Couët J, Lachance Y, Simard J, et al. Characterization of cdnas for human estradiol 17β-dehydrogenase and assignment of the gene to chromosome 17: evidence of two mrna species with distinct 5'-termini in human placenta. *Mol Endocrinol*. (1989) 3:1301–9. doi: 10.1210/mend-3-8-1301
124. Dumont M, Luu-The V, de Launoit Y, Labrie F. Expression of human 17 beta-hydroxysteroid dehydrogenase in mammalian cells. *J Steroid Biochem Mol Biol*. (1992) 41:605–8. doi: 10.1016/0960-0760(92)90391-U
125. Wu L, Einstein M, Geissler WM, Chan HK, Elliston KO, Andersson S. Expression cloning and characterization of human 17 beta-hydroxysteroid dehydrogenase type 2, a microsomal enzyme possessing 20 alpha-hydroxysteroid dehydrogenase activity. *J Biol Chem*. (1993) 268:12964–9.
126. Wang CL, Ying SJ, Wang ZY, Xing HJ, Wang LZ, He DY, et al. Molecular cloning and expression of 17β-hydroxysteroid dehydrogenase type 2 gene in Hu sheep. *Mol Biol Rep*. (2013) 40:1073–80. doi: 10.1007/s11033-012-2149-z
127. Lee JH, Gurney S, Pang D, Temkin A, Park N, Janicki SC, et al. Polymorphisms in HSD17B1: early onset and increased risk of Alzheimer's disease in women with down syndrome. *Curr Gerontol Geriatr Res*. (2012) 2012:361218. doi: 10.1155/2012/361218
128. Bae YH, Hwang JY, Kim YH, Koh JY. Anti-oxidative neuroprotection by estrogens in mouse cortical cultures. *J Korean Med Sci*. (2000) 15:327–36. doi: 10.3346/jkms.2000.15.3.327
129. Perez E, Liu R, Yang S-H, Cai ZY, Covey DF, Simpkins JW. Neuroprotective effects of an estratriene analog are estrogen receptor independent *in vitro* and *in vivo*. *Brain Res*. (2005) 1038:216–22. doi: 10.1016/j.brainres.2005.01.026
130. Regan RF, Guo Y. Estrogens attenuate neuronal injury due to hemoglobin, chemical hypoxia, and excitatory amino acids in murine cortical cultures. *Brain Res*. (1997) 764:133–40. doi: 10.1016/S0006-8993(97)00437-X
131. Blair IA. Analysis of estrogens in serum and plasma from postmenopausal women: past present, and future. *Steroids*. (2010) 75:297–306. doi: 10.1016/j.steroids.2010.01.012
132. Cirigliano M. Bioidentical hormone therapy: a review of the evidence. *J Women's Heal*. (2007) 16:600–31. doi: 10.1089/jwh.2006.0311
133. Stoffel-Wagner B, Watzka M, Steckelbroeck S, Schramm J, Bidlingmaier F, Klingmüller D. Expression of 17β-hydroxysteroid dehydrogenase types 1, 2, 3 and 4 in the human temporal lobe. *J Endocrinol*. (1999) 160:119–26. doi: 10.1677/joe.0.1600119
134. Tsutsui KSBA. Steroidogenesis in the avian brain. In: Dawson ACCM, editor. *Avian Endocrinology* (New Delhi: Narosa Publishing House), 59–77.
135. Mindnich R, Möller G, Adamski J. The role of 17 beta-hydroxysteroid dehydrogenases. *Mol Cell Endocrinol*. (2004) 218:7–20. doi: 10.1016/j.mce.2003.12.006
136. Stein DG. Progesterone exerts neuroprotective effects after brain injury. *Brain Res Rev*. (2008) 57:386–97. doi: 10.1016/j.brainresrev.2007.06.012
137. Stein DG, Wright DW, Kellermann AL. Does progesterone have neuroprotective properties? *Ann Emerg Med*. (2008) 51:164–72. doi: 10.1016/j.annemergmed.2007.05.001
138. Stein DG. Embracing failure: what the phase III progesterone studies can teach about TBI clinical trials. *Brain Inj*. (2015) 29:1259–72. doi: 10.3109/02699052.2015.1065344
139. Engler-Chiurazzi EB, Covey DF, Simpkins JW. A novel mechanism of non-feminizing estrogens in neuroprotection. *Exp Gerontol*. (2017) 94:99–102. doi: 10.1016/j.exger.2016.10.013
140. Engler-Chiurazzi EB, Brown CM, Povroznik JM, Simpkins JW. Estrogens as neuroprotectants: estrogenic actions in the context of cognitive aging and brain injury. *Prog Neurobiol*. (2016) 157:188–211. doi: 10.1016/j.pneurobio.2015.12.008

Conflict of Interest: 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

SYSTEMATIC REVIEW
published: 02 June 2020
doi: 10.3389/fendo.2020.00311

Higher Circulating Cortisol in the Follicular vs. Luteal Phase of the Menstrual Cycle: A Meta-Analysis

Ajna Hamidovic[1]*, *Kristina Karapetyan*[1], *Fadila Serdarevic*[2], *So Hee Choi*[1], *Tory Eisenlohr-Moul*[3] *and Graziano Pinna*[4]

[1] Department of Pharmacy Practice, College of Pharmacy, University of Illinois at Chicago, Chicago, IL, United States, [2] Department of Epidemiology, Erasmus Medical Centre Rotterdam, Rotterdam, Netherlands, [3] Department of Psychiatry, College of Medicine, University of Illinois at Chicago, Chicago, IL, United States, [4] The Psychiatric Institute, Department of Psychiatry, College of Medicine, University of Illinois at Chicago, Chicago, IL, United States

Although results of animal research show that interactions between stress and sex hormones are implicated in the development of affective disorders in women, translation of these findings to patients has been scarce. As a basic step toward advancing this field of research, we analyzed findings of studies which reported circulating cortisol levels in healthy women in the follicular *vs.* luteal phase of the menstrual cycle. We deemed this analysis critical not only to advance our understanding of basic physiology, but also as an important contrast to the findings of future studies evaluating stress and sex hormones in women with affective disorders. We hypothesized that cortisol levels would be lower in the follicular phase based on the proposition that changes in levels of potent GABAergic neurosteroids, including allopregnanolone, during the menstrual cycle dynamically change in the opposite direction relative to cortisol levels. Implementing strict inclusion criteria, we compiled results of high-quality studies involving 778 study participants to derive a standardized mean difference between circulating cortisol levels in the follicular vs. luteal phase of the menstrual cycle. In line with our hypothesis, our meta-analysis found that women in the follicular phase had higher cortisol levels than women in the luteal phase, with an overall Hedges' g of 0.13 ($p < 0.01$) for the random effects model. No significant between-study difference was detected, with the level of heterogeneity in the small range. Furthermore, there was no evidence of publication bias. As cortisol regulation is a delicate process, we review some of the basic mechanisms by which progesterone, its potent metabolites, and estradiol regulate cortisol output and circulation to contribute to the net effect of higher cortisol in the follicular phase.

Keywords: cortisol, hypothalamic-pituitary-gonadal (HPG) axis, hypothalamic-pituitary-adrenal (HPA) axis, menstrual cycle, follicular, luteal

OPEN ACCESS

Edited by:
Deborah Suchecki, Federal University of São Paulo, Brazil

Reviewed by:
William R. Lovallo, Independent Researcher, Oklahoma City, United States
Richard G. Hunter, University of Massachusetts Boston, United States

***Correspondence:**
Ajna Hamidovic
ahamidov@uic.edu

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

Received: *10 February 2020*
Accepted: *24 April 2020*
Published: *02 June 2020*

Citation:
Hamidovic A, Karapetyan K, Serdarevic F, Choi SH, Eisenlohr-Moul T and Pinna G (2020) Higher Circulating Cortisol in the Follicular vs. Luteal Phase of the Menstrual Cycle: A Meta-Analysis. Front. Endocrinol. 11:311. doi: 10.3389/fendo.2020.00311

INTRODUCTION

Women exhibit high prevalence of stress-related disorders, such as major depressive disorder (MDD) and anxiety spectrum disorder (1–9). Importantly, the increase in prevalence of these disorders is observed during periods of drastic hormonal changes, such as puberty, the pre-menstrual period, pregnancy, postpartum and menopause (10–12). These observations suggest that interactions between sex hormones, regulated by the hypothalamic-pituitary-gonadal (HPG)

axis, and cortisol, a stress hormone under the control of the hypothalamic-pituitary-adrenal (HPA) axis, may be critical determinants of stress-related disorder development and progression.

Research evaluating stress effects in MDD and anxiety disorders demonstrates a blunted cortisol response to psychosocial stress in female patients compared to their respective controls [for a meta-analysis, see (13)]. However, although these research studies provide valuable information, they only examine function of the HPA axis, without evaluating how sex hormones influence it.

The number of studies evaluating interactions between the provoked HPA and the HPG axes is limited in both diseased as well as healthy participants. Results of studies comparing reactivity to psychosocial stress in healthy women suggest that cortisol output is higher in the luteal *vs.* follicular phase of the menstrual cycle (14, 15). However, their small sample size and opposite findings from other studies (16–19) indicate that more research needs to be completed before a conclusion can be drawn. Additional studies, implementing strict verification of menstrual cycle phase, stress manipulation and participants' healthy status, are needed.

An even more fundamental question, though, is related to the physiological relationship between the HPA and HPG axes under unprovoked, tonic conditions. However, results from human laboratory and observational studies in healthy volunteers evaluating basal cortisol levels across the menstrual cycle range broadly. Thus, we focused on the function of the HPA axis with the hypothesis that there would be a higher physiological output of cortisol during the follicular compared to the luteal phase of the menstrual cycle. Our hypothesis was based on the finding that the progesterone metabolite allopregnanolone positively modulates gamma-aminobutyric acid $(GABA)_A$ receptors via an allosteric binding site to potentiate inhibitory signaling (20) and enhance the negative feedback on the HPA axis (21, 22). Therefore, during the luteal phase, when allopregnanolone levels are high, cortisol levels would be expected to decrease relative to the follicular phase, when allopregnanolone levels are low.

Tonic levels of cortisol across the menstrual cycle have been reported as higher for example, (23, 24) or unchanged (25, 26) in the follicular vs. luteal phase. These discrepancies are rooted in marked methodological differences across studies. Hence, our analysis only included high-quality research studies implementing strict criteria and phase identification. Based on mechanistic considerations of basic research studies (reviewed in the Discussion section) that have reported the effects of neuroactive steroids on the HPA axis function, we predicted a surge in cortisol during the early/mid-follicular phase. Our meta-analysis, indeed, shows that circulating cortisol levels change dynamically as a function of menstrual cycle phase, suggesting cortisol is specifically required during the early/mid follicular phase to mediate adaptive physiological processes in response to environmental stimuli, when both estradiol and progesterone are low.

METHODS

Search Strategy

We conducted a literature search in PubMed, Web of Knowledge and PsychInfo, and included eligible studies published through December 5th, 2019. Two authors (AH and KK) completed their search independently according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (27). Any discrepancies were reconciled by reviewing the literature jointly for specific points of difference. We used the following search string: [("Cortisol") AND ("Menstrual" OR "Luteal" OR "Follicular") for (DOCUMENT TYPE: (Article); LANGUAGE: English; SUBJECTS: Human)]. We compiled the results in EndNote X8.

Inclusion/Exclusion Criteria

This meta-analysis evaluated tonic peripheral cortisol levels of healthy menstruating female study participants across follicular vs. luteal phases of the menstrual cycle. Studies were considered eligible if a baseline value was provided prior to a laboratory intervention (for example, psychosocial stress procedure or exercise), if samples were collected longitudinally in a naturalistic (or a laboratory) setting across the menstrual cycle, if an experimental design evaluating a disease state included a healthy control or if an intervention included a placebo control. The exclusionary criteria implementation was carried out in a two-step approach.

In the first step, study abstracts ($N = 2{,}225$) were excluded if they were: (1) abstracts, review papers or case studies, (2) animal studies, or evaluation of cell lines, (3) male-only evaluations, (4) abstracts which only mentioned one menstrual phase (luteal or follicular) as a means of controlling for menstrual cycle phase (i.e., not as a comparison of the two phases), (5) studies evaluating a diseased population (including smokers or other substance use disorder population) or implementing a menstrual phase-specific intervention (without a placebo control). This category also included abstracts describing pregnant as well as women in the peri- or post-menstrual phases, as well as women who were on oral contraceptives. Finally, abstracts describing athletes or women who experienced early life trauma were also coded in this category. The remaining abstracts were excluded if they: (6) did not mention cortisol (blood, salivary or urinary), (7) were overlapping study participants with an already published study, and (8) if they described a procedure (such as IV fertilization, for example) which could cause changes in circulating cortisol due to anticipation.

In the second round of exclusion criteria implementation, we evaluated full articles. We excluded papers which did not evaluate groups according to menstrual cycle phases, measure cortisol, have a healthy, non-athletic control group, or report mean and/or variance and were published more than 25 years ago. Furthermore, we excluded papers which re-administered stress (as this can distort baseline cortisol levels), performed non-linear cortisol modeling, were non-original, written in languages other than English, or had overlapping participants. Given the rapid decline of cortisol in the morning, studies which collected

cortisol during morning times that varied ≥2 h or did not mention what time of the day cortisol sample collection -took place were excluded, as well as studies which determined cycle phase using self-report.

Data Extraction

Information on the following variables was collected: (1) age, (2) BMI, (3) day of follicular and luteal phase of cortisol collection, (4) phase estimation method, (5) time of day of cortisol collection, and (6) physiological source of cortisol level. Regarding the length of phase variable, whereas some studies reported a range of days, others reported multiple, exact days of the cycle on which cortisol was reported. In the event that a study reported cortisol values across multiple days of the menstrual cycle, data from the day closest to the beginning of the cycle (day 1) for follicular phase, and day 21 of the luteal phase were extracted to reflect the greatest contrast of estradiol and progesterone levels across the cycle. In the event that cortisol was reported on multiple "sub-phases" (for example, early-follicular, mid-follicular), again, data from the day closest to the beginning of the cycle (day 1) for follicular phase, and day 21 of the luteal phase were extracted. If cortisol was collected across multiple menstrual cycles (for example, two menstrual cycles), values from the last cycle were reported. In the event that studies reported cortisol values at multiple times of the day (for example, morning and evening), or multiple sources (for example, salivary

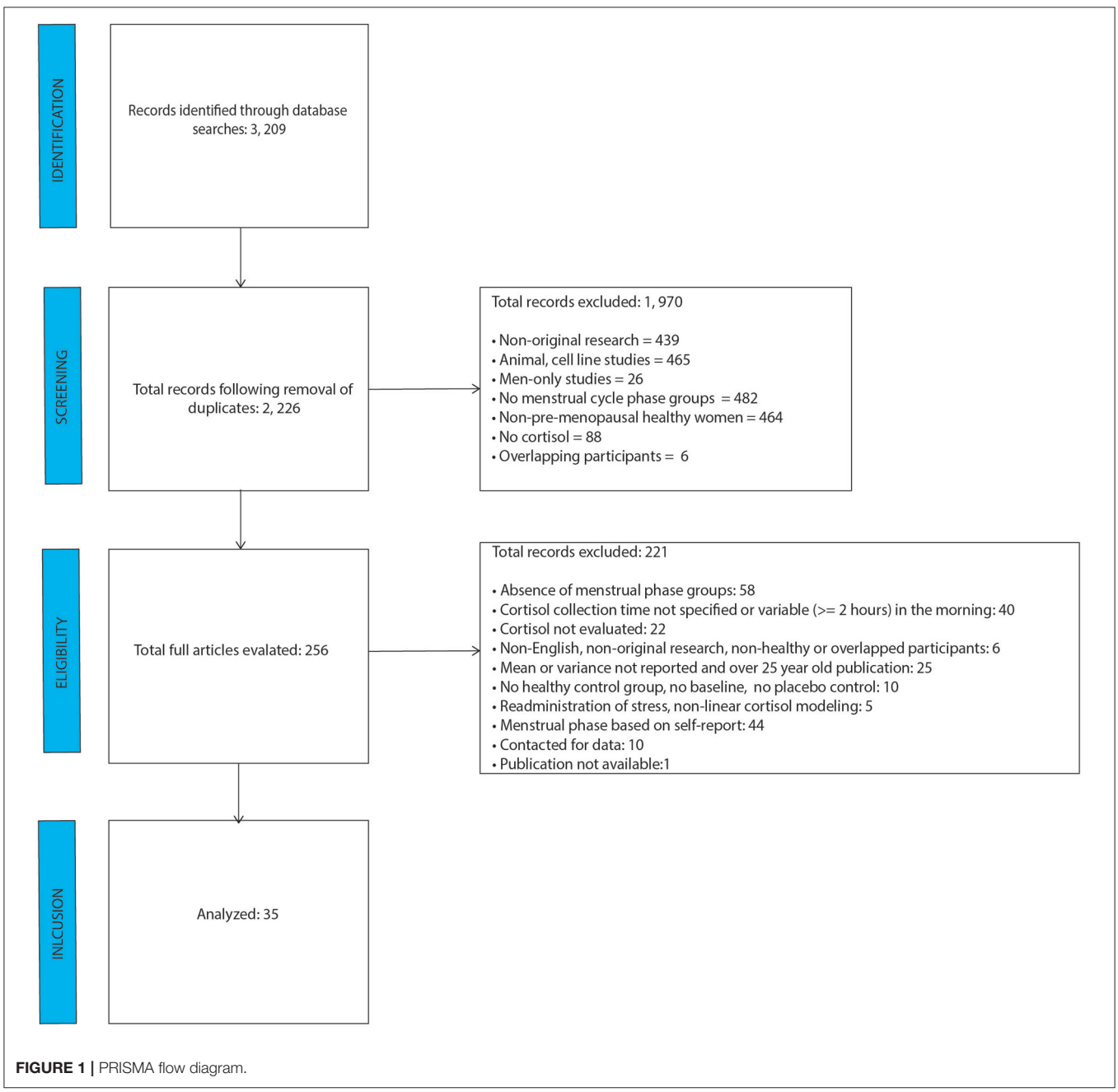

FIGURE 1 | PRISMA flow diagram.

and plasma), the most frequently reported time (morning) and source (blood) in the remaining studies were used to extract cortisol values.

Data Analysis

Analyses of were carried out by first calculating the Cohen's *d* effect size (28). In the event mean and variance values were provided for sub-groups of women in follicular and luteal phases, those were combined using the following formulas:

$$M = \frac{N_1 M_1 + N_2 M_2}{N_1 + N_2}$$

$$SD = \sqrt{\frac{(N_1 - 1)\, SD_1^2 + (N_2 - 1)\, SD_2^2 + \frac{N_1 N_2}{N_1 + N_2}\left(M_1^2 + M_2^2 - 2M_1 M_2\right)}{N_1 + N_2 - 1}}$$

where, N_1 = sample size group 1, N_2 = sample size group 2, M_1 = mean group 1, M_2 = mean group 2, SD_1 = standard deviation group 1, SD_2 = standard deviation group 2.

We divided the mean difference between the two groups by the pooled standard deviation. Next, we used the J-correction factor to obtain the Hedges' *g* effect size, which corrects for small samples (29) and is considered small, medium, and large for values 0.2, 0.5, and 0.8, respectively. Within- and between-subject study designs were combined as described in Morris et al. (30). We used a random effects model to calculate the pooled effect size with an associated 95% CI and a *p*-value (31). We assessed source-study heterogeneity using the χ^2-based Q test with its associated *p*-value. A statistically significant Q statistic suggests different effect sizes across studies, implying that methodological or population sample differences may be introducing variance across individual studies. We quantified heterogeneity using I^2 with values 25, 50, and 75% suggestive of small, medium and large heterogeneity and calculated potential publication bias using the Classical Tests (32). We completed sub-analyses according to source (saliva vs. plasma) and time of day (morning vs. afternoon). The meta-analysis was performed using the "escalc" function, and publication bias was assessed using the "ranktest" function in "metafor" package (33) in R.

RESULTS

Characteristics of Individual Studies

After removal of duplicate studies, literature search identified 2, 226 individual abstracts as shown in the PRISMA figure (**Figure 1**). Those abstracts were screened for relevance and coded for exclusion reasons. The greatest number of abstracts excluded was based on absence of menstrual cycle groups, followed by cell line/animal research studies. A total of 256 full-text articles were reviewed for relevance, of which 221 were excluded. Of the 221 studies, 44 were excluded based on the self-report nature of menstrual cycle phase determination, and 40 were excluded because they either didn't mention cortisol collection time, or the morning sample was collected at times

TABLE 1 | Participant information from individual studies.

References	Sample size (*N*)*		Age [mean (SD)]	BMI [mean (SD)]
	Follicular Phase	Luteal Phase		
Andreano et al. (34)	20	24	___	___
Barbarino et al. (35)	5	6	___	___
Beck et al. (36)	20		___	___
Hoeger Bement et al. (37)	20		20.9 (1.0)	23.0
Bricout et al. (38)	11		25.5 (7.6)	19.9
Cannon et al. (39)	7	8	___	___
Carr et al. (40)	4		___	___
Caufriez et al. (41)	10		30.0	21.8 (0.9)
Childs et al. (42)	29	23	21.9 (0.8)	22.3 (0.3)
Collins et al. (43)	15		29.5	___
Espin et al. (14)	30	30	19.3 (1.7)	21.7 (4.1)
Genazzani et al. (44)	5		___	___
Heitkemper et al. (45)	25		33.1 (5.3)	23.6 (4.9)
Huang et al. (46)	18	18	22.0 (2.4)	20.0 (2.8)
Inoue et al. (26)	9		23.7 (5.6)	___
Judd et al. (47)	6	6	___	___
Kasa-Vubu et al. (48)	10	14	29.4 (8.5)	24.0 (4.3)
Kerdelhué et al. (23)	11		___	___
Kirschbaum et al. (17)	19	21	23.4 (3.3)	21.7 (2.4)
LeRoux et al. (49)	9	9	21.8 (2.3)	22.5 (2.4)
Liu et al. (50)	6		___	___
Lombardi et al. (51)	20		26.2	___
Maki et al. (52)	20	20	27.0 (5.6)	25.0 (5.0)
Ohara et al. (53)	7		22.3 (1.0)	20.5 (2.1)
Paoletti et al. (54)	14		31.5 (2.7)	24.2 (2.0)
Parry et al. (55)	30		37.2 (5.8)	___
Rasgon et al. (56)	5		27.0 (4.0)	___
Reynolds et al. (57)	61		21.7 (3.4)	___
Roche and King (58)	23	23	24.2 (3.9)	23.6 (3.8)
Stewart et al. (59)	4		24.6 (4.5)	24.7 (2.1)
Su et al. (60)	10		30.8 (4.9)	___
Timon et al. (25)	20		___	21.3 (2.1)
Tulenheimo et al. (61)	14		___	___
Villada et al. (62)	13	17	19.0 (1.5)	21.3 (4.0)
Wolfram et al. (63)	29		26.3 (3.9)	22.1 (2.9)

**Only one sample size (for follicular and luteal phases) is listed for within subject design studies. The total sample size is 778.*

TABLE 2 | Menstrual cycle and outcome measure information from individual studies.

References	Menstrual cycle			Cortisol	
	Follicular phase	Luteal phase	Phase estimation	Time	Source
Andreano et al. (34)	1–7	18–24	Estradiol and progesterone	Afternoon	Saliva
Barbarino et al. (35)	4–8	20–24	Estradiol and progesterone	Morning	Plasma
Beck et al. (36)	10	24	LH surge	Morning	Plasma
Hoeger Bement et al. (37)	"Mid-follicular"	"Mid-luteal"	LH surge	Afternoon	Saliva
Bricout et al. (38)	"Mid-follicular"	"Mid-luteal"	Estradiol and progesterone	24-h	Urine
Cannon et al. (39)	1–14	15–28	Progesterone	24-h	Urine
Carr et al. (40)	1	21	LH surge	Morning	Plasma
Caufriez et al. (41)	3–8	23–28	Basal body temperature	24-h	Urine
Childs et al. (42)	3–10	16–24	LH ovulation test	Morning	Plasma
Collins et al. (43)	5–7	22–25	Basal body temperature	Morning	Plasma
Espin et al. (14)	5–8	20–24	Basal body temperature	Afternoon	Saliva
Genazzani et al. (44)	1	21	LH surge	Morning	Plasma
Heitkemper et al. (45)	1	22	LH ovulation test	Morning	Urine
Huang et al. (46)	1–4	24–28	Estradiol and progesterone	Afternoon	Saliva
Inoue et al. (26)	1–14	21–28	Estradiol and progesterone	Morning	Plasma
Judd et al. (47)	3–5	20–24	LH ovulation test	10-h	Serum
Kasa-Vubu et al. (48)	1–14	15–28	LH and progesterone	24-h	Plasma
Kerdelhué et al. (23)	1	21	LH surge	Morning	Serum
Kirschbaum et al. (17)	4–7	21–25	Estradiol and progesterone	Afternoon	Plasma
LeRoux et al. (49)	8–10	20–22	Estradiol and progesterone	Morning	Saliva
Liu et al. (50)	1–5	20–22	Pelvic Ultrasound	Morning	Plasma
Lombardi et al. (51)	5–7	22–26	LH surge and progesterone	Morning	Serum
Maki et al. (52)	2–4	22–24	LH ovulation test	Afternoon	Saliva
Ohara et al. (53)	1–14	15–28	LH ovulation test	Morning	Saliva
Paoletti et al. (54)	5–8	21–24	Basal body temperature	Morning	Serum
Parry et al. (55)	6–8	26–28	LH ovulation test	Morning	Plasma
Rasgon et al. (56)	2–9	7–14	LH ovulation test	Morning	Plasma
Reynolds et al. (57)	7–10	20–23	LH ovulation test	Afternoon	Saliva
Roche and King (58)	1–14	15to 28	Estradiol and progesterone	Morning	Plasma
Stewart et al. (59)	7	21	Progesterone	12-h	Plasma
Su et al. (60)	3–7 days after the end of menses	21	Progesterone	Morning	Plasma
Timon et al. (25)	1–2	21–22	Basal body temperature	Morning	Urine
Tulenheimo et al. (61)	6–9	21–24	Progesterone	Morning	Plasma
Villada et al. (62)	5–8	20–24	Basal body temperature	Afternoon	Saliva
Wolfram et al. (63)	2–6	21–24	LH ovulation test	CAR	Saliva

CAR, Cortisol Awakening Response.

which varied by two or more hours. The analysis included data from 35 final studies.

As shown in **Table 1**, most of the studies included participants in their 20s, with a BMI below 25. Whereas, some studies incorporated fine-grained sub-phases of follicular and luteal phases (see **Table 2**), others defined the phases as day 1–14 and 15–28. Phase estimation was determined via progesterone level acquisition, LH surge measurement, basal body temperature measurement, or pelvic ultrasound. Times of cortisol collection were in the morning, afternoon/evening or over several hours, and the source was saliva, plasma or urine. Whereas, **Supplementary Table 1** shows all the days/phases of menstrual cycle, time of collection and sources of cortisol across all the studies, **Table 2** only shows the actual day (or range of days), time and source of cortisol which were included in the analysis. These two tables are provided in order to increase transparency of reporting, as several studies reported several values, for which we implemented the rules as specified in the Methods Section Data Extraction.

Evaluation of Standardized Mean Difference in Cortisol Levels Across Menstrual Cycle

Women in the follicular phase had higher cortisol levels than women in the luteal phase, with an overall Hedges' *g* of

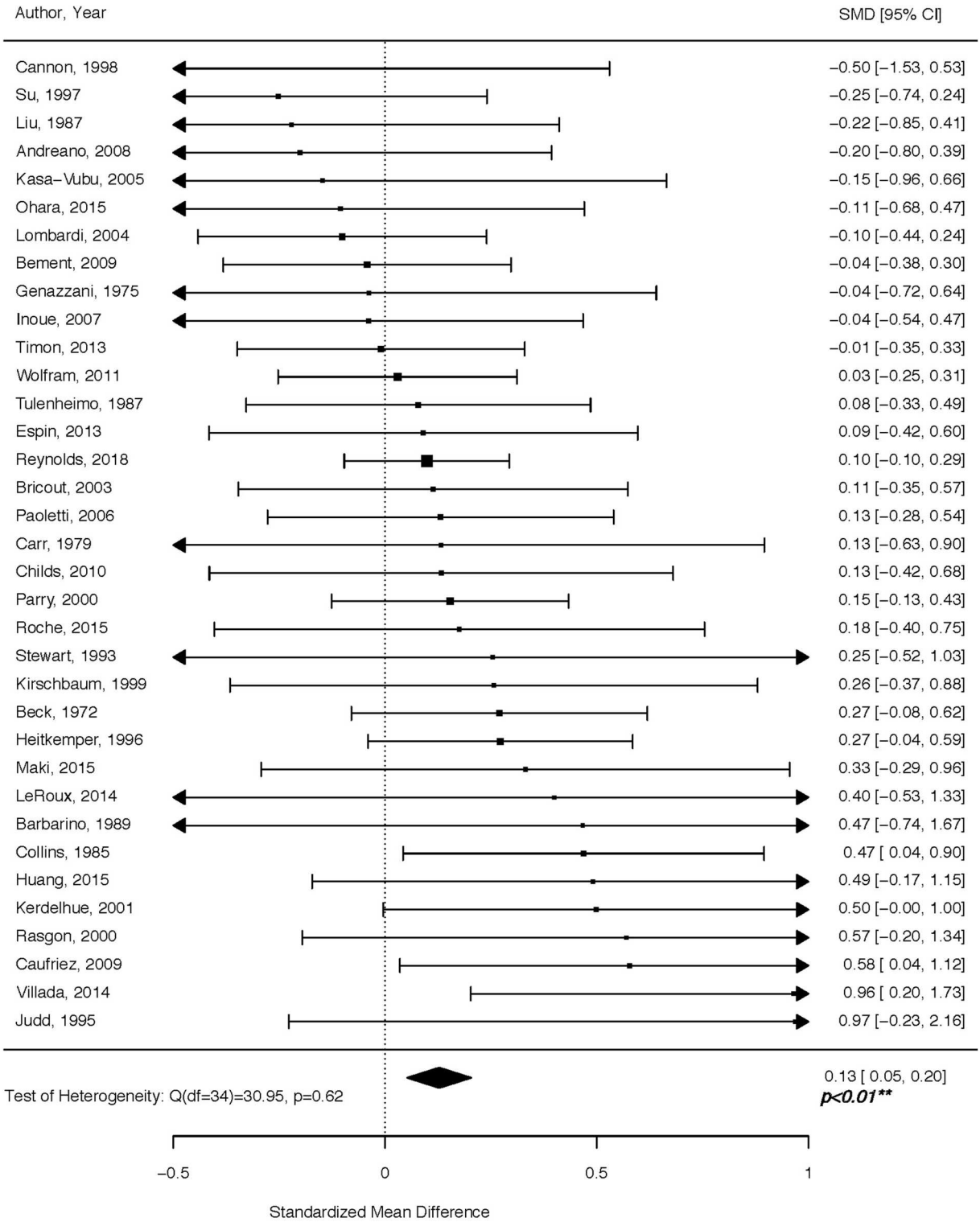

FIGURE 2 | Forest plot of cortisol levels across the menstrual cycle. Positive standardized mean difference (SMD) means that cortisol levels were higher in the follicular vs. luteal phase.

0.13 ($p < 0.01$) for the random effects model (**Figure 2**). The confidence interval range was between 0.05 and 0.20. No significant between-study difference was detected (tau^2 =0; H = 1.0), with the level of heterogeneity in the small range (I^2 = 0%, Q = 30.69; p = 0.63). The Rank Correlation Test for Funnel Plot Asymmetry resulted in

TABLE 3 | Results of sub-analyses according to the biospecimen source and time of day.

Factor	SMD						Q		
	Value	SE	Z value	*p*-value	CI.LB	CI.UB	df	Value	*p*-value
Source									
Plasma	0.12	0.0583	2.0907	0.0366	0.0076	0.2361	18	13.2214	0.7783
Saliva	0.1179	0.0615	1.9182	0.0551	−0.0026	0.2384	8	7.872	0.446
Time of day									
Morning	0.1363	0.0485	2.8088	0.005	0.0412	0.2314	21	16.6924	0.7296
Afternoon	0.0966	0.0716	1.348	0.1777	−0.0438	0.237	7	9.1638	0.2411

Kendall's tau = 0.14 ($p = 0.23$), indicating absence of small study effects.

Sub-analysis of Cortisol Levels According to Source and Time of Day

Sub-analysis according to the biospecimen source showed a significant effect of plasma ($p = 0.036$) and marginally significant effect of saliva ($p = 0.055$). The time of day sub-analysis showed a significant effect of morning ($p = 0.005$), but not afternoon ($p = 0.177$). **Table 3** displays all relevant sub-analyses statistics, including standardized mean difference, standard error, z and *p*-values, confidence intervals, degrees of freedom, Q statistic and its associated *p*-value.

DISCUSSION

For decades, literature on cortisol has yielded mixed results with respect to its concentration in the follicular vs. luteal phase of the menstrual cycle. Implementing a comprehensive search of high-quality studies spanning a period of almost 50 years of research, we show that circulating cortisol levels are higher in the follicular vs. luteal phase. Cortisol regulation is a delicate process of extensive physiological processes working in concert to adjust responses to environmental stimuli. Below, we review mechanisms driving circulating cortisol levels to both increase and decrease across various menstrual cycle time-points, while noting that the net effect of these, or other, still unidentified processes, is a higher circulating cortisol during the follicular compared to the luteal phase, as reported in our analysis (**Figure 2**).

The paraventricular nucleus (PVN) of the hypothalamus integrates numerous circadian and environmental inputs to funnel information through neurons expressing corticotropin-releasing hormone (CRH). The release of CRH into the hypophyseal portal vasostructure enhances the synthesis and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which, in turn, stimulates adrenal glands to synthesize and release cortisol to adapt metabolic processes and behavioral responses.

Intriguingly, PVN neurons express high levels of estrogen receptor beta (ER-β) and low levels of estrogen receptor alpha (ER-α) (64–66). Several research studies have demonstrated that estradiol, through its near equivalent affinity for the two estrogen receptor subtypes, can selectively decrease or increase HPA axis function. A high number of ER-β-expressing cells in the PVN are oxytocin and vasopressin immunoreactive (67–69), which complement the CRH neurons that also express ER-β (64). Stimulation of ER-β in the PVN results in a reduction of cortisol levels. In accord, both centrally- and peripherally-delivered ER-β selective agonists inhibit the HPA function (70). ER-α occupancy, on the other hand, has an indirect, trans-synaptic activation in the PVN. The peri-PVN region contains ER-α neurons, and their activation can impair glucocorticoid-mediated negative feedback regulation of the HPA axis (71). These opposing actions of estradiol—with ER-α amplifying, and ER-β reducing HPA function—are in agreement with laboratory observations that estradiol both enhances (72, 73) and inhibits (74, 75) HPA function. Hence, in the luteal phase, when estradiol levels are higher compared to the early/mid follicular phase, theoretically, depending on the extent of ER-β or ER-α expression and activation in or near the PVN, estradiol can either decrease or increase circulating cortisol levels.

The activity of CRH neurons in the PVN is tightly regulated by inhibitory GABAergic interneuron populations (76). Allopregnanolone, a progesterone derivative resulting from conversion by 5α-reductase type I and 3α-hydroxysteroid dehydrogenase, is an endogenous neurosteroid and a potent, positive, allosteric modulator of the action of the inhibitory neurotransmitter GABA at $GABA_A$ receptor. Studies in rodents show an inhibitory effect of allopregnanolone on the function of the HPA axis (77–79). This effect of allopregnanolone seems to be exerted through its action at $GABA_A$ receptors, and subsequent inhibition on PVN neurons (80) under both basal and stressful conditions (81). In support of these findings, in addition to allopregnanolone, another potent $GABA_A$ receptor modulator and deoxycorticosterone-derived steroid, tetrahydrodeoxycorticosterone (TH-DOC), also attenuates the HPA axis function (77, 82, 83). Therefore, in the luteal phase, under the physiological milieu of higher circulating progesterone levels and, most importantly, of its potent $GABA_A$ receptor-modulating metabolite, allopregnanolone, lower circulating cortisol levels can be expected relative to the follicular phase. Not surprisingly, our meta-analysis has confirmed this expectation (**Figure 3**).

Once released in the blood flow, ∼80% of circulatory cortisol is bound to corticosteroid-binding globulin (CBG), leaving ∼5% of cortisol in the free form (84). CBG is primarily

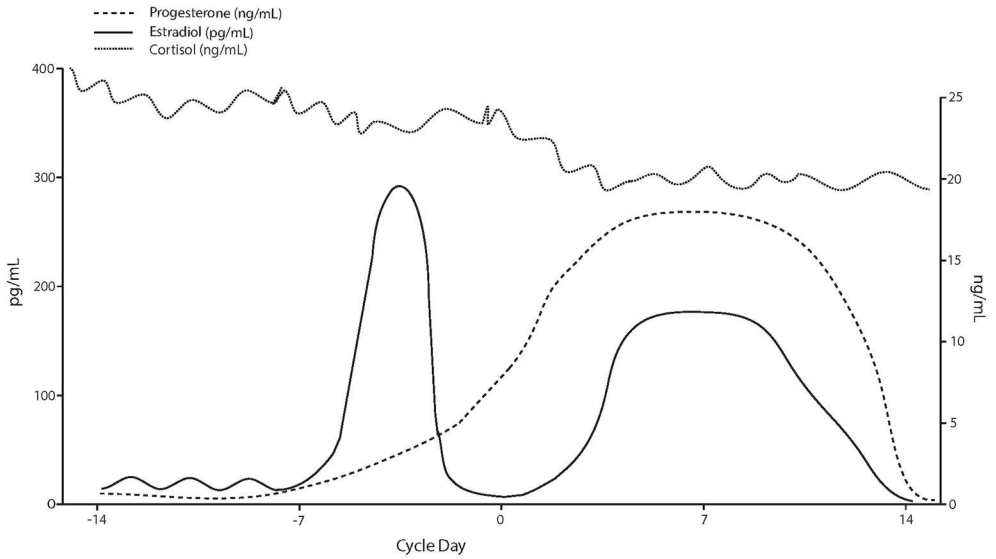

FIGURE 3 | Hypothetical cortisol levels across the menstrual cycle based on values reported in the analyzed studies.

synthesized by the liver and secreted into the bloodstream, where it binds and provides a pool of circulating cortisol. It enhances the availability of cortisol to be released on demand both systemically and at a tissue level. Whereas plasma cortisol reflects total cortisol (i.e., bound and unbound), the salivary levels only reflect unbound/free cortisol. Changes in circulating CBG have a significant impact on total, but not free, cortisol concentrations (85, 86). The CBG is under a tight regulation by estradiol, with women expressing greater CBG basal concentrations than men (87). Interestingly, ethinyl estradiol, found in oral contraceptives (OC), dose-dependently increases CBG serum levels (88). Ethynyl estradiol is also a potent ER modulator, with 194 and 151% of the affinity of estradiol for the ER-α and ER-β, respectively (89). It is, however, unclear whether changes in estradiol across the menstrual cycles can alter CBG. Nenke et al. (90) report that total CBG concentration reaches ∼1,000 nmol/L in pregnancy as well as during the active phase of pill cycle containing ethinyl estradiol. These expression levels are substantially higher than the CBG levels of ∼500 nmol/L determined in non-pregnant, non-OC taking individuals (90). Unfortunately, this study did not account for menstrual cycle phase, which would have provided valuable information regarding CBG changes, if any, across the menstrual cycle. The question of potential CBG changes across the menstrual cycle due to changes in estradiol concentrations should be evaluated in future studies by carefully examining the potential anti-estrogenic and blunting effect of luteal progesterone on estradiol-induced increase in CBG (91).

There are several limitations to consider in the present meta-analysis. First, our sub-analyses may have been underpowered to detect effects of time variations. For example, the "time of day" sub-analysis showed a significant effect of morning, but not afternoon menstrual phase cortisol (**Table 3**). However, degrees of freedom for morning vs. afternoon sub-analyses were 21 and 7, respectively, with the afternoon sample possibly underpowered to detect a significant effect. Importantly, our sub-analysis of "source" (plasma vs. serum) was consistent with our main study results, showing higher follicular cortisol in both the free and total form. Furthermore, given that the overall sample was fairly homogenous, with most women in the same age range (20–29 years old) and a normal BMI (**Table 1**), we were unable to perform a meta-regression, which would have provided meaningful information related to the direction of future mechanistic studies.

As the research evaluating interacting effects between HPA and HPG axes unfolds, there are several issues to consider. Perhaps the greatest pitfall of menstrual cycle research is the inadequate assessment of the menstrual cycle phase. In the full-article evaluation step of our meta-analysis, we excluded findings from 44 studies because menstrual cycle phase was estimated based on self-report. Retrospective reports of menstrual cycle "start" and "duration" are plagued by profound phase misinformation (92) and prospective measures confirming both ovulation and luteal phase status are essential. Menstrual cycle phase determination was also based on self-report in approximately half of the studies evaluating the HPA reactivity in the follicular vs. luteal phase (16, 18, 19), contributing to the inability to make meaningful conclusions regarding the direction of effect.

HPA axis dysfunction is strongly implicated in the etiopathology of affective disorders (93–95) with women at an increased risk (96, 97). Yet, basic questions related to the function of the HPA axis throughout stages of the menstrual cycle under acute or prolonged stress conditions remain largely unanswered. Whereas, our meta-analysis reflects a single time point in the cortisol diurnal cycle, it is still unknown whether there are phase-specific effects

on the shape of the diurnal curve. This assessment could be easily implemented given the availability of the salivary (unbound) cortisol assay, and would provide a comprehensive picture of daily cortisol trajectory and its potential mean, amplitude and/or phase shift as the hormonal milieu changes across the menstrual cycle. In this case, the awakening and morning cortisol should be taken more frequently than the late afternoon/evening samples.

It is strongly recommended that future studies employ a fine-grained approach (i.e., evaluating early/mid follicular, ovulatory, early, mid and late luteal phases) to advance women's mental health research, rather than broadly defining phases as "follicular" vs. "luteal."

In summary, the aim of our meta-analysis was to summarize findings of a period spanning over 50 years of research and test whether circulating cortisol levels change as a function of menstrual phase. With respect to this objective, we showed higher cortisol levels in the follicular *vs.* luteal phase of the menstrual cycle. By completing this aim, our hope is to simultaneously increase awareness of the poor state of women's neuroendocrine science research. Experimental protocols designed to study effects that influence the HPA and HGA axis function need to be specifically designed to account for women's physiological requirements. The joint protocols previously designed for both sexes rarely apply to studies involving women. Whereas, participant recruitment, evaluation and analysis are more rapid in many research scenarios involving men, given the dynamic nature of the menstrual cycle and the need for prospective data collection, the same parameters should not be applied to research involving women. Resources and completion expectations need to be adjusted as such. Once implemented, these changes will contribute meaningful information to progress our understanding of rhythmic hormonal changes, which are crucial for understanding the now well-established sex difference in affective disorder development and progression.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AH, KK, SC, and TE-M conceptualized the question, completed literature search and data gathering. AH and FS completed statistical analysis. AH and GP wrote the manuscript.

FUNDING

Research reported in this publication was supported by the National Institute of Mental Health of the National Institutes of Health under Award Number R21MH119642.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Ann Caufriez (Laboratoire de Physiologie et Pharmacologie, Université libre de Bruxelles, Brussels, Belgium) for sending a complete dataset and Dr. Scott Morris (Department of Psychology Illinois Institute of Technology. Chicago, Illinois, USA) for statistical consultation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00311/full#supplementary-material

REFERENCES

1. Albert PR. Why is depression more prevalent in women? *J Psychiatry Neurosci*. (2015) 40:219–21. doi: 10.1503/jpn.150205
2. Angst J, Gamma A, Gastpar M, Lepine JP, Mendlewicz J, Tylee A, et al. Gender differences in depression. Epidemiological findings from the European DEPRES I and II studies. *Eur Arch Psychiatry Clin Neurosci*. (2002) 252:201–9. doi: 10.1007/s00406-002-0381-6
3. Barnes VA, Treiber FA, Ludwig DA. African-American adolescents' stress responses after the 9/11/01 terrorist attacks. *J Adolesc Health*. (2005) 36:201–7. doi: 10.1016/j.jadohealth.2004.02.035
4. Breslau N, Schultz L, Peterson E. Sex differences in depression: a role for preexisting anxiety. *Psychiatr Res*. (1995) 58:1–12. doi: 10.1016/0165-1781(95)02765-O
5. Cahill L. Why sex matters for neuroscience. *Nat Rev Neurosci*. (2006) 7:477–84. doi: 10.1038/nrn1909
6. Kessler RC, McGonagle KA, Nelson CB, Hughes M, Swartz M, Blazer DG. Sex and depression in the National Comorbidity Survey. II: Cohort effects. *J Affect Disord*. (1994) 30:15–26. doi: 10.1016/0165-0327(94)90147-3
7. McLean CP, Asnaani A, Litz BT, Hofmann SG. Gender differences in anxiety disorders: prevalence, course of illness, comorbidity and burden of illness. *J Psychiatr Res*. (2011) 45:1027–35. doi: 10.1016/j.jpsychires.2011.03.006
8. Olff M. Sex and gender differences in post-traumatic stress disorder: an update. *Eur J Psychotraumatol*. (2017) 8:1351204. doi: 10.1080/20008198.2017.1351204
9. Seeman MV. Psychopathology in women and men: focus on female hormones. *Am J Psychiatry*. (1997) 154:1641–7. doi: 10.1176/ajp.154.12.1641
10. Beddig T, Kuhner C. Current aspects of premenstrual dysphoric disorder - a review. *Psychother Psychosom Med Psychol*. (2017) 67:504–13. doi: 10.1055/s-0043-113816
11. Biaggi A, Conroy S, Pawlby S, Pariante CM. Identifying the women at risk of antenatal anxiety and depression: A systematic review. *J Affect Disord*. (2016) 191:62–77. doi: 10.1016/j.jad.2015.11.014
12. Birkhauser M. Depression, menopause and estrogens: is there a correlation? *Maturitas*. (2002) 41:S3–8. doi: 10.1016/S0378-5122(02)00009-9
13. Zorn JV, Schur RR, Boks MP, Kahn RS, Joels M, Vinkers CH. Cortisol stress reactivity across psychiatric disorders: a systematic review and meta-analysis. *Psychoneuroendocrinology*. (2017) 77:25–36. doi: 10.1016/j.psyneuen.2016.11.036
14. Espin L, Almela M, Hidalgo V, Villada C, Salvador A, Gomez-Amor J. Acute pre-learning stress and declarative memory: impact of sex, cortisol response and menstrual cycle phase. *Horm Behav*. (2013) 63:759–65. doi: 10.1016/j.yhbeh.2013.03.013
15. Villada C, Hidalgo V, Almela M, Salvador A. Assessing performance on an evaluated speaking task. *J Psychophysiol*. (2018) 32:64–74. doi: 10.1027/0269-8803/a000185
16. Montero-Lopez E, Santos-Ruiz A, Garcia-Rios MC, Rodriguez-Blazquez M, Rogers HL, Peralta-Ramirez MI. The relationship between the menstrual cycle and cortisol secretion: Daily and stress-invoked cortisol patterns. *Int J Psychophysiol*. (2018) 131:67–72. doi: 10.1016/j.ijpsycho.2018.03.021

17. Kirschbaum C, Kudielka BM, Gaab J, Schommer NC, Hellhammer DH. Impact of gender, menstrual cycle phase, and oral contraceptives on the activity of the hypothalamus-pituitary-adrenal axis. *Psychosom Med*. (1999) 61:154–62. doi: 10.1097/00006842-199903000-00006
18. Duchesne A, Tessera E, Dedovic K, Engert V, Pruessner JC. Effects of panel sex composition on the physiological stress responses to psychosocial stress in healthy young men and women. *Biol Psychol*. (2012) 89:99–106. doi: 10.1016/j.biopsycho.2011.09.009
19. Duchesne A, Pruessner JC. Association between subjective and cortisol stress response depends on the menstrual cycle phase. *Psychoneuroendocrinology*. (2013) 38:3155–9. doi: 10.1016/j.psyneuen.2013.08.009
20. Girdler SS, Klatzkin R. Neurosteroids in the context of stress: implications for depressive disorders. *Pharmacol Ther*. (2007) 116:125–39. doi: 10.1016/j.pharmthera.2007.05.006
21. Brunton PJ. Neuroactive steroids and stress axis regulation: Pregnancy and beyond. *J Steroid Biochem Mol Biol*. (2016) 160:160–8. doi: 10.1016/j.jsbmb.2015.08.003
22. Gunn BG, Cunningham SG, Mitchell JD, Swinny JJ, Lambert D, Belellie D. GABAA receptor-acting neurosteroids: a role in the development and regulation of the stress response. *Front Neuroendocrinol*. (2015) 35:28–48. doi: 10.1016/j.yfrne.2014.06.001
23. Kerdelhué B, Brown S, Lenoir V, Queenan JT, Jones GS, Scholler R, et al. Timing of Initiation of the preovulatory luteinizing hormone surge and its relationship with the circadian cortisol rhythm in the human. *Neuroendocrinology*. (2002) 75:158–63. doi: 10.1159/000048233
24. Villada C, Hidalgo V, Almela M, Mastorci F, Sgoifo A, Salvador A. Coping with an acute psychosocial challenge: behavioral and physiological responses in young women. *PLoS ONE*. (2014) 9:e114640. doi: 10.1371/journal.pone.0114640
25. Timon R, Corvillo M, Brazo J, Robles MC, Maynar M. Strength training effects on urinary steroid profile across the menstrual cycle in healthy women. *Eur J Appl Physiol*. (2013) 113:1469–75. doi: 10.1007/s00421-012-2575-6
26. Inoue Y, Terao T, Iwata N, Okamoto K, Kojima H, Okamoto T, et al. Fluctuating serotonergic function in premenstrual dysphoric disorder and premenstrual syndrome: findings from neuroendocrine challenge tests. *Psychopharmacology*. (2007) 190:213–9. doi: 10.1007/s00213-006-0607-9
27. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gotzsche PC, Ioannidis JP, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *PLoS Med*. (2009) 6:e1000100. doi: 10.1371/journal.pmed.1000100
28. Cohen J. *Statistical Power Analysis for the Behavioral Sciences*. New York, NY: Routledge Academic (1988).
29. Hedges L, Olkin I. *Statistical Methods for Meta-Analysis*. San Diego, CA: Academic Press (1985).
30. Morris SB, DeShon RP. Combining effect size estimates in meta-analysis with repeated measures and independent-groups designs. *Psychol Methods*. (2002) 7:105–25. doi: 10.1037/1082-989x.7.1.105
31. Borenstein M. Effect sizes for continuous data. In: Cooper H, Hedges L, Valentine J, editors. *The Handbook of Research Synthesis and Meta Analysis*. New York, NY: Russell Sage Foundation (2009).
32. Egger M, Smith G, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. *Br Med J*. (1997) 315:629–34. doi: 10.1136/bmj.315.7109.629
33. Schwarzer G, Antes G, Schumacher M. A test for publication bias in meta-analysis with sparse binary data. *Stat Med*. (2007) 26:721–33. doi: 10.1002/sim.2588
34. Andreano JM, Arjomandi H, Cahill L. Menstrual cycle modulation of the relationship between cortisol and long-term memory. *Psychoneuroendocrinology*. (2008) 33:874–82. doi: 10.1016/j.psyneuen.2008.03.009
35. Barbarino A, De Marinis L, Folli G, Tofani A, Della Casa S, D'Amico C, et al. Corticotropin-releasing hormone inhibition of gonadotropin secretion during the menstrual cycle. *Metabolism*. (1989) 38:504–6. doi: 10.1016/0026-0495(89)90208-4
36. Beck RP, Morcos F, Fawcett D, Watanabe M. Adrenocortical function studies during the normal menstrual cycle and in women receiving norethindrone with and without mestranol. *Am J Obstet Gynecol*. (1972) 112:364–8. doi: 10.1016/0002-9378(72)90478-4
37. Hoeger Bement MK, Rasiarmos RL, DiCapo JM, Lewis A, Keller ML, Harkins AL, et al. The role of the menstrual cycle phase in pain perception before and after an isometric fatiguing contraction. *Eur J Appl Physiol*. (2009) 106:105–12. doi: 10.1007/s00421-009-0995-8
38. Bricout VA, Wright F, Lagoguey M. Urinary profile of androgen metabolites in a population of sportswomen during the menstrual cycle. *Int J Sports Med*. (2003) 24:197–202. doi: 10.1055/s-2003-39090
39. Cannon JG, Angel JB, Abad LW, O'Grady J, Lundgren N, Fagioli L, et al. Hormonal influences on stress-induced neutrophil mobilization in health and chronic fatigue syndrome. *J Clin Immunol*. (1998) 18:291–8. doi: 10.1023/A:1027389907780
40. Carr BR, Parker CR, Madden JD, MacDonald PC, Porter JC. Plasma levels of adrenocorticotropin and cortisol in women receiving oral contraceptive steroid treatment. *J Clin Endocrinol Metab*. (1979). 49:346–9. doi: 10.1210/jcem-49-3-346
41. Caufriez A, Leproult R, L'Hermite-Baleriaux M, Moreno-Reyes R, Copinschi G. A potential role of endogenous progesterone in modulation of GH, prolactin and thyrotrophin secretion during normal menstrual cycle. *Clin Endocrinol*. (2009) 71:535–42. doi: 10.1111/j.1365-2265.2009.03539.x
42. Childs E, Dlugos A, De Wit H. Cardiovascular, hormonal, and emotional responses to the TSST in relation to sex and menstrual cycle phase. *Psychophysiology*. (2010) 47:550–9. doi: 10.1111/j.1469-8986.2009.00961.x
43. Collins A, Eneroth P, Landgren BM. Psychoneuroendocrine stress responses and mood as related to the menstrual cycle. *Psychosom Med*. (1985) 47:512–27. doi: 10.1097/00006842-198511000-00002
44. Genazzani AR, Lemarchand-Béraud T, Aubert ML, Felber JP. Pattern of plasma ACTH, hGH, and cortisol during menstrual cycle. *J Clin Endocrinol Metab*. (1975) 41:431–7. doi: 10.1210/jcem-41-3-431
45. Heitkemper M, Jarrett M, Cain K, Shaver J, Bond E, Woods NF, et al. Increased urine catecholamines and cortisol in women with irritable bowel syndrome. *Am J Gastroenterol*. (1996) 91:906–13.
46. Huang Y, Zhou R, Wu M, Wang Q, Zhao Y. Premenstrual syndrome is associated with blunted cortisol reactivity to the TSST. *Stress*. (2015) 18:160–8. doi: 10.3109/10253890.2014.999234
47. Judd SJ, Wong J, Saloniklis S, Maiden M, Yeap B, Filmer S, et al. The effect of alprazolam on serum cortisol and luteinizing hormone pulsatility in normal women and in women with stress-related anovulation. *J Clin Endocrinol Metab*. (1995) 80:818–23. doi: 10.1210/jcem.80.3.7883836
48. Kasa-Vubu JZ, Dimaraki EV, Young EA. The pattern of growth hormone secretion during the menstrual cycle in normal and depressed women. *Clin Endocrinol*. (2005) 62:656–60. doi: 10.1111/j.1365-2265.2005.02274.x
49. LeRoux A, Wright L, Perrot T, Rusak B. Impact of menstrual cycle phase on endocrine effects of partial sleep restriction in healthy women. *Psychoneuroendocrinology*. (2014) 49:34–46. doi: 10.1016/j.psyneuen.2014.06.002
50. Liu JH, Rasmussen DD, Rivier J, Vale W, Yen SS. Pituitary responses to synthetic corticotropin-releasing hormone: absence of modulatory effects by estrogen and progestin. *Am J Obstet Gynecol*. (1987) 157:1387–91. doi: 10.1016/S0002-9378(87)80229-6
51. Lombardi I, Luisi S, Quirici B, Monteleone P, Bernardi F, Liut M, et al. Adrenal response to adrenocorticotropic hormone stimulation in patients with premenstrual syndrome. *Gynecol Endocrinol*. (2004) 18:79–87. doi: 10.1080/09513590310001652955
52. Maki PM, Mordecai KL, Rubin LH, Sundermann E, Savarese A, Eatough E, et al. Menstrual cycle effects on cortisol responsivity and emotional retrieval following a psychosocial stressor. *Horm Behav*. (2015) 74:201–8. doi: 10.1016/j.yhbeh.2015.06.023
53. Ohara K, Okita Y, Kouda K, Mase T, Miyawaki C, Nakamura H. Cardiovascular response to short-term fasting in menstrual phases in young women: an observational study. *BMC Womens Health*. (2015) 15:67. doi: 10.1186/s12905-015-0224-z
54. Paoletti AM, Romagnino S, Contu R, Orru MM, Marotto MF, Zedda P, et al. Observational study on the stability of the psychological status during normal pregnancy and increased blood levels of neuroactive steroids with GABA-A receptor agonist activity. *Psychoneuroendocrinology*. (2006) 31:485–92. doi: 10.1016/j.psyneuen.2005.11.006
55. Parry BL, Javeed S, Laughlin GA, Hauger R, Clopton P. Cortisol circadian rhythms during the menstrual cycle and with sleep deprivation in

premenstrual dysphoric disorder and normal control subjects. *Biol Psychiatry*. (2000) 48:920–31. doi: 10.1016/S0006-3223(00)00876-3
56. Rasgon N, McGuire M, Tanavoli S, Fairbanks L, Rapkin A. Neuroendocrine response to an intravenous L-tryptophan challenge in women with premenstrual syndrome. *Fertil Steril*. (2000) 73:144–9. doi: 10.1016/S0015-0282(99)00452-5
57. Reynolds TA, Makhanova A, Marcinkowska UM, Jasienska G, McNulty JK, Eckel LA, et al. Progesterone and women's anxiety across the menstrual cycle. *Horm Behav*. (2018) 102:34–40. doi: 10.1016/j.yhbeh.2018.04.008
58. Roche DJ, King AC. Sex differences in acute hormonal and subjective response to naltrexone: the impact of menstrual cycle phase. *Psychoneuroendocrinology*. (2015) 52:59–71. doi: 10.1016/j.psyneuen.2014.10.013
59. Stewart PM, Penn R, Holder R, Parton A, Ratcliffe JG, London DR. The hypothalamo-pituitary-adrenal axis across the normal menstrual cycle and in polycystic ovary syndrome. *Clin Endocrinol*. (1993) 38:387–91. doi: 10.1111/j.1365-2265.1993.tb00519.x
60. Su TP, Schmidt PJ, Danaceau M, Murphy DL, Rubinow DR. Effect of menstrual cycle phase on neuroendocrine and behavioral responses to the serotonin agonist M-chlorophenylpiperazine in women with premenstrual syndrome and controls. *J Clin Endocrinol Metab*. (1997) 82:1220–8. doi: 10.1210/jc.82.4.1220
61. Tulenheimo A, Laatikainen T, Salminen K. Plasma Beta-Endorphin Immunoreactivity in Premenstrual Tension. *Br J Obstet Gynaecol*. (1987) 94:26–9. doi: 10.1111/j.1471-0528.1987.tb02247.x
62. Villada C, Espin L, Hidalgo V, Rubagotti S, Sgoifo A, Salvador A. The influence of coping strategies and behavior on the physiological response to social stress in women: the role of age and menstrual cycle phase. *Physiol Behav*. (2017) 170:37–46. doi: 10.1016/j.physbeh.2016.12.011
63. Wolfram M, Bellingrath S, Kudielka BM. The cortisol awakening response (CAR) across the female menstrual cycle. *Psychoneuroendocrinology*. (2011) 36:905–12. doi: 10.1016/j.psyneuen.2010.12.006
64. Suzuki S, Handa RJ. Estrogen receptor-beta, but not estrogen receptor-alpha, is expressed in prolactin neurons of the female rat paraventricular and supraoptic nuclei: comparison with other neuropeptides. *J Comp Neurol*. (2005) 484:28–42. doi: 10.1002/cne.20457
65. Simerly RB, Chang C, Muramatsu M, Swanson LW. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. *J Comp Neurol*. (1990) 294:76–95. doi: 10.1002/cne.902940107
66. Estacio MA, Tsukamura H, Yamada S, Tsukahara S, Hirunagi K, Maeda K. Vagus nerve mediates the increase in estrogen receptors in the hypothalamic paraventricular nucleus and nucleus of the solitary tract during fasting in ovariectomized rats. *Neurosci Lett*. (1996) 208:25–8. doi: 10.1016/0304-3940(96)12534-9
67. Somponpun SJ, Sladek CD. Osmotic regulation of estrogen receptor-beta in rat vasopressin and oxytocin neurons. *J Neurosci*. (2003) 23:4261–9. doi: 10.1523/JNEUROSCI.23-10-04261.2003
68. Hrabovszky E, Kalló I, Hajszán T, Shughrue PJ, Merchenthaler I, Liposits Z. Expression of estrogen receptor-beta messenger ribonucleic acid in oxytocin and vasopressin neurons of the rat supraoptic and paraventricular nuclei. *Endocrinology*. (1998) 139:2600–4. doi: 10.1210/endo.139.5.6024
69. Alves SE, Lopez V, McEweb BS, Weiland NG. Differential colocalization of estrogen receptor b (ER b) with oxytocin and vasopressin in the paraventricular and supraoptic nuclei of the female rat brain: An immunocytochemical study. *Proc Natl Acad Sci USA*. (1998) 95:3281–6. doi: 10.1073/pnas.95.6.3281
70. Lund TD, Hinds LR, Handa RJ. The androgen 5alpha-dihydrotestosterone and its metabolite 5alpha-androstan-3beta, 17beta-diol inhibit the hypothalamo-pituitary-adrenal response to stress by acting through estrogen receptor beta-expressing neurons in the hypothalamus. *J Neurosci*. (2006) 26:1448–56. doi: 10.1523/JNEUROSCI.3777-05.2006
71. Weiser MJ, Handa RJ. Estrogen impairs glucocorticoid dependent negative feedback on the hypothalamic-pituitary-adrenal axis via estrogen receptor alpha within the hypothalamus. *Neuroscience*. (2009) 159:883–95. doi: 10.1016/j.neuroscience.2008.12.058
72. Gaskin JH, Kitay JI. Adrenocortical function in the hamster. Sex differences and effects of gonadal hormones. *Endocrinology*. (1970) 87:779–86. doi: 10.1210/endo-87-4-779
73. Burgess LH, Handa RJ. Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats. *Endocrinology*. (1992) 131:1261–9. doi: 10.1210/endo.131.3.1324155
74. Ochedalski T, Subburaju S, Wynn PC, Aguilera G. Interaction between oestrogen and oxytocin on hypothalamic-pituitary-adrenal axis activity. *J Neuroendocrinol*. (2007) 19:189–97. doi: 10.1111/j.1365-2826.2006.01525.x
75. Ter Horst GJ, Wichmann R, Gerrits M, Westenbroek C, Lin Y. Sex differences in stress responses: focus on ovarian hormones. *Physiol Behav*. (2009) 97:239–49. doi: 10.1016/j.physbeh.2009.02.036
76. Boudaba C, Szabo K, Tasker JG. Physiological mapping of local inhibitory inputs to the hypothalamic paraventricular nucleus. *J Neurosci*. (1996) 16:7151–60. doi: 10.1523/JNEUROSCI.16-22-07151.1996
77. Brunton PJ, McKay AJ, Ochedalski T, Piastowska A, Rebas E, Lachowicz A, et al. Central opioid inhibition of neuroendocrine stress responses in pregnancy in the rat is induced by the neurosteroid allopregnanolone. *J Neurosci*. (2009) 29:6449–60. doi: 10.1523/JNEUROSCI.0708-09.2009
78. Gunn BG, Cunningham L, Cooper MA, Corteen NL, Seifi M, Swinny JD, et al. Dysfunctional astrocytic and synaptic regulation of hypothalamic glutamatergic transmission in a mouse model of early-life adversity: relevance to neurosteroids and programming of the stress response. *J Neurosci*. (2013) 33:19534–54. doi: 10.1523/JNEUROSCI.1337-13.2013
79. Biggio G, Pisu MG, Biggio F, Serra M. Allopregnanolone modulation of HPA axis function in the adult rat. *Psychopharmacology*. (2014) 231:3437–44. doi: 10.1007/s00213-014-3521-6
80. Sarkar J, Wakefield S, MacKenzie G, Moss SJ, Maguire J. Neurosteroidogenesis is required for the physiological response to stress: role of neurosteroid-sensitive GABAA receptors. *J Neurosci*. (2011) 31:18198–210. doi: 10.1523/JNEUROSCI.2560-11.2011
81. Misztal T, Mlotkowska P, Marciniak E, Misztal A. Allopregnanolone reduces neuroendocrine response to acute stressful stimuli in sheep. *J Endocrinol*. (2020) 244:201–11. doi: 10.1530/JOE-19-0376
82. Owens MJ, Ritchie JC, Nemeroff CB. 5a-Pregnane-3a,21-diol-20-one (THDOC) attenuates mild stress-induced increases in plasma corticosterone via a non-glucocorticoid mechanism: comparison with alprazolam. *Brain Res*. (1992) 573:353–5. doi: 10.1016/0006-8993(92)90788-B
83. Patchev VK, Hassan AH, Holsboer DF, Almeida OF. The neurosteroid tetrahydroprogesterone attenuates the endocrine response to stress and exerts glucocorticoid-like effects on vasopressin gene transcription in the rat hypothalamus. *Neuropsychopharmacology*. (1996) 15:533–40. doi: 10.1016/S0893-133X(96)00096-6
84. Siiteri PK, Murai JT, Hammond GL, Nisker JA, Raymoure WJ, Kuhn RW. The serum transport of steroid hormones. *Recent Prog Horm Res*. (1982) 38:457–510. doi: 10.1016/B978-0-12-571138-8.50016-0
85. Coolens JL, Van Baelen H, Heyns W. Clinical use of unbound plasma cortisol as calculated from total cortisol and corticosteroid-binding globulin. *J Steroid Biochem*. (1987) 26:197–202. doi: 10.1016/0022-4731(87)90071-9
86. Vinning RF, McGinley RA, Maksvytis J, Ho KY. Salivary cortisol: a better measure ofadrenal cortical function than serum cortisol. *Ann Clin Biochem*. (1983) 20:329–35. doi: 10.1177/000456328302000601
87. Westphal U. Steroid-protein interactions. *Monogr Endocrinol*. (1971) 4:1–567. doi: 10.1007/978-3-642-46262-7_1
88. Moore DE, Kawagoe S, Davajan V, Nakamura RM, Mishell DR. An in vivo system in man for quantitation of estrogenicity. II Pharmacologic changes in binding capacity of serum corticosteroid-binding globulin induced by conjugated estrogens, mestranol, and ethinyl estradiol. *Am J Obstet Gynecol*. (1978) 130:482–6. doi: 10.1016/0002-9378(78)90294-6
89. Jeyakumar M, Carlson KE, Gunther JR, Katzenellenbogen JA. Exploration of dimensions of estrogen potency: parsing ligand binding and coactivator binding affinities. *J Biol Chem*. (2011) 286:12971–82. doi: 10.1074/jbc.M110.205112
90. Nenke MA, Zeng A, Meyer EJ, Lewis JG, Rankin W, Johnston J, et al. Differential effects of estrogen on corticosteroid-binding globulin forms suggests reduced cleavage in pregnancy. *J Endocr Soc*. (2017) 1:202–10. doi: 10.1210/js.2016-1094
91. Herrera AY, Faude S, Nielsen SE, Locke M, Mather M. Effects of hormonal contraceptive phase and progestin generation on stress-induced

cortisol and progesterone release. *Neurobiol Stress*. (2019) 10:100151. doi: 10.1016/j.ynstr.2019.100151
92. Hampson E. A brief guide to the menstrual cycle and oral contraceptive use for researchers in behavioral endocrinology. *Horm Behav*. (2019) 119:104655. doi: 10.1016/j.yhbeh.2019.104655
93. Daskalakis NP, Cohen H, Nievergelt CM, Baker DG, Buxbaum JD, Russo SJ, et al. New translational perspectives for blood-based biomarkers of PTSD: From glucocorticoid to immune mediators of stress susceptibility. *Exp Neurol*. (2016) 284:133–40. doi: 10.1016/j.expneurol.2016.07.024
94. Plotsky PM, Owens MJ, Nemeroff CB. Psychoneuroendocrinology of depression. Hypothalamic-pituitary-adrenal axis. *Psychiatr Clin North Am*. (1998) 21:293–307. doi: 10.1016/S0193-953X(05)70006-X
95. Sze Y, Brunton PJ. Sex, stress and steroids. *Eur J Neurosci*. (2019). doi: 10.1111/ejn.14615. [Epub ahead of print].
96. Altemus M, Sarvaiya N, Neill Epperson C. Sex differences in anxiety and depression clinical perspectives. *Front Neuroendocrinol*. (2014) 35:320–30. doi: 10.1016/j.yfrne.2014.05.004
97. Bangasser DA, Valentino RJ. Sex differences in stress-related psychiatric disorders: neurobiological perspectives. *Front Neuroendocrinol*. (2014) 35:303–19. doi: 10.1016/j.yfrne.2014.03.008

Disclaimer: The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 03 June 2020
doi: 10.3389/fendo.2020.00306

ARP-1 Regulates the Transcriptional Activity of the Aromatase Gene in the Mouse Brain

Shin-ichiro Honda[1]* *and Nobuhiro Harada*[2]

[1] Department of Biochemistry, Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka, Japan, [2] Department of Biochemistry, School of Medicine, Fujita Health University, Toyoake, Japan

An important function of aromatase in the brain is conversion of testosterone secreted from the testis into estradiol. Estradiol produced in the brain is thought to be deeply involved in the formation of sexually dimorphic nuclei and sexual behavior as a neurosteroid. We analyzed the brain-specific promoter to elucidate the control mechanisms of brain aromatase expression that may be highly involved in sexual differentiation of the brain. The 202-bp upstream region of the brain-specific exon 1 has three types of *cis*-acting elements, aro-AI, AII, and B. We isolated ARP-1 as an aro-AII-binding protein by yeast one-hybrid screening from a cDNA library of mouse fetal brains. ARP-1 is a member of the nuclear receptor superfamily and functions as an orphan-type transcription factor. ARP-1 protein synthesized *in vitro* showed the same binding property to the aro-AII site as nuclear extract from fetal brains. To determine how the promoter is involved in brain-specific transcription of the aromatase gene, we first detected the *in vivo* occupancy of the aro-AII site by ARP-1 using chromatin immunoprecipitation assays. Diencephalic regions of fetal brains at embryonic day 16 were analyzed, which revealed ARP-1 recruitment to the aro-AII site. To analyze the effects of ARP-1 on transcriptional regulation of the brain-specific aromatase promoter, a luciferase reporter plasmid driven by the brain-specific promoter was transfected into CV-1 cells together with a plasmid expressing ARP-1 protein. These analyses revealed that ARP-1 induced promoter activity in a dose-dependent manner. Furthermore, to determine whether ARP-1 is required for aromatase expression in neurons, ARP-1 knockdown was conducted in neuronal cell primary culture. Knockdown of ARP-1 significantly suppressed the increase in aromatase mRNA observed in cultured neurons. These results indicate that ARP-1 is involved in the transcriptional regulation of the brain-specific promoter of the aromatase gene.

Keywords: aromatase, steroid hormone, sexual differentiation, estrogen, chicken ovalbumin upstream promoter transcription factor, nuclear receptor

OPEN ACCESS

Edited by:
Gaetano Santulli, Columbia University, United States

Reviewed by:
Janine Prange-Kiel, University of Texas Southwestern Medical Center, United States
Maria Carmen Iglesias-Osma, University of Salamanca, Spain
Rachel E. Cohen, Minnesota State University, Mankato, United States

***Correspondence:**
Shin-ichiro Honda
sihonda@fukuoka-u.ac.jp

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

Received: *27 February 2020*
Accepted: *22 April 2020*
Published: *03 June 2020*

Citation:
Honda S and Harada N (2020) ARP-1 Regulates the Transcriptional Activity of the Aromatase Gene in the Mouse Brain. Front. Endocrinol. 11:306. doi: 10.3389/fendo.2020.00306

INTRODUCTION

The physiological functions of neurosteroids have been investigated in many laboratories (1–3). Neurosteroids facilitate a wide variety of biological activities in the brain either through the action of a canonical nuclear receptor or through interaction with membrane-bound receptors (4–6). The neurosteroid estrogen has been proposed to play critical roles in a variety of reproductive

behaviors. Aromatase, also called estrogen synthase, is mainly expressed in the gonads (7–9) and brain (10–13) in rodents. We have shown that aromatase plays an important role in the formation of morphological, neuroendocrinological, and behavioral sex differences. In fact, an experimental animal model of estrogen deficiency was generated in mice by targeted disruption of the aromatase gene (14–16), and the roles of estrogen in reproductive behaviors were extensively investigated (17–19). In our previous study, transgenic mice specifically expressing human aromatase in the brain were generated and crossed with aromatase knock-out (ArKO) mice, resulting in the creation of mice with brain-specific recovery of estrogen production (ArKO/bsArTG) (20). The ArKO/bsArTG mice exhibited significant restoration of impaired behaviors, suggesting that brain-restricted expression of aromatase is sufficient for the display of reproductive behavior. Thus, expression of aromatase in the brain is suggested to be essential for reproductive behavior in mice.

Transcription of the aromatase gene is governed by multiple tissue-specific promoter regions. In the brain, expression of the aromatase gene varies depending on the developmental stage, with a transient peak during the perinatal period, which is consistent with the critical period known as neonatal imprinting of sexual differentiation. We have identified a brain-specific exon 1 in human and mouse aromatase genes, and its use was restricted to neurons by alternative splicing of the multiple exons 1 (21–26). The promoter analyses revealed that the 202-bp upstream region of the mouse brain–specific exon 1 has strong promoter activity in primary culture of diencephalic neurons from fetal mouse brains. We have shown the functions of *cis*-acting elements responsible for the brain-specific spatiotemporal expression of the mouse aromatase gene. The 202-bp upstream region has three *cis*-elements: aro-AI (Arom-Aα), aro-AII (Arom-Aβ), and aro-B (Arom-B) (27, 28). Our previous study indicated the homeodomain-containing transcription factor Lhx2 as a binding protein to the aro-B site and a potential transcriptional regulator of brain-specific expression of the aromatase gene (28). Lhx2 can mediate transcriptional activity of the brain-specific aromatase gene and exhibits a transient peak during the perinatal period. In concordance with previous findings, it is highly likely that unidentified transcription factors, which bind to other *cis*-acting elements, including aro-AI and aro-AII, are also involved in the transcriptional regulation of aromatase in the brain.

In the present study, we provide evidence that a member of the nuclear receptor super family, ARP-1, can bind to the aro-AII site of the brain-specific promoter 1f and positively regulate aromatase expression.

MATERIALS AND METHODS

Yeast One-Hybrid Screening

The Matchmaker One-Hybrid System was used to isolate the cDNA encoding a protein that binds to the aro-AII element of the brain-specific promoter of the mouse aromatase gene. The procedures were performed according to the manufacturer's protocols (Clontech, Mountain View, CA, USA). Four tandem repeats of double stranded aro-AII (5′-TTATGTTGGCCCCTGACATATATATT-3′) nucleotides were subcloned into the upstream regions of the minimal promoters of pHISi-1 and pLacZ reporter plasmids. These plasmids were then linearized and transformed for integration into a YM4271 yeast genome to generate reporter yeast strains that were designated as YM4271/aro-AII-His and YM4271/aro-AII-LacZ. YM4271/aro-AII-His was further checked for growth on medium lacking histidine (His^- medium) in the presence of 45 mM 3-amino-1,2,4-triazol (3-AT). The YM4271/aro-AII-His yeast strain was transformed with a MATCHMAKER cDNA library constructed from embryonic day 17 (E17) mice and subsequently cloned into the vector pACT2 (Clontech). The transformed yeast colonies ($\sim 1.5 \times 10^6$) were screened, and three positive transformants, which were grown on SD medium plates lacking histidine and leucine with 45 mM 3-AT, were isolated. To exclude pseudo-positive clones, plasmids were recovered from selected clones and rescreened by transforming them into YM4271/aro-AII-LacZ cultures on SD medium plates lacking uracil and leucine. The filter replica method—using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 μg/ml)—was used to measure β-galactosidase activity according to the manufacturer's protocols. DNA sequences of plasmids from three individual positive blue colonies were sequenced and subsequently analyzed for homology using a BLAST DNA search.

Gel Shift Assay

Gel shift assays were carried out as described previously (29). For the gel shift assay, the 5′-protruding ends of the double-stranded aro-AII probe (5′-gTTATGTTGGCCCCTGACATATATATT-3′/5′-gAATATATATGTCAGGGGCCAACATAA-3′) were labeled with [α-^{32}P] dCTP using a Klenow fragment of DNA polymerase. Five micrograms of nuclear protein or aliquots of proteins synthesized *in vitro* were mixed with the DNA probes and incubated for 20 min on ice. The reaction mixtures were analyzed using a 5% polyacrylamide gel. The synthetic mutant oligonucleotides, AIIM1 and AIIM3, were also described in a previous paper (29). For competition experiments, a 200-fold molar excess of unlabeled nucleotides was added to the reaction mixture. For supershift experiments, an anti-ARP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-COUP-TFI (Chicken Ovalbumin Upstream Promoter-Transcription Factor I) antibody (Santa Cruz Biotechnology) was used to specifically recognize corresponding isoforms.

Plasmid constructs bearing ARP-1 that were suitable for *in vitro* translation were prepared as follows. ARP-1 cDNA obtained was subcloned into a pCI-neo vector (Promega, Madison, WI, USA), resulting in pCI-neoARP-1. The plasmid was linearized at the 3′ end of the coding region and transcribed by T7 RNA polymerase (Takara, Kyoto, Japan). The resulting ARP-1 mRNA was verified by 1% agarose gel electrophoresis. ARP-1 mRNA was translated using an *in vitro* protein synthesis kit (Promega) with rabbit reticulocyte lysate.

Animals

All experimental procedures using animals were approved by the Committee for Animal Experiments of Fukuoka University (reference no. 1705049).

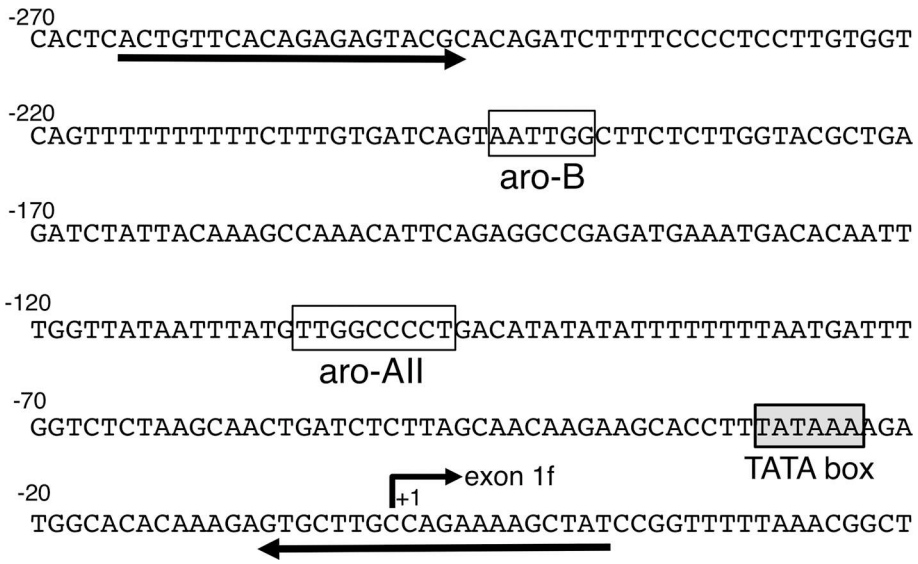

FIGURE 1 | Brain-specific exon 1 and its promoter region in the mouse aromatase gene. The number +1 corresponds to a potential transcription start site. A TATA box is shown in the shadowed box. The open boxes indicate the aro-AII and aro-B sites found in previous studies (27). The two primers used in the chromatin immunoprecipitation assay are also indicated in the figure by the arrows.

Chromatin Immunoprecipitation (ChIP) Assay

The chromatin immunoprecipitation assay was carried out using a ChIP assay kit (Upstate Cell Signaling Solutions, Charlottesville, VA, USA) as described previously. The diencephalic regions of E16 mouse fetal brains were treated with 1% formaldehyde to cross-link proteins to DNA. Then, the samples were homogenized in lysis buffer and sonicated to yield an average DNA size of 500 bp. Sonicated extracts were precleared with protein G-agarose/salmon sperm DNA (Upstate Cell Signaling Solutions) and divided into two fractions. Then, 5 μg of non-immunized goat immunoglobulin G (preimmune IgG) or anti-ARP-1 antibody (Santa Cruz Biotechnology) was applied. The immunoprecipitated products were eluted, and DNA–protein complexes were dissociated by heating at 65°C. The resulting DNA fraction was purified by phenol/chloroform extraction and ethanol precipitation and subsequently subjected to PCR amplification using the following aromatase gene-specific primers: MB-AR-N1, 5′-TCACTGTTCACAGAGAGTAC-3′; MB-AR-0R, 5′-ATAGCTTTTCTGGCAAGCAC-3′ (**Figure 1**).

Aromatase Gene Promoter Assay Using a Luciferase Reporter

CV-1 and HepG-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Luciferase reporter plasmids were constructed by cloning the fragments of brain-specific promoters into the pGL3-Basic luciferase vector (Promega, Madison, WI). To obtain fragments of the promoter region, we amplified the fragments by polymerase chain reaction (PCR) using mouse genomic DNA as a template and oligonucleotide pairs; the brain-specific promoter region of the mouse aromatase gene was amplified with the following primer pair: MB-AR-N1 (5′-TCACTGTTCACAGAGAGTAC-3′) and MB-AR-1R (5′-GGACTCTTGAAGATGGTGAG-3′), and the mouse apolipoprotein AI promoter region was amplified with the following primer pair: mo-apoA1-2 (5′-TGGGACCCCTGGAGTCTGC-3′) and mo-apoA1-R1 (5′-GGACGCTCTCCGACAGTCT-3′). The PCR products were subcloned into the SmaI site of the pGL3-Basic plasmid (Promega), resulting in the pGL3aroBr and pGL3apoAI plasmids. The cDNA clone of mouse ARP-1 was subcloned into the Bam HI and Not I sites of the p3XFLAG-myc-CMV-26 expression vector (Sigma-Aldrich, St. Louis, MO, USA), resulting in the pFLAG-ARP-1 expression plasmid.

The pFLAG-ARP-1 expression plasmid (50, 100, 250, or 500 ng), 500 ng of reporter plasmid, and 50 ng of phRluc-TK control vector (Promega) were mixed with 50 μl of antibiotic-free DMEM containing 8 μl of Plus Reagent (Invitrogen) and incubated for 15 min at room temperature. Next, 50 μl of antibiotic-free DMEM containing 2 μl of Lipofectamine Reagent (Invitrogen) was added to the mixture and incubated for 15 min at room temperature. The mixture was added onto CV-1 cell monolayers preincubated under serum-free conditions. After 5 h of incubation, the DNA–liposome complex was replaced with the complete medium and cultured for 48 h. For the reporter assay with HepG2 cells, 100, 250, or 500 ng of pFLAG-ARP-1 expression plasmid, 500 ng of pGL3aroBr or pGL3apoAI plasmid, and 50 ng of phRluc-TK control vector (Promega) were mixed and transfected in cells according to the procedure described above. The cells were solubilized with 150 μl of Passive Lysis Buffer (Promega). Promoter activity was measured using a

Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's protocol. Each experiment was performed in duplicate and repeated at least three times.

ARP-1 Knockdown of Fetal Neuronal Cells

Diencephalic neurons were prepared from E13 mouse fetal brains according to Abe-Dohmae et al. (30), and the cells were used to analyze the effects of ARP-1 knockdown on diencephalic aromatase expression. The neuronal cells were cultured in DF medium (50% DMEM and 50% Ham's F12 medium, supplemented with 5 μg/ml of insulin, 5 μg/ml of human transferrin, 5 ng/ml of sodium selenite, 20 nM of progesterone, 100 units/ml of penicillin G, and 100 μg/ml of streptomycin sulfate) in a poly-L-Lys-coated 12-well-plate (1.5×10^6 cells/well). Twenty-four hours after starting the culture, transfection was performed using the HiPerFect Transfection reagent (Qiagen, Valencia, CA). For transfection, the cells were washed twice with antibiotic-free DF medium and subsequently incubated in 1.2 ml of antibiotic-free DF medium containing 25 nM Silencer Select siRNA (siRNA ID: s102050) according to the manufacturer's instructions. Forty-eight hours after starting the transfection, the cells were harvested to prepare separate RNA and protein samples. Total RNA was prepared using the TRIzol regent (Invitrogen) and was then analyzed by RT-qPCR. The reduction in ARP-1 protein was estimated by western blot analysis using an anti-ARP-1 antibody (Santa Cruz Biotechnology) and an anti-β-actin antibody (Proteintech, Rosemont, IL, USA). For the western blotting analysis, the cells were washed with PBS, lysed in RIPA buffer [150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 50 mM Tris (pH 7.6)], then centrifuged at 14,000 × g for 10 min. The protein concentration was determined using a BCA protein assay kit (Bio-Rad, Hercules, CA, USA). Five micrograms of the protein lysate was separated on a 9% sodium dodecyl sulfate-polyacrylamide gel, then electro-transferred to a PVDF membrane. The membrane was blocked in Blocking One solution (Nacalai Tesque, Kyoto, Japan) for a duration ranging from 1 h to overnight, then incubated with anti-ARP-1 antibody at a dilution of 1:5,000 or anti-β-actin antibody at a dilution of 1:5,000 for 1 h at room temperature. The membrane was then incubated with the appropriate peroxidase-conjugated secondary antibody at a dilution of 1:20,000 (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Can Get Signal Immunoreaction Enhancer Solution (TOYOBO, Osaka, Japan) was used to dilute the primary and secondary antibodies. Chemiluminescence was detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). The immunoreactive signals were visualized and quantified using a ChemiDoc XRS instrument (Bio-Rad).

RT-qPCR

The reverse transcription reaction was performed using an AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA). Briefly, total RNA (3 μg) was reverse transcribed using random primers according to the manufacturer's instructions. cDNA aliquots were used for quantitative PCR analysis. Real-time PCR was performed using TaqMan probes with Brilliant II QPCR Master Mix (Agilent) according to the manufacturer's instructions. TaqMan Gene Expression Assay reagents (Thermo Fisher Scientific, Waltham, MA, USA) for aromatase (Assay ID: Mm00484049_m1) and β-actin (Assay ID: Mm01205647_g1) were used as TaqMan probes. Real-time PCR was performed using a two-step cycling protocol consisting of 45 cycles of 20 s at 95°C and 60 s at 60°C on an Mx3000P QPCR System (Agilent). All reactions included controls lacking the template. After the reactions, the Ct values were determined using fixed-threshold settings. The ΔΔCT method was used to determine the mRNA fold change, which was normalized to β-actin mRNA level. Each experiment was performed in duplicate and repeated at least three times.

Statistical Analysis

The results obtained from triplicate experiments are expressed as the mean ± S.E.M. All data analyses were performed using JMP® software (SAS institute Inc., Cary, NC, USA). Statistical analysis of the data was performed using one way ANOVA with *post-hoc* Tukey–Kramer correction, and differences were considered significant if the $p < 0.05$.

RESULTS

Identification of a Protein That Interacts With the aro-AII Site of the Brain-Specific Promoter 1f of the Aromatase Gene

To obtain a potential transcription factor that binds to the aro-AII sequence, we performed screening of an E17 mouse cDNA library using reporter yeast strains, designated as YM4271/aro-AII-His and YM4271/aro-AII-Lac.

Three positive clones were obtained from a one-hybrid screening of the cDNA library from E17 mice. Comparison of the sequences with GeneBank data using a BLAST DNA search revealed a sequence that was 100% identical to the previously identified transcription factor apolipoprotein AI Regulatory Protein 1 (ARP-1, also called COUP-TFII). These clones did not cover the full length of the ARP-1 protein, and an even longer clone was lacking nine amino acids of the N-terminus.

ARP-1 Protein Binds to the aro-AII Site

To assess ARP-1 binding activity to the aro-AII sequence, we carried out gel shift analyses with nuclear extracts prepared from the diencephalic region of E15 mouse brains. The sequence required for aro-AII to interact with the binding protein is shown in **Figures 1**,**2A**. In a gel shift assay using an AII probe that included the TTGGCCCCT sequence of the promoter region, the nuclear protein formed a specific mobility-shifted complex on the AII probe, and this complex did not form under competition with a 200-molar excess of an unlabeled AII probe. The aro-AII-binding protein recognized a nine-base-pair sequence "TTGGCCCCT" as shown in **Figure 2A**. When a mutation was introduced into this nine-base-pair sequence, the interaction with the protein was lost. As previously shown by our group (29) and indicated in **Figure 2A**, an oligonucleotide with a mutant introduced inside the "TT*AT*CCCCT" (AIIM3) sequence did not compete with the complex, but an oligonucleotide

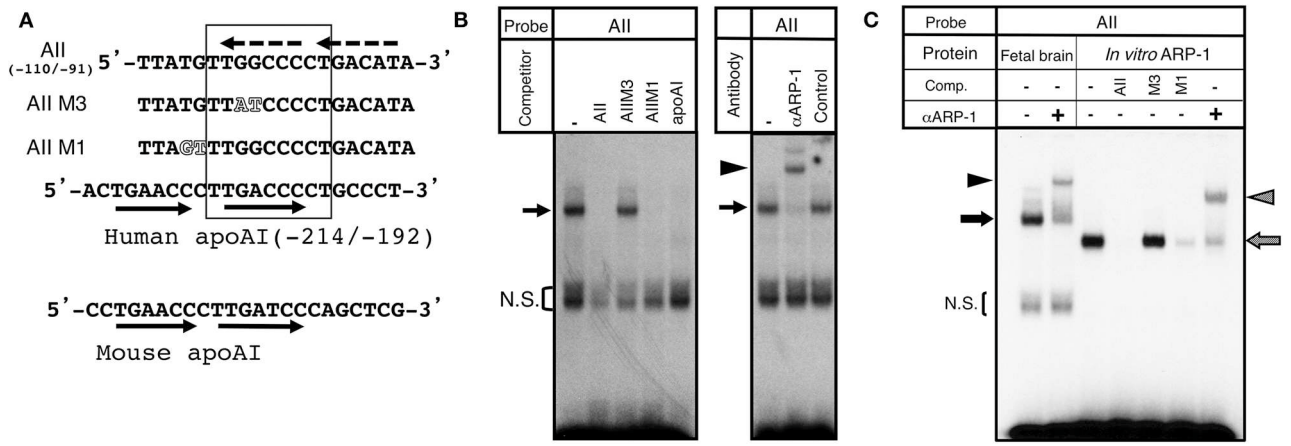

FIGURE 2 | Identification of ARP-1 as a candidate binding protein of the aro-AII site. **(A)** The aro-AII-binding protein recognized the sequence "TTGGCCCCT," which is shown inside the square. The wild-type and mutant oligonucleotide probes, AII, AIIM1, and AIIM3, are shown. Mutations introduced into the aro-AII oligonucleotide are shown by outlined characters. The nucleotide sequence of the ARP-1 binding site in the promoter region of the human and mouse ApoAI gene is also shown in the figure. Tandem repeats of human and mouse ApoAI oligonucleotides are shown in the oligonucleotide by arrows. The box indicates the portion of nucleotides essential for ARP-1 binding as described in our previous report. The putative tandem repeats in the AII probe are shown by broken arrows. **(B)** A gel shift assay was performed using 5 μg of nuclear protein. A 200-fold molar excess of unlabeled probe was used in the competition assay. The specific signals are indicated by the arrow on left side. The bracket shows the non-specific signals (N.S.). **(C)** For supershift assays, nuclear protein was incubated with 2 μg of the indicated antibody on ice for 30 min before the addition of a radiolabeled probe. The probe/nuclear protein complex and supershift signals are indicated by the arrow and arrowhead, respectively, on the left side.

mutated outside the sequence (AIIM1) could compete with the complex (**Figure 2B**, left panel). ARP-1 was found to be a transcriptional regulator of apolipoprotein A1 (31). A gel shift assay was performed using the ARP-1-binding site present in the promoter sequence of mouse apolipoprotein as a competitor (**Figure 2B**, left panel). A similar competition was also observed when the sequence present in the apolipoprotein gene was used as a competitor oligonucleotide. Moreover, the ARP-1 antibody super-shifted the complex formed by aro-AII and the nuclear factor on the gel shift assay (**Figure 2B**, right panel). To further conform the binding ability of ARP-1 to aro-AII, similar gel shift experiments were conducted using ARP-1 protein synthesized *in vitro* from the cDNA obtained by one-hybrid screening. As shown in **Figure 2C**, the ARP-1 synthesized *in vitro* had a slightly higher mobility complex than the nuclear protein, presumably because of the nine missing N-terminal amino acids from the wild-type ARP-1. The binding properties of the synthesized ARP-1 protein were apparently identical to those of the nuclear protein in brain extract. These results suggested that the binding protein of aro-AII is ARP-1.

ARP-1 Binds to aro-AII in the Brain-Specific Promoter 1f Region of the Mouse Aromatase Gene *in vivo*

In the E15 mouse brain, COUP-TFI, a protein with high homology and similar binding sites to ARP-1, is also observed. Thus, considering the possibility that COUP-TFI can also occupy the aro-AII site, we conducted a supershift analysis with specific antibodies by gel shift assay using fetal brain extract. As shown in **Figure 3A**, when the anti-ARP-1 antibody was added, a clear supershift band was observed, whereas only a faint supershift band was visible with the anti-COUP-TFI antibody. These results suggested that the majority of the protein that binds at the aro-AII site in the E15 fetal brain is ARP-1. To confirm that ARP-1 binds to the aro-AII site in the promoter 1f of the aromatase gene *in vivo*, we conducted a ChIP-PCR assay using the diencephalic region of E16 fetal mouse brains. As shown in **Figure 3B**, anti-ARP-1 IgG immunoprecipitated the promoter region containing the aro-AII site, resulting in an amplified DNA product on ChIP-PCR. In contrast, no PCR product was observed in the assay using control IgG. The results suggested that ARP-1 binds to the aro-AII *cis*-element in the 1f promoter region *in vivo*.

Regulatory Function of ARP-1 for the Brain-Specific Promoter 1f of the Aromatase Gene

To determine the regulatory effects of ARP-1 on the transcription from the promoter 1f of the aromatase gene, we performed a luciferase reporter assay. A luciferase reporter plasmid, pGL3aroBr, was transfected into CV-1 cells together with increasing amounts of ARP-1 expression plasmid. As shown in **Figure 4A**, ARP-1 dose-dependently enhanced luciferase reporter activity, reflecting the transcriptional activity of the promoter 1f, showing an ∼40-fold enhancement with 500 ng of ARP-1 expression plasmid. ARP-1 has been reported to repress the expression of the apoAI gene in human hepatoma-derived HepG2 cells (31). To examine whether the effects of ARP-1 on activity of the brain-specific aromatase promoter depend on the properties of cell lines, we conducted a luciferase reporter assay using HepG2 cells. ARP-1 repressed the promoter activity of the pGL3apoAI-containing apoAI promoter and enhancer in HepG2 cells. ARP-1, however, still showed the ability to enhance

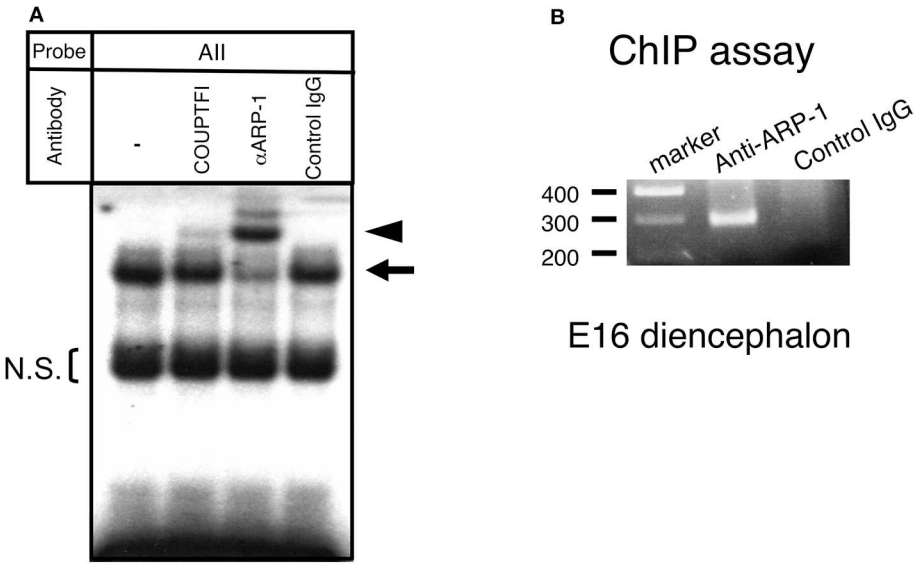

FIGURE 3 | ARP-1 protein binds to the aro-AII sequence *in vivo*. **(A)** A gel shift assay was performed using 5 μg of nuclear protein or an aliquot of *in vitro* synthesized ARP-1 protein. For supershift assays, nuclear protein was incubated with 2 μg of the indicated antibody on ice for 30 min before the addition of a radiolabeled probe. The probe/nuclear protein complex and supershift signals are indicated by the arrow and arrowhead, respectively, on the left side. The bracket shows the non-specific signals (N.S.). A 200-fold molar excess of unlabeled probe was used in the competition assay. The probe/ARP-1 complex and supershift signals are indicated by the hatched arrow and arrowhead, respectively, on the right side. **(B)** A chromatin immunoprecipitation assay confirmed that ARP-1 could associate with the aro-AII site in the fetal mouse brain *in vivo*. Fresh diencephalic regions of E16 mouse brains were treated with 1% formaldehyde. The fixed tissues were dissolved, and the DNA was sheared and immunoprecipitated with anti-ARP-1 antibody or preimmune IgG. The recovered genomic DNA was subjected to PCR with primers specific for the mouse aromatase gene as shown in **Figure 1**.

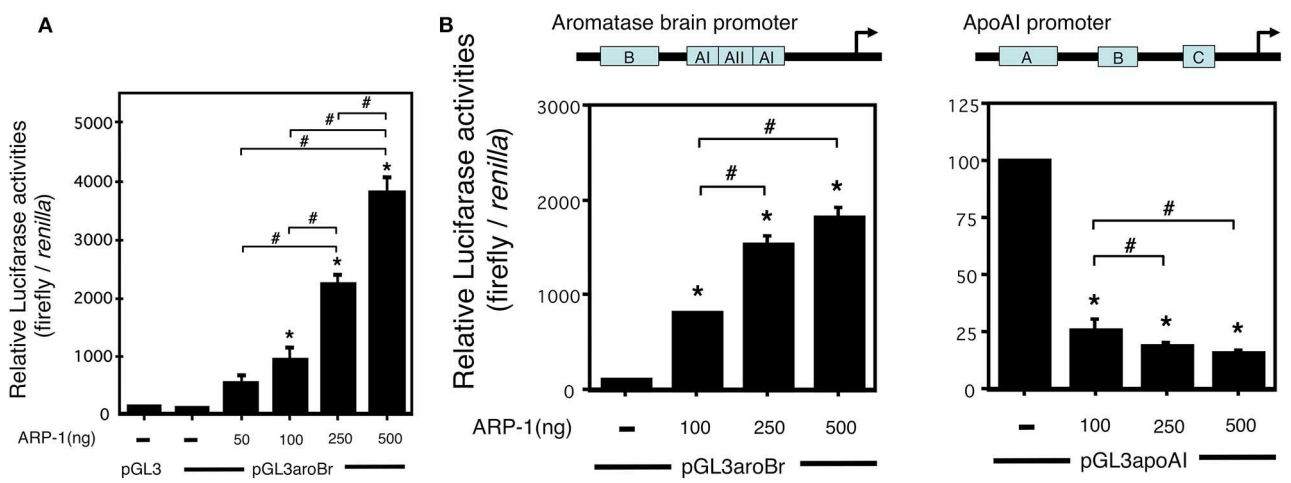

FIGURE 4 | ARP-1 increases brain-specific promoter activity of the mouse aromatase gene. **(A)** ARP-1 increases the promoter activity in a dose-dependent manner. The reporter plasmid (500 ng), pGL3aroBr, was co-transfected with the indicated amounts of pFLAG-ARP-1 plasmid (50, 100, 250, and 500 ng) and 50 ng of the internal control plasmid into CV-1 cells. The total amount of expression plasmid was adjusted to 500 ng with an empty plasmid (p3XFLAG-myc-CMV-26). The cells were harvested after 48 h, and a Dual-Luciferase Reporter Assay was carried out as described in the Materials and Methods section. **(B)** Effects of ARP-1 on the activity of aromatase and apolipoprotein AI promoters in HepG2 cells. A Dual-Luciferase Reporter Assay was conducted as described in **(A)**. The mean ± SEM of at least three independent experiments is shown in the figure. One-way ANOVA showed a significantly different distribution ($p < 0.0001$ for **(A,B)**. The p-value of the Tukey–Kramer test is indicated with the symbols as follows. Asterisks indicate statistically significant differences in relative promoter activity between the empty plasmid alone and that after co-transfection with the ARP-1 expression plasmid ($p < 0.05$). Pound signs indicate statistically significant differences in comparisons between indicated pairs ($p < 0.05$).

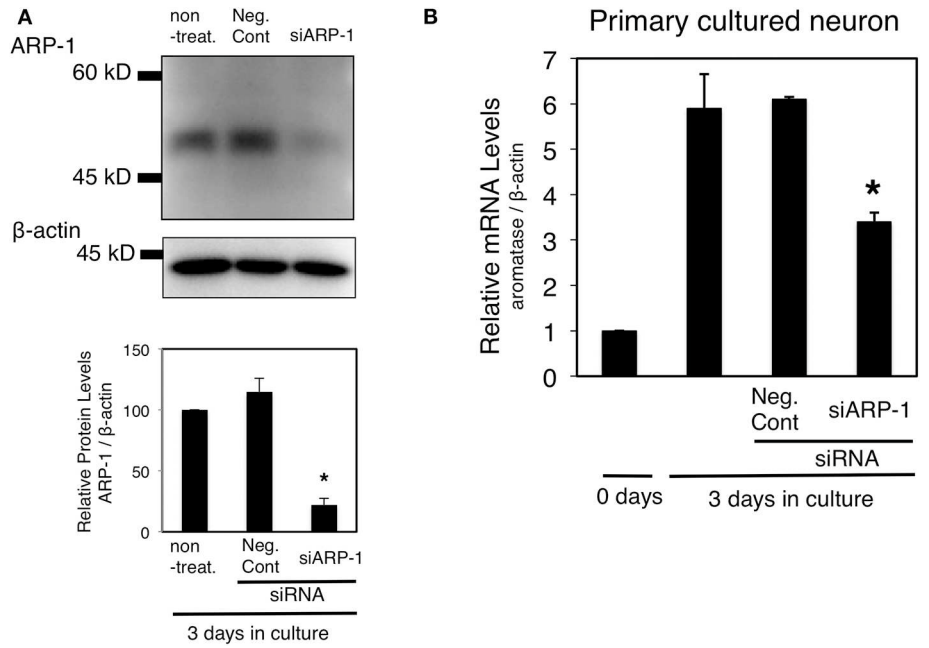

FIGURE 5 | Effect of ARP-1-targeting siRNA on expression of the endogenous aromatase gene in primary cultured neural cells. **(A)** Diencephalic neurons were transfected with ARP-1-targeting siRNA and cultured for 48 h, and then the lysates were subjected to western blotting with an anti-ARP-1 antibody and an anti-β-actin antibody (top panel). The amount of ARP-1 protein is quantitated and expressed as the relative ARP-1/β-actin value (lower panel). The mean ± SEM of three independent experiments is shown in the figure. One-way ANOVA showed a significantly different distribution ($p < 0.0002$). The p-value of the Tukey–Kramer test is indicated with the symbol as follows. Asterisks indicate statistically significant differences in ARP-1 protein levels between the ARP-1 siRNA and negative control siRNA groups ($p < 0.05$). **(B)** Effect of ARP-1 knockdown on the aromatase mRNA level in neural cells. Diencephalic neurons were prepared as in **(A)**, and total RNA was extracted from the cultured cells. The total RNA was analyzed by RT-qPCR to determine the amount of aromatase mRNA as described in the Materials and Methods section. The results are presented as the mean ± SEM of three independent experiments. One-way ANOVA showed a significantly different distribution ($p < 0.0002$). The p-value of the Tukey–Kramer test is indicated with the symbol as follows. Asterisks indicate statistically significant differences in aromatase mRNA levels between the ARP-1 siRNA and the negative control siRNA groups ($p < 0.05$).

promoter activity of the brain-specific aromatase in HepG2 cells (**Figure 4B**).

Previous studies have shown that *in vitro* cultured fetal diencephalic neurons can express aromatase mRNA (30). The mRNA expression level increased in a time-dependent manner for 3 days in E13 neurons prepared from the fetal diencephalon (32). To determine whether ARP-1 is required for aromatase expression in neurons, ARP-1 knockdown followed by real-time PCR was conducted. Addition of an siRNA against ARP-1 led to a significant decrease in the ARP-1 protein level in primary cultured neurons (**Figure 5A**). The aromatase mRNA level on day 3 in cultured neural cells was increased by ∼6-fold compared with the level on day 0 (in primary cultures derived from E13 mice). As shown in **Figure 5B**, ARP-1 knockdown by ARP-1-targeting siRNA decreased the increment of the aromatase mRNA level in cultured nerve cells by 43%.

DISCUSSION

We have previously analyzed the transcriptional mechanisms that regulate the brain-specific expression of the mouse aromatase gene, revealing three *cis*-elements in the promoter region. In the present study, we demonstrated that ARP-1, a member of the nuclear receptor superfamily, binds to the aro-AII site and is a positive transcriptional modulator of brain-specific expression of the aromatase gene. Cloning of ARP-1, also called COUP-TFII, revealed that it is highly homologous to COUP-TFI (33), and it was found to repress transcription of the apolipoprotein A1 gene via site A in its promoter region (31). The gel shift assays indicated that the ARP-1 protein was involved in aro-AII site binding. ARP-1 has been shown to bind to a wide spectrum of response elements encompassing AGGTCA direct repeats with various spacings, while it has the highest affinity for direct repeats of AGGTCA with one nucleotide spacing (DR1 element) (34). Our previous study revealed the essential nucleotide sequence for binding to the aro-AII site (29). The putative DR-1 was found in the nucleotide positions −110/−91 of the promoter region, although no apparent sequence of the direct repeat was observed within the essential nucleotide sequence for binding (**Figure 2A**). However, aro-AII with a mutation in one of the repeats (5′-ATGTCA to 5′-ATGcaA) retained binding activity with nuclear protein (29). Additional analysis is necessary to determine of the features of ARP-1 binding to the aro-AII site.

Expression of the aromatase gene in the mouse brain exhibits a transient peak during the perinatal period (35). A similar transient peak of expression was also observed in the *in*

vitro system, even if diencephalic tissues were dissociated and dispersed as individual neural cells (32). In this study, the luciferase reporter gene assay showed that ARP-1 activated transcription of the reporter gene in a dose-dependent manner (**Figure 4**). A decrease in ARP-1 protein caused by RNA interference reduced the spontaneous increase in aromatase mRNA levels in cultured neural cells from the diencephalic region of the fetal mouse brain (**Figure 5**). Moreover, ChIP-PCR analysis indicated that endogenous ARP-1 protein bound to the aro-AII site *in vivo* (**Figure 3**). These results suggest that ARP-1 is a transcription factor that positively regulates aromatase expression in the mouse brain via specific binding to the aro-AII site on the promoter 1f of the gene. ARP-1 can either positively or negatively modulate the expression of downstream genes through different mechanisms (34, 36, 37). ARP-1 exhibits positive regulation of the brain-specific promoter of the aromatase gene in both in CV-1 and HepG2 cells, suggesting that the function of ARP-1 in transcriptional regulation may depend on the target promoter context. Earlier, we demonstrated that a *LacZ* reporter gene driven by the −6.5-kb promoter region of the exon 1f showed almost the same spatiotemporal expression as the endogenous aromatase gene using transgenic mice (38). The −0.2-kb promoter region, however, partially reproduced endogenous aromatase expression, while the reporter gene was also observed in the extra-brain tissues (our unpublished observations (39). Moreover, a mutation introduced into the aro-AII site of the −6.5-kb promoter in the *LacZ* reporter caused a significant decrease in brain expression, and ectopic expression was observed. These results suggest that the aro-AII element is necessary, but not sufficient, for spatiotemporal expression of the aromatase gene in the brain.

ARP-1 is predominantly expressed in mesenchymal cells during organogenesis. The spatiotemporal expression of mouse COUP-TFs, including ARP-1, in the brain has also been determined (40). ARP-1 is first observed at approximately E8.5, peaks at E14–E15, and then decreases after birth (41, 42). In our previous report, an analysis of aro-AII binding activity in the brain at the perinatal, neonatal, and adult stages showed that the binding activity to nuclear protein was extremely reduced at the adult stage (29). These results are very consistent with the endogenous ARP-1 expression pattern. ARP-1 in the brain is predominantly localized in the diencephalon (43, 44, 61), while high expression of ARP-1 is also detected in the amygdaloid nucleus (45–47). Aromatase expression is initially observed at E13–14 in the mouse diencephalon; it subsequently increases at E16–17 and then decreases gradually to the adult expression level, with a transient peak during the perinatal period. The expression patterns of aromatase and ARP-1 in the brain are not completely identical, but these patterns are indeed overlapping.

Homozygous ARP-1 mutant mice exhibit various morphological abnormalities, such as defective angiogenesis and vascular remodeling, which result in death by E9.5 because of severe hemorrhage and edema in the brain and heart (48). Moreover, no homozygous ARP-1 mutant mice are detected at E11.5, and even if born, two-thirds of heterozygous mutants die before weaning (48). Recently, ventral forebrain–specific disruption of the ARP-1 gene has been shown to cause agenesis of the basomedial amygdala nucleus, indicating that the ARP-1 gene directs neuronal progenitor cells to generate the basomedial amygdala nucleus (47). Moreover, mice with a ventromedial hypothalamus–specific ARP-1 mutant gene have also been generated using *Cre* driven by the Ad4BP/SF-1 promoter (49). Unfortunately, the homozygous hypothalamus–specific mutants displayed embryonic lethality. New model animals may be needed to determine the interaction of ARP-1 with the brain-specific aromatase gene *in vivo*.

Estradiol-17β has been reported to serve as a regulatory factor that controls the expression of aromatase and its enzymatic activity in the brain (50–55). Yilmaz et al. investigated the brain-specific promoter using a murine hypothalamic neuronal cell line. They showed that estradiol regulated brain-specific aromatase transcription, and the −200/−1 region of promoter 1f participated in the estrogen responsiveness. Typical palindromic estrogen–responsive elements or their half-sites were not identified in the promoter region, while two AP-1-binding sites in the region might be essential for induction of transcriptional activity by estradiol. Binding of estrogen receptor α and c-Jun to AP-1-binding sites might positively regulate the transcriptional activity of the promoter 1f (56). Bulun and colleagues also reported functions of progesterone and glucocorticoid receptors as negative and positive regulators, respectively, for activity of the promoter 1f (57, 58). The inverse regulation of 1f promoter activity by progesterone and glucocorticoid is interesting considering that progesterone and glucocorticoid response elements share the same sequence. Interestingly, Cisternas et al. reported that estradiol increased aromatase mRNA and protein levels only in female neuronal cells from the anterior amygdala of embryonic brains (59). Estrogen receptor β is involved in the incremental expression of aromatase by estradiol and binds 5α-androstane-3β,17β-diol as a ligand in addition to estradiol (60). The aromatase expression patterns, which are specific for developmental stages or brain regions, may be produced using functional complexes consisting of multiple transcription factors and various hormonal factors. The local production of estrogen in the brain may serve as a neurosteroid during organization of the neuronal network, including sexually dimorphic nuclei, and in motivation/activation of sexual behaviors.

Taken together, these data support that ARP-1 is a transcription factor that regulates aromatase expression in the brain by binding to the aro-AII site on the promoter. Further studies on the transcription factors and their cofactors should be performed to elucidate the molecular processes of the spatiotemporal expression of brain aromatase and the biological processes of the organization and activation effects of estrogen.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are included in this published article.

AUTHOR CONTRIBUTIONS

SH and NH designed this work and wrote the manuscript. SH performed the experiments and analyzed the data.

FUNDING

This work was supported in part by a Grant-in-Aid for Scientific Research (C) from Japan Society for the Promotion of Science (KAKENHI Grant No 26461389).

ACKNOWLEDGMENTS

We thank Lisa Kreiner, PhD, from Edanz Group (https://en-author-services.edanzgroup.com/) for editing a draft of this manuscript.

REFERENCES

1. Baulieu EE. Neurosteroids: of the nervous system, by the nervous system, for the nervous system. *Recent Prog Horm Res*. (1997) 52:1–32.
2. Tsutsui K, Ukena K, Takase M, Kohchi C, Lea RW. Neurosteroid biosynthesis in vertebrate brains. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol*. (1999) 124:121–9. doi: 10.1016/S0742-8413(99)00065-1
3. Tsutsui K, Ukena K, Usui M, Sakamoto H, Takase M. Novel brain function: biosynthesis and actions of neurosteroids in neurons. *Neurosci Res*. (2000) 36:261–73. doi: 10.1016/S0168-0102(99)00132-7
4. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science*. (2005) 307:1625–30. doi: 10.1126/science.1106943
5. Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology*. (2005) 146:624–32. doi: 10.1210/en.2004-1064
6. Prossnitz ER, Barton M. The G-protein-coupled estrogen receptor GPER in health and disease. *Nat Rev Endocrinol*. (2011) 7:715–26. doi: 10.1038/nrendo.2011.122
7. Genissel C, Carreau S. Regulation of the aromatase gene expression in mature rat Leydig cells. *Mol Cell Endocrinol*. (2001) 178:141–6. doi: 10.1016/S0303-7207(01)00409-9
8. Carreau S, Lambard S, Delalande C, Denis-Galeraud I, Bilinska B, Bourguiba S. Aromatase expression and role of estrogens in male gonad: a review. *Reprod Biol Endocrinol*. (2003) 1:35. doi: 10.1186/1477-7827-1-35
9. Stocco C. Aromatase expression in the ovary: hormonal and molecular regulation. *Steroids*. (2008) 73:473–87. doi: 10.1016/j.steroids.2008.01.017
10. Roselli CE, Horton LE, Resko JA. Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system. *Endocrinology*. (1985) 117:2471–7. doi: 10.1210/endo-117-6-2471
11. Balthazart J, Foidart A, Surlemont C, Harada N. Distribution of aromatase-immunoreactive cells in the mouse forebrain. *Cell Tissue Res*. (1991) 263:71–9. doi: 10.1007/BF00318401
12. Lauber ME, Lichtensteiger W. Pre- and postnatal ontogeny of aromatase cytochrome P450 messenger ribonucleic acid expression in the male rat brain studied by *in situ* hybridization. *Endocrinology*. (1994) 135:1661–8. doi: 10.1210/endo.135.4.7925130
13. Shinoda K, Nagano M, Osawa Y. Neuronal aromatase expression in preoptic, strial, and amygdaloid regions during late prenatal and early postnatal development in the rat. *J Comp Neurol*. (1994) 343:113–29. doi: 10.1002/cne.903430109
14. Fisher CR, Graves KH, Parlow AF, Simpson ER. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proc Natl Acad Sci USA*. (1998) 95:6965–70. doi: 10.1073/pnas.95.12.6965
15. Honda S, Harada N, Ito S, Takagi Y, Maeda S. Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the cyp19 gene. *Biochem Biophys Res Commun*. (1998) 252:445–9. doi: 10.1006/bbrc.1998.9672
16. Toda K, Takeda K, Okada T, Akira S, Saibara T, Kaname T, et al. Targeted disruption of the aromatase P450 gene (Cyp19) in mice and their ovarian and uterine responses to 17beta-oestradiol. *J Endocrinol*. (2001) 170:99–111. doi: 10.1677/joe.0.1700099
17. Robertson KM, Simpson ER, Lacham-Kaplan O, Jones ME. Characterization of the fertility of male aromatase knockout mice. *J Androl*. (2001) 22:825–30. doi: 10.1002/j.1939-4640.2001.tb02587.x
18. Toda K, Okada T, Takeda K, Akira S, Saibara T, Shiraishi M, et al. Oestrogen at the neonatal stage is critical for the reproductive ability of male mice as revealed by supplementation with 17beta-oestradiol to aromatase gene (Cyp19) knockout mice. *J Endocrinol*. (2001) 168:455–63. doi: 10.1677/joe.0.1680455
19. Matsumoto T, Honda S, Harada N. Alteration in sex-specific behaviors in male mice lacking the aromatase gene. *Neuroendocrinology*. (2003) 77:416–24. doi: 10.1159/000071313
20. Honda S-I, Wakatsuki T, Harada N. Behavioral analysis of genetically modified mice indicates essential roles of neurosteroidal estrogen. *Front Endocrin*. (2011) 2:40. doi: 10.3389/fendo.2011.00040
21. Means GD, Kilgore MW, Mahendroo MS, Mendelson CR, Simpson ER. Tissue-specific promoters regulate aromatase cytochrome P450 gene expression in human ovary and fetal tissues. *Mol Endocrinol*. (1991) 5:2005–13. doi: 10.1210/mend-5-12-2005
22. Harada N, Utsumi T, Takagi Y. Tissue-specific expression of the human aromatase cytochrome P-450 gene by alternative use of multiple exons 1 and promoters, and switching of tissue-specific exons 1 in carcinogenesis. *Proc Natl Acad Sci USA*. (1993) 90:11312–6. doi: 10.1073/pnas.90.23.11312
23. Mahendroo MS, Mendelson CR, Simpson ER. Tissue-specific and hormonally controlled alternative promoters regulate aromatase cytochrome P450 gene expression in human adipose tissue. *J Biol Chem*. (1993) 268:19463–70.
24. Honda S, Harada N, Takagi Y. Novel exon 1 of the aromatase gene specific for aromatase transcripts in human brain. *Biochem Biophys Res Commun*. (1994) 198:1153–60. doi: 10.1006/bbrc.1994.1163
25. Honda S, Harada N, Takagi Y. The alternative exons 1 of the mouse aromatase cytochrome P-450 gene. *Biochim Biophys Acta*. (1996) 1305:145–50. doi: 10.1016/0167-4781(95)00200-6
26. Simpson ER, Michael MD, Agarwal VR, Hinshelwood MM, Bulun SE, Zhao Y. Cytochromes P450 11: expression of the CYP19 (aromatase) gene: an unusual case of alternative promoter usage. *FASEB J*. (1997) 11:29–36. doi: 10.1096/fasebj.11.1.9034163
27. Honda S, Harada N, Abe-Dohmae S, Takagi Y. Identification of cis-acting elements in the proximal promoter region for brain-specific exon 1 of the mouse aromatase gene. *Brain Res Mol Brain Res*. (1999) 66:122–32. doi: 10.1016/S0169-328X(99)00017-0
28. Honda S, Kozako T, Shimeno H, Soeda S, Harada N. LIM-homeodomain transcription factor, Lhx2, is involved in transcriptional control of brain-specific promoter/exon 1f of the mouse aromatase gene. *J Neuroendocrinol*. (2012) 24:1367–74. doi: 10.1111/j.1365-2826.2012.02356.x
29. Honda SI, Matsumoto T, Harada N. Characterization and purification of a protein binding to the cis-acting element for brain-specific exon 1 of the mouse aromatase gene. *J Steroid Biochem Mol Biol*. (2001) 79:255–60. doi: 10.1016/S0960-0760(01)00142-X
30. Abe-Dohmae S, Tanaka R, Harada N. Cell type- and region-specific expression of aromatase mRNA in cultured brain cells. *Brain Res Mol Brain Res*. (1994) 24:153–8. doi: 10.1016/0169-328X(94)90127-9
31. Ladias JA, Karathanasis SK. Regulation of the apolipoprotein AI gene by ARP-1, a novel member of the steroid receptor superfamily. *Science*. (1991) 251:561–5. doi: 10.1126/science.1899293
32. Abe-Dohmae S, Tanaka R, Takagi Y, Harada N. *In vitro* increase of aromatase mRNA in diencephalic neurons. *Neuroendocrinology*. (1996) 63:46–52. doi: 10.1159/000126934
33. Wang LH, Ing NH, Tsai SY, O'malley BW, Tsai MJ. The COUP-TFs compose a family of functionally related transcription factors. *Gene Expr*. (1991) 1:207–16.
34. Tsai SY, Tsai MJ. Chick ovalbumin upstream promoter-transcription factors (COUP-TFs): coming of age. *Endocr Rev*. (1997) 18:229–40. doi: 10.1210/er.18.2.229

35. Harada N, Yamada K. Ontogeny of aromatase messenger ribonucleic acid in mouse brain: fluorometrical quantitation by polymerase chain reaction. *Endocrinology*. (1992) 131:2306–12. doi: 10.1210/endo.131.5.1425429
36. Lin FJ, Qin J, Tang K, Tsai SY, Tsai MJ. Coup d'Etat: an orphan takes control. *Endocr Rev*. (2011) 32:404–21. doi: 10.1210/er.2010-0021
37. Tang K, Tsai SY, Tsai MJ. COUP-TFs and eye development. *Biochim Biophys Acta*. (2015) 1849:201–9. doi: 10.1016/j.bbagrm.2014.05.022
38. Harada N, Honda S. Analysis of spatiotemporal regulation of aromatase in the brain using transgenic mice. *J Steroid Biochem Mol Biol*. (2005) 95:49–55. doi: 10.1016/j.jsbmb.2005.04.003
39. Nausch N, Manteuffel G, Vanselow J. 0.2kb promoter sequence of the murine Cyp19 gene target beta-galactosidase expression to specific brain areas of transgenic mice. *J Steroid Biochem Mol Biol*. (2007) 103:119–28. doi: 10.1016/j.jsbmb.2006.08.007
40. Yang X, Feng S, Tang K. COUP-TF genes, human diseases, and the development of the central nervous system in murine models. *Curr Top Dev Biol*. (2017) 125:275–301. doi: 10.1016/bs.ctdb.2016.12.002
41. Jonk LJ, De Jonge ME, Pals CE, Wissink S, Vervaart JM, Schoorlemmer J, et al. Cloning and expression during development of three murine members of the COUP family of nuclear orphan receptors. *Mech Dev*. (1994) 47:81–97. doi: 10.1016/0925-4773(94)90098-1
42. Qiu Y, Cooney AJ, Kuratani S, Demayo FJ, Tsai SY, Tsai MJ. Spatiotemporal expression patterns of chicken ovalbumin upstream promoter-transcription factors in the developing mouse central nervous system: evidence for a role in segmental patterning of the diencephalon. *Proc Natl Acad Sci USA*. (1994) 91:4451–5. doi: 10.1073/pnas.91.10.4451
43. Tripodi M, Filosa A, Armentano M, Studer M. The COUP-TF nuclear receptors regulate cell migration in the mammalian basal forebrain. *Development*. (2004) 131:6119–29. doi: 10.1242/dev.01530
44. Kurrasch DM, Cheung CC, Lee FY, Tran PV, Hata K, Ingraham HA. The neonatal ventromedial hypothalamus transcriptome reveals novel markers with spatially distinct patterning. *J Neurosci*. (2007) 27:13624–34. doi: 10.1523/JNEUROSCI.2858-07.2007
45. Kanatani S, Yozu M, Tabata H, Nakajima K. COUP-TFII is preferentially expressed in the caudal ganglionic eminence and is involved in the caudal migratory stream. *J Neurosci*. (2008) 28:13582–91. doi: 10.1523/JNEUROSCI.2132-08.2008
46. Willi-Monnerat S, Migliavacca E, Surdez D, Delorenzi M, Luthi-Carter R, Terskikh AV. Comprehensive spatiotemporal transcriptomic analyses of the ganglionic eminences demonstrate the uniqueness of its caudal subdivision. *Mol Cell Neurosci*. (2008) 37:845–56. doi: 10.1016/j.mcn.2008.01.009
47. Tang K, Rubenstein JL, Tsai SY, Tsai MJ. COUP-TFII controls amygdala patterning by regulating neuropilin expression. *Development*. (2012) 139:1630–9. doi: 10.1242/dev.075564
48. Pereira FA, Qiu Y, Zhou G, Tsai MJ, Tsai SY. The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. *Genes Dev*. (1999) 13:1037–49. doi: 10.1101/gad.13.8.1037
49. Sabra-Makke L, Maritan M, Planchais J, Boutant M, Pegorier JP, Even PC, et al. Hypothalamic ventromedial COUP-TFII protects against hypoglycemia-associated autonomic failure. *Proc Natl Acad Sci USA*. (2013) 110:4333–8. doi: 10.1073/pnas.1219262110
50. Reddy VV, Naftolin F, Ryan KJ. Aromatization in the central nervous system of rabbits: effects of castration and hormone treatment. *Endocrinology*. (1973) 92:589–94. doi: 10.1210/endo-92-2-589
51. Pasmanik M, Callard GV. Changes in brain aromatase and 5 alpha-reductase activities correlate significantly with seasonal reproductive cycles in goldfish (*Carassius Auratus*). *Endocrinology*. (1988) 122:1349–56. doi: 10.1210/endo-122-4-1349
52. Negri-Cesi P, Colciago A, Motta M, Martini L, Celotti F. Aromatase expression and activity in male and female cultured rat hypothalamic neurons: effect of androgens. *Mol Cell Endocrinol*. (2001) 178:1–10. doi: 10.1016/S0303-7207(01)00442-7
53. Kretz O, Fester L, Wehrenberg U, Zhou L, Brauckmann S, Zhao S, et al. Hippocampal synapses depend on hippocampal estrogen synthesis. *J Neurosci*. (2004) 24:5913–21. doi: 10.1523/JNEUROSCI.5186-03.2004
54. Iivonen S, Heikkinen T, Puolivali J, Helisalmi S, Hiltunen M, Soininen H, et al. Effects of estradiol on spatial learning, hippocampal cytochrome P450 19, and estrogen alpha and beta mRNA levels in ovariectomized female mice. *Neuroscience*. (2006) 137:1143–52. doi: 10.1016/j.neuroscience.2005.10.023
55. Zhao C, Fujinaga R, Tanaka M, Yanai A, Nakahama K, Shinoda K. Region-specific expression and sex-steroidal regulation on aromatase and its mRNA in the male rat brain: immunohistochemical and *in situ* hybridization analyses. *J Comp Neurol*. (2007) 500:557–73. doi: 10.1002/cne.21193
56. Yilmaz MB, Wolfe A, Cheng YH, Glidewell-Kenney C, Jameson JL, Bulun SE. Aromatase promoter I.f is regulated by estrogen receptor alpha (ESR1) in mouse hypothalamic neuronal cell lines. *Biol Reprod*. (2009) 81:956–65. doi: 10.1095/biolreprod.109.077206
57. Yilmaz MB, Wolfe A, Zhao H, Brooks C, Bulun SE. Aromatase promoter I.f is regulated by progesterone receptor (Pgr) in mouse hypothalamic neuronal cell lines. *J Mol Endocrinol*. (2011) 47:69–80. doi: 10.1530/JME-10-0149
58. Brooks DC, Zhao H, Yilmaz MB, Coon VJ, Bulun SE. Glucocorticoid-induction of hypothalamic aromatase via its brain-specific promoter. *Mol Cell Endocrinol*. (2012) 362:85–90. doi: 10.1016/j.mce.2012.05.012
59. Cisternas CD, Garcia-Segura LM, Cambiasso MJ. Hormonal and genetic factors interact to control aromatase expression in the developing brain. *J Neuroendocrinol*. (2018) 30:e12535. doi: 10.1111/jne.12535
60. Cisternas CD, Cabrera Zapata LE, Arevalo MA, Garcia-Segura LM, Cambiasso MJ. Regulation of aromatase expression in the anterior amygdala of the developing mouse brain depends on ERbeta and sex chromosome complement. *Sci Rep*. (2017) 7:5320. doi: 10.1038/s41598-017-05658-6
61. Lopes Da Silva S, Cox JJ, Jonk LJ, Kruijer W, Burbach JP. Localization of transcripts of the related nuclear orphan receptors COUP-TF I and ARP-1 in the adult mouse brain. *Brain Res Mol Brain Res*. (1995) 30:131–6. doi: 10.1016/0169-328X(94)00289-Q

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.

frontiers in Endocrinology

PERSPECTIVE
published: 24 June 2020
doi: 10.3389/fendo.2020.00417

Anti-apoptotic Actions of Allopregnanolone and Ganaxolone Mediated Through Membrane Progesterone Receptors (PAQRs) in Neuronal Cells

Peter Thomas and Yefei Pang*

Marine Science Institute, University of Texas at Austin, Port Aransas, TX, United States

OPEN ACCESS

Edited by:
Hubert Vaudry,
Université de Rouen, France

Reviewed by:
Neil James MacLusky,
University of Guelph, Canada
David Chatenet,
Institut National de la Recherche Scientifique (INRS), Canada
Suchitra Joshi,
University of Virginia, United States

****Correspondence:***
Peter Thomas
peter.thomas@utexas.edu

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

Received: *25 March 2020*
Accepted: *26 May 2020*
Published: *24 June 2020*

Citation:
Thomas P and Pang Y (2020) Anti-apoptotic Actions of Allopregnanolone and Ganaxolone Mediated Through Membrane Progesterone Receptors (PAQRs) in Neuronal Cells. Front. Endocrinol. 11:417. doi: 10.3389/fendo.2020.00417

The neurosteroids progesterone and allopregnanolone regulate numerous neuroprotective functions in neural tissues including inhibition of epileptic seizures and cell death. Many of progesterone's actions are mediated through the nuclear progesterone receptor (PR), while allopregnanolone is widely considered to be devoid of hormonal activity and instead acts through modulation of GABA-$_A$ receptor activity. However, allopregnanolone can also exert hormonal actions in neuronal cells through binding and activating membrane progesterone receptors (mPRs) belonging to the progestin and adipoQ receptor (PAQR) family. The distribution and functions of the five mPR subtypes (α, β, γ, δ, ε) in neural tissues are briefly reviewed. mPRδ has the highest binding affinity for allopregnanolone and is highly expressed throughout the human brain. Low concentrations (20 nM) of allopregnanolone act through mPRδ to stimulate G protein (G_s)-dependent signaling pathways resulting in reduced cell death and apoptosis in mPRδ-transfected cells. The 3-methylated synthetic analog of allopregnanolone, ganaxolone, is currently undergoing clinical trials as a promising GABA-$_A$ receptor-selective antiepileptic drug (AED). New data show that low concentrations (20 nM) of ganaxolone also activate mPRδ signaling and exert anti-apoptotic actions through this receptor. Preliminary evidence suggests that ganaxolone can also exert neuroprotective effects by activating inhibitory G protein (G_i)-dependent signaling through mPRα and/or mPRβ in neuronal cells. The results indicate that mPRs are likely intermediaries in multiple actions of natural and synthetic neurosteroids in the brain. Potential off-target effects of ganaxolone through activation of mPRs in patients receiving long-term treatment for epilepsy and other disorders should be considered and warrant further investigation.

Keywords: membrane progesterone receptors, PAQR agonists, ganaxolone, allopregnanolone, inhibition apoptosis, neuronal cells, AED, neuroprotective

INTRODUCTION

Progesterone and its metabolites produced in neural tissues (neurosteroids, **Figure 1A**) such as allopregnanolone mediate a wide variety of actions in the brain including neuroprotection, anti-apoptosis, inhibition of epileptic seizures, reproductive behaviors, neuroendocrine control of reproduction, and both pro-tumorigenesis and anti-tumorigenesis (1–3). Many genomic actions of progesterone in neural tissues are mediated through PR whereas the neurosteroid allopregnanolone has negligible binding affinity for the PR and instead interacts with GABA-$_A$ receptors resulting in decreases in their activities and also activates the pregnane X receptor (PXR) (3–6). However, progesterone actions have also been observed in the brain which are PR-independent (i.e., persist in PR knockout mice) and in neuronal cells which have low expression of PRs (e.g., GnRH-producing GT1-7 cells) (7–9). Evidence has accumulated that some of these actions may be mediated through membrane progesterone receptors (mPRs) (4, 10, 11), 7-transmembrane receptors coupled to G proteins belonging to the progestin and adipoQ receptor (PAQR) family which is unrelated to the GPCR superfamily (12, 13). Moreover, recent studies with cultured neuronal cells show that low concentrations of progesterone and allopregnanolone exert hormonal actions through binding and activating mPRs, resulting in rapid induction of intracellular signaling pathways and anti-apoptosis (14, 15). Collectively, these results suggest that mPRs are likely intermediaries of progesterone and allopregnanolone actions in neural tissues, with potential implications for human health and disease.

The mechanisms by which progesterone, allopregnanolone, and an antiepileptic drug, ganaxolone, exert their protective actions in epilepsy are summarized here. The characteristics of mPRs, their distribution in brains of humans and rodents, and their proposed functions in the central nervous system are briefly discussed. The anti-apoptotic actions of allopregnanolone in neuronal cells and in mPR-transfected cancer cells that are mediated through mPR-dependent signaling pathways are reviewed. Ganaxolone, a synthetic analog of allopregnanolone, is currently undergoing clinical trials as a third generation AED that targets GABA-$_A$ receptors (16, 17). New data are presented showing that ganaxolone binds to mPRs and mimics the anti-apoptotic actions of allopregnanolone and progesterone in these cultured cells. These results indicate that clinical studies with ganaxolone should include investigations of possible additional unexpected, off-target effects of the drug through activation of mPRs.

PROTECTIVE EFFECTS OF NEUROSTEROIDS AGAINST EPILEPTIC SEIZURES

Epilepsy is a severe neurological disorder that affects over 50 million people throughout the world (16, 18). Progesterone exerts anticonvulsant effects in animal epilepsy models through a PR-independent mechanism as they are not decreased in PR knockout (PRKO) mice (19). Instead, progesterone's anticonvulsant potency is increased in PRKO mice which is consistent with results showing activation of PR in a status epilepticus rat model increases seizure frequency (19, 20). Progesterone's antiseizure actions are dependent on its conversion to allopregnanolone since cotreatment with the 5α-reductase inhibitor, finasteride, blocks progesterone's actions (19). Allopregnanolone displays very weak binding affinity for PRs (21) and exerts its protective effects against seizures through a PR-independent mechanism. Allopregnanolone acts through positive allosteric modulation of GABA-$_A$ receptor activity, and can also activate the receptors at higher concentrations in the absence of GABA (22, 23). Positive modulation of GABA-$_A$ receptors by allopregnanolone enhances inhibitory chloride conductance which in turn decreases neuronal excitability and reduces the incidence of seizures (19, 24). Although over 20 AEDs have been used to treat this disease, these treatments are ineffective in controlling seizures in a third of epileptic patients and long-term treatment with enzyme-modulating AEDs can cause endocrine, metabolic, and reproductive disorders (25). The 3β-methylated synthetic analog of allopregnanolone, ganaxolone (3α-hydroxy-3β-methyl-5α-pregnan-20-one, **Figure 1A**) is a promising fourth generation AED that is currently completing phase III clinical trials (16). Ganaxolone has been shown to have activity in several animal epilepsy models and is effective in infants with spasm and in adults with partial-onset seizures (17). Ganaxolone is also potentially useful for treatment of mood and anxiety disorders (16) and has been shown to improve sociability in a rodent model of autism spectrum disorder, which indicates it may increase sociability in autistic patients (26). Although ganaxolone can cause sedation in epilepsy patients, few other adverse effects of long-term administration of the drug have been observed to date in clinical trials. Methylation at the 3β position of ganaxolone impairs its metabolism to inactive metabolites, thereby increasing its period of effectiveness in inhibiting seizures compared to allopregnanolone (26). Like allopregnanolone, ganaxolone is an allosteric modulator of GABA-$_A$ receptors and acts through different allosteric binding sites to that of benzodiazepines, as revealed by ligand binding assays and receptor mutational analysis (17, 27, 28).

MEMBRANE PROGESTERONE RECEPTORS (mPRs, PAQRs)

Progesterone exerts hormonal actions in numerous cell and animal models through activation of membrane progesterone receptors (mPRs) belonging to the progesterone and adipoQ receptor (PAQR) family (29). These novel 7-transmembrane receptors were initially discovered in teleost fish gonads and their homologs were subsequently identified in other vertebrate classes (30, 31). mPRs mediate rapid, non-classical progesterone actions, which are frequently non-genomic, by activating G proteins and modulation of intracellular signaling pathways. The five mPR members of the PAQR family, mPRα (PAQR7), mPRβ (PAQR8), mPRγ (PAQR5), mPRδ (PAQR6), and mPRε (PAQR9), have different tissue distributions, progestin binding

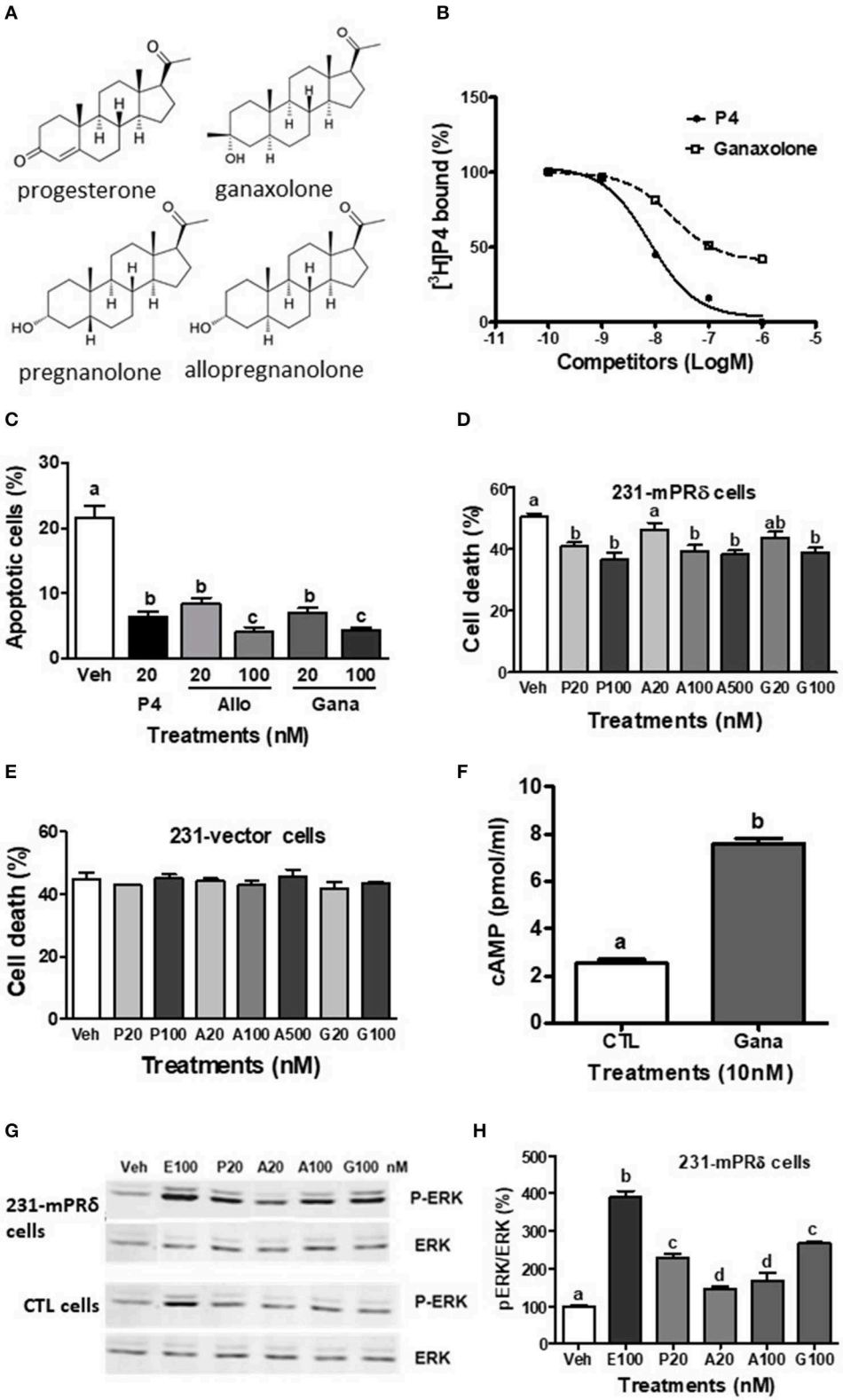

FIGURE 1 | Interactions of ganaxolone with human mPRδ (PAQR6). **(A)** Structures of ganaxolone and several other neurosteroids. **(B)** Representative competition curve of ganaxolone binding to plasma membranes of mPRδ-transfected MDA-MB-231 cells (231-mPRδ) expressed as percentage of maximum [^{3}H]-progesterone binding. Ganaxolone was added to the assay buffer dissolved in ethanol. Ethanol was 0.1% of total volume, which did not affect [^{3}H]-progesterone binding. P4,

(Continued)

FIGURE 1 | progesterone. **(C,E,F)** Effects of 4 days treatment with progesterone (P4), allopregnanolone (Allo) and ganaxolone (Gana) on serum starvation-induced percent apoptotic cells detected with a TUNEL assay kit **(C)** and percent cell death detected by trypan blue staining **(E,F)** of the vector-transfected MDA MB-231 cells and 231-mPRδ cells, $N = 3$. **(D)** Effect of treatment with ganaxolone (10 nM) for 15 min. on cellular cAMP levels in 231-mPRδ cells. $N = 3$. **(G,H)** Representative Western blot analysis and quantification of effects of 20-min treatments with progesterone (P), allopregnanolone (A), and ganaxolone (G) on activation of ERK. P-ERK: phosphorylated ERK, ERK: total ERK in the vector- (CTL cells), and 231-mPRδ cells. E100: 100 nM EGF as a positive control. The bar graph shows relative densitometry changes of the bands in Western blot images ($N = 3$). Results were analyzed by one-way ANOVA, followed by Newman-Keul's multiple comparison test. Treatment groups that are significantly different from each other in the *post hoc* test ($P < 0.05$) are indicated by different letters. Experiments were repeated three or more times, and similar results and similar significant differences between treatment groups were obtained on each occasion. See Pang et al. (15) for descriptions of reagents, culture conditions and assay procedures.

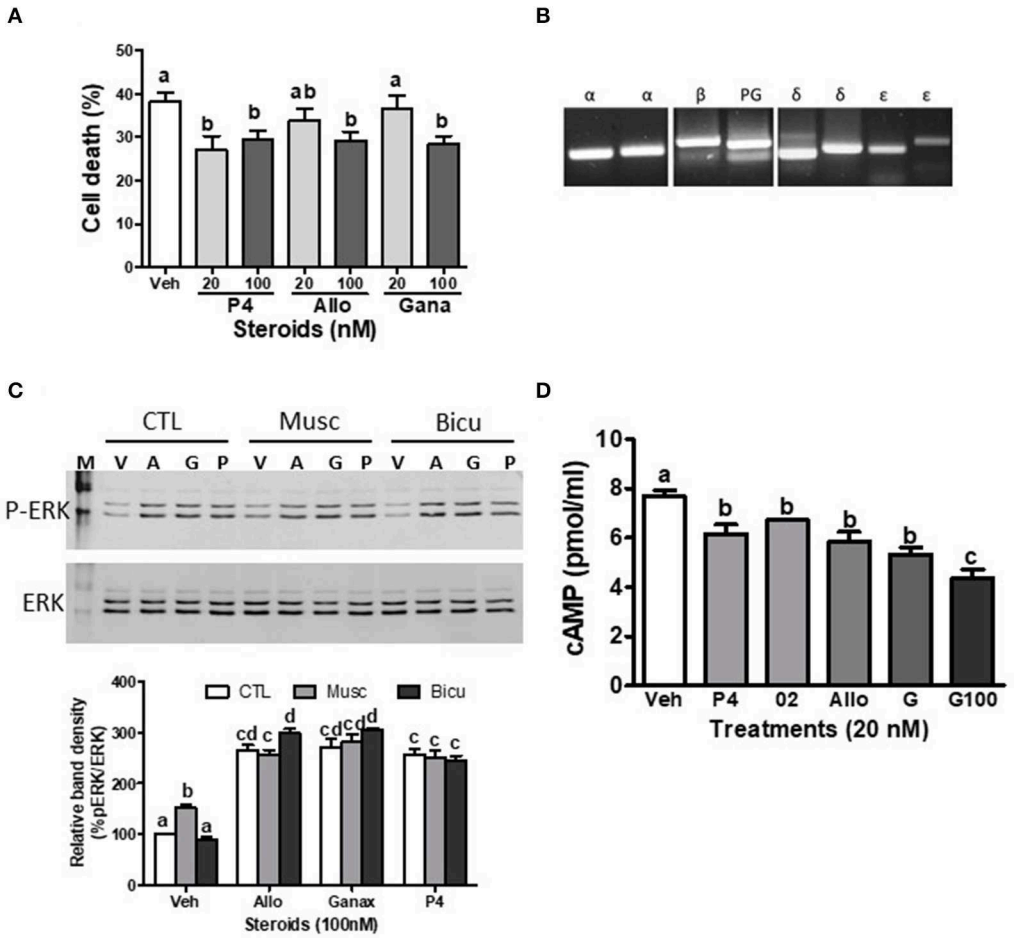

FIGURE 2 | Effects of ganaxolone on rodent neuronal cell lines. **(A)** Effects of 4 days treatment with 20 nM and 100 nM progesterone (P4), allopregnanolone (Allo) and ganaxolone (Gana) on cell death of mouse hypothalamic GT1-7 cells. $N = 3$. **(B)** Detection of mPRα (α), mPRβ (β), mPRδ (δ), mPRε (ε), and progesterone receptor membrane component 1, PGRMC1 (PG) mRNA expression by RT-PCR in immortalized rat hippocampal H19-7 cells. **(C)** Representative Western blot analysis of effects of pre-incubation with muscimol (Musc, 100 μM) and bicuculline (Bicu, 1 μM) for 20 min on neurosteroid-induced (100 nM, for 20 min.) activation of ERK. P-ERK, phosphorylated ERK; ERK, total ERK in H19-7 cells; V, vehicle control; A, allopregnanolone; G, ganaxolone; P, progesterone. The bar graph shows relative densitometry changes of the bands in Western blot images ($N = 3$). **(D)** Effects of 15 min. treatments with 20 nM progesterone (P4), Org OD 02-0 (02), allopregnanolone (Allo) and ganaxolone (G, 20 and 100 nM) on cAMP levels in H19-7 cells. ($N = 3$). Results were analyzed by one-way ANOVA, followed by Newman-Keul's multiple comparison test. Treatment groups that are significantly different from each other in the *post hoc* test ($P < 0.05$) are indicated by different letters. Experiments were repeated three or more times, and similar results and similar significant differences between treatment groups were obtained on each occasion. See Pang et al. (15) for descriptions of reagents, culture conditions, and assay procedures.

specificities, signal transduction pathways, and functions in vertebrate cells and tissues (12–15). mPRα is the predominant mPR isoform expressed in most progesterone target tissues with the exception of the brain and is the primary mPR that regulates several critical reproductive and non-reproductive progestin functions. For example, mPRα mediates oocyte meiotic maturation and sperm motility in fish, anti-apoptosis in fish ovarian granulosa cells and in human breast cancer

cells (32, 33), relaxation of human myometrial and vascular muscle cells (34, 35), reversal of epithelial to mesenchymal transition in breast cancer cells (36), and inhibition of prolactin release from rat lactotrophs through activation of TGFβ1 (37).

LOCALIZATION OF mPRs IN THE BRAIN AND PERIPHERAL NERVOUS SYSTEM

All five mPRs subtypes are expressed throughout the human brain and relative expression of mPRδ mRNA is highest among all the mPRs in nearly all brain regions, with greatest expression in the corpus callosum, hypothalamus, and spinal cord. Furthermore, mPRδ mRNA expression is greater than the mRNA expression of the other mPRs in the neocortex lobes, the limbic system (amygdala, hippocampus, nucleus accumbens), thalamus, as well as in the caudate and putamen, substantia nigra, medulla, and pons, brain regions involved in memory and movement, reward, and autonomic functions (15). Expression of mPRβ and mPRε genes is also high in many of these regions, including the hypothalamus, hippocampus, caudate, cerebellum, pons, and spinal cord, whereas mPRα expression is lower in most brain regions with highest expression in the temporal lobe, medulla, and spinal cord and mPRγ expression is low throughout the brain with the exception of the pons, spinal cord and choroid plexus. The finding that PR mRNA is also expressed throughout the human brain indicates the potential for interactions between mPRs and PR in progesterone regulation of brain functions. However, PR mRNA expression is lower than that of the mPRs in all brain regions except in the pituitary gland which also expresses high levels of mPRε (15).

Unfortunately, the only information currently available on mPRδ and mPRε expression in rodent brains is in the mouse hypothalamus, where low mRNA levels of these subtypes and mPRγ were detected, <10% those of mPRα and mPRβ (38). However, mPRα and mPRβ are broadly distributed in rat and mouse brains (4), with mPRα expression detected in the hippocampus, cerebellum, hypothalamus, thalamus, cortex, striatum, and olfactory bulb (39). Higher mRNA expression of mPRβ compared to that of mPRα and high immunoreactive mPRβ protein expression have been reported in the cortex, paraventricular, and preoptic regions of the hypothalamus, the oculomotor nucleus in the mesencephalon, with substantial expression also in the telencephalon, hippocampus, thalamus, and pons of female rat brains (40, 41). Although these two mPR subtypes are expressed only in neurons under normal conditions, mPRα is also expressed in oligodendrocytes, astrocytes and glial cells after traumatic brain injury, suggesting a potential role for the receptor in inflammatory responses and myelin repair (39). Similarly, mPRα is expressed in astrocytes, oligodendrocytes and their progenitor cells as well as in neurons throughout the spinal cord, whereas mPRβ has a more limited distribution and is mainly located in ventral horn neurons and neurites (42). Interestingly, in the peripheral nervous system all five mPR isoforms have been detected in Schwann cells (43). Collectively, these results indicate that progesterone and possibly allopregnanolone can act in all human brain regions through mPRs and suggest that different mPR subtypes are major intermediaries in these neurosteroid actions within distinct brain regions.

FUNCTIONS OF mPRs IN THE BRAIN AND PERIPHERAL NERVOUS SYSTEM

Although relatively few studies have been conducted so far on mPR functions in the brain, there is emerging evidence that they are intermediaries in several important progesterone neural functions. Experiments in new-born rats with the mPR-selective agonist, Org OD 02-0 (02-0) and in adults injected with mPRβ si-RNA show that the receptor is involved in stabilizing breathing and reducing apnea (9, 44, 45). Knockdown of mPRβ and mPRα mRNAs in the midbrains of female adult rodents by injection of antisense oligonucleotides into the lateral ventricle decreased reproductive behaviors (lordosis and aggression/rejection), whereas other behaviors were not affected (10, 46). The mPR agonist, 02-0, increases dopamine release from hypothalamic explants of rodent prolactinoma models resulting in decreased prolactin secretion and also exerts a direct action on mPRα in pituitary lactotrophs to decrease prolactin secretion through TGFβ-1 (37, 38). These results suggest mPR agonists are potentially of therapeutic use for treating pathological hyperprolactinemia (47). However, the role of mPRs in tumorigenesis in the brain remains unclear. Whereas, proliferation and invasion of glioblastoma cells was stimulated by 02-0 and decreased when mPRα expression was silenced (48), progesterone has been shown to inhibit the growth and metastasis of PR-null breast cancer cells through mPRα in the brains of nude mice (49). Several mPR functions have also been identified in the peripheral nervous system. A recent study showed migration and proliferation of primary rat Schwann cells *in vitro* were increased by treatment with the mPR agonist, 02-0 (50). This treatment also increased expression of differentiation markers and caused morphological changes characteristic of the repair phenotype, indicating a potential role of mPRs in peripheral nerve regeneration following injury (50). Activation of mPRs has also been shown to promote neurite growth in PC12 cells (51).

INTERACTIONS OF ALLOPREGNANOLONE WITH mPRs

We have shown that allopregnanolone exerts protective effects through mPR-dependent signaling pathways in cultured breast cancer cells that do not express PR or GABA-$_A$ receptors as well as in neuronal cells (14, 15). Among the mPRs allopregnanolone displays the highest binding affinity for mPRδ in transfected MDA-MB-231 triple negative breast cancer cells (231-mPRδ) with an IC50 of 151 nM and a relative binding affinity (RBA) of 33.6% that of progesterone.

Allopregnanolone is also an effective competitor for [^{3}H]-progesterone binding to mPRα and mPRβ, with IC50s of 400 and 550 nM, respectively, whereas it has negligible binding affinity for mPRε. Another neurosteroid, 5α-dihydroprogesterone, also displays high binding affinity for mPRα (13). Interestingly, the neurosteroids, dehydroepiandrosterone and pregnanolone (52) (**Figure 1A**), have relatively high binding affinities for mPRδ, with IC50s of 780 and 346 nM, respectively (15). A low concentration of allopregnanolone (20 nM) was shown to mimic the stimulatory actions of progesterone on cAMP production and ERK phosphorylation and also attenuate serum starvation-induced cell death and apoptosis in 231-mPRδ cells cultured *in vitro*. Allopregnanolone (20 nM) also decreased apoptosis of rat hippocampal neuronal (H19-7) cells which express mPRδ and mPRε, whereas the PR agonist R5020 was ineffective. Interestingly, allopregnanolone also mimicked the actions of progesterone in cultured rat GnRH secreting GT1-7 cells which express high levels of mPRα and mPRβ and lower expression of mPRδ mRNA to decrease cAMP production and attenuate cell death and apoptosis (14). The finding that allopregnanolone causes a decrease in cAMP production in GT1-7 cells suggests it is acting through mPRα and/or mPRβ which activate a Gi in these cells, rather than through mPRδ which activates a stimulatory G protein (7, 15). Collectively, these results suggest that low physiological concentrations of allopregnanolone can also potentially act through mPRα/mPRβ to influence their neural functions. On the basis of these findings we hypothesized that ganaxolone can similarly alter neuronal cell functions mediated by mPRs. Therefore, in the present study we investigated whether ganaxolone binds to mPRs, activates mPR-dependent signaling, and exerts anti-apoptotic actions in several neuronal cell lines and in mPR-transfected cells. Experiments were conducted primarily with 231-mPRδ cells, since mPRδ displays the highest binding affinity for allopregnanolone, following experimental procedures described in detail previously (15).

INTERACTIONS OF GANAXOLONE WITH mPRs

The present results show that ganaxolone also binds to mPRs and displays agonist activity in 231-mPRδ cells that do not express GABA-$_A$ receptors (**Figure 1**). A representative competitive binding assay showed that ganaxolone displaced up to 60% of [^{3}H]-progesterone binding to cell membranes of 231-mPRδ cells (**Figure 1B**) with an approximate IC50 of 100 nM, similar to that for allopregnanolone (15). However, the ganaxolone competition curve was not parallel to that of progesterone and higher ganaxolone concentrations (10^{-7} and 10^{-6}M) were ineffective in displacing the remaining ∼40% of [^{3}H]-progesterone binding. Similarly, a previous study showed that higher concentrations of allopregnanolone (10^{-6} and 10^{-5} M) did not replace the residual 30% [^{3}H]-progesterone binding to mPRδ (15). One possible interpretation of the results is that these two neurosteroids do not occupy all the progesterone binding sites on mPRδ. However, additional research on their potential binding to allosteric sites as well as their interactions with progesterone binding to orthosteric sites will be required to determine the nature of their interactions with mPRδ, and whether, for example, they act as ago-allosteric ligands (28, 53, 54). The results indicate that ganaxolone, like allopregnanolone, can potentially influence progesterone signaling through mPRδ. The finding that a low concentration of ganaxolone (20 nM) mimicked the effects of 20 nM progesterone and allopregnanolone on inhibition of serum starvation-induced apoptosis (**Figure 1C**) and cell death in 231-mPRδ cells (**Figure 1E**), whereas it was ineffective in reducing cell death in vector-transfected 231 cells (231-vector, **Figure 1F**) demonstrates that ganaxolone has a mPRδ agonist activity at low nM concentrations. While the progesterone-induced decreases in the two assays were similar (10–12% of the cells), percent cell death measured by trypan blue exclusion was higher than the percent apoptotic cells, which was expected because this assay does not distinguish between cell mortality and cell morbidity after serum starvation, whereas the TUNEL assay is a more robust measure of cells undergoing programed cell death (33). Ganaxolone triggers the same intracellular signaling pathways as those activated by progesterone and allopregnanolone. Ganaxolone treatment (10 nM) increased cAMP levels more than two-fold over no treatment control values in 231-mPRδ cells, consistent with previous results showing mPRδ activates a stimulatory G protein (**Figure 1D**) (15). Ganaxolone (100 nM) ganaxolone mimicked the effects of progesterone and allopregnanolone on phosphorylation of ERK in 231-mPRδ cells (**Figures 1G,H**). At the higher concentration (100 nM), ganaxolone also mimicked the inhibitory effects of progesterone and allopregnanolone on serum starvation-induced cell death in rat hypothalamic GT1-7 cells (**Figure 2A**). GT1-7 cells do not express appreciable amounts of PR mRNA in the absence of estrogen priming, but display significant expression of mPRα and mPRβ which have relatively high binding affinities for allopregnanolone, and lower expression of mPRδ (7, 14, 15). Rat hippocampal neuronal H19-7 cells, in which progesterone and allopregnanolone have previously been shown to inhibit serum starvation-induced cell death (15), have low expression of the PR and high expression of mPRα, mPRβ and mPRδ (**Figure 2B**). All three neurosteroids caused significant phosphorylation of ERK in H19-7 cells (**Figure 2C**). Moreover, MAP kinase signaling and its activation by ganaxolone and the two other neurosteroids was not altered by pretreatment with 100 μM muscimol, a GABA-$_A$ receptor agonist, or with 1 μM bicuculline, a GABA-$_A$ receptor antagonist (**Figure 2C**), confirming that activation of this pathway by these neurosteroids in H19-7 cells is not mediated through a GABA-$_A$ receptor. Moreover, the finding that the PR agonist R5020 does not have the anti-apoptotic effects observed with progesterone and allopregnanolone in H19-7 cells (15), suggests the PR is not involved in this response. Interestingly, treatments with 20 nM ganaxolone and allopregnanolone mimicked the effects of progesterone and the mPR-selective agonist, 02-0, to decrease cAMP production in H19-7 cells (**Figure 2D**), indicating an inhibitory G protein is activated. These results suggest these neurosteroids act

through mPRα and/or mPRβ in H19-7 cells, rather than through mPRδ, since mPRα and mPRβ activate inhibitory G proteins.

DISCUSSION

There is an extensive body of literature describing neuroprotective functions of progesterone and allopregnanolone mediated through the PR and GABA-$_A$ receptors, respectively. Our results suggest that allopregnanolone and the synthetic neurosteroid, ganaxolone, can also exert protective functions in cultured neuronal cells through activation of mPRs to attenuate cell death and apoptosis. However, details of the signaling pathways activated by these steroids through mPRs in neuronal cells are lacking. Moreover, only limited information is currently available on the functions of mPRs in the central nervous system and there is an urgent need to determine whether these neurosteroids exert similar neuroprotective functions through mPRs in *in vivo* models of neurodegenerative diseases. Information is also lacking on possible interactions between mPR and other progesterone and allopregnanolone signaling pathways mediating neuroprotective functions in neural tissues. For example, progesterone membrane component 1, which is abundant in many brain regions (4) and has been proposed to mediate progesterone neuroprotective actions (55), acts as an adaptor protein for mPRα in breast cancer cells, by coupling to mPRα and facilitating its transport to the cell surface where it mediates its membrane receptor functions (56). In addition, cross-talk between mPR and PR signaling has been shown in human myometrial cells and in rat Schwann cells. Activation of mPR in myometrial cells causes transactivation of PR and modulation of PR coactivator expression (34). On the other hand, experiments with the PR agonist, R5020, show mPRα and mPRβ expression in rat primary Schwann cells can be upregulated through the PR (50). Finally, it is important to obtain a clearer understanding of possible off-target effects of ganaxolone through activation of mPRs which would indicate several additional physiological functions that should be monitored in future clinical trials as well as suggesting potential medical complications for some epilepsy patients after long-term ganaxolone treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

The manuscript was written by PT and edited by YP. This study was designed by PT and the experiments were conducted and analyzed by YP. The interpretation of the results was conducted by PT and YP. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the H.E.B. Endowed Chair in Marine Science to PT.

ACKNOWLEDGMENTS

We thank Ms. Jing Dong for assistance with cell culture and experiments and Drs. Luca Castelnovo, Aubrey Converse, and Laura Jenkins for their helpful comments on the manuscript.

REFERENCES

1. Mani SK, Blaustein JD. Neural progestin receptors and female sexual behavior. *Neuroendocrinology*. (2012) 96:152–61. doi: 10.1159/000338668
2. Melcangi RC, Panzica GC. Allopregnanolone: state of the art. *Prog Neurobiol.* (2014) 113:1–5. doi: 10.1016/j.pneurobio.2013.09.005
3. González SL, Coronel MF, Raggio MC, Labombarda F. Progesterone receptor-mediated actions and the treatment of central system disorders: an update of the known and the challenge of the unknown. *Steroids.* (2020) 153:108525. doi: 10.1016/j.steroids.2019.108525
4. Guennoun R, Labombarda F, Gonzalez Deniselle MC, Liere P, De Nicola AF, Schumacher M. Progesterone and allopregnanolone in the central nervous system: response to injury and implication for neuroprotection. *J. Steroid Biochem. Mol. Biol.* (2015) 146:48–61. doi: 10.1016/j.jsbmb.2014.09.001
5. Lamba V, Yasuda K, Lamba JK, Assem M, Davila J, Strom S, et al. PXR (NR1I2): splice variants in human tissues, including brain, and identification of neurosteroids and nicotine as PXR activators. *Toxicol. Appl. Pharmacol.* (2004) 199:251–65. doi: 10.1016/j.taap.2003.12.027
6. Langmade SJ, Gale SE, Frolov A, Mohri I, Suzuki K, Mellon SH, et al. Pregnane X receptor (PXR) activation: a mechanism for neuroprotection in a mouse model of Niemann-Pick C disease. *Proc. Natl. Acad. Sci. USA.* (2006) 103:13807–12. doi: 10.1073/pnas.0606218103
7. Sleiter N, Pang Y, Park C, Horton TH, Dong J, Thomas P, et al. Progesterone receptor A (PRA) and PRB-independent effects of progesterone on gonadotropin-releasing hormone release. *Endocrinology.* (2009) 150:3833–44. doi: 10.1210/en.2008-0774
8. Reddy DS, Gangisetty O, Wu X. PR-independent neurosteroid regulation of α2-GABA-A receptors in three hippocampus subfields. *Brain Res.* (2017) 1659:142–7. doi: 10.1016/j.brainres.2017.01.030
9. Joseph V, Uppari NP, Kouci H, De Bruyn C, Boukari R, Bairam A. Respiratory regulation by steroids in newborn rats: a sex-specific balance between allopregnanolone and progesterone receptors. *Exp. Physiol.* (2018) 103:276–90. doi: 10.1113/EP086716
10. Frye CA, Walf AC, Kohtz AS, Zhu Y. Progesterone-facilitated lordosis of estrogen-primed mice is attenuated by knocking down membrane progestin receptors in the midbrain. *Steroids.* (2014) 81:17–25. doi: 10.1016/j.steroids.2013.11.009
11. Liu B, Arbogast LA. Gene expression profiles of intracellular and membrane progesterone receptor isoforms in the medial basal hypothalamus during pro-oestrus. *J. Neuroendocrinol.* (2009) 21:993–1000. doi: 10.1111/j.1365-2826.2009.01920.x
12. Tang YT, Hu T, Arterburn M, Boyle B, Bright JM, Emtage PC, et al. PAQR proteins: a novel membrane receptor family defined by an ancient 7-transmembrane pass motif. *J Mol Evol.* (2005) 61:372–80. doi: 10.1007/s00239-004-0375-2
13. Thomas P, Pang Y, Dong J, Groenen P, Kelder J, de Vlieg J, et al. Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor α subtypes and their evolutionary origins. *Endocrinology.* (2007) 148:705–18. doi: 10.1210/en.2006-0974

14. Thomas P, Pang Y. Membrane progesterone receptors (mPRs): evidence for neuroprotective neurosteroid signaling and neuroendocrine functions in neuronal cells. *Neuroendocrinology*. (2012) 96:162–71. doi: 10.1159/000339822
15. Pang Y, Dong J, Thomas P. Characterization, neurosteroid binding and brain distribution of human membrane progesterone receptors δ and ε (mPRδ and mPRε) and mPRδ involvement in neurosteroid inhibition of apoptosis. *Endocrinology*. (2013) 154:283–95. doi: 10.1210/en.2012-1772
16. Mula M. Emerging drugs for focal epilepsy. *Expert Opin Emerg Drugs*. (2018) 23:243–9. doi: 10.1080/14728214.2018.1527903
17. Nohria V, Giller E. Ganaxolone. *Neurotherapeutics*. (2007) 4:102–5. doi: 10.1016/j.nurt.2006.11.003
18. Tomson T, Walczak T, Sillanpaa M, Sander JW. Sudden unexpected death in epilepsy: a review of incidence and risk factors. *Epilepsia*. (2005) 46(Suppl 11):54–61. doi: 10.1111/j.1528-1167.2005.00411.x
19. Reddy DS, Castaneda DC, O'Malley BW, Rogawski MA. Anticonvulsant activity of progesterone and neurosteroids in progesterone receptor knockout mice. *J Pharmacol Exp Ther*. (2004) 310:230–9. doi: 10.1124/jpet.104.065268
20. Shiono S, Williamson J, Kapur J, Joshi S. Progesterone receptor activation regulates seizure susceptibility. *Ann Clin Translat Neurol*. (2019) 6:1302–10. doi: 10.1002/acn3.50830
21. Iswari S, Colas AE, Karavolas HJ. Binding of 5α-dihydroprogesterone and other progestins to female rat anterior pituitary nuclear extracts. *Steroids*. (1986) 47:189–203. doi: 10.1016/0039-128X(86)90088-7
22. Belelli D, Lambert JJ. Neurosteroids: endogenous regulators of the $GABA_A$ receptor. *Nat Rev Neurosci*. (2005) 6:565–75. doi: 10.1038/nrn1703
23. Wu YV, Burnham WM. Progesterone, 5α-dihydroprogesterone and allopregnanolone's effects on seizures: a review of animal and clinical studies. *Seizure-Eur J Epilep*. (2018) 63:26–36. doi: 10.1016/j.seizure.2018.10.012
24. Joshi S, Kapur J. Neurosteroid regulation of $GABA_A$ receptors: a role in catamenial epilepsy. *Brain Res*. (2019) 1703:311–40. doi: 10.1016/j.brainres.2018.02.031
25. Luef G, Rauchenzauner M. Epilepsy and hormones: a critical review. *Epilepsy Behav*. (2009) 15:73–7. doi: 10.1016/j.yebeh.2009.02.022
26. Saporito MS, Gruner JA, DiCamillo A, Hinchliffe R, Barker-Haliski M, White HS. Intravenously administered ganaxolone blocks diazepin-resistant lithium-pilocaprine-induced status epilepticus in rats: comparison with allopregnanolone. *J Pharmacol Exp Ther*. (2019) 368:326–37. doi: 10.1124/jpet.118.252155
27. Chuang SH, Reddy DS. 3β-methyl-neurosteroid analogs are preferential positive allosteric modulators and direct activators of extrasynaptic γ-aminobutyric acid type A receptors in the hippocampus dentate gyrus subfield. *J Pharmacol Exp Ther*. (2018) 365:583–601. doi: 10.1124/jpet.117.246660
28. Alanis BAV, Iorio MT, Silva LL, Bampali K, Ernst M, Schnürch M, et al. Allosteric GABAa receptor modulators-A review on the most recent heterocyclic chemotypes and their synthetic accessibility. *Molecules*. (2020). 25:999. doi: 10.3390/molecules25040999
29. Thomas P. Characteristics of membrane progestin receptor alpha (mPRα) and progesterone membrane receptor component one (PGMRC1) and their roles in mediating rapid progestin actions. *Front Neuroendocrinol*. (2008) 29:292–312. doi: 10.1016/j.yfrne.2008.01.001
30. Zhu Y, Rice CD, Pang Y, Pace M, Thomas P. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc Natl Acad Sci USA*. (2003) 100:2231–6. doi: 10.1073/pnas.0336132100
31. Zhu Y, Bond J, Thomas P. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. *Proc Natl Acad Sci USA*. (2003) 100:2237–42. doi: 10.1073/pnas.0436133100
32. Dressing GE, Pang Y, Dong J, Thomas P. Progestin signaling through mPRα in Atlantic croaker granulosa/theca cell cocultures and its involvement in progestin inhibition of apoptosis. *Endocrinology*. (2010) 151:5916–26. doi: 10.1210/en.2010-0165
33. Dressing GE, Alyea R, Pang Y, Thomas P. Membrane progesterone receptors (mPRs) mediate antimorbidity in breast cancer cells and are expressed in human breast tumors. *Horm Cancer*. (2012) 3:101–12. doi: 10.1007/s12672-012-0106-x
34. Karteris E, Zervou S, Pang Y, Dong J, Hillhouse EW, Randeva HS, et al. Progesterone signaling in human myometrium through two novel membrane G protein-coupled receptors: potential role in functional progesterone withdrawal at term. *Mol Endocrinol*. (2006) 20:1519–34. doi: 10.1210/me.2005-0243
35. Pang Y, Thomas P. Progesterone induces relaxation of vascular smooth muscle cells through mPRα (PAQR7). *Mol Cell Endocrinol*. (2018) 474:20–34. doi: 10.1016/j.mce.2018.02.003
36. Zuo L, Li W, You S. Progesterone reverses the mesenchymal phenotypes of basal phenotype breast cancer cells via a membrane progesterone receptor pathway. *Breast Cancer Res*. (2010) 12:R34. doi: 10.1186/bcr2588
37. Camilletti MA, Ferraris J, Abeledo-Machado A, Converse A, Faraoni E, Pisera D, et al. Participation of membrane progesterone receptor α in the inhibitory effect of progesterone on prolactin secretion. *J Neuroendocrinol*. (2018) 30:1–15. doi: 10.1111/jne.12614
38. Camilletti MA, Abeledo-Machado A, Perez PA, Faraoni EY, De Fino F, Rulli SB, et al. mPRs represent a novel target for PRL inhibition in experimental prolactinomas. *Endocr Relat Cancer*. (2019) 26:497–510. doi: 10.1530/ERC-18-0409
39. Meffre D, Labombarda F, Delespierre A, Chastre A, De Nicola AF, Stein DG, et al. Distribution of membrane progesterone receptor alpha in the male mouse and rat brain and its regulation after traumatic brain injury. *Neuroscience*. (2013) 231:111–24. doi: 10.1016/j.neuroscience.2012.11.039
40. Zuloaga DG, Yahn SL, Pang Y, Quihuis AM, Oyola MG, Reyan A, et al. Distribution and estrogen regulation of membrane progesterone receptor-β in the female rat brain. *Endocrinology*. (2012) 153:4432–43. doi: 10.1210/en.2012-1469
41. Intlekofer KA, Petersen SL. Distribution of mRNAs encoding classical progestin receptor, progesterone membrane components 1 &2, serpin mRNA binding protein 1, and progestin and adipoQ receptor family members 7 and 8 in rat forebrain. *Neuroscience*. (2011) 172:55065. doi: 10.1016/j.neuroscience.2010.10.051
42. Labombarda F, Meffre D, Delespierre B, Krivokapic-Blondiaux S, Chastre A, Thomas P, et al. Membrane progesterone receptors localization in the mouse spinal cord. *Neuroscience*. (2010) 166:94–106. doi: 10.1016/j.neuroscience.2009.12.012
43. Castelnovo LF, Magnaghi V, Thomas P. Expression of membrane progesterone receptors (mPRs) in rat peripheral glial cell membranes and their potential role in the modulation of cell migration and protein expression. *Steroids*. (2019) 142:6–13. doi: 10.1016/j.steroids.2017.09.009
44. Bairam A, Boukari R, Joseph V. Targeting progesterone receptors in newborn males and females: From the animal model to a new perspective for the treatment of apnea of prematurity. *Respir Physiol Neurobiol*. (2019) 263:55–61. doi: 10.1016/j.resp.2019.03.004
45. Boukari R, Rossignol O, Baldy C, Marcouiller F, Bairam A, Joseph V. Membrane progesterone receptor-β, but not -α, in dorsal brain stem establishes sex-specific chemoreflex responses and reduces apnea frequency in adult mice. *J Appl Physiol*. (2016) 121:781–91. doi: 10.1152/japplphysiol.00397.2016
46. Frye CA, Walf AA, Kohtz AS, Zhu Y. Membrane progestin receptors in the midbrain ventral tegmental area are required for progesterone-facilitated lordosis of rats. *Horm Behav*. (2013) 64:539–45. doi: 10.1016/j.yhbeh.2013.05.012
47. Camilletti MA, Abeledo-Macahdo A, Faraoni EY, Thomas P, Díaz-Torga G. New insight into progesterone actions on prolactin secretion and prolactinoma development. *Steroids*. (2019) 152:108496. doi: 10.1016/j.steroids.2019.108496
48. Gonzalez-Oroxco JC, Hansberg-Pastor V, Valdez-Cosmes P, Nicolas-Ortega W, Bastida-Beristain Y, Fuente-Granada M, et al. Activation of membrane progesterone receptor-alpha increases proliferation, migration, and invasion of human glioblastoma cells. *Mol Cell Endocrinol*. (2018) 477:81–9. doi: 10.1016/j.mce.2018.06.004
49. Zhou L, Zhou W, Zhang H, Hu Y, Yu L, Zhang Y, et al. Progesterone suppresses triple-negative breast cancer growth and metastasis to brain via membrane progesterone receptor α. *Int J Mol Med*. (2017) 40:755–61. doi: 10.3892/ijmm.2017.3060

50. Castelnovo LF, Caffino L, Fumagalli F, Thomas P, Magnaghi V. Membrane progesterone receptors (mPRs/PAQRs) regulate migration, proliferation and differentiation in rat Schwann cells. *J Mol Neurosci.* (2020) 70:433–48. doi: 10.1007/s12031-019-01433-6
51. Kasubuchi M, Watanabe K, Hirano K, Inoue D, Li X, Terasawa K, et al. Membrane progesterone receptor beta (mPRβ/Paqr8) promotes progesterone-dependent neurite outgrowth in PC12 neuronal cells via non-G protein-coupled receptor (GPCR) signaling. *Sci Rep.* 7:5168. doi: 10.1038/s41598-017-05423-9
52. Baulieu EE. Steroids and brain, a rising bio-medical domain: a perspective. *Front Endocrinol.* 9:316. doi: 10.3389/fendo.2018.00316
53. Wenthur CJ, Gentry PR, Mathews TP, Lindley CW. Drugs for allosteric sites on receptors. *Ann Rev Pharmacol Toxicol.* (2014) 54:165–84. doi: 10.1146/annurev-pharmtox-010611-134525
54. Guo D, Heitman LH, IJzerman A. Kinetic aspects of the interaction between ligand and G protein-coupled receptor: the case of adenosine receptors. *Chem Rev*. (2016) 117:38–66. doi: 10.1021/acs.chemrev.6b00025
55. Cooke PS, Nanjappa MK, Yang Z, Wang KKW. Therapeutic effect of progesterone and its metabolites in traumatic brain injury may involve non-classical signaling mechanisms. *Front Neurosci.* (2013) 7:108. doi: 10.3389/fnins.2013.00108
56. Thomas P, Pang Y, Dong J. Enhancement of cell surface expression and receptor functions of membrane progestin receptor α (mPRα) by progesterone receptor membrane component 1 (PGRMC1): evidence for a role of PGRMC1 as an adaptor protein for steroid receptors. *Endocrinology*. (2014) 155:1107–19. doi: 10.1210/en.2013-1991

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.

frontiers in Endocrinology

REVIEW
published: 26 June 2020
doi: 10.3389/fendo.2020.00420

Hypothalamic Astrocyte Development and Physiology for Neuroprogesterone Induction of the Luteinizing Hormone Surge

Kevin Sinchak[1]*, *Margaret A. Mohr*[2] *and Paul E Micevych*[2]

[1] *Department of Biological Sciences, California State University, Long Beach, Long Beach, CA, United States,* [2] *The Laboratory of Neuroendocrinology, Department of Neurobiology, David Geffen School of Medicine at UCLA, Brain Research Institute, University of California, Los Angeles, Los Angeles, CA, United States*

Neural circuits in female rats sequentially exposed to estradiol and progesterone underlie so-called estrogen positive feedback that induce the surge release of pituitary luteinizing hormone (LH) leading to ovulation and luteinization of the corpus hemorrhagicum. It is now well-established that gonadotropin releasing hormone (GnRH) neurons express neither the reproductively critical estrogen receptor-α (ERα) nor classical progesterone receptor (PGR). Estradiol from developing ovarian follicles acts on ERα-expressing kisspeptin neurons in the rostral periventricular region of the third ventricle (RP3V) to induce PGR expression, and kisspeptin release. Circulating estradiol levels that induce positive feedback also induce neuroprogesterone (neuroP) synthesis in hypothalamic astrocytes. This local neuroP acts on kisspeptin neurons that express PGR to augment kisspeptin expression and release needed to stimulate GnRH release, triggering the LH surge. *In vitro* and *in vivo* studies demonstrate that neuroP signaling in kisspeptin neurons occurs through membrane PGR activation of Src family kinase (Src). This signaling cascade has been also implicated in PGR signaling in the arcuate nucleus of the hypothalamus, suggesting that Src may be a common mode of membrane PGR signaling. Sexual maturation requires that signaling between neuroP synthesizing astrocytes, kisspeptin and GnRH neurons be established. Prior to puberty, estradiol does not facilitate the synthesis of neuroP in hypothalamic astrocytes. During pubertal development, levels of membrane ERα increase in astrocytes coincident with an increase of PKA phosphorylation needed for neuroP synthesis. Currently, it is not clear whether these developmental changes occur in existing astrocytes or are due to a new population of astrocytes born during puberty. However, strong evidence suggests that it is the former. Blocking new cell addition during puberty attenuates the LH surge. Together these results demonstrate the importance of pubertal maturation involving hypothalamic astrocytes, estradiol-induced neuroP synthesis and membrane-initiated progesterone signaling for the CNS control of ovulation and reproduction.

Keywords: neuroprogesterone, astrocyte, Src kinase, kisspeptin, RP3V, AVPV, estradiol, LH surge

OPEN ACCESS

Edited by:
Takayoshi Ubuka, Waseda University, Japan

Reviewed by:
William Henry Colledge, University of Cambridge, United Kingdom
Yoshihisa Uenoyama, Nagoya University, Japan

***Correspondence:**
Kevin Sinchak
Kevin.sinchak@csulb.edu

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

Received: *24 March 2020*
Accepted: *26 May 2020*
Published: *26 June 2020*

Citation:
Sinchak K, Mohr MA and Micevych PE (2020) Hypothalamic Astrocyte Development and Physiology for Neuroprogesterone Induction of the Luteinizing Hormone Surge. Front. Endocrinol. 11:420. doi: 10.3389/fendo.2020.00420

INTRODUCTION

Successful reproduction in female rodents depends on the interaction of steroidogenesis in the ovaries and brain. Almost 40 years ago Baulieu's group discovered that nervous tissue synthesizes steroids *de novo* from cholesterol and named them *neurosteroids* (1–4). Unraveling the physiology and actions of neurosteroids in the nervous system has been challenging because they are synthesized in specific locations, their actions must be differentiated from actions of circulating steroids, and in many cases the actions of peripheral steroids and neurosteroids are interdependent. Neurosteroids have been implicated in the myelination of peripheral nerves (5–8) neurogenesis (9) [reviewed in (10)], epilepsy, traumatic brain injury (11–13), and memory (14–18). Our research has concentrated on the role of the neurosteroid, neuroprogesterone (neuroP), which is synthesized *de novo* in hypothalamic astrocytes as part of the mechanism of estrogen positive feedback needed to stimulate the luteinizing hormone (LH) surge, inducing ovulation. This review considers estradiol signaling in the context of facilitating neuroP synthesis in astrocytes, and the integration of estradiol and neuroP signaling in regulating kisspeptin neurons in the rostral periventricular region of the third ventricle (RP3V). As with other steroid receptors, more recent findings indicate that in addition of nuclear localization and action, these receptors are trafficked to the plasma membrane where they are coupled to cell signaling cascades. The activation of nuclear progesterone receptor (PGR) at the cell membrane has recently been reviewed (19). In this review, we are primarily concerned with experimental evidence gathered in rodents. When appropriate, we indicate that the results were from different species. Kisspeptin is the most potent activator of neurons that release gonadotropin releasing hormone (GnRH) into the hypothalmo-hypophyseal portal circulation, generating a surge of pituitary LH into the systemic circulation. An LH surge is the trigger for ovulation and the formation of the corpus luteum—central events for reproduction.

POSITIVE FEEDBACK, THE LH SURGE, AND OVULATION

Hormones of the hypothalamic-pituitary-gonadal axis coordinate events that lead to maturation of ovarian follicles. The pivotal event is the LH surge that induces ovulation and reprograms the ovary to produce large amounts of progesterone as well as estradiol. These ovarian hormones are necessary to: (i) facilitate female sexual receptivity to maximize the potential of fertilization, (ii) induce the secretory phase of the stratum functionale completing the preparation of the uterine endometrium for implantation of the zygote should fertilization occur, and (iii) supporting the initial stage of pregnancy until the placenta develops.

Orchestrated actions of estradiol, progesterone and kisspeptin in the brain are critical for triggering the LH surge. GnRH neurons of the diagonal band of Broca (DBB) and medial septum project to the median eminence and release GnRH into the hypothalamo-hypophyseal portal system. GnRH regulates the release of follicle stimulating hormone (FSH) and LH from gonadotrophin cells in the anterior pituitary. Differential regulation of LH and FSH is accomplished by changes in GnRH release: low frequency and amplitude favor FSH release, whereas elevated amplitude and frequency preferentially release LH. Within the ovary, gonadotropins are critical for maturation of follicles, which become dependent on their stimulation. LH acts on the thecal and granular cells of the ovarian follicles and later the corpora lutea to regulate estradiol and progesterone synthesis throughout the cycle. At the beginning of the estrous cycle (diestrus I and II) as ovarian follicles mature, circulating estradiol levels slowly rise and produce negative feedback in the hypothalamus and pituitary retarding the release of gonadotropins. The main effects of negative feedback regulating GnRH release appear to be mediated through kisspeptin, neurokinin B, and dynorphin expressing (KNDy) neurons of the arcuate nucleus of the hypothalamus (ARH) (20) [reviewed in (21, 22)]. The mechanism of estrogen positive feedback requires the action of estradiol and progesterone, and yet, GnRH neurons do not express ERα or classical PGR (23–25). Therefore, estradiol and progesterone must signal through neurons upstream of the GnRH neuron. The majority of anterior hypothalamic kisspeptin neurons express ERα and PGR, providing a platform for integrating steroid actions that modulate the excitation of GnRH neurons (26–28). In rodents, positive feedback actions of steroids are mediated by kisspeptin neurons in the RP3V, which contains the anteroventral periventricular (AVPV) and rostral periventricular zone (25, 29–33). The AVPV is a site critical for estrogen positive feedback signaling in rodents. Lesioning or implanting anti-estrogens into the AVPV blocks the LH surge (34–36).

GnRH neurons in the DBB receive input from RP3V (including AVPV) kisspeptin neurons and are activated by kisspeptin to increase the frequency and amplitude of GnRH release inducing an LH surge from the pituitary (23–27, 29–31, 37–39). Infusion of exogenous kisspeptin excites GnRH neurons and induces levels of LH that mimic surge levels. GnRH neurons in the DBB express Kiss1R (formerly GPR54), the cognate receptor for kisspeptin (40–42). GnRH neuronal activation and the LH surge are lacking in female Kiss1R knockout mice (37). Activation of Kiss1R in GnRH neurons produces robust depolarizing currents and induces GnRH release (43–47). The timing of the LH surge requires the stimulatory action of kisspeptin and the removal of RFamide-related peptide 3 (RFRP-3; also known as gonadotropin-inhibitory hormone—GnIH) (48–53). In this model, the daily afternoon increase in GnRH and LH is due to suppression of the RFRP-3 inhibitory input to the GnRH neurons by the suprachiasmatic nucleus (SCN). We propose that estrogen positive feedback surge release of LH requires an amalgamation of circadian and kisspeptin models. It is only when the diurnal release of RFRP-3 inhibition of GnRH coincides with estradiol and neuroP stimulation of kisspeptin release that a GnRH–LH surge occurs–once every 4 days during the estrous cycle (52).

As estradiol levels rise rapidly and peak on the afternoon of proestrus, positive feedback predominates (54). Because

estradiol treatments induce the LH surge in ovariectomized and adrenalectomized (OVX/ADX) rats, progesterone was not thought to be required for the LH surge and the phenomenon was called "estrogen positive feedback" (55, 56). A large number of studies unequivocally demonstrated that in addition to estradiol, "estrogen" positive feedback requires PGR and progesterone (55, 57–64). It turned out that the needed progesterone, neuroP, is synthesized in the hypothalamus (32, 65–68). Rising estradiol levels during diestrus 1 to proestrus induce the expression of PGR and kisspeptin in RP3V neurons that are critical for the LH surge (26, 28, 33, 37, 63, 69–71). This initial kisspeptin induction is dependent on ERα (70, 71). *In vivo* experiments did not differentiate between effects of estradiol that induced PGR and kisspeptin since both require ERα. Moreover, *in vivo* experiments did not segregate estradiol effects directly on kisspeptin neurons from neuroP-PGR effects on kisspeptin neurons. Our *in vitro* experiments allowed us to tease apart these overlapping effects. Proestrous (positive feedback) levels of estradiol stimulate hypothalamic astrocytes to synthesize neuroP that acts on the estradiol-induced PGR in kisspeptin neurons, which augments the synthesis and release of kisspeptin needed for the GnRH-LH surge (19, 32, 33, 65, 68, 72) [reviewed in (73)]. Thus, a critical component of positive feedback is estrogen-facilitated neuroP signaling through ERα and PGR expressing kisspeptin neurons.

SYNTHESIS OF neuroP BY HYPOTHALAMIC ASTROCYTES

Depending on the final bioactive steroid, neurosteroidogenesis may involve one or a combination of astrocytes, oligodendrocytes, and neurons (74). This is because each cell type expresses certain enzymes within the steroidogenic pathway (74). The *de novo* synthesis of neurosteroids that are further down the pathway from cholesterol (e.g., estradiol) require shuttling through multiple cell types in order to be synthesized. However, neuroP only requires two enzymes to be synthesized from cholesterol, and astrocytes express both of these enzymes (4, 74) (**Figure 1**). The synthesis of progesterone is initiated by transport of cholesterol into the inner mitochondrial membrane through the interaction of translocator protein (TSPO) and steroid acute regulatory protein (StAR) (76–78) [but see (79–81)]. Cholesterol is converted to pregnenolone by the enzyme CYP11A1 (previously P450 side chain cleavage; P450scc) that is associated with the inner mitochondrial membrane. 3β-hydroxysteroid dehydrogenase (3β-HSD or HSD3B1) converts pregnenolone to progesterone, which diffuses out of astrocytes to activate local PGR-expressing kisspeptin neurons of the RP3V (19, 82) facilitating the LH surge.

Positive feedback levels of estradiol induce neuroP synthesis in hypothalamic astrocytes. Proestrous levels of estradiol activate membrane ERα (mERα) that is complexed with and transactivates metabotropic glutamate receptor-1a (mGluR1a; **Figure 1**) to rapidly induce phosphorylation events that regulate cholesterol transport (65, 76, 83, 84). Estradiol activation of the mERα-mGluR1a complex, signaling through Gαq, activates the phospholipase C-inositol trisphosphate (IP3) signaling pathway that produces a robust release/increase of intracellular free calcium ($[Ca^{2+}]_i$) from intracellular stores (83, 85). This activates a calcium-sensitive adenylate cyclase-protein kinase A pathway that increases the phosphorylation of TSPO and StAR in hypothalamic astrocytes, which is necessary for inducing neuroP synthesis (83, 84, 86). These results suggest that proestrous levels of estradiol increase neuroP synthesis by increasing the cholesterol transport into mitochondria and access to P450scc for conversion to pregnenolone. *In vivo*, estradiol increases hypothalamic expression and activity of the second enzyme in the neuroP synthesis, 3β-HSD (85, 87). The estradiol-induced increase in brain progesterone levels are sex- and site-specific: present in the adult female hypothalamus but absent in the male hypothalamus (72). Moreover, blocking 3β-HSD activity in the hypothalamus of adult OVX/ADX rats prevents the estradiol-induced LH surge (72). We further demonstrated that neuroP is important for the LH surge in gonadally intact rats by blocking hypothalamic neuroP synthesis on the morning of proestrus by third ventricular (3V) administration of aminoglutethimide (AGT), a P450scc inhibitor (67). The estrous cycle is arrested in proestrus prior to the LH surge even though peripheral estradiol levels, a marker of ovarian steroidogenesis, are unaffected in AGT-treated rats. In these animals, the uterus is swollen with fluid and there are no corpora lutea in the ovaries—all indicating the absence of an LH surge (67). Thus, estradiol-induced hypothalamic neuroP, rather than ovarian or adrenal progesterone, mediates the triggering of the LH surge during positive feedback. Dose and duration of estradiol exposure during negative and positive feedback regulate the mechanisms of neuroP synthesis by astrocytes, and properly coordinate the timing of neuroP synthesis with the priming of the rest of the Kisspeptin-GnRH-LH system.

neuroP SIGNALING THROUGH MEMBRANE PGR TO REGULATE KISSPEPTIN

neuroP actions appear to be mediated through PGR signaling in RP3V kisspeptin neurons. Estradiol-induced RP3V PGRs are required to initiate and reach the full magnitude and duration of the LH surge (62, 88). Likewise, the LH surge cannot be induced in PGR knockout mice (89). Female mice with PGR knocked out specifically in kisspeptin neurons are less fertile (i.e., fewer births with smaller litters), and lack an estradiol-induced LH surge and the associated AVPV c-Fos induction (90, 91). Furthermore, activation of RP3V PGR with R5020 (PGR specific agonist) induced an LH surge in estradiol-primed rats (33). Accumulating evidence supports that it is neuroP signaling through PGR in kisspeptin neurons that is required for the LH surge. While blocking neuroP synthesis with a 3β-HSD inhibitor attenuates the estradiol-induced LH surge in OVX/ADX rats, progesterone treatment or site-specific injections of kisspeptin into the DBB rescued the LH surge, demonstrating that estradiol induction of neuroP synthesis and the actions of neuroP occur first and are required for kisspeptin release (32).

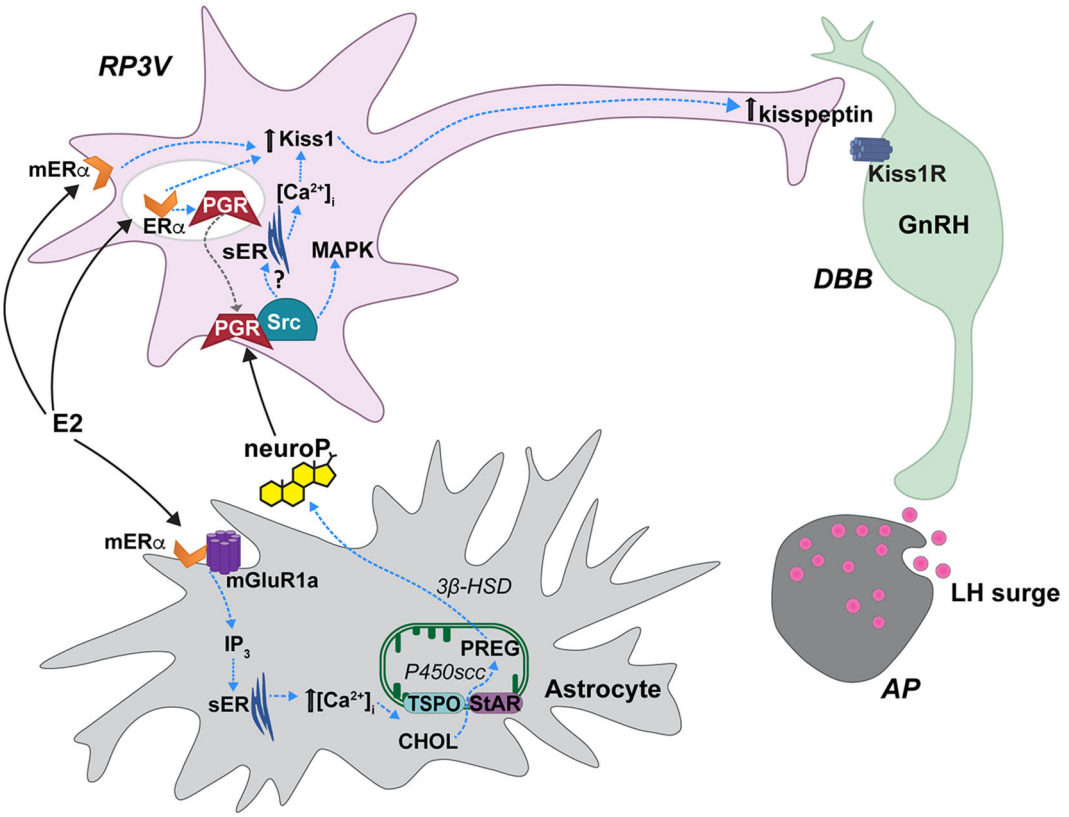

FIGURE 1 | Proposed estradiol-induced hypothalamic astrocyte steroidogenesis of neuroP that activates a membrane classical progesterone receptor (PGR)-Src tyrosine kinase (Src) signaling pathway in RP3V kisspeptin neurons to trigger the luteinizing (LH) surge. In hypothalamic astrocytes, proestrous levels of estradiol (E2) activate membrane estrogen receptor-α (mERα) that complex with and signal through metabotropic glutamate receptor-1a type (mGluR1a). mERα-mGluR1a signals through a PKC-IP3 pathway to increase intracellular calcium concentrations [(Ca^{2+})]. This releases Ca^{2+} from the smooth endoplasmic reticulum (sER). Within the mitochondrion, cholesterol (CHOL) is converted to pregnenolone (PREG) by P450 side chain cleavage (P450scc). PREG is then converted to neuroprogesterone (neuroP) by 3β-hydroxysteroid dehydrogenase (3β-HSD). The neuroP is secreted from the astrocytes to activate ERα-mediated, E2-induced PGR in RP3V kisspeptin neurons. Concurrently, E2 increases Kiss1 mRNA and kisspeptin expression via a mERα initiated mechanism [but see (70)]. neuroP rapidly augments the E2-induced Kiss1 mRNA and kisspeptin expression, potentially through PGR-Src signaling. PGR complexes with and signals through Src to activate a MAPK pathway. Further, a membrane PGR can initiate signaling that increases intracellular Ca^{2+} from sER stores. PGR-Src signaling also mediates the release of kisspeptin from neurons that project to diagonal band of Broca (DBB) GnRH neurons. Kisspeptin then binds to its cognate receptor, Kiss1R stimulating GnRH release into the median eminence that triggers the LH surge from gonadotrophs in the anterior pituitary (AP). Steroid acute regulatory protein (StAR), translocator protein (TSPO). Modified from Micevych et al. (75).

RP3V kisspeptin neurons are modeled *in vitro* using mHypoA51 cells that are derived from adult female hypothalamic kisspeptin neurons (19, 82). Estradiol induces PGR expression in these cells (19), which is observed in the RP3V where PGR expression is increased in areas that overlap with kisspeptin neurons by estradiol treatment in OVX rats or on proestrus (28, 33). Estradiol increases kisspeptin expression in mHypoA51 neurons, and subsequent progesterone further augments this expression (19). In co-culture experiments where mHypoA51 and adult female hypothalamic astrocytes are separated (i.e., not in direct contact) but share media, estradiol treatment induces neuroP synthesis in astrocytes and increases kisspeptin expression in the mHypoA51 neurons. neuroP secretion from astrocytes stimulates mHypoA51 neurons to rapidly increase kisspeptin release (19, 82). Importantly, mHypoA51 neurons express membrane progesterone receptors (mPR), including mPRα, mPRβ (see more below and **Figure 2**), and membrane-localized PGR. This membrane-localized PGR increases with estradiol treatment (19).

Although classified as a transcription factor and normally thought to be associated with the nucleus, PGR can be trafficked to the plasma membrane via palmitoylation, a mechanism seen in ERα trafficking (92). At the membrane, PGR can interact with and signal through other proteins to initiate rapid signaling, altering neuronal activity (33, 93, 94) (**Figure 2**). PGRs that are trafficked to the plasma membrane complex with and signal through Src kinase, a non-receptor tyrosine kinase (Src) (33, 93–95). PGRs have two distinct isoforms that are transcribed from a single gene: PGR-A and PGR-B. PGR-A lacks 164 amino acids in the N-domain, and is considered

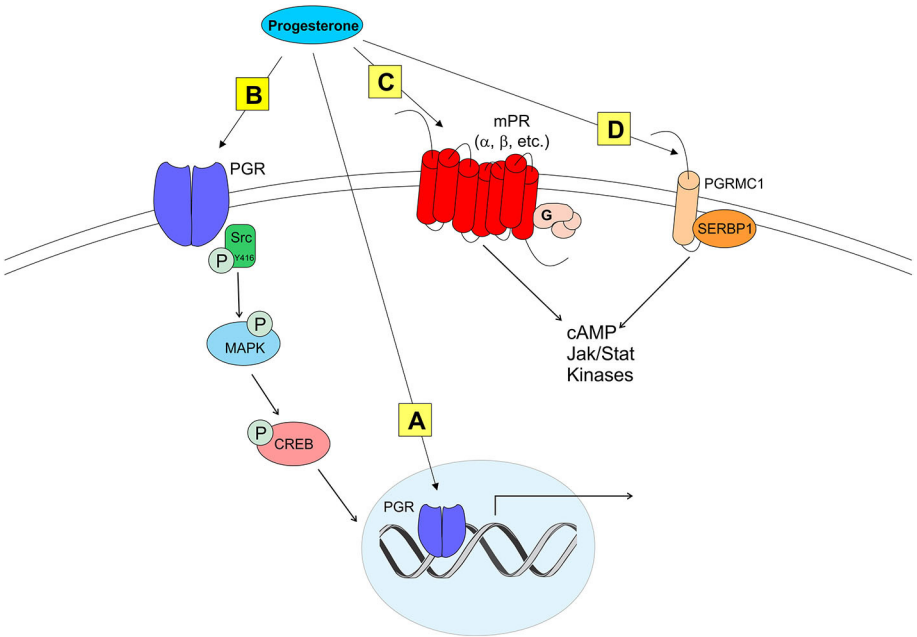

FIGURE 2 | Modes of progesterone signaling in the rodent. Classical progesterone receptor (PGR) can mediate progesterone signaling classically **(A)**, by binding to DNA progesterone response elements. PGR can also be trafficked to the plasma membrane [as in **(B)**] where it can activate rapid intracellular signaling cascades involving kinases such as Src. It is unknown whether membrane PGR transactivates another receptor like an mGluR as estrogen receptors have been shown to do. Multiple novel membrane progesterone receptors (mPRs) have been recently discovered and described, such as mPRs α, β, δ, and γ **(C)**. mPRs can activate signaling cascades via G proteins, which go on to affect cyclic AMP (cAMP) pathways. Finally, progestins can bind to progesterone receptor membrane component 1 [PGRMC1 **(D)**]. PGRMC1 can work in concert with SERBP1 to affect cAMP, Jak/Stat, and multiple kinase pathways (73).

the truncated form of PGR-B (96). A poly-proline rich region (amino acids 421-428, PPPPLPPR) near the N-domain of PGRs is responsible for binding and signaling through the Src SH3 domain (93). Although this region is conserved in PGR-A, and both isoforms display hormone-dependent binding to SH3, only PGR-B activates Src (94). Significantly, PGR-B is also the reproductively relevant isoform.

In vitro and *in vivo* experiments indicate that neuroP signals through PGR-Src complexes to activate kisspeptin neurons, and that the PGR-Src signaling is interdependent. Nearly all mHypoA51 neurons express kisspeptin, and most express PGR and Src (19, 82). Activation of either PGR or Src in mHypoA51 neurons induces kisspeptin release while inhibiting Src activation blocks progesterone activation of MAPK and kisspeptin release, implying that progesterone and Src interact to stimulate kisspeptin release via activation of a MAPK pathway (**Figure 1**) (19). Another potential rapid PGR initiated pathway for kisspeptin release is through release on intracellular stores of $[Ca^{2+}]$ (**Figure 1**) (82). In mHypoA51 neurons, progesterone induced a rapid increase in $[Ca^{2+}]$ that was blocked by pretreatment with RU486, a PGR antagonist (82). However, further studies are required to determine the physiological outcomes of both of these signaling pathways. *In vivo* data further support that neuroP induces Src-mediated PGR signaling. PGR and Src are co-expressed in neurons of the RP3V of female rats (33). Further, using the Duolink proximity ligation assay that uses specific antibodies to two selected proteins/antigens and then produces punctate staining if these proteins are in close proximity (<40 nm), we observed that estradiol-priming increases the levels of PGR and Src staining in RP3V neurons suggestive of an estradiol-induced increase in PGR-Src interactions (33). Similarly, in the ARH, a region important for facilitation of lordosis, we have observed a similar colocalization and estradiol-induced increase in PGR-Src proximity (97). In the RP3V, PGR and Src exhibit interdependent signaling in the induction of the LH surge. Bilateral infusion of either a classical PGR agonist (R5020) or Src family activator induced a robust LH surge in estradiol-primed OVX/ADX rats (97). However, bilateral RP3V infusion of either a PGR antagonist (RU486) or a Src inhibitor (PP2) blocked the induction of the LH surge by activation of either PGR (progesterone or R5020) or Src (Src activator). The ability of antagonizing either PGR or Src to block the signaling of both PGR and Src indicates that PGR-Src signaling is interdependent. It is likely that PGR is transactivating Src to initiate signaling. Even though Src is "downstream" of PGR, and activation of either one will induce the LH surge, for signaling to occur neither can be occupied by an antagonist, which likely produces a conformational change that prevents Src activation and signaling. This antagonist effect was also seen with interactions of PGR and dopamine receptors (98). However, in the absence of antagonist binding either PGR or Src, it appears the activation of either PGR or Src can initiate the signaling cascade. The similarities of the PGR-Src signaling cascade in the ARH, RP3V and mHypoA51 neurons suggests that PGR-Src

signaling may be a common mode of membrane PGR signaling. Together, the *in vivo* and *in vitro* findings indicate that membrane PGR-Src signaling mediates the neuro P activation of kisspeptin neurons to activate GnRH neurons to trigger the LH surge.

NON-CLASSICAL PROGESTERONE RECEPTORS IN KISSPEPTIN NEURONS

Although PGRs are essential to induce the LH surge, other types of mPRs have been proposed to modulate neuroP/progesterone actions through membrane initiated signaling [reviewed in (73); see **Figure 2**]. For example, during progesterone negative feedback, PGR knockout mice respond to positive feedback levels of progesterone to suppress GnRH release, suggesting that progesterone may also signal through non-classical mPRs (99). However, the role of non-classical mPR in the LH surge remains unknown (19, 100). Two families of mPRs have been discovered that initiate progesterone signaling at the plasma membrane. One group of these mPRs is in the Class II progestin and adipoQ receptor (PAQR) family (101–103). These mPRs have a classic 7-transmembrane protein structure and behave similarly to G protein-coupled receptors by rapidly facilitating progesterone action. *In vitro*, mHypoA51a neurons express subtypes of mPRs: mPRα and mPRβ (19). *In vivo*, estradiol upregulates mPRβ expression in the anterior hypothalamus (104). The distribution and estrogen regulation of mPRβ in the female rat brain (104), but not in mHypoA51 neurons (19), suggests that this *in vivo* upregulation occurs in non-kisspeptin cells. Although these mPRs are expressed in the RP3V, little is known about the role of these non-classical mPRs in regulating the neuroP induction of the LH surge. Another protein that binds progesterone and initiates signaling at the plasma membrane is progesterone receptor membrane components (PGRMC) (105–109). Two PGRMC subtypes have been discovered: PGRMC1 (aka 25-DX) and PGRMC2 (105, 106, 108, 110, 111). These PGRMC have been implicated in normal mammalian ovarian function including primordial follicle development, luteal vascularization and normal onset reproductive senescence (107, 110, 112). Young women with reduced PGRMC2 expression in granulosa cells have been diagnosed with diminished ovarian reserve (113). Similarly, reduced expression of PGRMC1 (via point mutation) has been associated with women exhibiting primary ovarian insufficiency (114). PGRMC mRNAs are expressed in the AVPV (109, 111, 115). However, only PGRMC2 mRNA levels are upregulated by the sequential treatment with estradiol and progesterone (115). Although their expression in the AVPV does not appear to be essential, mPRs and PGRMC's may influence PGR actions. A resolution of this issue requires further experimentation.

DEVELOPMENT OF ESTROGEN POSITIVE FEEDBACK DURING PUBERTY

Maturation of reproductive circuits in females that results in ovulation involves a multitude of changes in the brain during puberty. This is represented by the increase in GnRH pulsatility throughout puberty, which is needed for the surge release of LH [reviewed in (116)]. As with estrogen positive feedback and the facilitation of neuroP synthesis, kisspeptin neurons in the rodent AVPV are sexually dimorphic; females have more kisspeptin neurons in the AVPV compared with males (30). In female mice, the number of presumptive kisspeptin neurons in the AVPV increases across pubertal development (117). The increase in GnRH pulsatility and the increase in the number of kisspeptin neurons are crucial to the development of estrogen positive feedback, but the maturation of these two systems do not fully explain the development of estrogen positive feedback during puberty.

PUBERTAL DEVELOPMENT OF ESTRADIOL-INDUCED neuroP SYNTHESIS IN THE HYPOTHALAMUS

Much like the rest of the system that controls estrogen positive feedback, the ability of the hypothalamus to coordinate estradiol-induced neuroP synthesis is something that develops across puberty in the rodent. It was previously observed that primary hypothalamic astrocyte cultures did not increase progesterone synthesis in response to estradiol if harvested from neonatal female or male mice of any age, and maturation *in vitro* did not make these astrocytes respond to estradiol with neuroP synthesis (65). At the time it was thought that something about the pubertal transition made astrocytes competent to respond to estradiol, but this idea was not formally tested until recently. Mohr et al., showed that estradiol-facilitated neuroP synthesis in the hypothalamus develops during puberty in the female rat (118). First, hypothalamic tissues, collected from gonadally intact rats either on postnatal day 17, (PND 17; prepuberty), PND 35, (peripuberty), or on the afternoon of proestrus around PND 60 (adulthood), were assayed for neuroP levels with liquid chromatography tandem mass spectrometry (LC-MS/MS). NeuroP significantly increases during puberty in gonad-intact female rats, from prepuberty to adulthood. Then, in OVX rats of the same ages, estradiol treatment only in adulthood reliably facilitates neuroP synthesis. The prepubertal female hypothalamus is insensitive to estradiol in terms of neuroP synthesis. However, during puberty (peripuberty) the ability of estradiol to stimulate neuroP synthesis develops. The pubertal development of neuroP synthesis is yet another way that the brain changes during puberty to allow for estrogen positive feedback signaling.

Adult female hypothalamic astrocytes are the source of estradiol-facilitated neuroP synthesis that contributes to estrogen positive feedback (68). Corresponding to the pubertal increase in estradiol-facilitated neuroP synthesis *in vivo*, there is an increase in the amount of mERα in hypothalamic astrocyte cultures. In these primary female astrocyte cultures, there is also an increase in caveolin-1 protein, a scaffolding protein that participates in the trafficking of ERα to the cell membrane and coupling with mGluR1a (118). Because membrane-initiated estradiol signaling is necessary to augment neuroP synthesis in astrocytes in adulthood [reviewed in (119)], the lack of mERα provides an explanation as to why pre-pubertal hypothalamic

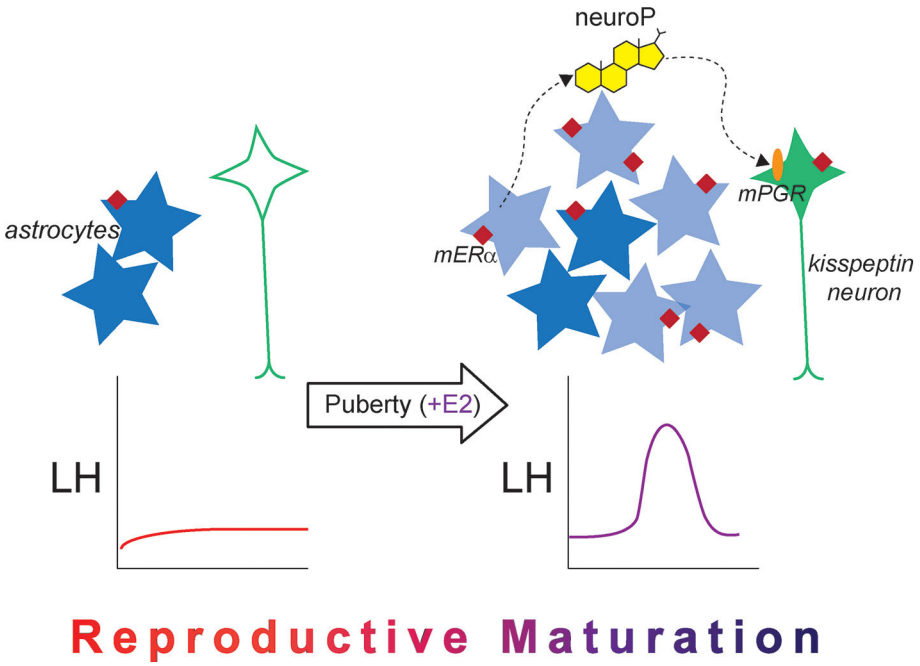

FIGURE 3 | The new astrocyte model for the pubertal development of rodent estrogen positive feedback. Prior to puberty, kisspeptin expression is low in the female AVPV (green neuron), and mERα expression (maroon diamond) in hypothalamic astrocytes (dark blue stars) is low. The LH surge does not occur naturally and cannot be induced with exogenous estradiol administration. After puberty occurs and once estrogen signaling begins, estrogen-responsive newborn astrocytes are added to the AVPV (light blue stars), which are necessary for the LH surge in adulthood. There is also an increase in mERα expression in hypothalamic astrocytes, and estradiol-induced neuroP synthesis occurs, which acts on membrane-associated PGR (orange oval) to stimulate kisspeptin release. Together, the changes in cellular machinery across pubertal development alter estradiol responsiveness and permit the LH surge to occur, indicating that reproductive maturation is complete.

astrocytes are incapable of estradiol-induced neuroP synthesis. It appears that pubertal expression of caveolin-1 that shepherds ERα to the membrane may be key to the development of estrogen positive feedback that induces neuroP synthesis to trigger the LH surge.

PUBERTALLY BORN ASTROCYTES IN THE AVPV: KEY TO THE DEVELOPMENT OF neuroP SYNTHESIS?

Another explanation of how estradiol-induced neuroP synthesis develops in the hypothalamus is the "new astrocyte model" (**Figure 3**). Accordingly, estradiol-responsive astrocytes are not present in the prepubertal hypothalamus. During and after puberty, populations of new cells are added to the female rat AVPV, and a large majority of these newly born cells express markers for astrocytes (GFAP) (120, 121). These newborn AVPV cells are more numerous in females compared with males, and this sex difference in pubertal cell addition is dependent on gonadal hormones (122). Pubertal cell addition to the AVPV mirrors the overall sex difference observed in the rodent AVPV, considering that the female AVPV is larger and contains more neurons in females compared with males (123). This sex difference in structure likely contributes to the functional sex difference of this brain region, because only female rodents are capable of estrogen positive feedback (124). That females have higher amounts of cells added to the AVPV compared with males during peripuberty may indicate that these newborn cells are needed for estrogen positive feedback signaling.

Indeed, these newly born cells are crucially important for estrogen positive feedback induction of the LH surge. When cell proliferation is blocked with cytrabine (AraC), a pyrimidine analog, either during puberty or in early adulthood, the estradiol + progesterone-induced LH surge is diminished in female rats (120). A majority of these newborn cells are astrocytes, suggesting that these newborn AVPV astrocytes are the source of estradiol-responsive hypothalamic astrocytes that synthesize neuroP necessary for estrogen positive feedback. In the 2017 study by Mohr et al., both estradiol and progesterone were used to elicit the LH surge (120). In this study, the LH surge was not eliminated entirely, which may be explained by several factors: AraC did not eliminate *all* newborn AVPV cells, and therefore, some neuroP-producing astrocytes were present to produce neuroP and elicit some LH release, or more likely, administration of progesterone on the morning of the day of the surge bypassed hypothalamic neuroP, eliciting some LH release. Had only estrogen been used, which can also elicit the LH surge in rats (72), the effect of AraC may have been more dramatic on the LH surge because the only source of progesterone would have been from hypothalamic astrocytes (neuroP).

More studies are required to determine the exact role of pubertally born astrocytes in the development of estrogen positive feedback signaling. However, it seems likely that the

birth of astrocytes contributes to the maturation of reproductive circuits controlling estrogen positive feedback signaling. There may be a developmental difference in the birth and maturation of astrocytes if they are born while circulating estradiol is elevated (i.e., after puberty compared with before puberty) that makes them competent to respond to estradiol with neuroP synthesis. These newborn astrocytes could have higher levels of mERα, and caveolin-1, resulting in increased PKA phosphorylation, making them more proficient in estradiol-induced neuroP synthesis.

CONCLUSIONS

The regulation of ovulation is the central event in mammalian reproduction and during puberty. Indeed, neural circuits controlling reproduction in females are considered mature when ovulation can occur. In rodents, at the very least, this critical physiological process requires the coordination of the hypothalamo-pituitary-ovarian axis with the SCN circadian clock. Regulation of the surge release of LH requires a complex neuronal and glial circuitry that directs various peripheral and central hormones onto kisspeptin neurons. In turn, circadian-regulated inputs interact with GnRH neurons, activating the anterior pituitary to release a surge of LH. This mechanism has been dubbed "estrogen positive feedback" for the importance of estrogen, but it is far from the only critical hormonal participant in this process. Developing ovarian follicles synthesize ever increasing levels of estradiol that induce PGR expression in kisspeptin neurons. As estradiol levels peak on the afternoon of proestrus, neuroP synthesis is rapidly facilitated in hypothalamic astrocytes, many of which may be born after the initiation of puberty. Together, estradiol and neuroP stimulate kisspeptin expression and release. When this hormonal activation of kisspeptin coincides with the release of circadian inhibition–a physiological LH surge occurs (i.e., one that stimulates ovulation). We now understand the signaling involved in regulating both the synthesis of neuroP in astrocytes and the neuroP signaling in kisspeptin neurons. In astrocytes, mERα transactivates mGluR1a to induce neuroP synthesis. In kisspeptin neurons, a portion of estradiol-induced PGR are trafficked to the cell membrane where neuroP activates them, augmenting both kisspeptin expression and release. NeuroP signaling in kisspeptin neurons involves Src activation and the release of intracellular calcium. Thus, the brain does not passively respond to ovarian hormones but is an active participant in triggering the LH surge to induce ovulation.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing and editing of this manuscript.

FUNDING

Research was supported by NIH grants HD042635 (PM), HD058638 (KS), 5R25GM071638 (NIH RISE – CSULB), NIH grants HD04612, and HD097965 (MM).

ACKNOWLEDGMENTS

We thank Timbora Chuon for his technical support.

REFERENCES

1. Baulieu EE. Steroid hormones in the brain: several mechanisms? In: Fuxe K, Gustafsson JA, editors. *Steroid Hormone Regulation of the Brain*. Oxford: Pergamon (1981). p. 3–14. doi: 10.1016/B978-0-08-026864-4.50007-4
2. Baulieu EE. Neurosteroids: a new function in the brain. *Biol Cell.* (1991) 71:3–10. doi: 10.1016/0248-4900(91)90045-O
3. Baulieu EE. Neurosteroids: a novel function of the brain. *Psychoneuroendocrinology.* (1998) 23:963–87. doi: 10.1016/S0306-4530(98)00071-7
4. Corpechot C, Young J, Calvel M, Wehrey C, Veltz JN, Touyer G, et al. Neurosteroids: 3 alpha-hydroxy-5 alpha-pregnan-20-one and its precursors in the brain, plasma, and steroidogenic glands of male and female rats. *Endocrinology.* (1993) 133:1003–9. doi: 10.1210/endo.133.3.8365352
5. Schumacher M, Akwa Y, Guennoun R, Robert F, Labombarda F, Desarnaud F, et al. Steroid synthesis and metabolism in the nervous system: trophic and protective effects. *J Neurocytol.* (2000) 29:307–26. doi: 10.1023/A:1007152904926
6. Schumacher M, Guennoun R, Robert F, Carelli C, Gago N, Ghoumari A, et al. Local synthesis and dual actions of progesterone in the nervous system: neuroprotection and myelination. *Growth Hormone IGF Res.* (2004) 14(Suppl A):S18–33. doi: 10.1016/j.ghir.2004.03.007
7. Schumacher M, Weill-Engerer S, Liere P, Robert F, Franklin RJ, Garcia-Segura LM, et al. Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. *Prog Neurobiol.* (2003) 71:3–29. doi: 10.1016/j.pneurobio.2003.09.004
8. Guennoun R, Labombarda F, Gonzalez Deniselle MC, Liere P, De Nicola AF, Schumacher M. Progesterone and allopregnanolone in the central nervous system: response to injury and implication for neuroprotection. *J Ster Biochem Mol Biol.* (2015) 146:48–61. doi: 10.1016/j.jsbmb.2014.09.001
9. Zhang P, Xie MQ, Ding YQ, Liao M, Qi SS, Chen SX, et al. Allopregnanolone enhances the neurogenesis of midbrain dopaminergic neurons in APPswe/PSEN1 mice. *Neuroscience.* (2015) 290:214–26. doi: 10.1016/j.neuroscience.2015.01.019
10. Cai H, Cao T, Zhou X, Yao JK. Neurosteroids in schizophrenia: pathogenic and therapeutic implications. *Front Psychiatry.* (2018) 9:73. doi: 10.3389/fpsyt.2018.00073
11. Reddy DS, Rogawski MA. Neurosteroid replacement therapy for catamenial epilepsy. *Neurotherapeutics.* (2009) 6:392–401. doi: 10.1016/j.nurt.2009.01.006
12. Morrow AL. Recent developments in the significance and therapeutic relevance of neuroactive steroids–Introduction to the special issue. *Pharmacol Therap.* (2007) 116:1–6. doi: 10.1016/j.pharmthera.2007.04.003
13. Dubrovsky BO. Steroids, neuroactive steroids and neurosteroids in psychopathology. *Prog Neuropsychopharmacol Biol Psychiatry.* (2005) 29:169–92. doi: 10.1016/j.pnpbp.2004.11.001
14. Mayo W, Dellu F, Robel P, Cherkaoui J, Le Moal M, Baulieu EE, et al. Infusion of neurosteroids into the nucleus basalis magnocellularis affects cognitive processes in the rat. *Brain Res.* (1993) 607:324–8. doi: 10.1016/0006-8993(93)91524-V
15. Vallee M, Mayo W, Darnaudery M, Corpechot C, Young J, Koehl M, et al. Neurosteroids: deficient cognitive performance in aged rats depends on low

pregnenolone sulfate levels in the hippocampus. *Proc Natl Acad Sci USA*.. (1997) 94:14865–70. doi: 10.1073/pnas.94.26.14865
16. Vallee M, Mayo W, Le Moal M. Role of pregnenolone, dehydroepiandrosterone and their sulfate esters on learning and memory in cognitive aging. *Brain Res Brain Res Rev.* (2001) 37:301–12. doi: 10.1016/S0165-0173(01)00135-7
17. Vallee M, Mayo W, Koob GF, Le Moal M. Neurosteroids in learning and memory processes. *Int Rev Neurobiol.* (2001) 46:273–320. doi: 10.1016/S0074-7742(01)46066-1
18. Ladurelle N, Eychenne B, Denton D, Blair-West J, Schumacher M, Robel P, et al. Prolonged intracerebroventricular infusion of neurosteroids affects cognitive performances in the mouse. *Brain Res.* (2000) 858:371–9. doi: 10.1016/S0006-8993(00)01953-3
19. Mittelman-Smith MA, Wong AM, Micevych PE. Estrogen and progesterone integration in an *in vitro* model of RP3V kisspeptin neurons. *Neuroendocrinology.* (2018) 106:101–15. doi: 10.1159/000471878
20. Mittelman-Smith MA, Williams H, Krajewski-Hall SJ, Lai J, Ciofi P, McMullen NT, et al. Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. *Endocrinology.* (2012) 153:2800–12. doi: 10.1210/en.2012-1045
21. Rance NE, Dacks PA, Mittelman-Smith MA, Romanovsky AA, Krajewski-Hall SJ. Modulation of body temperature and LH secretion by hypothalamic KNDy (kisspeptin, neurokinin B and dynorphin) neurons: a novel hypothesis on the mechanism of hot flushes. *Front Neuroendocrinol.* (2013) 34:211–27. doi: 10.1016/j.yfrne.2013.07.003
22. Navarro VM. New insights into the control of pulsatile GnRH release: the role of Kiss1/neurokinin B neurons. *Front Endocrinol.* (2012) 3:48. doi: 10.3389/fendo.2012.00048
23. Herbison AE, Theodosis DT. Localization of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone-releasing hormone in the male and female rat. *Neuroscience.* (1992) 50:283–98. doi: 10.1016/0306-4522(92)90423-Y
24. Shivers BD, Harlan RE, Morrell JI, Pfaff DW. Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. *Nature.* (1983) 304:345–7. doi: 10.1038/304345a0
25. Wintermantel TM, Campbell RE, Porteous R, Bock D, Grone HJ, Todman MG, et al. Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. *Neuron.* (2006) 52:271–80. doi: 10.1016/j.neuron.2006.07.023
26. Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA. Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology.* (2005) 146:3686–92. doi: 10.1210/en.2005-0488
27. Lehman MN, Merkley CM, Coolen LM, Goodman RL. Anatomy of the kisspeptin neural network in mammals. *Brain Res.* (2010) 1364:90–102. doi: 10.1016/j.brainres.2010.09.020
28. Zhang J, Yang L, Lin N, Pan X, Zhu Y, Chen X. Aging-related changes in RP3V kisspeptin neurons predate the reduced activation of GnRH neurons during the early reproductive decline in female mice. *Neurobiol Aging.* (2014) 35:655–68. doi: 10.1016/j.neurobiolaging.2013.08.038
29. Han SK, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, et al. Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. *J Neurosci.* (2005) 25:11349–56. doi: 10.1523/JNEUROSCI.3328-05.2005
30. Clarkson J, Herbison AE. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinol.* (2006) 147:5817–25. doi: 10.1210/en.2006-0787
31. Liu X, Lee K, Herbison AE. Kisspeptin excites gonadotropin-releasing hormone neurons through a phospholipase C/calcium-dependent pathway regulating multiple ion channels. *Endocrinology.* (2008) 149:4605–14. doi: 10.1210/en.2008-0321
32. Delhousay LK, Chuon T, Mittleman-Smith M, Micevych P, Sinchak K. RP3V kisspeptin neurons mediate neuroprogesterone induction of the luteinizing hormone surge in female rat. *bioRxiv.* (2019) 2019:700435. doi: 10.1101/700435
33. Chuon T, Feri M, Carlson C, Ondrejik S, Micevych P, Sinchak K. Neuroprogesterone signals through Src kinase within RP3V neurons to induce the luteinizing hormone surge in female rats. *bioRxiv.* (2019) 2019:835470. doi: 10.1101/835470
34. Petersen SL, Barraclough CA. Suppression of spontaneous LH surges in estrogen-treated ovariectomized rats by microimplants of antiestrogens into the preoptic brain. *Brain Res.* (1989) 484:279–89. doi: 10.1016/0006-8993(89)90371-5
35. Wiegand SJ, Terasawa E, Bridson WE. Persistent estrus and blockade of progesterone-induced LH release follows lesions which do not damage the suprachiasmatic nucleus. *Endocrinology.* (1978) 102:1645–8. doi: 10.1210/endo-102-5-1645
36. Wiegand SJ, Terasawa E. Discrete lesions reveal functional heterogeneity of suprachiasmatic structures in regulation of gonadotropin secretion in the female rat. *Neuroendocrinology.* (1982) 34:395–404. doi: 10.1159/000123335
37. Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE. Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *J Neurosci.* (2008) 28:8691–7. doi: 10.1523/JNEUROSCI.1775-08.2008
38. Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, Takase K, et al. Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. *J Reprod Dev.* (2007) 53:367–78. doi: 10.1262/jrd.18146
39. Williams WP, 3rd, Jarjisian SG, Mikkelsen JD, Kriegsfeld LJ. Circadian control of kisspeptin and a gated GnRH response mediate the preovulatory luteinizing hormone surge. *Endocrinology.* (2011) 152:595–606. doi: 10.1210/en.2010-0943
40. Herbison AE, de Tassigny X, Doran J, Colledge WH. Distribution and postnatal development of Gpr54 gene expression in mouse brain and gonadotropin-releasing hormone neurons. *Endocrinology.* (2010) 151:312–21. doi: 10.1210/en.2009-0552
41. Parhar IS, Ogawa S, Sakuma Y. Laser-captured single digoxigenin-labeled neurons of gonadotropin-releasing hormone types reveal a novel G protein-coupled receptor (Gpr54) during maturation in cichlid fish. *Endocrinology.* (2004) 145:3613–8. doi: 10.1210/en.2004-0395
42. Bosch MA, Tonsfeldt KJ, Ronnekleiv OK. mRNA expression of ion channels in GnRH neurons: subtype-specific regulation by 17beta-estradiol. *Mol Cell Endocrinol.* (2013) 367:85–97. doi: 10.1016/j.mce.2012.12.021
43. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, et al. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J Biol Chem.* (2001) 276:34631–6. doi: 10.1074/jbc.M104847200
44. Li Q, Jimenez-Krassel F, Ireland JJ, Smith GW. Gene expression profiling of bovine preovulatory follicles: gonadotropin surge and prostanoid-dependent up-regulation of genes potentially linked to the ovulatory process. *Reproduction.* (2009) 137:297–307. doi: 10.1530/REP-08-0308
45. Roseweir AK, Millar RP. The role of kisspeptin in the control of gonadotrophin secretion. *Hum Reprod Update.* (2009) 15:203–12. doi: 10.1093/humupd/dmn058
46. Kirilov M, Clarkson J, Liu X, Roa J, Campos P, Porteous R, et al. Dependence of fertility on kisspeptin-Gpr54 signaling at the GnRH neuron. *Nat Commun.* (2013) 4:2492. doi: 10.1038/ncomms3492
47. Novaira HJ, Sonko ML, Hoffman G, Koo Y, Ko C, Wolfe A, et al. Disrupted kisspeptin signaling in GnRH neurons leads to hypogonadotrophic hypogonadism. *Mol Endocrinol.* (2014) 28:225–38. doi: 10.1210/me.2013-1319
48. Gotlieb N, Moeller J, Kriegsfeld LJ. Circadian control of neuroendocrine function: implications for health and disease. *Curr Opin Physiol.* (2018) 5:133–40. doi: 10.1016/j.cophys.2018.11.001
49. Gotlieb N, Baker CN, Moeller J, Kriegsfeld LJ. Time-of-day-dependent sensitivity of the reproductive axis to RFamide-related peptide-3 inhibition in female Syrian hamsters. *J Neuroendocrinol.* (2019) 31:e12798. doi: 10.1111/jne.12798
50. Rizwan MZ, Poling MC, Corr M, Cornes PA, Augustine RA, Quennell JH, et al. RFamide-related peptide-3 receptor gene expression in GnRH and kisspeptin neurons and GnRH-dependent mechanism of action. *Endocrinology.* (2012) 153:3770–9. doi: 10.1210/en.2012-1133
51. Poling MC, Quennell JH, Anderson GM, Kauffman AS. Kisspeptin neurones do not directly signal to RFRP-3 neurones but RFRP-3 may directly modulate

a subset of hypothalamic kisspeptin cells in mice. *J Neuroendocrinol.* (2013) 25:876–86. doi: 10.1111/jne.12084
52. Gibson EM, Humber SA, Jain S, Williams WP 3rd, Zhao S, Bentley GE, et al. Alterations in RFamide-related peptide expression are coordinated with the preovulatory luteinizing hormone surge. *Endocrinology.* (2008) 149:4958–69. doi: 10.1210/en.2008-0316
53. Kriegsfeld LJ, Mei DF, Bentley GE, Ubuka T, Mason AO, Inoue K, et al. Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. *Proc Natl Acad Sci USA.* (2006) 103:2410–5. doi: 10.1073/pnas.0511003103
54. Chazal G, Faudon M, Gogan F, Laplante E. Negative and positive effects of oestradiol upon luteinizing hormone secretion in the female rat. *J Endocrinol.* (1974) 61:511–2. doi: 10.1677/joe.0.0610511
55. Mann DR, Korowitz CD, Macfarland LA, Cost MG. Interactions of the light-dark cycle, adrenal glands and time of steroid administration in determining the temporal sequence of LH and prolactin release in female rats. *Endocrinol.* (1976) 99:1252–62. doi: 10.1210/endo-99-5-1252
56. Sridaran R, Blake CA. Effects of long-term adrenalectomy on periovulatory increases in serum gonadotrophins and ovulation in rats. *J Endocrinol.* (1980) 84:75–82. doi: 10.1677/joe.0.0840075
57. DePaolo LV, Barraclough CA. Dose dependent effects of progesterone on the facilitation and inhibition of spontaneous gonadotropin surges in estrogen treated ovariectomized rats. *BiolReprod.* (1979) 21:1015–23. doi: 10.1095/biolreprod21.4.1015
58. Brom GM, Schwartz NB. Acute changes in the estrous cycle following ovariectomy in the golden hamster. *Neuroendocrinology.* (1968) 3:366–77. doi: 10.1159/000121725
59. Ferin M, Tempone A, Zimmering PE, Van de Wiele RL. Effect of antibodies to 17beta-estradiol and progesterone on the estrous cycle of the rat. *Endocrinology.* (1969) 85:1070–8. doi: 10.1210/endo-85-6-1070
60. Labhsetwar AP. Role of estrogens in ovulation: a study using the estrogen-antagonist, I.C.I. 46,474. *Endocrinology.* (1970) 87:542–51. doi: 10.1210/endo-87-3-542
61. Rao IM, Mahesh VB. Role of progesterone in the modulation of the preovulatory surge of gonadotropins and ovulation in the pregnant mare's serum gonadotropin-primed immature rat and the adult rat. *Biol Reprod.* (1986) 35:1154–61. doi: 10.1095/biolreprod35.5.1154
62. Mahesh VB, Brann DW. Regulation of the preovulatory gonadotropin surge by endogenous steroids. *Steroids.* (1998) 63:616–29. doi: 10.1016/S0039-128X(98)00075-0
63. Chappell PE, Levine JE. Stimulation of gonadotropin-releasing hormone surges by estrogen. I. Role of hypothalamic progesterone receptors. *Endocrinology.* (2000) 141:1477–85. doi: 10.1210/endo.141.4.7428
64. Nath R, Bhakta A, Moudgil VK. ZK98299–a new antiprogesterone: biochemical characterization of steroid binding parameters in the calf uterine cytosol. *Arch Biochem Biophys.* (1992) 292:303–10. doi: 10.1016/0003-9861(92)90083-9
65. Micevych PE, Chaban V, Ogi J, Dewing P, Lu JK, Sinchak K. Estradiol stimulates progesterone synthesis in hypothalamic astrocyte cultures. *Endocrinology.* (2007) 148:782–9. doi: 10.1210/en.2006-0774
66. Micevych P, Sinchak K. Synthesis and function of hypothalamic neuroprogesterone in reproduction. *Endocrinology.* (2008) 149:2739–42. doi: 10.1210/en.2008-0011
67. Micevych P, Sinchak K. Estradiol regulation of progesterone synthesis in the brain. *Mol Cell Endocrinol.* (2008) 290:44–50. doi: 10.1016/j.mce.2008.04.016
68. Micevych PE, Sinchak K. The neurosteroid progesterone underlies estrogen positive feedback of the LH surge. *Front Endocrinol.* (2011) 2:90. doi: 10.3389/fendo.2011.00090
69. Smith JT, Popa SM, Clifton DK, Hoffman GE, Steiner RA. Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. *J Neurosci.* (2006) 26:6687–94. doi: 10.1523/JNEUROSCI.1618-06.2006
70. Gottsch ML, Navarro VM, Zhao Z, Glidewell-Kenney C, Weiss J, Jameson JL, et al. Regulation of Kiss1 and dynorphin gene expression in the murine brain by classical and nonclassical estrogen receptor pathways. *J Neurosci.* (2009) 29:9390–5. doi: 10.1523/JNEUROSCI.0763-09.2009
71. Glidewell-Kenney C, Hurley LA, Pfaff L, Weiss J, Levine JE, Jameson JL. Nonclassical estrogen receptor alpha signaling mediates negative feedback in the female mouse reproductive axis. *Proc Natl Acad Sci USA.* (2007) 104:8173–7. doi: 10.1073/pnas.0611514104
72. Micevych P, Sinchak K, Mills RH, Tao L, LaPolt P, Lu JK. The luteinizing hormone surge is preceded by an estrogen-induced increase of hypothalamic progesterone in ovariectomized and adrenalectomized rats. *Neuroendocrinol.* (2003) 78:29–35. doi: 10.1159/000071703
73. Mittelman-Smith MA, Rudolph LM, Mohr MA, Micevych PE. Rodent models of non-classical progesterone action regulating ovulation. *Front Endocrinol.* (2017) 8:165. doi: 10.3389/fendo.2017.00165
74. Zwain IH, Yen SS. Neurosteroidogenesis in astrocytes, oligodendrocytes, and neurons of cerebral cortex of rat brain. *Endocrinol.* (1999) 140:3843–52. doi: 10.1210/endo.140.8.6907
75. Micevych PE, Wong AM, Mittelman-Smith MA. Estradiol membrane-initiated signaling and female reproduction. *Compr Physiol.* (2015) 5:1211–22. doi: 10.1002/cphy.c140056
76. Chen C, Kuo J, Wong A, Micevych P. Estradiol Modulates Translocator Protein (TSPO) and Steroid Acute Regulatory Protein (StAR) via Protein Kinase A (PKA) signaling in hypothalamic astrocytes. *Endocrinology.* (2014) 155:2976–85. doi: 10.1210/en.2013-1844
77. Itzhak Y, Baker L, Norenberg MD. Characterization of the peripheral-type benzodiazepine receptors in cultured astrocytes: evidence for multiplicity. *Glia.* (1993) 9:211–8. doi: 10.1002/glia.440090306
78. Papadopoulos V, Baraldi M, Guilarte TR, Knudsen TB, Lacapere JJ, Lindemann P, et al. Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. *Trends Pharmacol Sci.* (2006) 27:402–9. doi: 10.1016/j.tips.2006.06.005
79. Tu LN, Morohaku K, Manna PR, Pelton SH, Butler WR, Stocco DM, et al. Peripheral benzodiazepine receptor/translocator protein global knock-out mice are viable with no effects on steroid hormone biosynthesis. *J Biol Chem.* (2014) 289:27444–54. doi: 10.1074/jbc.M114.578286
80. Fan J, Campioli E, Midzak A, Culty M, Papadopoulos V. Conditional steroidogenic cell-targeted deletion of TSPO unveils a crucial role in viability and hormone-dependent steroid formation. *Proc Natl Acad Sci USA.* (2015) 112:7261–6. doi: 10.1073/pnas.1502670112
81. Barron AM, Ji B, Kito S, Suhara T, Higuchi M. Steroidogenic abnormalities in translocator protein knockout mice and significance in the aging male. *Biochem J.* (2018) 475:75–85. doi: 10.1042/BCJ20170645
82. Mittelman-Smith MA, Wong AM, Kathiresan AS, Micevych PE. Classical and membrane-initiated estrogen signaling in an *in vitro* model of anterior hypothalamic kisspeptin neurons. *Endocrinology.* (2015) 156:2162–73. doi: 10.1210/en.2014-1803
83. Chaban VV, Lakhter AJ, Micevych P. A membrane estrogen receptor mediates intracellular calcium release in astrocytes. *Endocrinology.* (2004) 145:3788–95. doi: 10.1210/en.2004-0149
84. Kuo J, Hariri OR, Bondar G, Ogi J, Micevych P. Membrane estrogen receptor-alpha interacts with metabotropic glutamate receptor type 1a to mobilize intracellular calcium in hypothalamic astrocytes. *Endocrinology.* (2009) 150:1369–76. doi: 10.1210/en.2008-0994
85. Micevych P, Soma KK, Sinchak K. Neuroprogesterone: Key to estrogen positive feedback? *Brain Res Rev.* (2008) 57:470–80. doi: 10.1016/j.brainresrev.2007.06.009
86. Kuo J, Hamid N, Bondar G, Prossnitz ER, Micevych P. Membrane estrogen receptors stimulate intracellular calcium release and progesterone synthesis in hypothalamic astrocytes. *J Neurosci.* (2010) 30:12950–7. doi: 10.1523/JNEUROSCI.1158-10.2010
87. Soma KK, Sinchak K, Lakhter A, Schlinger BA, Micevych PE. Neurosteroids and female reproduction: estrogen increases 3beta-HSD mRNA and activity in rat hypothalamus. *Endocrinology.* (2005) 146:4386–90. doi: 10.1210/en.2005-0569
88. Micevych PE, Matt DW, Go VLW. Concentrations of cholecystokinin, substance P, and bombesin in discrete regions of male and female rat brain: Sex differences and estrogen effects. *Exp Neurol.* (1981) 100:416–26. doi: 10.1016/0014-4886(88)90119-7
89. Chappell PE, Schneider JS, Kim P, Xu M, Lydon JP, O'Malley BW, et al. Absence of gonadotropin surges and gonadotropin-releasing hormone self- priming in ovariectomized (OVX), estrogen (E2)-treated,

progesterone receptor knockout (PRKO) mice. *Endocrinology.* (1999) 140:3653–8. doi: 10.1210/endo.140.8.6895

90. Gal A, Lin PC, Cacioppo JA, Hannon PR, Mahoney MM, Wolfe A, et al. Loss of fertility in the absence of progesterone receptor expression in kisspeptin neurons of female mice. *PLoS ONE.* (2016) 11:e0159534. doi: 10.1371/journal.pone.0159534
91. Stephens SB, Tolson KP, Rouse ML Jr, Poling MC, Hashimoto-Partyka MK, et al. Absent progesterone signaling in kisspeptin neurons disrupts the LH surge and impairs fertility in female mice. *Endocrinology.* (2015) 156:3091–7. doi: 10.1210/en.2015-1300
92. Pedram A, Razandi M, Sainson RC, Kim JK, Hughes CC, Levin ER. A conserved mechanism for steroid receptor translocation to the plasma membrane. *J Biol Chem.* (2007) 282:22278–88. doi: 10.1074/jbc.M611877200
93. Boonyaratanakornkit V, Scott MP, Ribon V, Sherman L, Anderson SM, Maller JL, et al. Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. *Mol cell.* (2001) 8:269–80. doi: 10.1016/S1097-2765(01)00304-5
94. Boonyaratanakornkit V, McGowan E, Sherman L, Mancini MA, Cheskis BJ, Edwards DP. The role of extranuclear signaling actions of progesterone receptor in mediating progesterone regulation of gene expression and the cell cycle. *Mol Endocrinol.* (2007) 21:359–75. doi: 10.1210/me.2006-0337
95. Pang DT, Wang JK, Valtorta F, Benfenati F, Greengard P. Protein tyrosine phosphorylation in synaptic vesicles. *Proc Natl Acad Sci USA.* (1988) 85:762–6. doi: 10.1073/pnas.85.3.762
96. Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, et al. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *EMBO J.* (1990) 9:1603–14. doi: 10.1002/j.1460-2075.1990.tb08280.x
97. Micevych PE, Sinchak K. Extranuclear signaling by ovarian steroids in the regulation of sexual receptivity. *Hormones Behav.* (2018) 104:4–14. doi: 10.1016/j.yhbeh.2018.05.001
98. Mani SK, Allen JM, Lydon JP, Mulac-Jericevic B, Blaustein JD, DeMayo FJ, et al. Dopamine requires the unoccupied progesterone receptor to induce sexual behavior in mice [published erratum appears in Mol Endocrinol 1997 Apr;11:423]. *MolEndocrinol.* (1996) 10:1728–37. doi: 10.1210/mend.10.12.8961281
99. Sleiter N, Pang Y, Park C, Horton TH, Dong J, Thomas P, et al. Progesterone receptor A (PRA) and PRB-independent effects of progesterone on gonadotropin-releasing hormone release. *Endocrinology.* (2009) 150:3833–44. doi: 10.1210/en.2008-0774
100. Mani SK, Blaustein JD. Neural progestin receptors and female sexual behavior. *Neuroendocrinology.* (2012) 96:152–61. doi: 10.1159/000338668
101. Zhu Y, Bond J, Thomas P. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. *Proc Natl Acad Sci USA.* (2003) 100:2237–42. doi: 10.1073/pnas.0436133100
102. Zhu Y, Rice CD, Pang Y, Pace M, Thomas P. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc Natl Acad Sci USA.* (2003) 100:2231–6. doi: 10.1073/pnas.0336132100
103. Pang Y, Dong J, Thomas P. Characterization, neurosteroid binding and brain distribution of human membrane progesterone receptors delta and {epsilon} (mPRdelta and mPR{epsilon) and mPRdelta involvement in neurosteroid inhibition of apoptosis. *Endocrinology.* (2013) 154:283–95. doi: 10.1210/en.2012-1772
104. Zuloaga DG, Yahn SL, Pang Y, Quihuis AM, Oyola MG, Reyna A, et al. Distribution and estrogen regulation of membrane progesterone receptor-beta in the female rat brain. *Endocrinology.* (2012) 153:4432–43. doi: 10.1210/en.2012-1469
105. Peluso JJ, Gawkowska A, Liu X, Shioda T, Pru JK. Progesterone receptor membrane component-1 regulates the development and Cisplatin sensitivity of human ovarian tumors in athymic nude mice. *Endocrinology.* (2009) 150:4846–54. doi: 10.1210/en.2009-0730
106. Peluso JJ, Liu X, Gawkowska A, Johnston-MacAnanny E. Progesterone activates a progesterone receptor membrane component 1-dependent mechanism that promotes human granulosa/luteal cell survival but not progesterone secretion. *J Clin Endocrinol Metab.* (2009) 94:2644–9. doi: 10.1210/jc.2009-0147
107. Peluso JJ, Liu X, Uliasz T, Pru CA, Kelp NC, Pru J. PGRMC1/2 promotes luteal vascularization and maintains the primordial follicles of mice. *Reproduction.* (2018) 156:365–73. doi: 10.1530/REP-18-0155
108. Peluso JJ, Romak J, Liu X. Progesterone receptor membrane component-1 (PGRMC1) is the mediator of progesterone's antiapoptotic action in spontaneously immortalized granulosa cells as revealed by PGRMC1 small interfering ribonucleic acid treatment and functional analysis of PGRMC1 mutations. *Endocrinology.* (2008) 149:534–43. doi: 10.1210/en.2007-1050
109. Intlekofer KA, Petersen SL. Distribution of mRNAs encoding classical progestin receptor, progesterone membrane components 1 and 2, serpine mRNA binding protein 1, and progestin and ADIPOQ receptor family members 7 and 8 in rat forebrain. *Neuroscience.* (2011) 172:55–65. doi: 10.1016/j.neuroscience.2010.10.051
110. Clark NC, Pru CA, Yee SP, Lydon JP, Peluso JJ, Pru JK. Conditional ablation of progesterone receptor membrane component 2 causes female premature reproductive senescence. *Endocrinology.* (2017) 158:640–51. doi: 10.1210/en.2016-1701
111. Krebs CJ, Jarvis ED, Chan J, Lydon JP, Ogawa S, Pfaff DW. A membrane-associated progesterone-binding protein, 25-Dx, is regulated by progesterone in brain regions involved in female reproductive behaviors. *Proc Natl Acad Sci USA.* (2000) 97:12816–21. doi: 10.1073/pnas.97.23.12816
112. Guo M, Zhang C, Wang Y, Feng L, Wang Z, Niu W, et al. Progesterone receptor membrane component 1 mediates progesterone-induced suppression of oocyte meiotic prophase i and primordial folliculogenesis. *Sci Rep.* (2016) 6:36869. doi: 10.1038/srep36869
113. Skiadas CC, Duan S, Correll M, Rubio R, Karaca N, Ginsburg ES, et al. Ovarian reserve status in young women is associated with altered gene expression in membrana granulosa cells. *Mol Hum Reprod.* (2012) 18:362–71. doi: 10.1093/molehr/gas008
114. Mansouri MR, Schuster J, Badhai J, Stattin EL, Losel R, Wehling M, et al. Alterations in the expression, structure and function of progesterone receptor membrane component-1 (PGRMC1) in premature ovarian failure. *Hum Mol Genet.* (2008) 17:3776–83. doi: 10.1093/hmg/ddn274
115. Intlekofer KA, Petersen SL. 17beta-estradiol and progesterone regulate multiple progestin signaling molecules in the anteroventral periventricular nucleus, ventromedial nucleus and sexually dimorphic nucleus of the preoptic area in female rats. *Neuroscience.* (2011) 176:86-92. doi: 10.1016/j.neuroscience.2010.12.033
116. Richter TA, Terasawa E. Neural mechanisms underlying the pubertal increase in LHRH release in the rhesus monkey. *Trends Endocrinol Metab.* (2001) 12:353–9. doi: 10.1016/S1043-2760(01)00442-8
117. Mayer C, Acosta-Martinez M, Dubois SL, Wolfe A, Radovick S, Boehm U, et al. Timing and completion of puberty in female mice depend on estrogen receptor alpha-signaling in kisspeptin neurons. *Proc Natl Acad Sci USA.* (2010) 107:22693–8. doi: 10.1073/pnas.1012406108
118. Mohr MA, Falcy BA, Laham BJ, Wong A, Micevych PE. Neuroprogesterone synthesis in hypothalamic astrocytes is associated with a pubertal increase in membrane ERα levels. *bioRxiv.* (2019) 2019:833434. doi: 10.1101/833434
119. Micevych PE, Mermelstein PG, Sinchak K. Estradiol membrane-initiated signaling in the brain mediates reproduction. *Trends Neurosci.* (2017) 40:654–66. doi: 10.1016/j.tins.2017.09.001
120. Mohr MA, DonCarlos LL, Sisk CL. Inhibiting production of new brain cells during puberty or adulthood blunts the hormonally induced surge of luteinizing hormone in female rats. *eNeuro.* (2017) 4, 1–13. doi: 10.1523/ENEURO.0133-17.2017
121. Mohr MA, Garcia FL, DonCarlos LL, Sisk CL. Neurons and glial cells are added to the female rat anteroventral periventricular nucleus during puberty. *Endocrinology.* (2016) 157:2393–402. doi: 10.1210/en.2015-2012

122. Ahmed EI, Zehr JL, Schulz KM, Lorenz BH, DonCarlos LL, Sisk CL. Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions. *Nat Neurosci.* (2008) 11:995–7. doi: 10.1038/nn.2178
123. Davis EC, Shryne JE, Gorski RA. Structural sexual dimorphisms in the anteroventral periventricular nucleus of the rat hypothalamus are sensitive to gonadal steroids perinatally, but develop peripubertally. *Neuroendocrinology.* (1996) 63:142–8. doi: 10.1159/000126950
124. Hoffman GE, Le WW, Schulterbrandt T, Legan SJ. Estrogen and progesterone do not activate Fos in AVPV or LHRH neurons in male rats. *Brain Res.* (2005) 1054:116–24. doi: 10.1016/j.brainres.2005.06.082

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.

frontiers in Endocrinology

MINI REVIEW
published: 23 July 2020
doi: 10.3389/fendo.2020.00468

A Teleost Fish Model to Understand Hormonal Mechanisms of Non-breeding Territorial Behavior

Ana C. Silva[1,2], *Lucía Zubizarreta*[2,3] *and Laura Quintana*[2]*

[1] Laboratorio de Neurociencias, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay, [2] Unidad Bases Neurales de la Conducta, Departamento de Neurofisiología Celular y Molecular, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay, [3] Laboratorio de Neurofisiología Celular y Sináptica, Departamento de Fisiología, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay

Aggressive behaviors occurring dissociated from the breeding season encourage the search of non-gonadal underlying regulatory mechanisms. Brain estrogen has been shown to be a key modulator of this behavior in bird and mammal species, and it remains to be understood if this is a common mechanism across vertebrates. This review focuses on the contributions of *Gymnotus omarorum*, the first teleost species in which estrogenic modulation of non-breeding aggression has been demonstrated. *Gymnotus omarorum* displays year-long aggression, which has been well characterized in the non-breeding season. In the natural habitat, territory size is independent of sex and determined by body size. During the breeding season, on the other hand, territory size no longer correlates to body size, but rather to circulating estrogens and gonadosomatic index in females, and 11-ketotestosterone in males. The hormonal mechanisms underlying non-breeding aggression have been explored in dyadic encounters in lab settings. Males and females display robust aggressive contests, whose outcome depends only on body size asymmetry. This agonistic behavior is independent of gonadal hormones and fast acting androgens. Nevertheless, it is dependent on fast acting estrogenic action, as acute aromatase blockers affect aggression engagement, intensity, and outcome. Transcriptomic profiling in the preoptic area region shows non-breeding individuals express aromatase and other steroidogenic enzyme transcripts. This teleost model reveals there is a role of brain estrogen in the control of non-breeding aggression which seems to be common among distant vertebrate species.

Keywords: ***Gymnotus omarorum*, non-breeding aggression, fadrozole, natural spacing, estrogen**

OPEN ACCESS

Edited by:
Kiran K. Soma,
University of British Columbia, Canada

Reviewed by:
Chelsea Anne Weitekamp,
United States Environmental Protection Agency, United States
Devaleena S. Pradhan,
Idaho State University, United States

***Correspondence:**
Laura Quintana
lquintana@iibce.edu.uy

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

Received: *01 March 2020*
Accepted: *15 June 2020*
Published: *23 July 2020*

Citation:
Silva AC, Zubizarreta L and Quintana L (2020) A Teleost Fish Model to Understand Hormonal Mechanisms of Non-breeding Territorial Behavior. Front. Endocrinol. 11:468. doi: 10.3389/fendo.2020.00468

INTRODUCTION

The study of territoriality can provide insight into how animals integrate social and environmental cues with their physiological context to produce behavioral responses. Steroid hormones are key in this integration, affecting behavior through the modulation of brain areas belonging to the social behavior network (1–3). Territoriality occurs when animals defend spatially associated resources against competing individuals, and it is frequently mediated by agonistic encounters (4–6). Animals will only defend resources when the benefits exceed the costs of defense, and this is key to understanding how spacing, mating, and social systems have evolved (7). Pioneering studies in the

field of behavioral ecology (8) have shown that optimal cost-benefit balance in territorial defense occurs when animals compete for mating opportunities, while the defense of resources unrelated to reproduction is less often observed. Nonetheless, in a few species territorial defense is present year-round (9–11), which may ensure access to foraging areas or protection from predators across seasons (4). Territorial aggression may thus occur in these unconventional cases uncoupled from a breeding physiology and independently from gonadal hormones.

Aggressive behaviors which occur dissociated from the breeding season, encourage the search of non-gonadal underlying regulatory mechanisms (12, 13). Early reports in wild birds established the independence of non-breeding aggression from circulating androgens (14–16). Many studies have shown that aggression may occur when gonads are regressed and even after castration in some species of birds, mammals, reptiles and fish (9, 11, 17–25). In addition, it has also been reported that territorial challenges during the non-breeding season do not affect circulating testosterone (22, 26, 27). Brain estrogens have been shown to have a forefront role in the regulation of non-breeding aggression. This was first postulated by pioneer research showing that estrogens promote aggression in non-breeding song sparrows (18, 28, 29) and in California mice subjected to a short photoperiod (30, 31). Aromatase, which converts androgens into estrogens, is present in brain regions related to aggression, and may display seasonal changes in activity (32, 33). This raises the question: is the role of brain estrogen underlying non-breeding aggression a general strategy across vertebrates?

Fish are an ideal group to approach this question as they are evolutionarily early vertebrates, they display diverse and elaborate social behaviors, brain areas related to social behavior are conserved, and they exhibit extraordinarily high levels of brain aromatase activity (34–38).

This review focuses on the contributions from a teleost fish model on the hormonal modulation of non-breeding territorial behavior, to better understand different mechanisms underlying aggression. South American weakly electric fish of the Order Gymnotiformes, constitute a highly diverse group. They produce electric organ discharges (EOD) that are used for active sensing and communication [reviewed in (39)], and their well-known electrogenic system is composed of discrete nuclei in the brainstem and spinal cord and a peripheral electric organ. This system has been shown to be hormone-sensitive in many of its components frequently producing sexually dimorphic communication signals, making these fish well established models to study steroid action on neural circuits underlying behavior (40–49). *Gymnotus omarorum* occurs naturally at the southern boundary of gymnotiform distribution in South America (Uruguay). It is a seasonal breeder, yet it displays year-round territoriality in both males and females (50). It allows the analysis of territorial aggression in the natural habitat as well as the exploration of its proximate mechanisms in lab settings. The fact that this behavior occurs when gonads are regressed and circulating sex steroids are low, puts the spotlight on brain synthesis of steroid hormones. This is the first teleost model that contributes to revealing common estrogenic roles in the control of non-breeding aggression, broadening the perspective of the current state of knowledge currently based mostly on bird and mammal models.

YEAR-LONG SPACING IN THE NATURAL HABITAT

The spacing patterns of *G. omarorum* in the natural habitat likely reflect year-long territorial defense in both males and females. Territorial defense, usually associated with breeding males, has been proposed to follow two general principles: (1) territory size depends on body size as it is the universal indicator of physical strength and resource holding power (51–53); and (2) territory size depends on individual reproductive state and may be related to circulating androgen levels (54–56). Sexual dimorphism in territory size during breeding can also be expected even in species in which both sexes display territoriality, as males and females may have asymmetries in their motivation and/or their fighting ability. This is the case of red squirrels (*Sciurus vulgaris*), for example, in which males often hold larger territories than females (57) or in the striped plateau lizard (*Sceloporus virgatus*), in which females are more territorial than males (58).

During the breeding season (corresponding to the austral spring-summer, from December to February), this sexually monomorphic species displays similar patterns of spatial arrangement for males and females (59). In resting diurnal conditions, both males and females are found occupying individual spots, distanced at least a meter away from their closest neighbor. A close analysis shows that sex is relevant in spatial arrangements, as animals are more likely to have an opposite-sex than a same-sex closest neighbor. Although males and females hold same-sized territories, when the size of each territory is normalized to its owner's body size, sexual dimorphism arises as females hold relatively larger territories. This interesting difference is probably due to sex-biased reproductive requirements associated to anisogamy, which may lead to higher metabolic requirements in females and thus the need for larger foraging grounds. In male *G. omarorum* gonadosomatic index (GSI) did not show correlation to territory sizes, but circulating 11-ketotestosterone (11-KT, the main bioactive androgen in teleost fish) marginally predicted territory size (59). This data falls in line with the well documented relationship between androgens and male territorial behavior (60–62). In contrast, both female GSI and circulating estradiol show high predicting power on territory size, which constitutes the first report to associate circulating estradiol and territory size in a vertebrate species (59). In the light of the evidence that estradiol promotes female aggression (63–65), ovarian estradiol is likely involved in the modulation of breeding territorial aggression in this species. In summary, during the breeding season, sexually dimorphic individual traits seem to influence motivation toward territory defense in *G. omarorum* impacting on individual spacing in the wild in a sex-dependent manner.

During the non-breeding season (corresponding to the austral autumn-winter, from June to August), adults of *G. omarorum* occupy individual spots in the wild separated at least one meter

from the closest neighbor. Sex of individuals does not bias spacing, as closest neighbors are randomly opposite-sex or same-sex. Body size, but not sex, correlates positively with territory size (59). Motivation to maintain territories in the non-breeding season may be related to the fact that these fish continuously produce electric signals as a means of communicating and imaging their world. Electrogeneration is an energetically expensive process which has been associated with high basal metabolic requirements (39, 66) and most likely imposes high year-long foraging demands. Equally sized territories between males and females may reflect the same energetic requirements in both sexes.

GONAD-INDEPENDENT AGONISTIC BEHAVIOR MEDIATES NON-BREEDING TERRITORIAL BEHAVIOR

G. omarorum is one of the few teleost species in which the hormonal regulation of non-breeding aggression has been studied [see also damselfish, (22, 27, 67)], and the only teleost species in which the determinants of natural non-breeding spacing have been explored in the field (59).

The acquisition and defense of territories in non-breeding *G. omarorum* have been empirically shown to be mediated by agonistic encounters in laboratory settings (68). When staging dyadic agonistic encounters using a neutral plain arena, all fish engage in rapid escalated conflicts in which the dominant-subordinate status is achieved in <5 min. Subordinates end the struggle when they decide to stop attacking and retreat. In addition, they further signal their surrender electrically: first interrupting their EOD to hide from the dominant, then emitting transient electric submission signals, and finally, adopting a lower post-resolution EOD basal rate (69, 70). The intensity of submission signals emitted by the subordinate individual is correlated to the aggression levels displayed by the dominant (71). Body size is the only predictor of contest outcome, while individual sex has no significant influence (69). After resolution, dominants monopolize the acquired territory and actively exclude subordinate fish to the periphery of the tank (68). Laboratory evidence falls in line with what is observed in the wild, where non-breeding territory sizes are determined by body size and are unrelated to sex. Several pieces of evidence support that the non-breeding agonistic behavior of *G. omarorum* is independent of gonadal hormones. First, intra and intersexual non-breeding agonistic contests are indistinguishable (69, 72). Secondly, aggressive challenges do not have an effect on circulating 11-ketotestosterone (72). Moreover, the clearest evidence of gonadal independence of non-breeding aggression in *G. omarorum* is that agonistic behavior persists unchanged after castration. Gonadectomized and control dyads do not differ in contest outcome, dynamics, aggression levels, nor submissive displays (21), demonstrating that the low levels of non-breeding circulating gonadal hormones are not necessary for the occurrence of this behavior.

NON-GONADAL ESTROGENS MODULATE NON-BREEDING AGONISTIC BEHAVIOR

Brain estrogens are critical regulators of non-breeding aggression. In the absence of high circulating testosterone, brain derived estrogens may be synthesized from circulating adrenal dehydroepiandrosterone (DHEA), proposed to have a key role underlying non-breeding aggression in mammals and birds. DHEA is reported to have higher plasmatic levels in the non-breeding season in birds (26, 73), its levels may respond to social challenges in birds and mammals (26, 74, 75) and it can be metabolized in the brain into active androgens and estrogens (76, 77). In contrast to the breeding season, in non-breeding mammalian and avian models estrogens exert rapid effects upon aggression which reflect non-genomic mechanisms (30, 31, 78, 79). In turn, aggressive interactions can produce changes in steroid hormone levels in specific brain areas of the songbird model (76, 80).

In *G. omarorum* the influence of gonadal hormones in the non-breeding aggression has been ruled out by castration experiments (21), and the role of extra-gonadal steroid hormones has been tested via pharmacological manipulations. Short term involvement of androgens and estrogens was explored focusing on the effects these hormones have on the rapid dynamics of conflict and resolution. Acutely impeding aromatase action by administration of its inhibitor (Fadrozole, 60 min pre contest) in intrasexual dyads had a profound effect in non-breeding agonistic encounters of *G. omarorum*. Overall, results from both male-male and female-female contests show that the inhibition of estrogen synthesis causes a decrease in aggressive displays revealed by an important delay in initiating overt aggression. In addition, it decreases aggression levels and prevents potential winners (larger fish) from achieving dominance (21, 81). Direct short-term effects of androgens were ruled out, since acute treatment with androgen receptor antagonists showed no influence upon conflict engagement, aggression dynamics nor the establishment of dominant-subordinate status (81). If androgens were directly involved in the modulation of non-breeding aggression in *G. omarorum*, their action may be evinced in a longer time frame, as has been observed in other non-breeding territorial fish in which chronic androgen receptor blocking decreases aggression (22).

To date, the expression pattern of brain aromatase has been identified in several teleost species (82–89); including a recent study in the weakly electric fish *Apteronotus leptorhynchus* (90), which also exhibits territorial aggression in non-breeding conditions (43, 91). Aromatase mRNA was mapped in non-breeding male and female *A. leptorhynchus* in the telencephalon, preoptic area, hypothalamus, and pituitary gland, showing a high degree of regional conservation with previous reports in teleosts. Reports of the presence of high levels of aromatase in the social behavior network strongly suggest these neural circuits are affected by local estrogen production. Moreover, testosterone aromatization has reported effects in social behavior electric displays in *Apteronotus* (42, 92). The first transcriptomic study carried out in *G. omarorum* during the non-breeding season shows that aromatase, as well as other steroidogenic

enzymes are expressed in the preoptic area (93). This node of the social brain has a well-documented role in aggressive behavior (1, 94–97). Moreover, it has already been shown that preoptic area neuropeptides have a status-dependent role in the modulation of non-breeding aggression in *G. omarorum* (70, 98). The analysis of local brain synthesis of estrogens and androgens in this region regulating non-breeding aggression is currently underway.

Overall, research in *G. omarorum* point to brain estrogen as an important modulator of non-breeding aggression acting in regions of the social brain through rapid mechanisms.

STATE OF THE ART AND PERSPECTIVES: NEUROSTEROIDS UNDERLYING NON-BREEDING AGGRESSION

Currently, *G. omarorum* is the strongest teleost model to approach neuroendocrine mechanisms underlying non-breeding aggression. Contributions in this model demonstrate that brain estrogens are key regulators of non-breeding aggression in a much broader sense than previously reported. Revised evidence, brought together from both laboratory and natural settings, shed light on the sequence of events and underlying mechanisms leading to territory acquisition and spatial distribution in the wild (**Figure 1**). Fish contenders competing for territory display a short evaluation time and engage in escalated conflicts from which a clear dominant-subordinate status emerges. Males and females show no difference in aggressive behavior, but outcome is biased by body size: the larger fish wins and acquires the disputed territory. Agonistic behavior is independent of gonadal hormones and fast acting androgens, although it is strongly dependent on estrogenic action, revealed by the rapid and dramatic effect of blocking estrogen synthesis upon conflict engagement, aggression intensity and establishment of dominance. Agonistic behavior is a key element for the non-breeding distribution of fish in the wild in which animals hold sexually monomorphic territories and body size is the strongest determinant for territory size.

The year-long territorial behavior of *G. omarorum* opens exciting avenues of research on steroid modulation of aggression, and in particular, the yet unexplored role of both circulating and brain-derived steroids in breeding territorial aggression. We have two hypotheses on potential seasonal plasticity in the role of steroids regulating aggression, which are leading our current research. First, we understand that non-breeding contests produce a fast rise in brain estrogen in regions of the social behavior network. This estrogen peak has a rapid, non-genomic effect, promoting aggressive behavior, the fast establishment of dominance, and ultimately, at least in a short time scale, it correlates to the size of the acquired territory in the natural habitat. In absence of high circulating sex steroids, we propose this brain hormonal signature is important in enabling stable territory distributions in natural populations. Secondly, based on the correlation between GSI and territory size in the breeding season, and the independence of aggression from

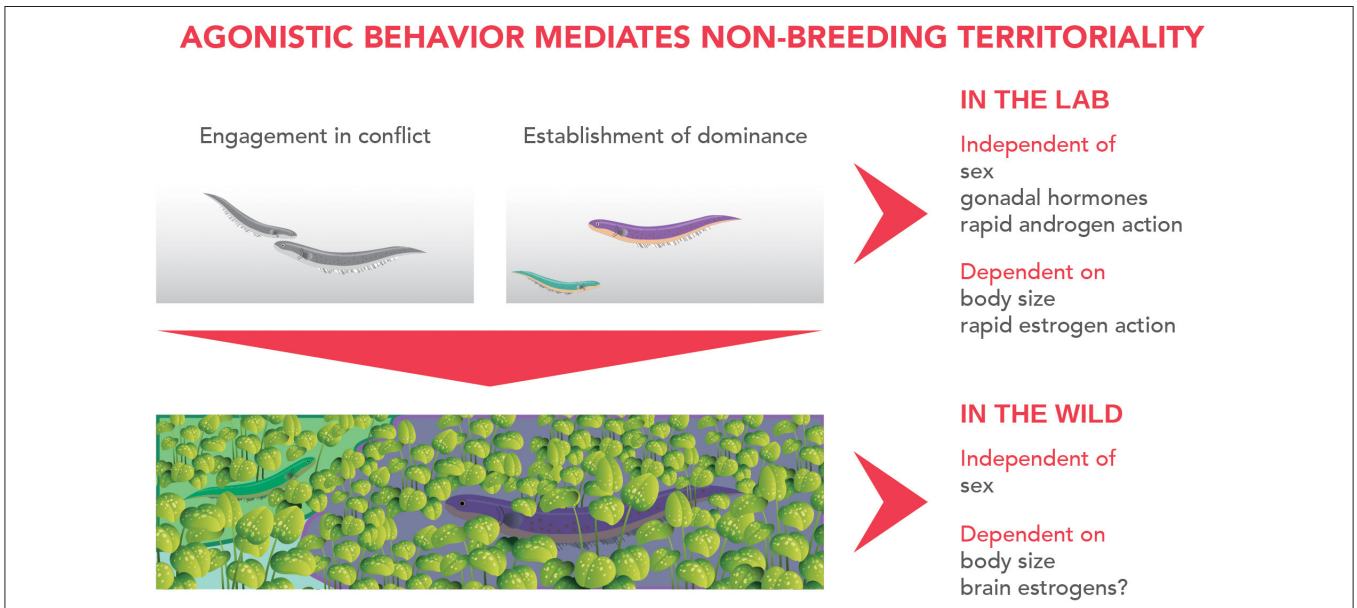

FIGURE 1 | Events and underlying mechanisms of non-breeding territoriality in *Gymnotus omarorum*. Laboratory evidence shows that agonistic behavior mediates territory acquisition, as after conflict resolution dominant animals monopolize the territory and actively exclude subordinate fish to the periphery of the tank. Body size, but not sex, is a strong predictor of conflict outcome. Aggression is maintained even in gonadectomized animals, indicating its independence of gonadal hormones. Behavioral pharmacology evinces aggression is also independent of rapid actions of androgens, although strongly dependent on rapid estrogenic action, as an aromatase inhibitor greatly influences conflict engagement, aggression intensity and establishment of dominance. This evidence suggests that brain-derived estrogens play a key role in agonistic behavior. Agonistic behavior most probably underlies territorial spacing in the natural habitat. Territory sizes are not sex-biased, but do depend on body size, and we propose that at least in the short-term after dominance establishment, they also correlate to brain estrogen levels.

gonads in the non-breeding season, we postulate that regulation of aggression varies seasonally. We hypothesize that estrogens and androgens maintain key roles as modulators, but their main sources alternate from the brain (in the non-breeding season) to the gonads (in the breeding season). In addition, we propose that non-breeding aggression depends exclusively upon brain-derived steroids, either produced *de novo* or from circulating precursors. Studies testing these two hypotheses are underway.

The contributions of *G. omarorum*, a teleost fish with persistent aggression uncoupled from seasonal breeding, expand concepts based on mammal and bird models to further understand the breadth of estrogenic regulation of aggression. Fish are the oldest and most diverse class of vertebrates. Thus, common regulation strategies suggest either a very strong conservation of the trait, or an independent evolution path arriving at the same solution, both underscoring the relevance and extensive impact of estrogens upon aggression.

AUTHOR CONTRIBUTIONS

AS and LQ conceived the general organization of the manuscript. AS, LZ, and LQ wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Funding agencies: Agencia Nacional de Investigación e Innovación Grant ANII_ FCE 136381; Comisión Sectorial de Investigación Científica, Universidad de la República, Uruguay, Grant Grupos I+D (#883158). Programa de Desarrollo de las Ciencias Básicas.

REFERENCES

1. Goodson JL, Kabelik D. Dynamic limbic networks and social diversity in vertebrates: From neural context to neuromodulatory patterning. *Front Neuroendocrinol*. (2009) 30:429–41. doi: 10.1016/j.yfrne.2009.05.007
2. Newman SW. The medial extended amygdala in male reproductive behavior A node in the mammalian social behavior network. *Ann NY Acad Sci*. (1999) 877:242–57. doi: 10.1111/j.1749-6632.1999.tb09271.x
3. O'Connell LA, Hofmann HA. Genes, hormones, and circuits: An integrative approach to study the evolution of social behavior. *Front Neuroendocrinol*. (2011) 32:320–35. doi: 10.1016/j.yfrne.2010.12.004
4. Kaufmann JH. On the definitions and functions of dominance and territoriality. *Biol Rev*. (1983) 58:1–20. doi: 10.1111/j.1469-185X.1983.tb00379.x
5. Nelson RJ. *Biology of Aggression*. New York, NY: Oxford University Press (2006).
6. Wilson EO. *Sociobiology: The New Synthesis*. Oxford: Belknap Press of Harvard U Press (1975).
7. Grant JWA. *Territoriality. Behavioural Ecology of Teleost Fishes*. Oxford: Oxford University Press (1997).
8. Huntingford FA, Turner AK, Downie LM. *Animal Conflict*. Boca Raton, FL: Chapman & Hall/CRC (1987).
9. Caldwell G, Glickman S, Smith E. Seasonal aggression independent of seasonal testosterone in wood rats. *Proc Natl Acad Sci USA*. (1984) 81:5255–7. doi: 10.1073/pnas.81.16.5255
10. Kricher J. Behavioral ecology of tropical birds. *Wilson Bull*. (2001) 113:357–8. doi: 10.1676/0043-5643(2001)113[0357:OL]2.0.CO;2
11. Wingfield JC, Hahn TP. Testosterone and territorial behaviour in sedentary and migratory sparrows. *Anim Behav*. (1994) 47:77–89. doi: 10.1006/anbe.1994.1009
12. Heimovics SA, Trainor BC, Soma KK. Rapid effects of estradiol on aggression in birds and mice: the fast and the furious. *Integr Comp Biol*. (2015) 55:281–93. doi: 10.1093/icb/icv048
13. Munley KM, Rendon NM, Demas GE. Neural androgen synthesis and aggression: insights from a seasonally breeding rodent. *Front Endocrinol*. (2018) 9:136. doi: 10.3389/fendo.2018.00136
14. Burger AE, Millar RP. Seasonal changes of sexual and territorial behaviour and plasma testosterone levels in male lesser sheathbills (*Chionis minor*). *Zeitschrift Tierpsychol*. (1980) 52:397–406. doi: 10.1111/j.1439-0310.1980.tb00726.x
15. Logan CA, Wingfield JC. Autumnal territorial aggression is independent of plasma testosterone in mockingbirds. *Hormones Behav*. (1990) 24:568–81. doi: 10.1016/0018-506X(90)90042-V
16. Schwabl H, Kriner E. Territorial aggression and song of male European robins (*Erithacus rubecula*) in autumn and spring: effects of antiandrogen treatment. *Hormones Behav*. (1991) 25:180–94. doi: 10.1016/0018-506X(91)90049-N
17. Gwinner E, Rödl T, Schwabl H. Pair territoriality of wintering stonechats: behaviour, function and hormones. *Behav Ecol Sociobiol*. (1994) 34:321–7. doi: 10.1007/BF00197002
18. Soma KK, Sullivan K, Wingfield J. Combined aromatase inhibitor and antiandrogen treatment decreases territorial aggression in a wild songbird during the nonbreeding season. *Gen Comp Endocrinol*. (1999) 115:442–53. doi: 10.1006/gcen.1999.7334
19. Wingfield JC. Regulation of territorial behavior in the sedentary song sparrow, melospiza melodia morphna. *Hormones Behav*. (1994) 28:1–15. doi: 10.1006/hbeh.1994.1001
20. Wingfield JC, Lynn S, Soma KK. Avoiding the 'costs' of testosterone: ecological bases of hormone-behavior interactions. *Brain Behav Evol*. (2001) 57:239–51. doi: 10.1159/000047243
21. Jalabert C, Quintana L, Pessina P, Silva A. Extra-gonadal steroids modulate non-breeding territorial aggression in weakly electric fish. *Horm Behav*. (2015) 72:60–7. doi: 10.1016/j.yhbeh.2015.05.003
22. Vullioud P, Bshary R, Ros AFH. Intra- and interspecific aggression do not modulate androgen levels in dusky gregories, yet male aggression is reduced by an androgen blocker. *Horm Behav*. (2013) 64:430–8. doi: 10.1016/j.yhbeh.2013.06.007
23. Moore MC, Marler CA. Effects of testosterone manipulations on nonbreeding season territorial aggression in free-living male lizards, Sceloporus jarrovi. *Gen Comp Endocrinol*. (1987) 65:225–32. doi: 10.1016/0016-6480(87)90170-5
24. Demas GE, Cooper MA, Albers HE, Soma KK. Novel mechanisms underlying neuroendocrine regulation of aggression: a synthesis of rodent, avian, and primate studies. In: *Handbook of Neurochemistry and Molecular Neurobiology*. (2007). p. 337–72.
25. Jasnow AM, Huhman KL, Bartness TJ, Demas GE. Short-day increases in aggression are inversely related to circulating testosterone concentrations in male Siberian hamsters (Phodopus sungorus). *Hormon Behav*. (2000) 38:102–10. doi: 10.1006/hbeh.2000.1604
26. Hau M, Stoddard ST, Soma KK. Territorial aggression and hormones during the non-breeding season in a tropical bird. *Hormones Behav*. (2004) 45:40–9. doi: 10.1016/j.yhbeh.2003.08.002
27. Ros AFH, Vullioud P, Bruintjes R, Vallat A, Bshary R. Intra- and interspecific challenges modulate cortisol but not androgen levels in a year-round territorial damselfish. *J Exp Biol*. (2014) 217:1768. doi: 10.1242/jeb.093666
28. Soma KK, Sullivan KA, Tramontin AD, Saldanha CJ, Schlinger BA, Wingfield JC. Acute and chronic effects of an aromatase inhibitor on territorial aggression in breeding and nonbreeding male song sparrows. *J Comp Physiol A*. (2000) 186:759–69. doi: 10.1007/s003590000129
29. Soma KK, Tramontin AD, Wingfield JC. Oestrogen regulates male aggression in the non-breeding season. *Proc Biol Sci*. (2000) 267:1089–96. doi: 10.1098/rspb.2000.1113

30. Trainor BC, Sima Finy M, Nelson RJ. Rapid effects of estradiol on male aggression depend on photoperiod in reproductively non-responsive mice. *Hormones Behav*. (2008) 53:192–9. doi: 10.1016/j.yhbeh.2007.09.016
31. Trainor BC, Lin S, Finy MS, Rowland MR, Nelson RJ. Photoperiod reverses the effects of estrogens on male aggression via genomic and nongenomic pathways. *Proc Natl Acad Sci USA*. (2007) 104:9840. doi: 10.1073/pnas.0701819104
32. Soma KK, Schlinger BA, Wingfield JC, Saldanha CJ. Brain aromatase, 5α-reductase, and 5β-reductase change seasonally in wild male song sparrows: relationship to aggressive and sexual behavior. *J Neurobiol*. (2003) 56:209–21. doi: 10.1002/neu.10225
33. Soma KK, Bindra RK, Gee J, Wingfield JC, Schlinger BA. Androgen-metabolizing enzymes show region-specific changes across the breeding season in the brain of a wild songbird. *J Neurobiol*. (1999) 41:176–88. doi: 10.1002/(SICI)1097-4695(19991105)41:2<176::AID-NEU2>3.0.CO;2-2
34. Diotel N, Do-Rego J-L, Anglade I, Vaillant C, Pellegrini E, Vaudry H, et al. The brain of teleost fish, a source, and a target of sexual steroids. *Front Neurosci*. (2011) 5:137. doi: 10.3389/fnins.2011.00137
35. Forlano PM, Bass AH. Seasonal plasticity of brain aromatase mRNA expression in glia: divergence across sex and vocal phenotypes. *J Neurobiol*. (2005) 65:37–49. doi: 10.1002/neu.20179
36. O'Connell LA, Hofmann HA. Evolution of a vertebrate social decision-making network. *Science*. (2012) 336:1154. doi: 10.1126/science.1218889
37. Diotel N, Page YL, Mouriec K, Tong S-K, Pellegrini E, Vaillant C, et al. Aromatase in the brain of teleost fish: expression, regulation and putative functions. *Front Neuroendocrinol*. (2010) 31:172–92. doi: 10.1016/j.yfrne.2010.01.003
38. Pasmanik M, Callard GV. Aromatase and 5α-reductase in the teleost brain, spinal cord, and pituitary gland. *Gen Comp Endocrinol*. (1985) 60:244–51. doi: 10.1016/0016-6480(85)90320-X
39. Markham MR, Ban Y, McCauley AG, Maltby R. Energetics of sensing and communication in electric fish: a blessing and a curse in the anthropocene? *Integr Comp Biol*. (2016) 56:889–900. doi: 10.1093/icb/icw104
40. Bass AH. A hormone-sensitive communication system in an electric fish. *J Neurobiol*. (1986) 17:131–55. doi: 10.1002/neu.480170303
41. Dulka J, Maler L, Ellis W. Androgen-induced changes in electrocommunicatory behavior are correlated with changes in substance P-like immunoreactivity in the brain of the electric fish Apteronotus leptorhynchus. *J Neurosci*. (1995) 15:1879. doi: 10.1523/JNEUROSCI.15-03-01879.1995
42. Dulka JG, Maler L. Testosterone modulates female chirping behavior in the weakly electric fish, Apteronotus leptorhynchus. *J Comp Physiol A*. (1994) 174:331–43. doi: 10.1007/BF00240215
43. Dunlap KD. Hormonal and body size correlates of electrocommunication behavior during dydadic interactions in a weakly electric fish, Apteronotus leptorhynchus. *Hormones Behav*. (2002) 41:187–94. doi: 10.1006/hbeh.2001.1744
44. Dunlap KD, Chung M, Castellano JF. Influence of long-term social interaction on chirping behavior, steroid levels and neurogenesis in weakly electric fish. *J Exp Biol*. (2013) 216:2434. doi: 10.1242/jeb.082875
45. Gavassa S, Goldina A, Silva AC, Stoddard PK. Behavioral ecology, endocrinology and signal reliability of electric communication. *J Exp Biol*. (2013) 216:2403. doi: 10.1242/jeb.082255
46. Pouso P, Quintana L, Bolatto C, Silva AC. Brain androgen receptor expression correlates with seasonal changes in the behavior of a weakly electric fish, *Brachyhypopomus gauderio*. *Hormones Behav*. (2010) 58:729–36. doi: 10.1016/j.yhbeh.2010.07.005
47. Smith GT. Evolution and hormonal regulation of sex differences in the electrocommunication behavior of ghost knifefishes (Apteronotidae). *J Exp Biol*. (2013) 216:2421. doi: 10.1242/jeb.082933
48. Zakon H. Weakly electric fish as model systems for studying long-term steroid action on neural circuits. *Brain Behav Evol*. (1993) 42:242–51. doi: 10.1159/000114158
49. Borde M, Quintana L, Comas V, Silva A. Hormone-mediated modulation of the electromotor CPG in pulse-type weakly electric fish. Commonalities and differences across species. *Dev Neurobiol*. (2020) 80:70–80. doi: 10.1002/dneu.22732
50. Black-Cleworth P. The role of electrical discharges in the non-reproductive social behaviour of *Gymnotus carapo* (Gymnotidae, Pisces). *Anim Behav Monographs*. (1970) 3:1–IN1. doi: 10.1016/S0066-1856(70)80001-2
51. Elliott JM. Mechanisms responsible for population regulation in young migratory trout, *Salmo trutta*. III The role of territorial behaviour. *J Anim Ecol*. (1990) 59:803–18. doi: 10.2307/5015
52. Keeley ER. An experimental analysis of territory size in juvenile steelhead trout. *Anim Behav*. (2000) 59:477–90. doi: 10.1006/anbe.1999.1288
53. Woodward G, Ebenman B, Emmerson M, Montoya JM, Olesen JM, Valido A, et al. Body size in ecological networks. *Trends Ecol Evol*. (2005) 20:402–9. doi: 10.1016/j.tree.2005.04.005
54. Adkins-Regan E. *Hormones and Animal Social Behavior*. New Jersey, NJ: Princeton University Press (2005).
55. Mougeot F, Redpath SM, Moss R, Matthiopoulos J, Hudson PJ. Territorial behaviour and population dynamics in red grouse Lagopus lagopus scoticus. I Population experiments. *J Anim Ecol*. (2003) 72:1073–82. doi: 10.1046/j.1365-2656.2003.00781.x
56. Watson A, Parr R. Hormone implants affecting territory size and aggressive and sexual behaviour in red grouse. *Scand J Ornithol*. (1981) 12:55–61. doi: 10.2307/3675905
57. Wauters L, Dhondt AA. Spacing behaviour of red squirrels, *Sciurus vulgaris*: variation between habitats and the sexes. *Anim Behav*. (1992) 43:297–311. doi: 10.1016/S0003-3472(05)80225-8
58. Smith DC. Home range and territory in the striped plateau lizard. (*Sceloporus virgatus*). *Anim Behav*. (1985) 33:417–27. doi: 10.1016/S0003-3472(85)80066-X
59. Zubizarreta L, Quintana L, Hernández D, Teixeira de Mello F, Meerhoff M, Massaaki Honji R, et al. (2020) Seasonal and social factors associated with spacing in a wild territorial electric fish. *PLoS ONE*. 15:e0228976. doi: 10.1371/journal.pone.0228976
60. Monaghan EP, Glickman SE. Hormones and aggressive behavior. *Behav Endocrinol*. (1992) 134:692–4.
61. Nelson RJ. *An Introduction to Behavioral Endocrinology*. 3rd ed. Sunderland, MA: Sinauer Associates (2005).
62. Simon NG, Lu S-F. Androgens and aggression. In: Nelson RJ, editor. *Biology of Aggression*. New York, NY: Oxford University Press (2006). p. 211–30.
63. Albert DJ, Jonik RH, Walsh ML. Hormone-dependent aggression in female rats: testosterone implants attenuate the decline in aggression following ovariectomy. *Physiol Behav*. (1990) 47:659–64. doi: 10.1016/0031-9384(90)90074-E
64. Rendon NM, Amez AC, Proffitt MR, Bauserman ER, Demas GE. Aggressive behaviours track transitions in seasonal phenotypes of female Siberian hamsters. *Funct Ecol*. (2017) 31:1071–81. doi: 10.1111/1365-2435.12816
65. Scaia MF, Morandini L, Noguera C, Trudeau VL, Somoza GM, Pandolfi M. Can estrogens be considered as key elements of the challenge hypothesis? The case of intrasexual aggression in a cichlid fish. *Physiol Behav*. (2018) 194:481–90. doi: 10.1016/j.physbeh.2018.06.028
66. Stoddard PK, Salazar VL. Energetic cost of communication. *J Exp Biol*. (2011) 214:200. doi: 10.1242/jeb.047910
67. Ros AFH, Damjanovic K, Glauser G, Bshary R. No scope for social modulation of steroid levels in a year-round territorial damselfish. *J Exp Zool Part A Ecol Genet Physiol*. (2015) 323:80–8. doi: 10.1002/jez.1900
68. Perrone R, Pedraja F, Valiño G, Tassino B, Silva A. Non-breeding territoriality and the effect of territory size on aggression in the weakly electric fish, *Gymnotus omarorum*. *Acta Ethol*. (2019) 22:79–89. doi: 10.1007/s10211-019-00309-7
69. Batista G, Zubizarreta L, Perrone R, Silva A. Non-sex-biased dominance in a sexually monomorphic electric fish: fight structure and submissive electric signalling. *Ethology*. (2012) 118:398–410. doi: 10.1111/j.1439-0310.2012.02022.x
70. Perrone R, Silva AC. Status-dependent vasotocin modulation of dominance and subordination in the weakly electric fish *Gymnotus omarorum*. *Front Behav Neurosci*. (2018) 12:1. doi: 10.3389/fnbeh.2018.00001
71. Zubizarreta L, Stoddard PK, Silva A. Aggression levels affect social interaction in the non-breeding territorial aggression of the weakly electric fish, *Gymnotus omarorum*. *Ethology*. (2015) 121:8–16. doi: 10.1111/eth.12299
72. Quintana L, Zubizarreta L, Jalabert C, Batista G, Perrone R, Silva A. Building the case for a novel teleost model of non-breeding

aggression and its neuroendocrine control. *J Physiol*. (2016) 110:224–32. doi: 10.1016/j.jphysparis.2016.11.009
73. Soma KK, Wingfield JC. Dehydroepiandrosterone in songbird plasma: seasonal regulation and relationship to territorial aggression. *Gen Comp Endocrinol*. (2001) 123:144–55. doi: 10.1006/gcen.2001.7657
74. Soma KK, Rendon NM, Boonstra R, Albers HE, Demas GE. DHEA effects on brain and behavior: insights from comparative studies of aggression. *J Steroid Biochem Mol Biol*. (2015) 145:261–72. doi: 10.1016/j.jsbmb.2014.05.011
75. Rendon NM, Rudolph LM, Sengelaub DR, Demas GE. The agonistic adrenal: melatonin elicits female aggression via regulation of adrenal androgens. *Proc R Soc B*. (2015) 282:20152080. doi: 10.1098/rspb.2015.2080
76. Heimovics SA, Prior NH, Ma C, Soma KK. Rapid effects of an aggressive interaction on dehydroepiandrosterone, testosterone and oestradiol levels in the male song sparrow brain: a seasonal comparison. *J Neuroendocrinol*. (2016) 28:12345. doi: 10.1111/jne.12345
77. Schlinger BA, Pradhan DS, Soma KK. 3β-HSD activates DHEA in the songbird brain. *Neurochem Int*. (2008) 52:611–20. doi: 10.1016/j.neuint.2007.05.003
78. Heimovics SA, Ferris JK, Soma KK. Non-invasive administration of 17β-estradiol rapidly increases aggressive behavior in non-breeding, but not breeding, male song sparrows. *Hormones Behav*. (2015) 69:31–8. doi: 10.1016/j.yhbeh.2014.11.012
79. Laredo SA, Villalon Landeros R, Dooley JC, Steinman MQ, Orr V, Silva AL, et al. Nongenomic effects of estradiol on aggression under short day photoperiods. *Hormones Behav*. (2013) 64:557–65. doi: 10.1016/j.yhbeh.2013.06.002
80. Charlier TD, Newman AEM, Heimovics SA, Po KWL, Saldanha CJ, Soma KK. Rapid effects of aggressive interactions on aromatase activity and oestradiol in discrete brain regions of wild male white-crowned sparrows. *J Neuroendocrinol*. (2011) 23:742–53. doi: 10.1111/j.1365-2826.2011.02170.x
81. Zubizarreta L, Silva AC, Quintana L. The estrogenic pathway modulates non-breeding female aggression in a teleost fish. *Physiol Behav*. (2020) 220:112883. doi: 10.1016/j.physbeh.2020.112883
82. Dong W, Wang L, Thornton C, Scheffler BE, Willett KL. Benzo(a)pyrene decreases brain and ovarian aromatase mRNA expression in *Fundulus heteroclitus*. *Aquat Toxicol*. (2008) 88:289–300. doi: 10.1016/j.aquatox.2008.05.006
83. Forlano PM, Deitcher DL, Myers DA, Bass AH. Anatomical distribution and cellular basis for high levels of aromatase activity in the brain of teleost fish: aromatase enzyme and mRNA expression identify glia as source. *J Neurosci*. (2001) 21:8943. doi: 10.1523/JNEUROSCI.21-22-08943.2001
84. Goto-Kazeto R, Kight KE, Zohar Y, Place AR, Trant JM. Localization and expression of aromatase mRNA in adult zebrafish. *Gen Comp Endocrinol*. (2004) 139:72–84. doi: 10.1016/j.ygcen.2004.07.003
85. Hallgren S, Olsén KH. Effects on guppy brain aromatase activity following short-term steroid and 4-nonylphenol exposures. *Environ Toxicol*. (2010) 25:261–71. doi: 10.1002/tox.20494
86. Jeng S-R, Pasquier J, Yueh W-S, Chen G-R, Lee Y-H, Dufour S, et al. Differential regulation of the expression of cytochrome P450 aromatase, estrogen and androgen receptor subtypes in the brain–pituitary–ovarian axis of the Japanese eel. (*Anguilla japonica*) reveals steroid dependent and independent mechanisms. *Gen Comp Endocrinol*. (2012) 175:163–72. doi: 10.1016/j.ygcen.2011.11.005
87. Menuet A, Pellegrini E, Brion F, Gueguen M-M, Anglade I, Pakdel F, et al. Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. *J Comp Neurol*. (2005) 485:304–20. doi: 10.1002/cne.20497
88. Menuet A, Anglade I, Le Guevel R, Pellegrini E, Pakdel F, Kah O. Distribution of aromatase mRNA and protein in the brain and pituitary of female rainbow trout: Comparison with estrogen receptor α. *J Comp Neurol*. (2003) 462:180–93. doi: 10.1002/cne.10726
89. Strobl-Mazzulla PH, Lethimonier C, Gueguen MM, Karube M, Fernandino JI, Yoshizaki G, et al. Brain aromatase (Cyp19A2) and estrogen receptors, in larvae and adult pejerrey fish *Odontesthes bonariensis*: neuroanatomical and functional relations. *Gen Comp Endocrinol*. (2008) 158:191–201. doi: 10.1016/j.ygcen.2008.07.006
90. Shaw K, Krahe R. Pattern of aromatase mRNA expression in the brain of a weakly electric fish, *Apteronotus leptorhynchus*. *J Chem Neuroanat*. (2018) 90:70–9. doi: 10.1016/j.jchemneu.2017.12.009
91. Triefenbach FA, Zakon HH. Changes in signalling during agonistic interactions between male weakly electric knifefish, *Apteronotus leptorhynchus*. *Anim Behav*. (2008) 75:1263–72. doi: 10.1016/j.anbehav.2007.09.027
92. Meyer JH, Leong M, Keller CH. Hormone-induced and maturational changes in electric organ discharges and electroreceptor tuning in the weakly electric fishApteronotus. *J Comp Physiol A*. (1987) 160:385–94. doi: 10.1007/BF00613028
93. Eastman G, Valiño G, Radío S, Young R, Quintana L, Zakon H, et al. Brain transcriptomics of agonistic behaviour in the weakly electric fish *Gymnotus omarorum*, a wild teleost model of non-breeding aggression. *Sci Rep*. (2020) 10:9496. doi: 10.1038/s41598-020-66494-9
94. Kelly AM, Goodson JL. Social functions of individual vasopressin-oxytocin cell groups in vertebrates: what do we really know? *Front Neuroendocrinol*. (2014) 35:512–29. doi: 10.1016/j.yfrne.2014.04.005
95. Moore FL, Lowry CA. Comparative neuroanatomy of vasotocin and vasopressin in amphibians and other vertebrates. *Comp Biochem Physiol C*. (1998) 119:251–60. doi: 10.1016/S0742-8413(98)00014-0
96. Santangelo N, Bass AH. New insights into neuropeptide modulation of aggression: field studies of arginine vasotocin in a territorial tropical damselfish. *Proc R Soc B Biol Sci*. (2006) 273:3085–92. doi: 10.1098/rspb.2006.3683
97. Veenema AH, Beiderbeck DI, Lukas M, Neumann ID. Distinct correlations of vasopressin release within the lateral septum and the bed nucleus of the stria terminalis with the display of intermale aggression. *Hormones Behav*. (2010) 58:273–81. doi: 10.1016/j.yhbeh.2010.03.006
98. Pouso P, Radmilovich M, Silva A. An immunohistochemical study on the distribution of vasotocin neurons in the brain of two weakly electric fish, *Gymnotus omarorum* and *Brachyhypopomus gauderio*. *Tissue Cell*. (2017) 49:257–69. doi: 10.1016/j.tice.2017.02.003

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.

frontiers in Neuroscience

MINI REVIEW
published: 29 July 2020
doi: 10.3389/fnins.2020.00797

Sexually Dimorphic Formation of the Preoptic Area and the Bed Nucleus of the Stria Terminalis by Neuroestrogens

Shinji Tsukahara and Masahiro Morishita*

Division of Life Science, Graduate School of Science and Engineering, Saitama University, Saitama, Japan

Testicular androgens during the perinatal period play an important role in the sexual differentiation of the brain of rodents. Testicular androgens transported into the brain act via androgen receptors or are the substrate of aromatase, which synthesizes neuroestrogens that act via estrogen receptors. The latter that occurs in the perinatal period significantly contributes to the sexual differentiation of the brain. The preoptic area (POA) and the bed nucleus of the stria terminalis (BNST) are sexually dimorphic brain regions that are involved in the regulation of sex-specific social behaviors and the reproductive neuroendocrine system. Here, we discuss how neuroestrogens of testicular origin act in the perinatal period to organize the sexually dimorphic structures of the POA and BNST. Accumulating data from rodent studies suggest that neuroestrogens induce the sex differences in glial and immune cells, which play an important role in the sexually dimorphic formation of the dendritic synapse patterning in the POA, and induce the sex differences in the cell number of specific neuronal cell groups in the POA and BNST, which may be established by controlling the number of cells dying by apoptosis or the phenotypic organization of living cells. Testicular androgens in the peripubertal period also contribute to the sexual differentiation of the POA and BNST, and thus their aromatization to estrogens may be unnecessary. Additionally, we discuss the notion that testicular androgens that do not aromatize to estrogens can also induce significant effects on the sexually dimorphic formation of the POA and BNST.

Keywords: sexual differentiation of the brain, sexually dimorphic nucleus, sex difference, androgens, estrogens, preoptic area, bed nucleus of the stria terminalis

OPEN ACCESS

Edited by:
Takayoshi Ubuka, Waseda University, Japan

Reviewed by:
Mitsuhiro Kawata, Kyoto Prefectural University of Medicine, Japan
Carol F. Elias, University of Michigan, United States

****Correspondence:***
Shinji Tsukahara
stsuka@mail.saitama-u.ac.jp

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

Received: *24 March 2020*
Accepted: *07 July 2020*
Published: *29 July 2020*

Citation:
Tsukahara S and Morishita M (2020) Sexually Dimorphic Formation of the Preoptic Area and the Bed Nucleus of the Stria Terminalis by Neuroestrogens. Front. Neurosci. 14:797. doi: 10.3389/fnins.2020.00797

INTRODUCTION

Sex differences in the structures of the brain are considered to underlie sex-specific functions of the brain and brain functions that differ between sexes or genders. The mechanisms by which the brain is sexually differentiated have not yet been completely elucidated; however, they have long been studied using animal models, especially rodents. Based on accumulated data, androgens secreted from the testes during the perinatal period are converted to estrogens in the brain, wherein the neuroestrogens masculinize and defeminize the brain. Neuroestrogens are essential but not sole factors in the sexual differentiation of the brain. There are other factors that significantly

contribute to the brain sexual differentiation. The processes of brain sexual differentiation require sex chromosome genes' expression in the brain (McCarthy and Arnold, 2011; Cox et al., 2014) and gonadal steroids secreted during the peripubertal period (Juraska et al., 2013; Schulz and Sisk, 2016). However, there is no doubt that neuroestrogens of testicular origin play an important role in the sexual differentiation of the brain. In this mini review, we focused on two sexually dimorphic brain regions: the preoptic area (POA) and the bed nucleus of the stria terminalis (BNST), which are involved in the regulation of sexually dimorphic social behaviors and reproductive neuroendocrine functions. First, we give an overview of the sex differences in the POA and BNST of the rodent brain. Second, we discuss how neuroestrogens masculinize or defeminize the POA and BNST. Third, we further discuss the notion that testicular androgens that do not aromatize into estrogens can also induce the sexually dimorphic formation of the POA and BNST.

SEX DIFFERENCES IN THE POA AND BNST

The POA and BNST show morphological sex differences that are related to sex-specific brain functions (**Figure 1**). The number of dendritic spine synapses in the POA is twofold greater in male rats than in females; the POA masculinized by neuroestrogens, resulting in a greater number of dendritic spine synapses, plays an important role in the control of male sexual behavior (Amateau and McCarthy, 2002a, 2004; Wright et al., 2008; Wright and McCarthy, 2009). The male-biased sex difference in dendritic spine synapses in the POA is established by the crosstalk between neuroendocrine and immune systems where microglia and mast cells have significant roles [see reviews (Arambula and McCarthy, 2020; McCarthy, 2020) and the next section].

In the POA of rats and mice, there are two sexually dimorphic nuclei that have been identified to date. The sexually dimorphic nucleus of the POA (SDN-POA) exhibits male-biased sex differences in volume and the number of neurons (Gorski et al., 1978, 1980). The SDN-POA of male rats has been suggested to be related to partner preference (Houtsmuller et al., 1994; Woodson et al., 2002) and sexual arousal (Arendash and Gorski, 1983; De Jonge et al., 1989; Maejima et al., 2018); however, the physiological functions of the SDN-POA require further investigation. Approximately half of the SDN-POA neurons express calbindin-D28K (Calb) (Morishita et al., 2017), a calcium-binding protein that functions as a buffer, sensor, and transporter of calcium (Schmidt, 2012). A cluster of Calb neurons in the SDN-POA is termed the calbindin-sexually dimorphic nucleus (CALB-SDN), which has more Calb neurons in males than in females (Sickel and McCarthy, 2000; Edelmann et al., 2007; Orikasa and Sakuma, 2010). Although the physiological roles of Calb neurons remain unclear, Calb neurons in male rats are activated during sexual behavior (Yamaguchi et al., 2018).

Another sexually dimorphic nucleus in the POA is the anteroventral periventricular nucleus (AVPV), which is larger and contains more neurons in females than in males (Bleier et al., 1982). The AVPV of rats contains neurons expressing kisspeptin, neurons producing dopamine, and neurons producing both GABA and glutamate, and exhibits a female-biased sex difference in the number of these neurons (Simerly et al., 1985a; Ottem et al., 2004; Kauffman et al., 2007). In the AVPV of mice, approximately half of kisspeptin neurons produce dopamine and vice versa (Clarkson and Herbison, 2011). In the female AVPV, kisspeptin neurons expressing estrogen receptor α (ERα) are a target of the positive feedback actions of ovarian estrogens to induce ovulation (Kauffman, 2009; Tsukamura et al., 2010). Furthermore, kisspeptin neurons in the AVPV of female mice are key players in orchestrating successful reproduction by synchronizing copulation with ovulation (Hellier et al., 2018). Dual-phenotype GABA/glutamate neurons in the AVPV of rats and mice interact with gonadotropin-releasing hormone neurons to excite or inhibit their activity (Ottem et al., 2004; Liu et al., 2011). Dopamine neurons in the AVPV of female mice enhance maternal behavior, whereas dopamine neurons in the AVPV of male mice do not affect parental behavior, but suppress intermale aggression (Scott et al., 2015).

The principal nucleus of the BNST (BNSTp) is a subnucleus of the BNST showing male-biased sex differences in size and neuron number (Hines et al., 1985, 1992). Like the SDN-POA, the BNSTp contains more Calb neurons in male mice than in female mice (Gilmore et al., 2012). The subregion of the BNSTp that contains many Calb neurons and exhibits the male-biased sex difference in the number of Calb neurons is hereinafter referred to as CALB-BNSTp. BNSTp neurons expressing aromatase in male mice are necessary to distinguish the conspecific sexes and ensure social interactions (Bayless et al., 2019). However, the physiological functions of Calb neurons in the BNSTp remain unclear. Unlike the CALB-BNSTp, the ventral part of the BNSTp (BNSTpv) contains few Calb neurons without sex differences, but the BNSTpv is larger and has more non-Calb neurons in female mice than in males (Moe et al., 2016; Morishita et al., 2017). Thus, the BNSTp is composed of a region exhibiting male-biased sex differences in Calb neurons and a region exhibiting female-biased sex differences in non-Calb neurons.

NEUROESTROGENS OF TESTICULAR ORIGIN ARE SIGNIFICANT FACTORS FOR SEXUALLY DIMORPHIC FORMATION OF THE POA AND BNST

Neuroestrogens originating from testicular androgens affect the POA and BNST in the perinatal period to organize sexually dimorphic structures in a variety of modes of action (**Figure 2**). As mentioned before, the POA of rats has more dendritic spines in males than in females. The increased number of dendritic spines in the male POA is induced by estrogens in the perinatal period (Amateau and McCarthy, 2002a, 2004). The mechanisms responsible for the masculinization of dendritic spine patterning by estrogens are considered to be as follows. First, neuroestrogens originating from testicular androgens during the perinatal period affect mast cells in the POA via ER to stimulate histamine

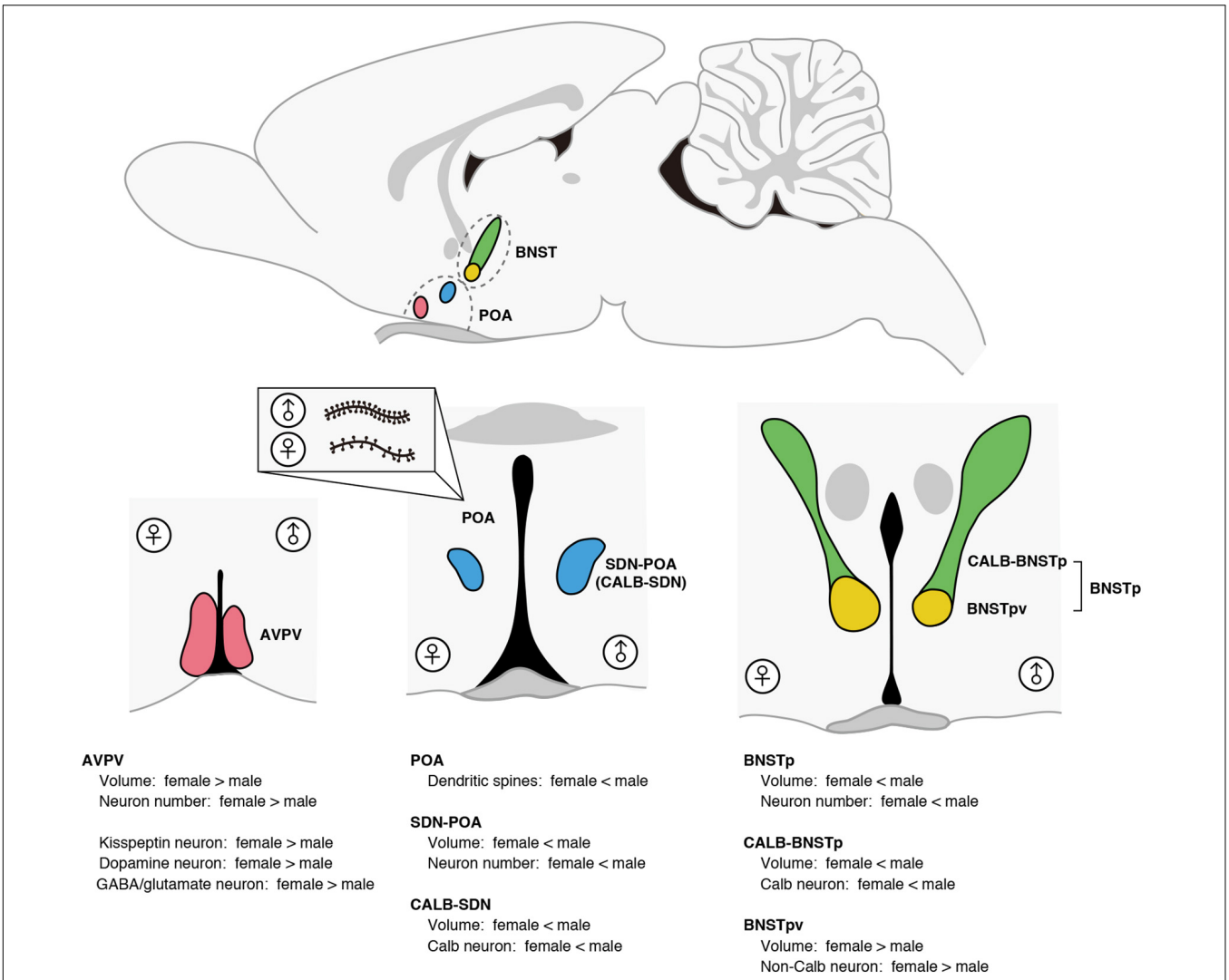

FIGURE 1 | Morphological sex differences in the POA and BNST of rats and mice. The AVPV exhibits female-biased sex differences in volume and neuron number. Compared with males, females have a greater number of kisspeptin neurons, dopamine neurons, and GABA/glutamate neurons in the AVPV. The SDN-POA exhibits male-biased sex differences in volume and neuron number. The SDN-POA contains a cluster of Calb neurons termed CALB-SDN. The CALB-SDN is larger in volume and contains a larger number of Calb neurons in males than in females. The POA shows a sex difference in the dendritic synapse pattern. POA neurons in males have more dendritic spines compared with POA neurons in females. The BNST contains a male-biased sexually dimorphic nucleus (BNSTp), which is composed of a sexually dimorphic subregion that is larger in volume and number of Calb neurons in males (CALB-BNSTp) and a sexually dimorphic subregion that is larger in volume and number of non-Calb neurons in females (BNSTpv).

release, which then stimulates microglia in the POA to release prostaglandin E2, which triggers POA neurons to increase dendritic spine synapses via induction of glutamate receptor signaling (Wright et al., 2008; Wright and McCarthy, 2009; Lenz et al., 2011, 2013, 2018). Thus, microglia and mast cells have critical roles in the masculinization of dendritic spine patterning. The POA of postnatal males has twice as many ameboid microglia, a class of microglia with a more activated morphological profile, compared with postnatal females (Lenz et al., 2013). The male-biased sex difference in ameboid microglia is regulated by neuroestrogens of testicular origin, because treatment with estradiol increased ameboid microglia in the POA of postnatal females (Lenz et al., 2013). The male POA has more activated mast cells than the female POA in the perinatal period, and approximately half of the mast cells in both sexes express ERα (Lenz et al., 2018). Additionally, astrocytes in the POA of postnatal rats exhibit a sex difference in morphology: astrocytes in males have longer and more primary processes, and the astrocyte morphology is masculinized by neuroestrogens in the postnatal period (Amateau and McCarthy, 2002b). Astrocytes release chemical transmitters, including glutamate, and are involved in synapse formation (Araque et al., 1999). Taken together, the sexually dimorphic synapse formation may follow the sexual differentiation of these non-neuronal cells.

The masculinization of the SDN-POA/CALB-SDN requires the actions of estrogens that are synthesized in the brain from

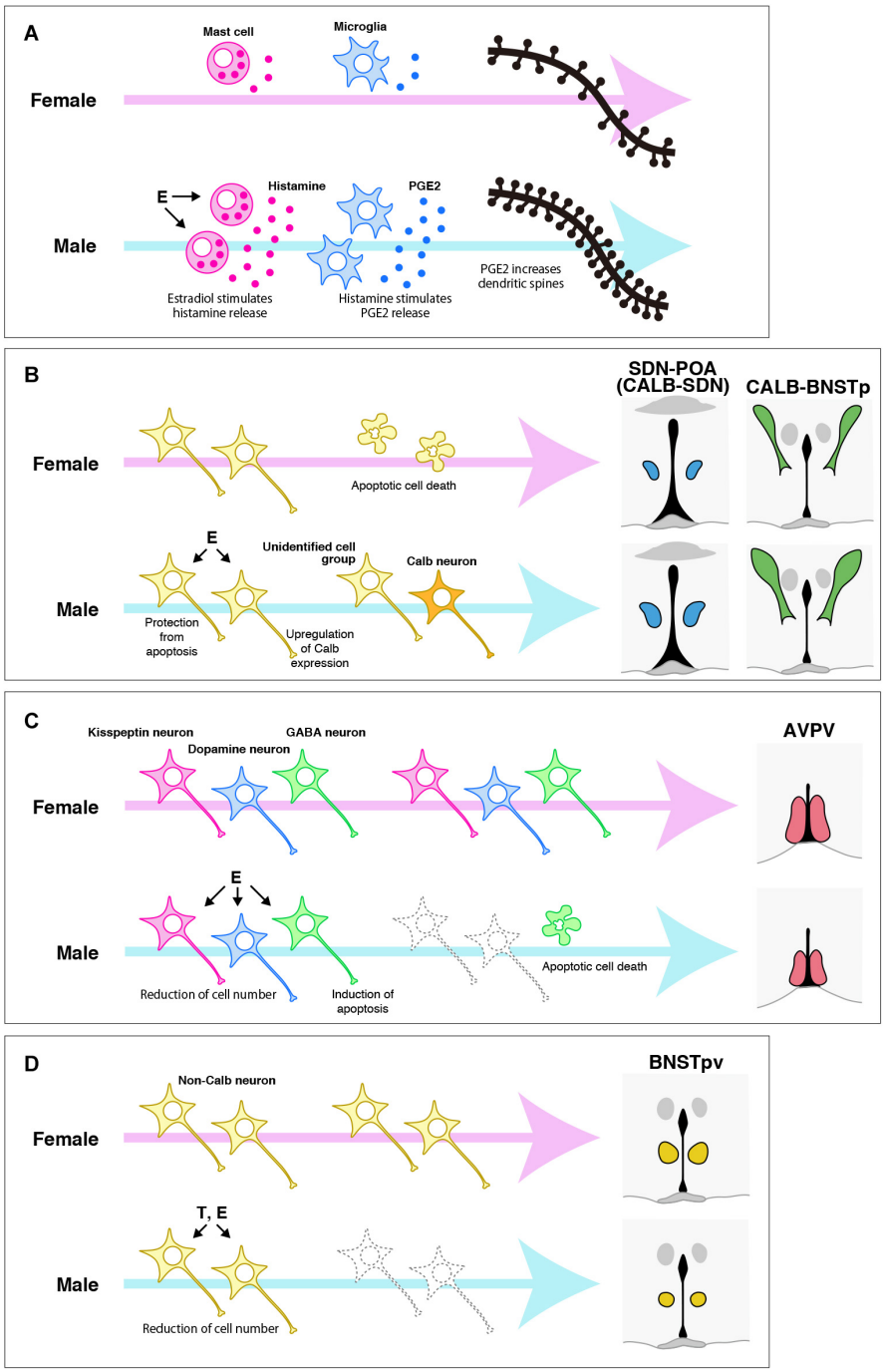

FIGURE 2 | Possible mechanisms for organizing the sexually dimorphic structures of the POA and BNST of rats and mice. **(A)** The mechanism responsible for the sexual differentiation of dendritic synapse patterning in the POA, which was proposed by McCarthy and colleagues (see reviews Arambula and McCarthy, 2020; McCarthy, 2020). Estradiol (E), which is synthetized in the brain from testicular testosterone (T) during the perinatal period, stimulates histamine release from mast cells, which then stimulates prostaglandin E2 (PGE2) to increase dendritic spines. **(B)** The mechanism for organizing the sexually dimorphic structures of the SDN-POA/CALB-SDN and CALB-BNSTp. E originating from testicular T in the perinatal period protects a population of neurons from apoptotic cell death, although the neurochemical properties of the cell population have not been identified. Additionally, E may upregulate Calb expression, followed by induction of a male-biased sex difference in the number of Calb neurons. **(C)** The mechanisms for inducing a female-biased sex difference in the number of cells in the specific neuronal cell groups in the AVPV. E originating from testicular T in the perinatal period induces the death of GABA neurons by apoptosis to reduce their number. E may also reduce the number of kisspeptin neurons and dopamine neurons by a mechanism other than apoptosis, although the mechanism remains unknown. **(D)** The roles of T and E during the perinatal period in the sexual differentiation of the BNSTpv. T and E reduce the number of BNSTpv neurons that do not express Calb. However, identification of the neurochemical properties and the mechanism responsible for inducing the sex difference require further investigation.

testicular androgens and act via ERα during the postnatal period (Gorski et al., 1978; Patchev et al., 2004; Orikasa and Sakuma, 2010; Gilmore et al., 2012; Morishita et al., 2017). How neuroestrogens masculinize the SDN-POA/CALB-SDN is not completely understood. However, controlling cell numbers by apoptosis during the postnatal period appears to produce a male-biased sex difference in the number of SDN-POA neurons. The number of cells generated during the late fetal period and incorporated into the SDN-POA during the neonatal period does not differ between sexes (Jacobson et al., 1985; Dodson et al., 1988; Kato et al., 2012), but the number of apoptotic cells in the SDN-POA of postnatal rats is smaller in males because of suppression of apoptosis by neuroestrogens of testicular origin (Arai et al., 1996; Davis et al., 1996; Chung et al., 2000). Postnatal apoptosis in the SDN-POA is regulated by the mitochondrial apoptotic pathway involving Bcl-2, Bax, and caspase-3. In the SDN-POA of postnatal rats, the expression of Bcl-2 and Bax is higher in males and in females, respectively, followed by higher activity of caspoase-3 in males (Tsukahara et al., 2006). The sex differences in Bcl-2 and Bax expression result from the upregulation of Bcl-2 expression and downregulation of Bax expression by neuroestrogens of testicular origin because estradiol treatment increased the Bcl-2 protein level and decreased the Bax protein level in the POA of postnatal female rats (Tsukahara et al., 2008). Nevertheless, the sex difference in the number of Calb neurons may occur independently of apoptotic regulation, because deletion of the *Bax* gene did not affect the number of Calb neurons in the CALB-SDN of mice in both sexes (Gilmore et al., 2012). Calb expression may be upregulated by neuroestrogens of testicular origin; the number of Calb neurons is increased in male mice, because the mouse *Calb* promoter possesses estrogen-responsive elements and is estrogen responsive (Gill and Christakos, 1995). Ca^{2+} is a key regulator of cellular functions in living cells, but it also induces apoptosis upon prolonged changes in its intercellular concentrations, including an increase in its cytoplasmic and mitochondrial concentrations (Hajnoczky et al., 2003). Calb protects neurons from cell death by chelating intercellular Ca^{2+} (Meier et al., 1998; D'Orlando et al., 2002; Fan et al., 2007). It also prevents neuronal cell death by inhibiting caspase-3 activity (Choi et al., 2008; Choi and Oh, 2014). These findings may support the notion that Calb upregulation by estrogens prevents apoptotic cell death.

Neuroestrogens originating from testicular androgens in the perinatal period reduce the total number of neurons to defeminize the AVPV in rats and mice (Patchev et al., 2004; Kanaya et al., 2014). Furthermore, these neuroestrogens defeminized specific neuronal cell groups in the AVPV by reducing their cell number. The number of kisspeptin neurons in the AVPV increased in male rats with neonatal castration and decreased in female rats with neonatal estradiol treatment (Kauffman et al., 2007; Homma et al., 2009). Perinatal or neonatal testosterone treatment reduced the number of dopamine neurons in the AVPV of female rats (Simerly et al., 1985b). The number of dopamine neurons in the AVPV of male mice increased upon the deletion of the genes for ERα and ERβ, resulting in the disappearance of the sex difference in dopamine neurons (Simerly et al., 1997; Bodo et al., 2006). Male rodents have a greater number of apoptotic cells in the AVPV during the perinatal period than female rodents do, which is attributed to the induction of apoptosis by estrogens (Sumida et al., 1993; Arai et al., 1996; Yoshida et al., 2000; Waters and Simerly, 2009). Controlling the number of neurons by apoptosis via Bcl-2 and Bax is required for the sexually dimorphic formation of the AVPV of mice (Zup et al., 2003; Forger et al., 2004). The AVPV of postnatal rats shows a male-biased sex difference in Bax expression and a female-biased sex difference in Bcl-2 expression, followed by higher activity of caspase-3 in the male AVPV (Tsukahara et al., 2006). In addition, the tumor necrosis factor α (TNF-α)-TNF receptor 2 (TNFR2)-NFκB cell survival pathway is activated in the AVPV of postnatal female rats to upregulate Bcl-2 expression, whereas this pathway is suppressed by TNF receptor-associated factor 2-inhibitng protein (TRIP) in the male AVPV, followed by an increase in the number of apoptotic cells (Krishnan et al., 2009). Postnatal apoptosis regulated by this pathway may result in a sex difference in GABA neurons of the AVPV (Krishnan et al., 2009). However, the sex differences in the number of dopamine and kisspeptin neurons in the AVPV of mice are independent of Bcl-2 and Bax (Zup et al., 2003; Forger et al., 2004; Semaan et al., 2010). There may be other mechanisms that establish the sex differences in dopamine and kisspeptin neurons, although they remain nuclear.

Estrogens that are synthesized in the brain from testicular androgens and act via ERα during the postnatal period masculinize the BNSTp by increasing the volume and neuron number in rats and mice (Guillamon et al., 1988; Chung et al., 2000; Hisasue et al., 2010; Tsukahara et al., 2011). The number of apoptotic cells in the BNSTp of postnatal rats is smaller in males because of the protection of cells from apoptosis by neonatal testicular androgens (Chung et al., 2000). This indicates that suppression of apoptotic cell death by testicular androgens contributes to the masculinization of the BNSTp. The apoptotic pathway involving Bax accounts for the male-biased sex difference in the number of BNSTp neurons in adulthood following the female-biased sex difference in postnatal apoptosis (Forger et al., 2004; Gotsiridze et al., 2007). Like the BNSTp, the CALB-BNSTp in mice is masculinized by postnatal testicular androgens, which act after aromatization (Morishita et al., 2017). However, Bax-dependent apoptosis may not be necessary for establishing the sex difference in the number of Calb neurons, because deletion of the *Bax* gene increased Calb neurons in both sexes, but did not eliminate the sex difference (Gilmore et al., 2012). As mentioned earlier, estradiol can induce Calb expression (Gill and Christakos, 1995). Phenotypic organization induced by estrogens is a possible mechanism for the sexual differentiation of Calb neurons, although this idea needs to be investigated. The volume and the number of non-CALB neurons in the BNSTpv increased in male mice with neonatal castration and decreased in female mice upon postnatal treatment with estradiol or dihydrotestosterone (Morishita et al., 2017), suggesting that testicular androgens affect the BNSTpv after aromatizing to estrogens, but they also affect this area without aromatization.

In rodents, the critical time window in which neuroestrogens effectively induce brain sexual differentiation is limited to the

perinatal period. Nevertheless, the effects of neuroestrogens persist until adulthood. The long-lasting effects are considered to be due to epigenetic changes in gene expression [see reviews (Forger, 2016, 2018; McCarthy, 2019)]. In fact, some of the aforementioned sex differences emerge via epigenetic regulation. Compared with postnatal males, postnatal female rats have higher DNA methyltransferase activity in the POA, which is followed by higher DNA methylation, and postnatal estradiol treatment reduced DNA methyltransferase activity and DNA methylation in the female POA (Nugent et al., 2015). Moreover, inhibition of DNA methyltransferase in the brain of neonatal females increased dendritic spines of POA neurons and masculinized sexual behavior (Nugent et al., 2015), and increased the number of Calb neurons in the CALB-SDN and CALB-BNSTp, resulting in elimination of the sex difference in Calb neurons (Mosley et al., 2017; Cisternas et al., 2020). Epigenetic regulation via histone modification also contributes to masculinization of the brain. Inhibition of histone deacetylase in the brain reduced the number of BNSTp neurons in male mice and neonatally testosterone-treated females (Murray et al., 2009) and reduced the activity of sexual behavior in male rats (Matsuda et al., 2011). ER is a ligand-activated transcription factor, and thereby estrogens binding to ER modulate the expression of the target genes at the transcriptional level. Therefore, though it may not be the whole story, epigenetic regulation by estrogens is an essential part of the molecular mechanisms of brain sexual differentiation.

SEXUALLY DIMORPHIC FORMATION OF THE POA AND BNST REQUIRES NEUROESTROGENS OF TESTICULAR ORIGIN AND TESTICULAR ANDROGENS

Masculinization of the BNSTp is disrupted in rats with reduced functional androgen receptors (ARs) (Durazzo et al., 2007) and AR-knockout mice (Kanaya et al., 2014), indicating that the masculinization of the BNSTp requires the actions of testicular androgens via AR. The BNSTp of mice begins to express AR from the neonatal period, but the expression level is low until one week after birth (Juntti et al., 2010; Kanaya et al., 2014). It seems likely that the androgen actions via AR mainly occur after the perinatal period. The sex difference in the number of Calb neurons in the CALB-SDN and CALB-BNSTp of mice emerges before puberty and becomes pronounced after puberty (Wittmann and McLennan, 2013a,b; Morishita et al., 2017). This is partly due to an increase in the number of Calb neurons during the peripubertal period, which is induced by testicular androgens via AR because the decrease in the number of Calb neurons by prepubertal castration was reversed by peripubertal treatment with dihydrotestosterone, but not with estradiol (Morishita et al., 2020). Thus, testicular androgens that are synthesized during the peripubertal period and act via ARs are necessary for the masculinization of Calb neurons. However, it cannot be excluded that masculinization of the brain requires the actions of neuroestrogens during puberty, because prepubertal knockdown of ERα in the medial amygdala, a male-biased sexually dimorphic nucleus, disrupts the masculinization of this nucleus in mice (Sano et al., 2016).

CONCLUSION

Perinatal testicular androgens induce masculinizing and defeminizing effects on the POA and BNST of rodents through binding to ER after conversion to estrogens in the brain rather than by binding to AR directly, resulting in sex differences in glial and immune cells, dendritic synapse patterning, and specific neuronal cell groups. Interactions among immune cells, glial cells, and neuronal cells under the influence of neuroestrogens is a prerequisite for producing the sex difference in dendritic synapse patterning in the POA. Sex differences in specific neuronal cell groups in the SDN-POA, AVPV, and BNSTp may be established by controlling the number of dying cells by apoptosis or phenotypic organization of living cells that are influenced by neuroestrogens. Neuroestrogens binding to ER modulate the expression of the target genes at the transcriptional level, but also modulate gene expression by epigenetic regulation, which ensures the long-lasting effects of neuroestrogens beyond the perinatal period. Testicular androgens in the peripubertal period also contribute to the sexual differentiation of the POA and BNST, but aromatizing them to estrogens may not be necessary. Thus, peripubertal testicular androgens can act via AR directly to masculinize the sexually dimorphic nuclei.

AUTHOR CONTRIBUTIONS

MM and ST prepared the manuscript. Both authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 20K07257) to ST and Grant for Basic Science Research Projects from the Sumitomo Foundation (No. 180084) to ST.

REFERENCES

Amateau, S. K., and McCarthy, M. M. (2002a). A novel mechanism of dendritic spine plasticity involving estradiol induction of prostaglandin-E2. *J. Neurosci.* 22, 8586–8596. doi: 10.1523/jneurosci.22-19-08586.2002

Amateau, S. K., and McCarthy, M. M. (2002b). Sexual differentiation of astrocyte morphology in the developing rat preoptic area. *J. Neuroendocrinol.* 14, 904–910. doi: 10.1046/j.1365-2826.2002.00858.x

Amateau, S. K., and McCarthy, M. M. (2004). Induction of PGE2 by estradiol mediates developmental masculinization of sex behavior. *Nat. Neurosci.* 7, 643–650. doi: 10.1038/nn1254

Arai, Y., Sekine, Y., and Murakami, S. (1996). Estrogen and apoptosis in the developing sexually dimorphic preoptic area in female rats. *Neurosci. Res.* 25, 403–407. doi: 10.1016/0168-0102(96)01070-x

Arambula, S. E., and McCarthy, M. M. (2020). Neuroendocrine-immune crosstalk shapes sex-specific brain development. *Endocrinology* 161:bqaa055. doi: 10.1210/endocr/bqaa055

Araque, A., Parpura, V., Sanzgiri, R. P., and Haydon, P. G. (1999). Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci.* 22, 208–215. doi: 10.1016/s0166-2236(98)01349-6

Arendash, G. W., and Gorski, R. A. (1983). Effects of discrete lesions of the sexually dimorphic nucleus of the preoptic area or other medial preoptic regions on the sexual behavior of male rats. *Brain Res. Bull.* 10, 147–154. doi: 10.1016/0361-9230(83)90086-2

Bayless, D. W., Yang, T., Mason, M. M., Susanto, A. A. T., Lobdell, A., and Shah, N. M. (2019). Limbic neurons shape sex recognition and social behavior in sexually naive males. *Cell* 176, 1190–1205.e20. doi: 10.1016/j.cell.2018.12.041

Bleier, R., Byne, W., and Siggelkow, I. (1982). Cytoarchitectonic sexual dimorphisms of the medial preoptic and anterior hypothalamic areas in guinea pig, rat, hamster, and mouse. *J. Comp. Neurol.* 212, 118–130. doi: 10.1002/cne.902120203

Bodo, C., Kudwa, A. E., and Rissman, E. F. (2006). Both estrogen receptor-alpha and -beta are required for sexual differentiation of the anteroventral periventricular area in mice. *Endocrinology* 147, 415–420. doi: 10.1210/en.2005-0834

Choi, W. S., Lee, E., Lim, J., and Oh, Y. J. (2008). Calbindin-D28K prevents drug-induced dopaminergic neuronal death by inhibiting caspase and calpain activity. *Biochem. Biophys. Res. Commun.* 371, 127–131. doi: 10.1016/j.bbrc.2008.04.020

Choi, W. S., and Oh, Y. J. (2014). Calbindin-D28K Prevents Staurosporin-induced Bax Cleavage and Membrane Permeabilization. *Exp. Neurobiol.* 23, 173–177. doi: 10.5607/en.2014.23.2.173

Chung, W. C., Swaab, D. F., and De Vries, G. J. (2000). Apoptosis during sexual differentiation of the bed nucleus of the stria terminalis in the rat brain. *J. Neurobiol.* 43, 234–243. doi: 10.1002/(sici)1097-4695(20000605)43:3<234::aid-neu2>3.0.co;2-3

Cisternas, C. D., Cortes, L. R., Golynker, I., Castillo-Ruiz, A., and Forger, N. G. (2020). Neonatal inhibition of DNA methylation disrupts testosterone-dependent masculinization of neurochemical phenotype. *Endocrinology* 161:bqz022. doi: 10.1210/endocr/bqz022

Clarkson, J., and Herbison, A. E. (2011). Dual phenotype kisspeptin-dopamine neurones of the rostral periventricular area of the third ventricle project to gonadotrophin-releasing hormone neurones. *J. Neuroendocrinol.* 23, 293–301. doi: 10.1111/j.1365-2826.2011.02107.x

Cox, K. H., Bonthuis, P. J., and Rissman, E. F. (2014). Mouse model systems to study sex chromosome genes and behavior: relevance to humans. *Front. Neuroendocrinol.* 35, 405–419. doi: 10.1016/j.yfrne.2013.12.004

Davis, E. C., Popper, P., and Gorski, R. A. (1996). The role of apoptosis in sexual differentiation of the rat sexually dimorphic nucleus of the preoptic area. *Brain Res.* 734, 10–18. doi: 10.1016/0006-8993(96)00298-3

De Jonge, F. H., Louwerse, A. L., Ooms, M. P., Evers, P., Endert, E., and van de Poll, N. E. (1989). Lesions of the SDN-POA inhibit sexual behavior of male Wistar rats. *Brain Res. Bull.* 23, 483–492. doi: 10.1016/0361-9230(89)90194-9

Dodson, R. E., Shryne, J. E., and Gorski, R. A. (1988). Hormonal modification of the number of total and late-arising neurons in the central part of the medial preoptic nucleus of the rat. *J. Comp. Neurol.* 275, 623–629. doi: 10.1002/cne.902750410

D'Orlando, C., Celio, M. R., and Schwaller, B. (2002). Calretinin and calbindin D-28k, but not parvalbumin protect against glutamate-induced delayed excitotoxicity in transfected N18-RE 105 neuroblastoma-retina hybrid cells. *Brain Res.* 945, 181–190. doi: 10.1016/s0006-8993(02)02753-1

Durazzo, A., Morris, J. A., Breedlove, S. M., and Jordan, C. L. (2007). Effects of the testicular feminization mutation (tfm) of the androgen receptor gene on BSTMPM volume and morphology in rats. *Neurosci. Lett.* 419, 168–171. doi: 10.1016/j.neulet.2007.04.033

Edelmann, M., Wolfe, C., Scordalakes, E. M., Rissman, E. F., and Tobet, S. (2007). Neuronal nitric oxide synthase and calbindin delineate sex differences in the developing hypothalamus and preoptic area. *Dev. Neurobiol.* 67, 1371–1381. doi: 10.1002/dneu.20507

Fan, Y., Shi, L., Gu, Y., Zhao, Y., Xie, J., Qiao, J., et al. (2007). Pretreatment with PTD-calbindin D 28k alleviates rat brain injury induced by ischemia and reperfusion. *J. Cereb. Blood Flow Metab.* 27, 719–728. doi: 10.1038/sj.jcbfm.9600373

Forger, N. G. (2016). Epigenetic mechanisms in sexual differentiation of the brain and behaviour. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 371:20150114. doi: 10.1098/rstb.2015.0114

Forger, N. G. (2018).). Past, present and future of epigenetics in brain sexual differentiation. *J. Neuroendocrinol.* 30:e12492. doi: 10.1111/jne.12492

Forger, N. G., Rosen, G. J., Waters, E. M., Jacob, D., Simerly, R. B., and de Vries, G. J. (2004). Deletion of Bax eliminates sex differences in the mouse forebrain. *Proc. Natl. Acad. Sci. U.S.A.* 101, 13666–13671. doi: 10.1073/pnas.0404644101

Gill, R. K., and Christakos, S. (1995). Regulation by estrogen through the 5'-flanking region of the mouse calbindin-D28k gene. *Mol. Endocrinol.* 9, 319–326. doi: 10.1210/mend.9.3.7776978

Gilmore, R. F., Varnum, M. M., and Forger, N. G. (2012). Effects of blocking developmental cell death on sexually dimorphic calbindin cell groups in the preoptic area and bed nucleus of the stria terminalis. *Biol. Sex Differ.* 3:5. doi: 10.1186/2042-6410-3-5

Gorski, R. A., Gordon, J. H., Shryne, J. E., and Southam, A. M. (1978). Evidence for a morphological sex difference within the medial preoptic area of the rat brain. *Brain Res.* 148, 333–346. doi: 10.1016/0006-8993(78)90723-0

Gorski, R. A., Harlan, R. E., Jacobson, C. D., Shryne, J. E., and Southam, A. M. (1980). Evidence for the existence of a sexually dimorphic nucleus in the preoptic area of the rat. *J. Comp. Neurol.* 193, 529–539. doi: 10.1002/cne.901930214

Gotsiridze, T., Kang, N., Jacob, D., and Forger, N. G. (2007). Development of sex differences in the principal nucleus of the bed nucleus of the stria terminalis of mice: role of Bax-dependent cell death. *Dev. Neurobiol.* 67, 355–362. doi: 10.1002/dneu.20353

Guillamon, A., Segovia, S., and del Abril, A. (1988). Early effects of gonadal steroids on the neuron number in the medial posterior region and the lateral division of the bed nucleus of the stria terminalis in the rat. *Brain Res. Dev. Brain Res.* 44, 281–290. doi: 10.1016/0165-3806(88)90226-x

Hajnoczky, G., Davies, E., and Madesh, M. (2003). Calcium signaling and apoptosis. *Biochem. Biophys. Res. Commun.* 304, 445–454. doi: 10.1016/s0006-291x(03)00616-8

Hellier, V., Brock, O., Candlish, M., Desroziers, E., Aoki, M., Mayer, C., et al. (2018). Female sexual behavior in mice is controlled by kisspeptin neurons. *Nat. Commun.* 9:400. doi: 10.1038/s41467-017-02797-2

Hines, M., Allen, L. S., and Gorski, R. A. (1992). Sex differences in subregions of the medial nucleus of the amygdala and the bed nucleus of the stria terminalis of the rat. *Brain Res.* 579, 321–326. doi: 10.1016/0006-8993(92)90068-k

Hines, M., Davis, F. C., Coquelin, A., Goy, R. W., and Gorski, R. A. (1985). Sexually dimorphic regions in the medial preoptic area and the bed nucleus of the stria terminalis of the guinea pig brain: a description and an investigation of their relationship to gonadal steroids in adulthood. *J. Neurosci.* 5, 40–47. doi: 10.1523/jneurosci.05-01-00040.1985

Hisasue, S., Seney, M. L., Immerman, E., and Forger, N. G. (2010). Control of cell number in the bed nucleus of the stria terminalis of mice: role of testosterone metabolites and estrogen receptor subtypes. *J. Sex Med.* 7, 1401–1409. doi: 10.1111/j.1743-6109.2009.01669.x

Homma, T., Sakakibara, M., Yamada, S., Kinoshita, M., Iwata, K., Tomikawa, J., et al. (2009). Significance of neonatal testicular sex steroids to defeminize anteroventral periventricular kisspeptin neurons and the GnRH/LH surge system in male rats. *Biol. Reprod.* 81, 1216–1225. doi: 10.1095/biolreprod.109.078311

Houtsmuller, E. J., Brand, T., de Jonge, F. H., Joosten, R. N., van de Poll, N. E., and Slob, A. K. (1994). SDN-POA volume, sexual behavior, and partner preference of male rats affected by perinatal treatment with ATD. *Physiol. Behav.* 56, 535–541. doi: 10.1016/0031-9384(94)90298-4

Jacobson, C. D., Davis, F. C., and Gorski, R. A. (1985). Formation of the sexually dimorphic nucleus of the preoptic area: neuronal growth, migration and changes in cell number. *Brain Res.* 353, 7–18. doi: 10.1016/0165-3806(85)90019-7

Juntti, S. A., Tollkuhn, J., Wu, M. V., Fraser, E. J., Soderborg, T., Tan, S., et al. (2010). The androgen receptor governs the execution, but not programming,

of male sexual and territorial behaviors. *Neuron* 66, 260–272. doi: 10.1016/j.neuron.2010.03.024

Juraska, J. M., Sisk, C. L., and DonCarlos, L. L. (2013). Sexual differentiation of the adolescent rodent brain: hormonal influences and developmental mechanisms. *Horm. Behav.* 64, 203–210. doi: 10.1016/j.yhbeh.2013.05.010

Kanaya, M., Tsuda, M. C., Sagoshi, S., Nagata, K., Morimoto, C., Thu, C. K., et al. (2014). Regional difference in sex steroid action on formation of morphological sex differences in the anteroventral periventricular nucleus and principal nucleus of the bed nucleus of the stria terminalis. *PLoS One* 9:e112616. doi: 10.1371/journal.pone.0112616

Kato, Y., Nakashima, S., Maekawa, F., and Tsukahara, S. (2012). Involvement of postnatal apoptosis on sex difference in number of cells generated during late fetal period in the sexually dimorphic nucleus of the preoptic area in rats. *Neurosci. Lett.* 516, 290–295. doi: 10.1016/j.neulet.2012.04.017

Kauffman, A. S. (2009). Sexual differentiation and the Kiss1 system: hormonal and developmental considerations. *Peptides* 30, 83–93. doi: 10.1016/j.peptides.2008.06.014

Kauffman, A. S., Gottsch, M. L., Roa, J., Byquist, A. C., Crown, A., Clifton, D. K., et al. (2007). Sexual differentiation of Kiss1 gene expression in the brain of the rat. *Endocrinology* 148, 1774–1783.

Krishnan, S., Intlekofer, K. A., Aggison, L. K., and Petersen, S. L. (2009). Central role of TRAF-interacting protein in a new model of brain sexual differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 106, 16692–16697. doi: 10.1073/pnas.0906293106

Lenz, K. M., Nugent, B. M., Haliyur, R., and McCarthy, M. M. (2013). Microglia are essential to masculinization of brain and behavior. *J. Neurosci.* 33, 2761–2772. doi: 10.1523/JNEUROSCI.1268-12.2013

Lenz, K. M., Pickett, L. A., Wright, C. L., Davis, K. T., Joshi, A., and McCarthy, M. M. (2018). Mast cells in the developing brain determine adult sexual behavior. *J. Neurosci.* 38, 8044–8059. doi: 10.1523/JNEUROSCI.1176-18.2018

Lenz, K. M., Wright, C. L., Martin, R. C., and McCarthy, M. M. (2011). Prostaglandin E(2) regulates AMPA receptor phosphorylation and promotes membrane insertion in preoptic area neurons and glia during sexual differentiation. *PLoS One* 6:e18500. doi: 10.1371/journal.pone.0018500

Liu, X., Porteous, R., d'Anglemont de Tassigny, X., Colledge, W. H., Millar, R., Petersen, S. L., et al. (2011). Frequency-dependent recruitment of fast amino acid and slow neuropeptide neurotransmitter release controls gonadotropin-releasing hormone neuron excitability. *J. Neurosci.* 31, 2421–2430. doi: 10.1523/JNEUROSCI.5759-10.2011

Maejima, S., Abe, Y., Yamaguchi, S., Musatov, S., Ogawa, S., Kondo, Y., et al. (2018). VGF in the medial preoptic nucleus increases sexual activity following sexual arousal induction in male rats. *Endocrinology* 159, 3993–4005. doi: 10.1210/en.2018-00804

Matsuda, K. I., Mori, H., Nugent, B. M., Pfaff, D. W., McCarthy, M. M., and Kawata, M. (2011). Histone deacetylation during brain development is essential for permanent masculinization of sexual behavior. *Endocrinology* 152, 2760–2767. doi: 10.1210/en.2011-0193

McCarthy, M. M. (2019). Is sexual differentiation of brain and behavior epigenetic? *Curr. Opin. Behav. Sci.* 25, 83–88. doi: 10.1016/j.cobeha.2018.10.005

McCarthy, M. M. (2020). A new view of sexual differentiation of mammalian brain. *J. Comp. Physiol. A Neuroethol. Sens. NeuralBehav. Physiol.* 206, 369–378. doi: 10.1007/s00359-019-01376-8

McCarthy, M. M., and Arnold, A. P. (2011). Reframing sexual differentiation of the brain. *Nat. Neurosci.* 14, 677–683. doi: 10.1038/nn.2834

Meier, T. J., Ho, D. Y., Park, T. S., and Sapolsky, R. M. (1998). Gene transfer of calbindin D28k cDNA via herpes simplex virus amplicon vector decreases cytoplasmic calcium ion response and enhances neuronal survival following glutamatergic challenge but not following cyanide. *J. Neurochem.* 71, 1013–1023. doi: 10.1046/j.1471-4159.1998.71031013.x

Moe, Y., Kyi-Tha-Thu, C., Tanaka, T., Ito, H., Yahashi, S., Matsuda, K. I., et al. (2016). A sexually dimorphic area of the dorsal hypothalamus in mice and common marmosets. *Endocrinology* 157, 4817–4828. doi: 10.1210/en.2016-1428

Morishita, M., Koiso, R., and Tsukahara, S. (2020). Actions of peripubertal gonadal steroids in the formation of sexually dimorphic brain regions in mice. *Endocrinology* 161:bqaa063. doi: 10.1210/endocr/bqaa063

Morishita, M., Maejima, S., and Tsukahara, S. (2017). Gonadal hormone-dependent sexual differentiation of a female-biased sexually dimorphic cell group in the principal nucleus of the bed nucleus of the Stria Terminalis in mice. *Endocrinology* 158, 3512–3525. doi: 10.1210/en.2017-00240

Mosley, M., Weathington, J., Cortes, L. R., Bruggeman, E., Castillo-Ruiz, A., Xue, B., et al. (2017). Neonatal inhibition of DNA methylation alters cell phenotype in sexually dimorphic regions of the mouse brain. *Endocrinology* 158, 1838–1848. doi: 10.1210/en.2017-00205

Murray, E. K., Hien, A., de Vries, G. J., and Forger, N. G. (2009). Epigenetic control of sexual differentiation of the bed nucleus of the stria terminalis. *Endocrinology* 150, 4241–4247. doi: 10.1210/en.2009-0458

Nugent, B. M., Wright, C. L., Shetty, A. C., Hodes, G. E., Lenz, K. M., Mahurkar, A., et al. (2015). Brain feminization requires active repression of masculinization via DNA methylation. *Nat. Neurosci.* 18, 690–697. doi: 10.1038/nn.3988

Orikasa, C., and Sakuma, Y. (2010). Estrogen configures sexual dimorphism in the preoptic area of C57BL/6J and ddN strains of mice. *J. Comp. Neurol.* 518, 3618–3629. doi: 10.1002/cne.22419

Ottem, E. N., Godwin, J. G., Krishnan, S., and Petersen, S. L. (2004). Dual-phenotype GABA/glutamate neurons in adult preoptic area: sexual dimorphism and function. *J. Neurosci.* 24, 8097–8105. doi: 10.1523/JNEUROSCI.2267-04.2004

Patchev, A. V., Gotz, F., and Rohde, W. (2004). Differential role of estrogen receptor isoforms in sex-specific brain organization. *FASEB J.* 18, 1568–1570. doi: 10.1096/fj.04-1959fje

Sano, K., Nakata, M., Musatov, S., Morishita, M., Sakamoto, T., Tsukahara, S., et al. (2016). Pubertal activation of estrogen receptor alpha in the medial amygdala is essential for the full expression of male social behavior in mice. *Proc. Natl. Acad. Sci. U.S.A.* 113, 7632–7637. doi: 10.1073/pnas.1524907113

Schmidt, H. (2012). Three functional facets of calbindin D-28k. *Front. Mol. Neurosci.* 5:25. doi: 10.3389/fnmol.2012.00025

Schulz, K. M., and Sisk, C. L. (2016). The organizing actions of adolescent gonadal steroid hormones on brain and behavioral development. *Neurosci. Biobehav. Rev.* 70, 148–158. doi: 10.1016/j.neubiorev.2016.07.036

Scott, N., Prigge, M., Yizhar, O., and Kimchi, T. (2015). A sexually dimorphic hypothalamic circuit controls maternal care and oxytocin secretion. *Nature* 525, 519–522. doi: 10.1038/nature15378

Semaan, S. J., Murray, E. K., Poling, M. C., Dhamija, S., Forger, N. G., and Kauffman, A. S. (2010). BAX-dependent and BAX-independent regulation of Kiss1 neuron development in mice. *Endocrinology* 151, 5807–5817. doi: 10.1210/en.2010-0783

Sickel, M. J., and McCarthy, M. M. (2000). Calbindin-D28k immunoreactivity is a marker for a subdivision of the sexually dimorphic nucleus of the preoptic area of the rat: developmental profile and gonadal steroid modulation. *J. Neuroendocrinol.* 12, 397–402. doi: 10.1046/j.1365-2826.2000.00474.x

Simerly, R. B., Swanson, L. W., and Gorski, R. A. (1985a). The distribution of monoaminergic cells and fibers in a periventricular preoptic nucleus involved in the control of gonadotropin release: immunohistochemical evidence for a dopaminergic sexual dimorphism. *Brain Res.* 330, 55–64. doi: 10.1016/0006-8993(85)90007-1

Simerly, R. B., Swanson, L. W., Handa, R. J., and Gorski, R. A. (1985b). Influence of perinatal androgen on the sexually dimorphic distribution of tyrosine hydroxylase-immunoreactive cells and fibers in the anteroventral periventricular nucleus of the rat. *Neuroendocrinology* 40, 501–510. doi: 10.1159/000124122

Simerly, R. B., Zee, M. C., Pendleton, J. W., Lubahn, D. B., and Korach, K. S. (1997). Estrogen receptor-dependent sexual differentiation of dopaminergic neurons in the preoptic region of the mouse. *Proc. Natl. Acad. Sci. U.S.A.* 94, 14077–14082. doi: 10.1073/pnas.94.25.14077

Sumida, H., Nishizuka, M., Kano, Y., and Arai, Y. (1993). Sex differences in the anteroventral periventricular nucleus of the preoptic area and in the related effects of androgen in prenatal rats. *Neurosci. Lett.* 151, 41–44. doi: 10.1016/0304-3940(93)90040-r

Tsukahara, S., Hojo, R., Kuroda, Y., and Fujimaki, H. (2008). Estrogen modulates Bcl-2 family protein expression in the sexually dimorphic nucleus of the preoptic area of postnatal rats. *Neurosci. Lett.* 432, 58–63. doi: 10.1016/j.neulet.2007.12.006

Tsukahara, S., Kakeyama, M., and Toyofuku, Y. (2006). Sex differences in the level of Bcl-2 family proteins and caspase-3 activation in the sexually dimorphic nuclei of the preoptic area in postnatal rats. *J. Neurobiol.* 66, 1411–1419. doi: 10.1002/neu.20276

Tsukahara, S., Tsuda, M. C., Kurihara, R., Kato, Y., Kuroda, Y., Nakata, M., et al. (2011). Effects of aromatase or estrogen receptor gene deletion on masculinization of the principal nucleus of the bed nucleus of the stria terminalis of mice. *Neuroendocrinology* 94, 137–147. doi: 10.1159/000327541

Tsukamura, H., Homma, T., Tomikawa, J., Uenoyama, Y., and Maeda, K. (2010). Sexual differentiation of kisspeptin neurons responsible for sex difference in gonadotropin release in rats. *Ann. N. Y. Acad. Sci.* 1200, 95–103. doi: 10.1111/j.1749-6632.2010.05645.x

Waters, E. M., and Simerly, R. B. (2009). Estrogen induces caspase-dependent cell death during hypothalamic development. *J. Neurosci.* 29, 9714–9718. doi: 10.1523/JNEUROSCI.0135-09.2009

Wittmann, W., and McLennan, I. S. (2013a). Anti-Mullerian hormone may regulate the number of calbindin-positive neurons in the sexually dimorphic nucleus of the preoptic area of male mice. *Biol. Sex Differ.* 4, 18. doi: 10.1186/2042-6410-4-18

Wittmann, W., and McLennan, I. S. (2013b). The bed nucleus of the stria terminalis has developmental and adult forms in mice, with the male bias in the developmental form being dependent on testicular AMH. *Horm. Behav.* 64, 605–610. doi: 10.1016/j.yhbeh.2013.08.017

Woodson, J. C., Balleine, B. W., and Gorski, R. A. (2002). Sexual experience interacts with steroid exposure to shape the partner preferences of rats. *Horm. Behav.* 42, 148–157. doi: 10.1006/hbeh.2002.1816

Wright, C. L., Burks, S. R., and McCarthy, M. M. (2008). Identification of prostaglandin E2 receptors mediating perinatal masculinization of adult sex behavior and neuroanatomical correlates. *Dev. Neurobiol.* 68, 1406–1419. doi: 10.1002/dneu.20665

Wright, C. L., and McCarthy, M. M. (2009). Prostaglandin E2-induced masculinization of brain and behavior requires protein kinase A, AMPA/kainate, and metabotropic glutamate receptor signaling. *J. Neurosci.* 29, 13274–13282. doi: 10.1523/JNEUROSCI.3603-09.2009

Yamaguchi, S., Abe, Y., Maejima, S., and Tsukahara, S. (2018). Sexual experience reduces neuronal activity in the central part of the medial preoptic nucleus in male rats during sexual behavior. *Neurosci. Lett.* 685, 155–159. doi: 10.1016/j.neulet.2018.08.037

Yoshida, M., Yuri, K., Kizaki, Z., Sawada, T., and Kawata, M. (2000). The distributions of apoptotic cells in the medial preoptic areas of male and female neonatal rats. *Neurosci. Res.* 36, 1–7. doi: 10.1016/s0168-0102(99)00100-5

Zup, S. L., Carrier, H., Waters, E. M., Tabor, A., Bengston, L., Rosen, G. J., et al. (2003). Overexpression of bcl-2 reduces sex differences in neuron number in the brain and spinal cord. *J. Neurosci.* 23, 2357–2362. doi: 10.1523/jneurosci.23-06-02357.2003

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.

frontiers in Neuroscience

MINI REVIEW
published: 29 July 2020
doi: 10.3389/fnins.2020.00796

Genomic and Non-genomic Action of Neurosteroids in the Peripheral Nervous System

*Alessandra Colciago, Veronica Bonalume, Valentina Melfi and Valerio Magnaghi**

Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan, Italy

Since the former evidence of biologic actions of neurosteroids in the central nervous system, also the peripheral nervous system (PNS) was reported as a structure affected by these substances. Indeed, neurosteroids are synthesized and active in the PNS, exerting many important actions on the different cell types of this system. PNS is a target for neurosteroids, in their native form or as metabolites. In particular, old and recent evidence indicates that the progesterone metabolite allopregnanolone possesses important functions in the PNS, thus contributing to its physiologic processes. In this review, we will survey the more recent findings on the genomic and non-genomic actions of neurosteroids in nerves, ganglia, and cells forming the PNS, focusing on the mechanisms regulating the peripheral neuron-glial crosstalk. Then, we will refer to the physiopathological significance of the neurosteroid signaling disturbances in the PNS, in to identify new molecular targets for promising pharmacotherapeutic approaches.

Keywords: neuroactive steroid, allopregnanolone, GABA, myelin, Schwann cell, dorsal root ganglia

OPEN ACCESS

Edited by:
Hubert Vaudry,
Université de Rouen, France

Reviewed by:
Michael Schumacher,
Institut National de la Santé et de la Recherche Médicale (INSERM), France
Shogo Haraguchi,
Showa University, Japan

***Correspondence:**
Valerio Magnaghi
valerio.magnaghi@unimi.it

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

Received: *25 March 2020*
Accepted: *07 July 2020*
Published: *29 July 2020*

Citation:
Colciago A, Bonalume V, Melfi V and Magnaghi V (2020) Genomic and Non-genomic Action of Neurosteroids in the Peripheral Nervous System. Front. Neurosci. 14:796. doi: 10.3389/fnins.2020.00796

INTRODUCTION

The importance of endogenous neurosteroids for the control of the peripheral nervous system (PNS) become increasingly relevant in the last decades. Since the 1980 last century, when Baulieu and colleagues (Baulieu, 1997) introduced the term "neurosteroids" to indicate steroids that were synthesized *de novo* in the brain, also the PNS has

Abbreviations: 3α-diol, 5α-androstane-3α,17β-diol; 3β-diol, 5α-androstane-3β,17β-diol; 3α-HSD, 3α-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 5α-R, 5α-reductase; $5HT_3$, 5-hydroxytryptamine type 3; ALLO, allopregnanolone; AR, androgen receptor; CREB, cAMP response element-binding protein; DHEA, dehydroepiandrosterone; DHP, dihydroprogesterone; DHT, dihydrotestosterone; DRG, dorsal root ganglia; E2, 17β-estradiol; EAAC1, excitatory amino acid transporter 1; EGR1/Krox-24 early growth response (EGR1/Krox-24); EGR2/Krox-20 early growth response 2 (EGR2/Krox-20); Egr3, early growth response 3; ER, estrogen receptor; ERK1/2, extracellular signal-regulated protein kinase 1 and 2; FAK, focal adhesion kinase; GABA, γ-aminobutirric acid; GABA-A, GABA type A receptor; GABA-B, GABA type B receptor; GAD67, glutamic acid decarboxylase of 67 kDa; GFAP, glial fibrillary acidic protein; GPCR, G protein-coupled receptor; GPR30, GPCR ER-1 or GPER1; GR, glucocorticoid receptor; LXR, liver X receptor; MAG, myelin associated glycoprotein; mAR, membrane androgen receptor; MEP, motor exit point; mER, membrane estrogen receptor; mGR, membrane glucocorticoids receptor; mPR, membrane progesterone receptor; MR, mineralocorticoid receptor; NCV, nerve conduction velocity; NMDA, N-Methyl-D-aspartate; Olig1, oligodendrocyte transcription factor 1; P0, glycoprotein P0; P450C17, 17α-hydroxylase/17,20-lyase; P450scc, P450 side-chain cleavage enzyme; p75-NTR, neurotrophin receptor p75; PAQR, progestin and adipoQ receptor family; PGRMC-1, PR membrane component-1; PHTPP, 2-Phenyl-3-(4-hydroxyphenyl)-5,7-bis(trifluoromethyl)-pyrazolo[1,5-a]pyrimidine; PK-A, protein kinase A; PK-C, protein kinase C; PMP22, peripheral myelin protein 22; PNS, peripheral nervous system; PR, progesterone receptor, PREG, pregnenolone; PROG, progesterone; SC, Schwann cells; Shh, sonic hedgehog; Src, tyrosine protein kinase Src; SRC1, steroid nuclear receptor coactivator; Sox-10, SRY-box10; StAR, steroidogenic acute regulatory protein; STZ, streptozotocin; T, testosterone; THDOC, tetrahydrodesoxycorticosterone; THP, tetrahydroprogesterone; TSPO, translocator protein.

been referred as an important target of their action. In the PNS, the neuroactive steroids (comprising the aforementioned neurosteroids as well as the hormonal steroids) are synthesized and/or metabolized in active forms and exert important physiopathologic functions.

The neurosteroidogenic machinery includes a set of enzymes (**Figure 1**), starting from P450 side-chain cleavage (P450scc), which convert cholesterol into pregnenolone (PREG) in mitochondria, moving to the 3β-hydroxysteroid-dehydrogenase (3β-HSD), that converts PREG into progesterone (PROG) or the 17α-hydroxylase/17,20-lyase (P450C17), converting PREG into dehydroepiandrosterone (DHEA). These steroids may be then metabolized into androgens, androstenediol, androstenedione and testosterone (T). Furthermore, by the action of the enzyme P450 aromatase the androgens androstenedione and T are converted into the estrogens estrone and 17β-estradiol (E2), respectively.

Most of these enzymes, as well as their metabolic products PROG, PREG, T, DHEA, and E2, have been found in PNS (Caruso et al., 2008). In particular, the glial Schwann cells (SC) of the PNS possess the enzymatic machinery required to produce the neurosteroids: P450scc, 3β-HSD, etc., (Celotti et al., 1992; Melcangi et al., 2001; Schumacher et al., 2001). The activity of some steroidogenic enzymes in SC is neuronal dependent, such as the 3β-HSD, that raised in the presence of neurons (Chan et al., 2000; Robert et al., 2001). Dorsal root ganglia (DRG) express the P450scc and 3β-HSD but not other steroidogenic enzymes, indicating that other autocrine or paracrine mechanisms might influence the steroidogenesis in the soma of primary sensory neurons (Schaeffer et al., 2010). Additionally, the enzymatic complexes formed by the 5α-reductase (5α-R) and the 3α-hydroxysteroid-dehydrogenase (3α-HSD) was found in the PNS (**Figure 1**), primarily in SC (Melcangi et al., 1990; Celotti et al., 1992; Melcangi et al., 1992, 1999b). This enzymatic complex converts steroids possessing the delta(4)-3-keto configuration into their more active 5α-3α-reduced metabolites, the so-called neuroactive steroids (Celotti et al., 1992). Thereby, the PROG is converted into dihydroprogesterone (DHP) and then into 5α-pregnan-3α-ol-20-one, also named tetrahydroprogesterone (THP) or allopregnanolone (ALLO); similarly, the steroid T is converted into dihydrotestosterone (DHT) then into 5α-androstane-3α,17β-diol (3α-diol). The 5α-reduced intermediate metabolite DHP and DHT can be further converted, by the enzyme 3β-HSD, into 5α-pregnan-3β-ol-20-one (also named isopregnanolone) or 5α-androstane-3β,17β-diol (3β-diol), respectively (Giatti et al., 2015; **Figure 1**).

The PNS showed also the presence of other important factors supporting *de novo* local synthesis of neuroactive steroids. For instance, those factors regulating the translocation of cholesterol into the mitochondria, likely the steroidogenic acute regulatory protein (StAR) and the translocator protein of 18 kDa (TSPO), as well as the liver X receptor (LXR), were found to be present and active in peripheral nerves. TSPO was formerly considered as a crucial protein for steroid biosynthesis (Li and Papadopoulos, 1998). More recent and debated observations, however, argued the significance of TSPO in steroidogenesis and evidenced its involvement in pathological conditions, like inflammation, apoptosis and neurological diseases (e.g., Alzheimer's disease or multiple sclerosis); indeed, TSPO seems to be expressed only in response to insults and pathological states (Bonsack and Sukumari-Ramesh, 2018). In the PNS, the activation of TSPO with the specific ligand Ro5-4864 improved the levels of neuroactive steroids and exerted neuroprotective effects in the peripheral nerves of streptozotocin (STZ)-induced diabetic rats (Giatti et al., 2009). LXR is a ligand activated transcription factor belonging to the nuclear receptor superfamily. It is important for cholesterol biosynthesis, serving as a sensor that prevents the excessive intracellular accumulation of cholesterol (Jakobsson et al., 2012). In the PNS, its activation by specific ligands induces neuroactive steroid synthesis in the sciatic nerve of STZ-induced diabetic rats, thus ameliorating diabetes-induced neuropathy (Cermenati et al., 2010).

Interestingly, the synthesis and the levels of neuroactive steroids proved sexually dimorphic, in physiologic states (Melcangi et al., 2016), as well as in peripheral neurodegenerative conditions, such as the diabetic neuropathy (Pesaresi et al., 2010). For instance, in the sciatic nerve of STZ-rats the levels of PREG, T, DHT, and 3α-diol decreased in males, whereas the levels of PROG, THP and isopregnanolone drop down only in female (Pesaresi et al., 2010). In the same rat model of diabetic neuropathy, the gonadectomy ameliorates the nerve alterations in females but not in males (Pesaresi et al., 2011a).

In this review, we will survey the recent findings of the classical and non-classical, genomic and non-genomic action of neuroactive steroids in peripheral nerves, ganglia and cells forming the PNS, thereby focusing on the mechanisms regulating the peripheral neuron-glial crosstalk.

MECHANISMS OF ACTION OF NEUROACTIVE STEROIDS

In the PNS, the neuroactive steroids exert several biologic functions, modulating the mitogenic activity, cell proliferation, myelination process, nerve repair, and axonal conduction.

The neuroactive steroid actions occur through either "classical" or "non-classical" receptors, which localized both in the neuronal and in the glial compartment (i.e., SC) of the PNS. The classical action is generally genomic and consists of the binding to intracellular receptors in the target cells, followed by the regulation of gene transcription (Slater et al., 1994). Conversely, the non-classic action is more rapid and involves the modulation of membrane receptors, such as neurotransmitter and neurotrophin receptors, ion channels or the newest membrane steroid receptors (Brann et al., 1995; Barabas et al., 2018). Commonly, among the neurotransmitter receptors affected by neuroactive steroids, there are the γ-aminobutyric acid (GABA) and the N-Methyl-D-aspartate (NMDA) receptors (Lambert et al., 1996; Rupprecht et al., 2001; Monnet and Maurice, 2006; Sedlacek et al., 2008). Moreover, the family of steroid membrane receptors includes specific receptors for estrogens, androgens, glucocorticoids and progestogens (Levin, 2011). Whether these receptors are the classical

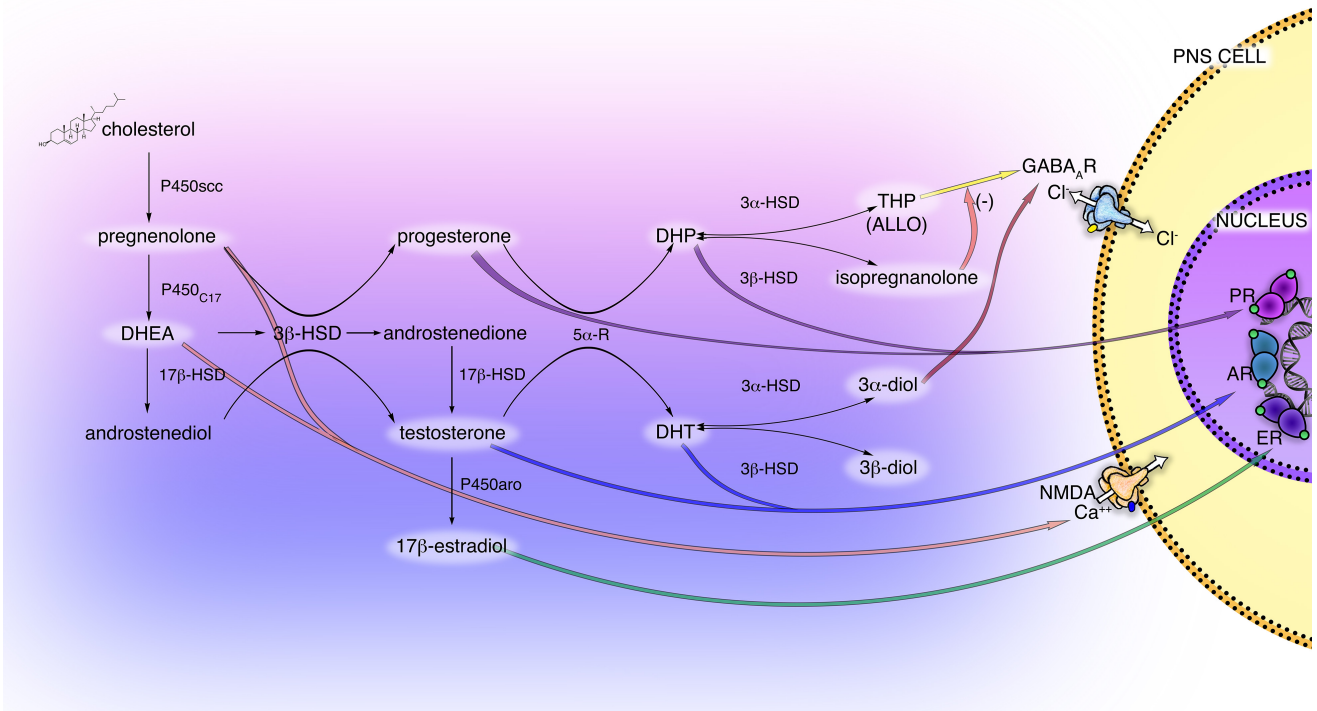

FIGURE 1 | Scheme of principal neuroactive steroid biosynthetic and metabolic pathways, and their receptor interactions in the PNS. The main steroidogenic enzymes, metabolites, and receptors modulating neuroactive steroid action are present in the cells of the PNS (i.e., Schwann cells and DRG neurons). These steroidogenic pathways, through the intermediate pregnenolone (PREG), dehydroepiandrosterone (DHEA) and androstenediol lead to the synthesis of progestogens (PROG, progesterone; DHP, dehydroprogesterone; THP, tetrahydroprogesterone also called ALLO, allopregnanolone; isopregnanolone), androgens (T, testosterone; DHT, dihydrotestosterone; 3α-diol, 5α-androstane-3α,17β-diol; 3β-diol, 5α-androstane-3α,17β-diol) and estrogens (E2, 17β-estradiol). P450 side-chain cleavage enzyme (P450scc); 17α-hydroxylase/17,20-lyase (P450C17); 17β-hydroxysteroid-dehydrogenase (17β-HSD); 5α-R, 5α-reductase; 3α-hydroxysteroid-dehydrogenase (3α-HSD); 3β-hydroxysteroid-dehydrogenase (3β-HSD); P450 aromatase (P450aro). These neuroactive steroids may act through classical receptor (PR, progesterone receptor; AR, androgen receptor; ER estrogen receptor) or non-classical receptor (GABA type A receptor; NMDA receptor), see text for details.

nuclear receptor, which localizes on the cell membrane, or distinct receptors characterized by different proteins is still a matter of debate.

Interestingly, the capability of neuroactive steroids to interact with classical rather than non-classical receptors is ancillary to their conversion into active compounds. For instance (**Figure 1**), the progestogens PROG and DHP mainly exert classical activity through the genomic PROG receptor (PR), while their metabolite ALLO fulfills a non-classical activity via the GABA type A (GABA-A) receptor. Indeed, ALLO is one of the most re-known and important GABA-A receptor modulators (Lambert et al., 2009; Faroni and Magnaghi, 2011), while the progestogen metabolite isopregnanolone has proved to antagonize the effect of ALLO at GABA-A receptor (Wang et al., 2002). Similarly, androgen metabolites exert classical and non-classical actions. For instance, (**Figure 1**) 3α-diol activates the GABA-A receptor, whereas 3β-diol is an agonist of the estrogen receptor (ER) beta (ERβ) (Lambert et al., 2003; Handa et al., 2008).

Evidence on the involvement of all these receptors in the different physiopathologic states affecting the PNS has been fully reported.

GENOMIC ACTIONS OF NEUROACTIVE STEROIDS IN PNS

Classical intracellular steroid receptors, such as PR, ER, androgen receptor (AR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) were found in peripheral nerves (Magnaghi et al., 1999, 2001; Jordan et al., 2002; Shaqura et al., 2016) as well as in SC (Neuberger et al., 1994; Jung-Testas et al., 1996; Groyer et al., 2006; Faroni and Magnaghi, 2011; Shaqura et al., 2016) and DRG (Luo et al., 2008; Dong et al., 2012; Shaqura et al., 2016; **Figure 1**). Generally, these classic receptors bind, respectively, the progestogens PROG and DHP, the estrogens E2 and estrone, the androgens DHEA, T and DHT, the gluco/mineralocorticoids corticosterone, dehydrocorticosterone, and deoxycorticosterone (Slater et al., 1994; Prough et al., 2016).

In the PNS, E2 promoted the proliferation and differentiation of SC, *in vitro* and *in vivo*, thus fostering the myelination process (Chen et al., 2016; Gu et al., 2018). By the way, some of these effects were inhibited by the antagonist of the genomic ER type α (ERα) and type β (ERβ) receptors, ICI182780 and 2-phenyl-3-(4-hydroxyphenyl)-5,7-bis(trifluoromethyl)-pyrazolo[1,5-a]pyrimidine (PHTPP), respectively, highlighting

a classical genomic mechanism. Nevertheless, the specific block of the intracellular signaling cascade of the extracellular signal-regulated protein kinase 1/2 (ERK1/2) or AKT (commonly downstream the activation of the membrane receptor) evidenced that also these pathways may occur, and suggested that the mechanisms are complicated, likely involving both genomic and non-genomic actions (Gu et al., 2018). Besides, also PROG was able to promote SC proliferation, and its effects appeared sex-specific (Jung-Testas et al., 1993). E2 was effective in males, while PROG promotes SC proliferation only in females. These actions implied genomic mechanisms since they were blocked by specific ERα and PR antagonists, ICI128780 and zk112994, respectively, (Fex Svenningsen and Kanje, 1999).

In the PNS, the effect of neuroactive steroids been extensively studied on the myelination and re-myelination processes has been extensively studied. The glucocorticoid corticosterone stimulated the expression of the two most important proteins of the peripheral myelin (Desarnaud et al., 2000): the glycoprotein P0 (P0) and the peripheral myelin protein of 22 kDa (PMP22). However, progestogens were proved as the more compelling steroids able to regulate the PNS myelination. PROG, DHP, and ALLO stimulated the expression of P0 and PMP22 in the sciatic nerve of young and old male rats (Melcangi et al., 1998, 1999a, 2000b; Faroni and Magnaghi, 2011). In *in vivo* models, PROG, DHP and ALLO proved able to reduce the age-associated myelin abnormalities in the sciatic nerve of elderly rats (Azcoitia et al., 2003) and stimulated the re-myelination of injured nerves, in a model of nerve cryolesion or transection (Koenig et al., 1995; Melcangi et al., 2000a). Furthermore, in a model of guided facial nerve regeneration, PROG increased the SC proliferation, myelination as well as the number of nerve fibers (Chavez-Delgado et al., 2005). The effects of PROG and DHP on P0 and PMP22 levels occurred also in SC cultures (Desarnaud et al., 1998; Melcangi et al., 1998, 1999a; Magnaghi et al., 2001), indicating a direct classical genomic effect of progestogens on these cells. In accordance, we proposed that the complicated and long-term effects of PROG, DHP and ALLO (after its retro-conversion into DHP, within PNS cells; see **Figure 1**) in modulating the expression of protein P0 are linked to the interaction with the PR expressed in SC (Magnaghi et al., 2001). The specific PR antagonist mifepristone (RU38486), indeed, blocked the effects of PROG, DHP and ALLO on the P0 levels (Melcangi et al., 2003), corroborating the genomic mechanism. Nevertheless, the rapid effect of ALLO on PMP22 levels seemed due to an interaction with the GABA-A receptor expressed in SC (see the chapter below).

The classical genomic effect on P0 was sustained by the presence of putative PROG responsive elements on the P0 gene (Magnaghi et al., 1999) and by the involvement of the steroid nuclear receptor coactivator SRC1 in the regulation of P0 expression (Cavarretta et al., 2004). Interestingly, the genomic action exerted by progestogens on the PNS myelin proteins, that is P0, PMP22 and myelin associated glycoprotein (MAG), was sex-specific; in fact male rats resulted more responsive to the genomic effects of PROG and DHP (Magnaghi et al., 2006b). It was highlighted that PROG coordinates also the initiation of the PNS myelination, because it increases the expression of some basic transcription factors priming the SC myelination, such as early growth response 2 EGR2 (EGR2/Krox-20), early growth response (EGR1/Krox-24), early growth response 3 (Egr-3), SRY-box10 (Sox10) and Fos B (Guennoun et al., 2001; Mercier et al., 2001; Magnaghi et al., 2007). At least in the case of Krox-20, the presence of putative PROG responsive elements in the gene promoter support a PR-mediated genomic effect (Magnaghi et al., 2007).

Other findings suggested that the glycoprotein P0 is also under the control of classical AR. Gonadectomy of adult male rats induced a decrease in myelin protein P0, whereas DHT enhanced the P0 levels in sciatic nerve of normal animals (Magnaghi et al., 1999). This effect attested the capacity of androgens to participate in the control of peripheral myelination, however, since the SC do not express AR (Magnaghi et al., 1999), the effect was supposed to be indirectly mediated via the neuronal compartment. The androgens efficacy may be ascribed to the crosstalk between SC and the axon, hypothesizing the transfer of vesicles likely containing the receptors (Grossfeld et al., 1988; Lopez-Verrilli and Court, 2012), or the involvement of motoneurons which express the AR (Jordan et al., 2002). The finding that genomic effects of neuroactive steroids on SC are indirectly mediated by the neuronal compartment is supported by the observation that the PR antagonist mifepristone induced an axonal impairment during the development, determining a significant reduction of axon diameter (Melcangi et al., 2003). In accordance, the PROG enhancement of myelin formation was shown in an *in vitro* co-culture model of SC-DRG neurons, corroborating the requirement of the neuronal compartment for the progestogen action (Chan et al., 2000).

Neuroactive steroids, mainly PROG and DHP, also exert neuroprotective and pro-regenerative effects in case of neurodegenerative pathologies of the PNS, such as nerve traumatic injuries (i.e., cryolesion, transection or crush) or diabetic neuropathy (Koenig et al., 1995; Melcangi et al., 2000a; Chavez-Delgado et al., 2005; Leonelli et al., 2007; Roglio et al., 2008). For instance, PROG and DHP, likely through genomic mechanisms involving the PR, are able to counteract the decrease of P0 and PMP22 expression induced in the STZ model of diabetic neuropathy (Leonelli et al., 2007). Both neuroactive steroids decreased the number of altered fibers (i.e., presenting myelin infoldings) in the sciatic nerve of STZ neuropathic rats (Veiga et al., 2006), still corroborating the neuroprotective role of PR. In parallel, also the androgen DHT proved able to increase the P0 mRNA levels in the sciatic nerve of STZ neuropathic rats, likely via AR-mediated mechanisms (Roglio et al., 2007). In the same model of STZ-induced neuropathy, DHP and DHT improved another hallmark of diabetic neuropathy, promoting the changes in Na^+-K^+ ATPase activity (Leonelli et al., 2007; Roglio et al., 2007). In diabetic rats, also the treatment with DHEA exerted neuroprotective effects, mostly in females rather than in male animals (Pesaresi et al., 2011b). DHEA was effective following rat sciatic nerve transection, whereas it reduced the extent of denervation atrophy stimulating the earlier onset of axonal regeneration (Ayhan et al., 2003). Following traumatic nerve crush injury, DHEA and E2 promote the fast recovery of

gait along with an enhancement of myelinated fibers (Gudemez et al., 2002; Islamov et al., 2002). Moreover, also T was capable to accelerate the functional recovery following rat sciatic nerve crush (Kujawa et al., 1993; Brown et al., 1999).

Evidence of GR-dependent induction of gene transcription was found in adult DRG neurite, which grew in response to stress or glucocorticoid treatment. This phenomenon exacerbates the effect of acute systemic stress on neuronal plasticity and PNS regeneration (Lerch et al., 2017). Importantly, a putative role of GR in regulating peripheral nociception has been proposed. This hypothesis was corroborated by the GR localization, which was found predominantly in peripheral nociceptive unmyelinated C-fiber and Aδ lightly myelinated fibers (Shaqura et al., 2016).

NON-GENOMIC ACTION OF NEUROACTIVE STEROIDS IN PNS: ROLE OF MEMBRANE STEROID RECEPTORS

The steroid membrane receptors mediate the rapid (second to minutes) non-classical, non-genomic action of neuroactive steroids, occurring at the cell surface of neurons and glial cells. To date, it consists of specific receptors for estrogens (membrane ER, mER), androgens (membrane AR, mAR) glucocorticoids (membrane GR, mGR) and progestogens (membrane PR, mPR). These receptors mostly belong to the G protein-coupled receptor (GPCR) family and activate a plethora of intracellular signaling cascade (Levin, 2011). Recent studies investigated the presence of some membrane receptors in the PNS, focusing primarily on the subfamilies mER and mPR.

The GPR30, named GPCR ER-1 (GPER1), is a non-nuclear ER located on the cell membrane, which binds E2 with high affinity and potency, thus mediating non-genomic events (Thomas et al., 2005). DRG, autonomic pelvic ganglia and sensory trigeminal ganglia express GPR30, which modulation by the specific G1 agonist induced a membrane depolarization (Dun et al., 2009). In the PNS, however, some rapid estrogenic effect seemed to be due to the non-genomic action of classic ERα, likely translocated to the cell membrane. For instance, mouse DRG neurons express membrane associated ERα, producing a rapid attenuation of ATP-induced Ca^{++} signaling, likely a mechanism involved in gender-specific pain perception (Chaban and Micevych, 2005). Another study underlined the cytoprotective potential of E2 on the transplanted SC in a model of spinal cord injury (Siriphorn et al., 2010). Protection was not inhibited by classical ER antagonist ICI 182780, suggesting that non-genomic mechanisms involving mER may occur (Siriphorn et al., 2010).

In the last decade, five subtypes of mPRs (mPRα−ε) were classified. These receptors are GPCRs, belong to the progestin and adipoQ receptor family (PAQR) and mediate rapid neuroprotective actions of progestogens (i.e., PROG and ALLO) in the nervous system (Thomas and Pang, 2012). The PR membrane component-1 (PGRMC-1; formerly named 25Dx) is another protein complexing with the plasminogen activator inhibitor 1 RNA binding protein and able to bind PROG (Peluso et al., 2008; Cooke et al., 2013). PGRMC1 was implicated in the neuroprotective effects of PROG following traumatic brain injury (Meffre et al., 2013) and spinal cord injury (De Nicola et al., 2009). Although it was found in S42 SC line (Castelnovo et al., 2019), the possible function in PNS was not further investigated. Very recently, some mPRs (primarily mPRα and mPRβ) were found in PNS and in SC *in vitro* (**Figure 2**), whereby they promote cell migration, proliferation and differentiation (Castelnovo et al., 2019, 2020). Indeed, in SC, mPR activation with the specific ligand O2 induced rapid downregulation of myelinating (i.e., Sox10 and Krox20) and non-myelinating [i.e., glial fibrillary acidic protein (GFAP) and neurotrophin receptor p75 (p75-NTR)] markers of SC. Contemporarily, other specific markers of repairing SC [i.e., oligodendrocyte transcription factor 1 (Olig1) and sonic hedgehog (Shh)] resulted up- and/or down-regulated following mPR activation (Castelnovo et al., 2020). These effects were mediated by an intracellular activation of phosphorylated AKT (**Figure 2**). Overall, these observations proved a direct control of SC by mPR, playing a promising role in the promotion of nerve re-growth (Castelnovo et al., 2019, 2020).

Recent work shed light on a peripheral glial population named "motor exit point" (MEP) glia, possessing some typical features of peripheral SC, that might be relevant in spinal cord regeneration (Fontenas and Kucenas, 2018). Theoretically, it could be speculated that mPR exerts a direct neuroprotective effect within the spinal cord through the mPR stimulation of MEP glial cells, although this hypothesis deserves further proof.

Apparently less investigated, also the mGR subfamily was studied in the PNS. Evidence of a putative non-genomic pathway including GR binding sites has been found in membrane fractions of DRG neurons, suggesting a potential rapid, GPCR-linked, non-genomic mechanism for mGR in mediating peripheral pain (Shaqura et al., 2016).

NON-GENOMIC ACTION OF NEUROACTIVE STEROIDS IN PNS: INVOLVEMENT OF GABA AND OTHER RECEPTORS

Besides the steroid membrane receptors, the non-classical action of neuroactive steroids comprises the modulation of other membrane receptors, for instance, the neurotransmitter receptors GABA-A, GABA type B (GABA-B), NMDA, 5-hydroxytryptamine type 3 (5-HT_3) and σ1 receptors (Lambert et al., 1996, 2009; Rupprecht et al., 2001; Monnet and Maurice, 2006; Sedlacek et al., 2008).

GABA-A receptor is a member of the ligand-gated ion channel family, permeable to a Cl^- flux and composed of five subunits from a repertoire of nineteen isoforms (i.e., α1-6, β1-3, γ1-3, δ, ε, π, θ, ρ1-3) (Whiting et al., 1995, 1997; Lambert et al., 2003). GABA-A receptor is allosterically activated by neuroactive steroids, mainly ALLO but also 3α-diol, 3β-diol and the corticosteroid 5α-3α metabolite tetrahydrodesoxycorticosterone (THDOC) (Reddy and Rogawski, 2002). In this regard, it is the most studied non-genomic mechanism of neuroactive steroids in the nervous system as well as in the PNS (Park-Chung et al.,

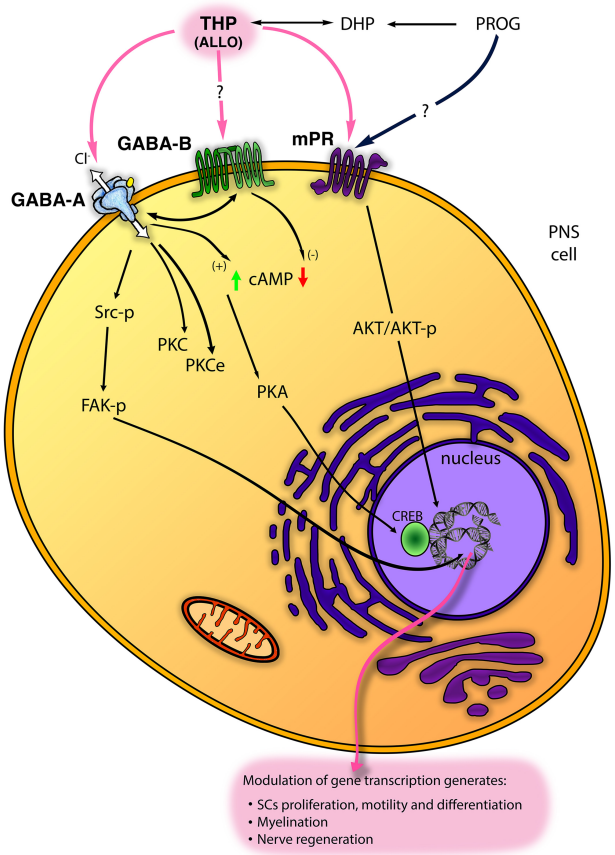

FIGURE 2 | Scheme of main non-classical mechanisms of progestogens (PROG, progesterone and THP, tetrahydroprogesterone also called ALLO, allopregnanolone) in the PNS. ALLO may interact with GABA type A receptor (GABA-A), although its modulation of GABA type B receptor (GABA-B) receptor has been proposed. In SC, ALLO and likely PROG interact with the membrane PROG receptor (mPR). Some intracellular signalings downstream these receptors (including Src/FAK kinases; protein kinase C, PKC, and PKCε; cyclic AMP, cAMP, and protein kinase A, PKA, etc.) are reported (see text for details).

1999; Belelli and Lambert, 2005). GABA-A receptor is classified in different subtypes based on its subunit composition. The receptor formed by one α (α2-5) plus β3 and γ2 subunits gives consistent potentiation to the ALLO-mediated GABA-activated currents (Hosie et al., 2009), while δ-containing subtype was classically described at extrasynaptic sites whereby it is highly sensitive to the 5α-3α-reduced metabolites (Mihalek et al., 1999; Belelli et al., 2002). In the PNS, GABA-A receptor is widely distributed in nerves, neurons, and glial cells. SC express the α2 and 3, β1, 2, 3 and γ2 subunits (Melcangi et al., 1999a; Magnaghi et al., 2006a), as well as the α4 and δ subunits, more characteristic of the extrasynaptic receptor (Faroni et al., 2019). In addition, we found most of these subunits in the mouse DRG neuronal cultures, with predominant expression of the synaptic subunits α1, α2, β1, β3, γ2 (Faroni et al., 2019). In general, in the PNS, the role of ALLO through GABA-A receptor has been widely characterized in the glial compartment, showing enhancement of SC proliferation, motility, differentiation and myelination (**Figure 2**; see also the following chapter). ALLO non-classical non-genomic effects were mimicked also by PROG and DHP, but only in prolonged (e.g., 24 h) treatment condition, when progestogens can be converted into their 5α-3α metabolite ALLO, then exerting its action via GABA-A receptor modulation (**Figure 2**). Recently, in DRG sensory neuron, GABA-A receptor has been characterized for its relevance in controlling peripheral pain (Chen et al., 2014; Zhang et al., 2015; Du et al., 2017), albeit ALLO's allosteric modulation of GABA-A receptor in pain was not yet fully investigated.

Interestingly, some evidence suggests that neuroactive steroids require a different route of access to the transmembrane-domain binding sites within GABA-A receptor (Shu et al., 2004; Akk et al., 2005; Hosie et al., 2006; Chisari et al., 2009). Neuroactive steroids may be entrapped in the intracellular compartment, then re-supply the cell membrane with ligands able to modulate the GABA-A receptor at later times; by this way, the kinetic of neuroactive steroid action at GABA-A receptor may be modulated by neuroactive steroids themselves (Akk et al., 2005). In accordance, the regulation of the lipid components of the peripheral myelin may be considered as a kind of non-classical non-genomic action of neuroactive steroids in the PNS. Although this uncommon mechanism was exerted by DHP, usually acting through the classic genomic PR action, it lies in the middle ground of a genomic/non-genomic mechanism. However, in a model of diabetic neuropathy, DHP proved able to promote fatty acid desaturation and to reduce the morphological alteration of nerves, reaffirming its neuroprotective role in PNS (Mitro et al., 2014).

In principle, the possibility of ALLO to interact with the metabotropic GABA-B receptor should not be completely excluded (**Figure 2**). To date, although a direct interaction of ALLO with GABA-B receptor was not clearly stated, several pieces of evidence highlighted a cross-regulation between GABA-A and GABA-B receptors in PNS (Magnaghi, 2007; Faroni et al., 2019). For instance, ALLO exerted a GABA-A mediated biphasic control of different GABA-B receptor subunits (Magnaghi et al., 2006a; Magnaghi, 2007). The metabotropic GABA-B receptor is a dimeric complex, a member of the GPCR superfamily (Bowery and Enna, 2000). In the PNS, GABA-B receptor subunits 1a, 1b and 2 were found in SC, sciatic nerve, satellite cells and DRG neurons (Magnaghi et al., 2004; Magnaghi, 2007; Faroni et al., 2014), where the functional receptor was proved to be negatively coupled to the adenylate cyclase system (**Figure 2**; Magnaghi et al., 2004). Its activation decreased SC proliferation and the expression of some important myelin proteins, like P0, PMP22, MAG and connexin 32 (Magnaghi et al., 2004). In a neuropathic model of partial sciatic ligation, a 7-day administration of specific GABA-B ligands (i.e., baclofen and the antagonist CGP56433) strongly improved the biochemical, morphological and behavioral outcomes of sciatic nerve (Magnaghi et al., 2014). Furthermore, studies in transgenic mice with a conditional deletion of GABA-B1 receptor in PNS demonstrated that some important GABA-A subunits, expressed in SC and DRG neurons, were cross-regulated by GABA-B receptor (Faroni et al., 2019).

Beside the GABA-A receptor, other neurotransmitter receptors, such as NMDA receptor, are affected by neuroactive

steroids (**Figure 1**). Indeed, it was shown that PREG, DHEA and DHEA sulfate activate allosterically NMDA receptor (**Figure 1**), while PREG sulfate acts as the negative modulator (Wu et al., 1991; Baulieu, 1997; Park-Chung et al., 1999). NMDA is an ionotropic glutamate receptor also distributed in the PNS, where it localizes in peripheral axons and SC (Evans et al., 1991, 1992; Carlton et al., 1998; Christensen et al., 2016; Campana et al., 2017). The PNS also has specific glutamate transporters and it synthesizes glutamate, which was found in sensory and motor neurons (Chen et al., 2017). Moreover, sensory cranial ganglia synthesize glutamate (Malet and Brumovsky, 2015), while the SC possess the enzymatic machinery able to uptake and synthesize glutamate, like the excitatory amino acid transporter 1 (EAAC1) and glutamine synthetase (Miller et al., 2002; Perego et al., 2012). The σ1 receptor is an intracellular protein that localizes in membranes of the endoplasmic reticulum, plasmalemma, nucleus, and mitochondria (Alonso et al., 2000) and it is able to enhance NMDA activity (Pabba and Sibille, 2015). Activation of this receptor rises intracellular Ca^{++} influx via NMDA (Hayashi et al., 2000), thus confirming the capability of σ1 to modulate NMDA receptor. Accordingly, it was shown that DHEA sulfate acts as σ1 agonist, inducing a clear σ1-like potentiation of NMDA response, while PREG sulfate exerted opposite effects; also PROG is an endogenous antagonist of σ1 receptor (Maurice et al., 1999). To date, evidence of neuroactive steroid actions through NMDA or σ1 receptor, specifically in the PNS, has not been yet provided.

ALLO ACTIVATION OF GABA-A RECEPTOR IN PNS: INTRACELLULAR SIGNALING

ALLO is the most re-known neuroactive steroid able to regulate the PNS, controlling glial proliferation, differentiation and myelination processes (**Figure 2**). ALLO non-genomic effects on GABA-A receptors usually occurred at nanomolar concentration, engaging an allosteric interaction that entails the presence of the endogenous ligand GABA. Instead, at high concentration (micromolar range) ALLO directly gates GABA-A receptor (Callachan et al., 1987), although it was shown that neuroactive steroids might directly gate GABA-A receptor even at 100 nM, likely attaining a relatively low kinetic (Shu et al., 2004).

In any case, the presence of endogenous GABA is a requisite for ALLO action. Following the former observation in the early 1980's proving GABAergic fibers in PNS (Brown and Marsh, 1978; Morris et al., 1983; Olsen et al., 1984), it was unequivocally demonstrated the presence of GABA and its synthetic machinery (glutamic acid decarboxylase of 67 kDa, GAD67) in SC (Magnaghi et al., 2010). An autocrine loop has been proposed, through which nanomolar concentration of ALLO was able to increase the GAD67 levels in SC, thus providing GABA as the endogenous ligand for the GABA-A receptor (Magnaghi et al., 2010). Therefore, the local GABA synthesis in peripheral nerves supports the allosteric action of ALLO in SC and neighboring compartments. In accordance, it was shown that SC possesses EAAC1, the active uptake system able to provide glutamate as a precursor for GABA synthesis (Perego et al., 2011, 2012). In SC, EAAC1 expression and activity were still controlled by ALLO, through a GABA-A mediated and protein kinase C (PKC) mechanisms (**Figure 2**) ALLO promoted the transport of EAAC1 from the intracellular stores into the SC membrane (in actin-rich cell tips), modifying their morphology (Perego et al., 2012).

ALLO was shown to increase SC proliferation (Perego et al., 2012; Melfi et al., 2017) and this action was GABA-A mediated because it was mimicked by the specific ligand muscimol and blocked by the specific antagonist bicuculline (Perego et al., 2012). It was highlighted that ALLO's control of SC proliferation was dependent on EAAC1 transport and activity at SC plasma membrane (Perego et al., 2012), once again confirming that GABA synthesis was necessary for ALLO effects. ALLO also stimulated morphologic changes and motility of SC, then promoting myelination (Melfi et al., 2017), which are fundamental processes for the development, maturation, and regeneration of PNS. Remarkably, ALLO participated in the control of peripheral myelin proteins (e.g., P0, MAG), being particularly active in enhancing the levels of PMP22, mRNA and protein (Melcangi et al., 1999a,b; Magnaghi et al., 2001). The specificity of this action was confirmed, respectively, by the capability of muscimol to replicate and of bicuculline to abolish the ALLO's effects on PMP22 (Magnaghi et al., 2001, 2006a). This confirmed the hypothesis that in SC protein PMP22 is controlled by GABA-A receptors. However, the capability to stimulate PMP22 expression was observed also with 3α-diol (Magnaghi et al., 2001), likely via the same allosteric GABA-A receptor modulation (Frye et al., 1996; **Figure 1**).

As expected, the intracellular mechanisms downstream the ALLO modulation of GABA-A receptors imply changes in intracellular Cl^- flux (**Figure 2**). Conversely, at least in PNS, most of ALLO effects reflected as transcriptional changes. In the last decades, some studies were addressed to clear this point. For instance, in the developing rat cortex, GABA-A receptor activation leads to an increase of Ca^{++} influx through L-type voltage-gated Ca^{++} channels. This leads to the phosphorylation and activation of the cAMP response element-binding protein (CREB) transcription factor, in turn regulating protein expression, for instance of the brain derived neurotrophic factor (Mantelas et al., 2003). Unfortunately, these mechanisms were not shown in the PNS, whereas the concomitant activation of ion channels (e.g., Ca^{++} channels), following neuroactive steroid binding to GABA-A receptor, is still questionable. Although the involvement of Ca^{++} channel is not clear, ALLO was proved able to modulate the protein kinase A (PK-A), through enhanced cAMP levels and CREB phosphorylation (Magnaghi et al., 2010), or the PKC pathway (Perego et al., 2012; **Figure 2**). These intracellular signalings were supposed to be downstream the allosteric action of ALLO at GABA-A receptor. Recently, another intracellular ALLO's pathway has been found. ALLO effects on SC proliferation, motility and myelination, indeed, imply tyrosine protein kinase Src (Src) and focal adhesion kinase (FAK) activation (Melfi et al., 2017), although other signaling pathways should not be excluded (**Figure 2**). ALLO effects on Src were mimicked by muscimol, counteracted by bicuculline and by the specific Src inhibitor PP2, suggesting that in SC ALLO activation of GABA-A induces an intracellular

phosphorylation cascade, leading to actin rearrangements of the cytoskeleton, enhancement of SC motility and myelination (Melfi et al., 2017; **Figure 2**).

One strategy that neuroactive steroids use to control GABA-A receptor function is to phosphorylate/de-phosphorylate its subunits by the recruitment of protein kinases or phosphatases (Belelli and Lambert, 2005). Phosphorylation of GABA-A can produce different effects, ranging from enhancement to inhibition, depending on the subunit targeted and on the location of sites being phosphorylated (Moss and Smart, 1996). In parallel, PKC phosphorylation of GABA-A receptor may influence the sensitivity to neuroactive steroids (Brussaard and Koksma, 2003; Vergnano et al., 2007). Accordingly, PKC-ε is considered as a novel isoform of PKC, regulating the sensitivity to neuroactive steroids. Indeed, animals lacking PKC-ε showed hypersensitivity to behavioral effects induced with allosteric GABA-A receptor modulation (Hodge et al., 2002). PKC-ε was found in SC and DRG neurons in culture (Puia et al., 2015). Interestingly, PKC-ε was upregulated in DRG neurons exposed to the culture medium from ALLO-treated SC, suggesting that these cells release one or more factors able to regulate PKC-ε in DRG neurons (Puia et al., 2015). Since PKC-ε is relevant in modulating some pain pathways, we speculated that these mechanisms identified novel putative circuits involved in the control of pain processes at PNS and spinal cord levels (Puia et al., 2015).

In the PNS, ALLO hired importance also during neurodegenerative conditions, likely implying the regulation of other nervous cells or structures (i.e., DRG neurons). In a model of STZ-induced diabetic neuropathy, ALLO and 3α-diol enhanced nerve conduction velocity (NCV) and intraepidermal nerve density, decreasing sensitivity to thermal pain (Leonelli et al., 2007; Roglio et al., 2007). Although the mechanism behind these effects was not elucidated, the involvement of non-genomic mechanisms through GABA-A receptor was hypothesized. In support of the non-genomic action of ALLO in neuropathic pain (Patte-Mensah et al., 2014), it should be highlighted that ALLO may regulate other channels and/or signaling pathways involved in neuropathic pain, such as T-type Ca^{++} channels, voltage-gated Na^{+} channels, purinergic receptor P2X3 and bradykinin signaling (Cho and Chaban, 2012; Ayoola et al., 2014).

CONCLUSION

In this review, we sum up most of the latest evidence on the effects of neuroactive steroids, either classical or non-classical, genomic or non-genomic, in the PNS. Neuroactive steroids exhibit important functions in the development, myelination, neuroprotection and nerve repair of the PNS. In particular, ALLO revealed the most well studied and incisive neuroactive steroid in regulating the biologic and physiologic functions of the PNS. Here we reported several steps forward in the identification of its mechanism of action. Some ALLO's effects may be ascribed to GABA-A (or likely GABA-B) activation, PKA, PKC or PKC-ε modulation, as well as to the Src/FAK kinases involvement. Besides, mPR or electrophysiological changes in ion channels, likely Cl^{-} flux, have been recently proposed to occur also in the PNS.

Interestingly, the neuroregenerative effects of ALLO via GABA-A receptor might be promising for the treatment of the peripheral neurodegenerative pathologies, particularly for traumatic injuries requiring the surgical application of bioengineered conduits. Indeed, *in vitro* testing of 2D silk fibroin scaffold, functionalized for controllable *in situ* delivery of ALLO, showed great potential for nerve repair (Gennari et al., 2018). Therefore, the administration of neuroactive steroids might represent a novel and promising strategy to prevent or treat different types of peripheral neuropathies and the associated neuropathic pain.

AUTHOR CONTRIBUTIONS

AC wrote and revised the whole manuscript. VB prepared the figures and revised the manuscript. VMe searched the bibliography and proofread the manuscript. VMa planned, wrote and revised the whole manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors are grateful to Astrid William, for her helpful discussion and reviewing of the manuscript.

REFERENCES

Akk, G., Shu, H. J., Wang, C., Steinbach, J. H., Zorumski, C. F., Covey, D. F., et al. (2005). Neurosteroid access to the GABAA receptor. *J. Neurosci.* 25, 11605–11613. doi: 10.1523/jneurosci.4173-05.2005

Alonso, G., Phan, V., Guillemain, I., Saunier, M., Legrand, A., Anoal, M., et al. (2000). Immunocytochemical localization of the sigma(1) receptor in the adult rat central nervous system. *Neuroscience* 97, 155–170. doi: 10.1016/s0306-4522(00)00014-2

Ayhan, S., Markal, N., Siemionow, K., Araneo, B., and Siemionow, M. (2003). Effect of subepineurial dehydroepiandrosterone treatment on healing of transected nerves repaired with the epineurial sleeve technique. *Microsurgery* 23, 49–55. doi: 10.1002/micr.10088

Ayoola, C., Hwang, S. M., Hong, S. J., Rose, K. E., Boyd, C., Bozic, N., et al. (2014). Inhibition of CaV3.2 T-type calcium channels in peripheral sensory neurons contributes to analgesic properties of epipregnanolone. *Psychopharmacology* 231, 3503–3515. doi: 10.1007/s00213-014-3588-0

Azcoitia, I., Leonelli, E., Magnaghi, V., Veiga, S., Garcia-Segura, L. M., and Melcangi, R. C. (2003). Progesterone and its derivatives dihydroprogesterone and tetrahydroprogesterone reduce myelin fiber morphological abnormalities and myelin fiber loss in the sciatic nerve of aged rats. *Neurobiol. Aging* 24, 853–860. doi: 10.1016/s0197-4580(02)00234-8

Barabas, K., Godo, S., Lengyel, F., Ernszt, D., Pal, J., and Abraham, I. M. (2018). Rapid non-classical effects of steroids on the membrane receptor dynamics and downstream signaling in neurons. *Horm. Behav.* 104, 183–191. doi: 10.1016/j.yhbeh.2018.05.008

Baulieu, E. E. (1997). Neurosteroids: of the nervous system, by the nervous system, for the nervous system. *Recent Prog. Horm. Res.* 52, 1–32.

Belelli, D., Casula, A., Ling, A., and Lambert, J. J. (2002). The influence of subunit composition on the interaction of neurosteroids with GABA(A)

receptors. *Neuropharmacology* 43, 651–661. doi: 10.1016/s0028-3908(02)00172-7

Belelli, D., and Lambert, J. J. (2005). Neurosteroids: endogenous regulators of the GABA(A) receptor. *Nat. Rev. Neurosci.* 6, 565–575. doi: 10.1038/nrn1703

Bonsack, F., and Sukumari-Ramesh, S. (2018). TSPO: an evolutionarily conserved protein with elusive functions. *Int. J. Mol. Sci.* 19:1694. doi: 10.3390/ijms19061694

Bowery, N. G., and Enna, S. J. (2000). gamma-aminobutyric acid(B) receptors: first of the functional metabotropic heterodimers. *J. Pharmacol. Exp. Ther.* 292, 2–7.

Brann, D. W., Hendry, L. B., and Mahesh, V. B. (1995). Emerging diversities in the mechanism of action of steroid hormones. *J. Steroid Biochem. Mol. Biol.* 52, 113–133. doi: 10.1016/0960-0760(94)00160-n

Brown, D. A., and Marsh, S. (1978). Axonal GABA-receptors in mammalian peripheral nerve trunks. *Brain Res.* 156, 187–191. doi: 10.1016/0006-8993(78)90098-7

Brown, T. J., Khan, T., and Jones, K. J. (1999). Androgen induced acceleration of functional recovery after rat sciatic nerve injury. *Restor. Neurol. Neurosci.* 15, 289–295.

Brussaard, A. B., and Koksma, J. J. (2003). Conditional regulation of neurosteroid sensitivity of GABAA receptors. *Ann. N. Y. Acad. Sci.* 1007, 29–36.

Callachan, H., Cottrell, G. A., Hather, N. Y., Lambert, J. J., Nooney, J. M., and Peters, J. A. (1987). Modulation of the GABAA receptor by progesterone metabolites. *Proc. R. Soc. Lond. B. Biol. Sci.* 231, 359–369. doi: 10.1098/rspb.1987.0049

Campana, W. M., Mantuano, E., Azmoon, P., Henry, K., Banki, M. A., Kim, J. H., et al. (2017). Ionotropic glutamate receptors activate cell signaling in response to glutamate in Schwann cells. *FASEB J.* 31, 1744–1755. doi: 10.1096/fj.201601121r

Carlton, S. M., Chung, K., Ding, Z., and Coggeshall, R. E. (1998). Glutamate receptors on postganglionic sympathetic axons. *Neuroscience* 83, 601–605. doi: 10.1016/s0306-4522(97)00406-5

Caruso, D., Scurati, S., Maschi, O., De Angelis, L., Roglio, I., Giatti, S., et al. (2008). Evaluation of neuroactive steroid levels by liquid chromatography-tandem mass spectrometry in central and peripheral nervous system: effect of diabetes. *Neurochem. Int.* 52, 560–568. doi: 10.1016/j.neuint.2007.06.004

Castelnovo, L. F., Caffino, L., Bonalume, V., Fumagalli, F., Thomas, P., and Magnaghi, V. (2020). Membrane progesterone receptors (mPRs/PAQRs) differently regulate migration, proliferation, and differentiation in rat schwann cells. *J. Mol. Neurosci.* 70, 433–448. doi: 10.1007/s12031-019-01433-6

Castelnovo, L. F., Magnaghi, V., and Thomas, P. (2019). Expression of membrane progesterone receptors (mPRs) in rat peripheral glial cell membranes and their potential role in the modulation of cell migration and protein expression. *Steroids* 142, 6–13. doi: 10.1016/j.steroids.2017.09.009

Cavarretta, I. T. R., Martini, L., Motta, M., Smith, C. L., and Melcangi, R. C. (2004). SRC-1 is involved in the control of the gene expression of myelin protein Po. *J. Mol. Neurosci.* 24, 217–226. doi: 10.1385/jmn:24:2:217

Celotti, F., Melcangi, R. C., and Martini, L. (1992). The 5 alpha-reductase in the brain: molecular aspects and relation to brain function. *Front. Neuroendocrinol.* 13, 163–215.

Cermenati, G., Giatti, S., Cavaletti, G., Bianchi, R., Maschi, O., Pesaresi, M., et al. (2010). Activation of the liver X receptor increases neuroactive steroid levels and protects from diabetes-induced peripheral neuropathy. *J. Neurosci.* 30, 11896–11901. doi: 10.1523/jneurosci.1898-10.2010

Chaban, V. V., and Micevych, P. E. (2005). Estrogen receptor-alpha mediates estradiol attenuation of ATP-induced Ca2+ signaling in mouse dorsal root ganglion neurons. *J. Neurosci. Res.* 81, 31–37. doi: 10.1002/jnr.20524

Chan, J. R., Rodriguez-Waitkus, P. M., Ng, B. K., Liang, P., and Glaser, M. (2000). Progesterone synthesized by Schwann cells during myelin formation regulates neuronal gene expression. *Mol. Biol. Cell* 11, 2283–2295. doi: 10.1091/mbc.11.7.2283

Chavez-Delgado, M. E., Gomez-Pinedo, U., Feria-Velasco, A., Huerta-Viera, M., Castaneda, S. C., Toral, F. A., et al. (2005). Ultrastructural analysis of guided nerve regeneration using progesterone- and pregnenolone-loaded chitosan prostheses. *J. Biomed. Mater. Res. B Appl. Biomater.* 74, 589–600. doi: 10.1002/jbm.b.30243

Chen, J. T., Guo, D., Campanelli, D., Frattini, F., Mayer, F., Zhou, L., et al. (2014). Presynaptic GABAergic inhibition regulated by BDNF contributes to neuropathic pain induction. *Nat. Commun.* 5:5331.

Chen, T. J., Frohlich, N., Kula, B., Barzan, R., and Kukley, M. (2017). Glutamate activates AMPA receptor conductance in the developing schwann cells of the mammalian peripheral nerves. *J. Neurosci.* 37, 11818–11834. doi: 10.1523/jneurosci.1168-17.2017

Chen, Y., Guo, W., Xu, L., Li, W., Cheng, M., Hu, Y., et al. (2016). 17beta-estradiol promotes schwann cell proliferation and differentiation, accelerating early remyelination in a mouse peripheral nerve injury model. *Biomed. Res. Int.* 2016:7891202.

Chisari, M., Eisenman, L. N., Krishnan, K., Bandyopadhyaya, A. K., Wang, C., and Taylor, A. (2009). The influence of neuroactive steroid lipophilicity on GABAA receptor modulation: evidence for a low-affinity interaction. *J. Neurophysiol.* 102, 1254–1264. doi: 10.1152/jn.00346.2009

Cho, T., and Chaban, V. V. (2012). Interaction between P2X3 and oestrogen receptor (ER)alpha/ERbeta in ATP-mediated calcium signalling in mice sensory neurones. *J. Neuroendocrinol.* 24, 789–797. doi: 10.1111/j.1365-2826.2011.02272.x

Christensen, P. C., Welch, N. C., Brideau, C., and Stys, P. K. (2016). Functional ionotropic glutamate receptors on peripheral axons and myelin. *Muscle Nerve* 54, 451–459. doi: 10.1002/mus.25078

Cooke, P. S., Nanjappa, M. K., Yang, Z., and Wang, K. K. (2013). Therapeutic effects of progesterone and its metabolites in traumatic brain injury may involve non-classical signaling mechanisms. *Front. Neurosci.* 7:108. doi: 10.3389/fnins.2013.00108

De Nicola, A. F., Labombarda, F., Gonzalez Deniselle, M. C., Gonzalez, S. L., Garay, L., Meyer, M., et al. (2009). Progesterone neuroprotection in traumatic CNS injury and motoneuron degeneration. *Front. Neuroendocrinol.* 30, 173–187. doi: 10.1016/j.yfrne.2009.03.001

Desarnaud, F., Bidichandani, S., Patel, P. I., Baulieu, E. E., and Schumacher, M. (2000). Glucocorticosteroids stimulate the activity of the promoters of peripheral myelin protein-22 and protein zero genes in schwann cells. *Brain Res.* 865, 12–16. doi: 10.1016/s0006-8993(00)02130-2

Desarnaud, F., Do Thi, A. N., Brown, A. M., Lemke, G., Suter, U., Baulieu, E. E., et al. (1998). Progesterone stimulates the activity of the promoters of peripheral myelin protein-22 and protein zero genes in schwann cells. *J. Neurochem.* 71, 1765–1768. doi: 10.1046/j.1471-4159.1998.71041765.x

Dong, F., Xie, W., Strong, J. A., and Zhang, J. M. (2012). Mineralocorticoid receptor blocker eplerenone reduces pain behaviors in vivo and decreases excitability in small-diameter sensory neurons from local inflamed dorsal root ganglia in vitro. *Anesthesiology* 117, 1102–1112. doi: 10.1097/aln.0b013e3182700383

Du, X., Hao, H., Yang, Y., Huang, S., Wang, C., Gigout, S., et al. (2017). Local GABAergic signaling within sensory ganglia controls peripheral nociceptive transmission. *J. Clin. Invest.* 127, 1741–1756. doi: 10.1172/jci86812

Dun, S. L., Brailoiu, G. C., Gao, X., Brailoiu, E., Arterburn, J. B., Prossnitz, E. R., et al. (2009). Expression of estrogen receptor GPR30 in the rat spinal cord and in autonomic and sensory ganglia. *J. Neurosci. Res.* 87, 1610–1619. doi: 10.1002/jnr.21980

Evans, P. D., Reale, V., Merzon, R. M., and Villegas, J. (1991). N-methyl-D-aspartate (n.d.) and non-NMDA type glutamate receptors are present on squid giant axon Schwann cells. *J. Exp. Biol.* 157, 593–600.

Evans, P. D., Reale, V., Merzon, R. M., and Villegas, J. (1992). N-methyl-D-aspartate (n.d.) and non-NMDA (metabotropic) type glutamate receptors modulate the membrane potential of the Schwann cell of the squid giant nerve fibre. *J. Exp. Biol.* 173, 229–249.

Faroni, A., Castelnovo, L. F., Procacci, P., Caffino, L., Fumagalli, F., Melfi, S., et al. (2014). Deletion of GABA-B receptor in Schwann cells regulates remak bundles and small nociceptive C-fibers. *Glia* 62, 548–565. doi: 10.1002/glia.22625

Faroni, A., and Magnaghi, V. (2011). The neurosteroid allopregnanolone modulates specific functions in central and peripheral glial cells. *Front. Endocrinol.* 2:103. doi: 10.3389/fnins.2013.00103

Faroni, A., Melfi, S., Castelnovo, L. F., Bonalume, V., Colleoni, D., Magni, P., et al. (2019). GABA-B1 receptor-null schwann cells exhibit compromised in vitro myelination. *Mol. Neurobiol.* 56, 1461–1474. doi: 10.1007/s12035-018-1158-x

Fex Svenningsen, A., and Kanje, M. (1999). Estrogen and progesterone stimulate Schwann cell proliferation in a sex- and age-dependent manner. *J. Neurosci. Res.* 57, 124–130. doi: 10.1002/(sici)1097-4547(19990701)57:1<124::aid-jnr13>3.0.co;2-p

Fontenas, L., and Kucenas, S. (2018). Motor exit point (MEP) glia: novel myelinating glia that bridge CNS and PNS myelin. *Front. Cell Neurosci.* 12:333. doi: 10.3389/fnins.2013.00333

Frye, C. A., Van Keuren, K. R., and Erskine, M. S. (1996). Behavioral effects of 3 alpha-androstanediol. I: modulation of sexual receptivity and promotion of GABA-stimulated chloride flux. *Behav. Brain Res.* 79, 109–118. doi: 10.1016/0166-4328(96)00004-6

Gennari, C. G., Cilurzo, F., Mitro, N., Caruso, D., Minghetti, P., and Magnaghi, V. (2018). In vitro and in vivo evaluation of silk fibroin functionalized with GABA and allopregnanolone for Schwann cell and neuron survival. *Regen. Med.* 13, 141–157. doi: 10.2217/rme-2017-0102

Giatti, S., Garcia-Segura, L. M., and Melcangi, R. C. (2015). New steps forward in the neuroactive steroid field. *J. Steroid Biochem. Mol. Biol.* 153, 127–134. doi: 10.1016/j.jsbmb.2015.03.002

Giatti, S., Pesaresi, M., Cavaletti, G., Bianchi, R., Carozzi, V., Lombardi, R., et al. (2009). Neuroprotective effects of a ligand of translocator protein-18 kDa (Ro5-4864) in experimental diabetic neuropathy. *Neuroscience* 164, 520–529. doi: 10.1016/j.neuroscience.2009.08.005

Grossfeld, R. M., Klinge, M. A., Lieberman, E. M., and Stewart, L. C. (1988). Axon-glia transfer of a protein and a carbohydrate. *Glia* 1, 292–300. doi: 10.1002/glia.440010409

Groyer, G., Eychenne, B., Girard, C., Rajkowski, K., Schumacher, M., and Cadepond, F. (2006). Expression and functional state of the corticosteroid receptors and 11 beta-hydroxysteroid dehydrogenase type 2 in schwann cells. *Endocrinology* 147, 4339–4350. doi: 10.1210/en.2005-1625

Gu, Y., Wu, Y., Su, W., Xing, L., Shen, Y., He, X., et al. (2018). 17beta-estradiol enhances schwann cell differentiation via the ERbeta-ERK1/2 signaling pathway and promotes remyelination in injured sciatic nerves. *Front. Pharmacol.* 9:1026. doi: 10.3389/fnins.2013.001026

Gudemez, E., Ozer, K., Cunningham, B., Siemionow, K., Browne, E., and Siemionow, M. (2002). Dehydroepiandrosterone as an enhancer of functional recovery following crush injury to rat sciatic nerve. *Microsurgery* 22, 234–241. doi: 10.1002/micr.10039

Guennoun, R., Benmessahel, Y., Delespierre, B., Gouezou, M., Rajkowski, K. M., Baulieu, E. E., et al. (2001). Progesterone stimulates Krox-20 gene expression in schwann cells. *Brain Res. Mol. Brain Res.* 90, 75–82. doi: 10.1016/s0169-328x(01)00094-8

Handa, R. J., Pak, T. R., Kudwa, A. E., Lund, T. D., and Hinds, L. (2008). An alternate pathway for androgen regulation of brain function: activation of estrogen receptor beta by the metabolite of dihydrotestosterone, 5alpha-androstane-3beta,17beta-diol. *Horm. Behav.* 53, 741–752. doi: 10.1016/j.yhbeh.2007.09.012

Hayashi, T., Maurice, T., and Su, T. P. (2000). Ca(2+) signaling via sigma(1)-receptors: novel regulatory mechanism affecting intracellular Ca(2+) concentration. *J. Pharmacol. Exp. Ther.* 293, 788–798.

Hodge, C. W., Raber, J., Mcmahon, T., Walter, H., Sanchez-Perez, A. M., Olive, M. F., et al. (2002). Decreased anxiety-like behavior, reduced stress hormones, and neurosteroid supersensitivity in mice lacking protein kinase cepsilon. *J. Clin. Invest.* 110, 1003–1010. doi: 10.1172/jci200215903

Hosie, A. M., Clarke, L., Da Silva, H., and Smart, T. G. (2009). Conserved site for neurosteroid modulation of GABA A receptors. *Neuropharmacology* 56, 149–154. doi: 10.1016/j.neuropharm.2008.07.050

Hosie, A. M., Wilkins, M. E., Da Silva, H. M., and Smart, T. G. (2006). Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. *Nature* 444, 486–489. doi: 10.1038/nature05324

Islamov, R. R., Hendricks, W. A., Jones, R. J., Lyall, G. J., Spanier, N. S., and Murashov, A. K. (2002). 17Beta-estradiol stimulates regeneration of sciatic nerve in female mice. *Brain Res.* 943, 283–286. doi: 10.1016/s0006-8993(02)02827-5

Jakobsson, T., Treuter, E., Gustafsson, J. A., and Steffensen, K. R. (2012). Liver X receptor biology and pharmacology: new pathways, challenges and opportunities. *Trends Pharmacol. Sci.* 33, 394–404. doi: 10.1016/j.tips.2012.03.013

Jordan, C. L., Price, R. H. Jr., and Handa, R. J. (2002). Androgen receptor messenger RNA and protein in adult rat sciatic nerve: implications for site of androgen action. *J. Neurosci. Res.* 69, 509–518. doi: 10.1002/jnr.10324

Jung-Testas, I., Schumacher, M., Bugnard, H., and Baulieu, E. E. (1993). Stimulation of rat schwann cell proliferation by estradiol: synergism between the estrogen and cAMP. *Brain Res. Dev. Brain Res.* 72, 282–290. doi: 10.1016/0165-3806(93)90194-f

Jung-Testas, I., Schumacher, M., Robel, P., and Baulieu, E. E. (1996). Demonstration of progesterone receptors in rat schwann cells. *J. Steroid Biochem. Mol. Biol.* 58, 77–82. doi: 10.1016/0960-0760(96)00009-x

Koenig, H. L., Schumacher, M., Ferzaz, B., Thi, A. N., Ressouches, A., Guennoun, R., et al. (1995). Progesterone synthesis and myelin formation by schwann cells. *Science* 268, 1500–1503. doi: 10.1126/science.7770777

Kujawa, K. A., Jacob, J. M., and Jones, K. J. (1993). Testosterone regulation of the regenerative properties of injured rat sciatic motor neurons. *J. Neurosci. Res.* 35, 268–273. doi: 10.1002/jnr.490350306

Lambert, J. J., Belelli, D., Hill-Venning, C., Callachan, H., and Peters, J. A. (1996). Neurosteroid modulation of native and recombinant GABAA receptors. *Cell Mol. Neurobiol.* 16, 155–174. doi: 10.1007/bf02088174

Lambert, J. J., Belelli, D., Peden, D. R., Vardy, A. W., and Peters, J. A. (2003). Neurosteroid modulation of GABAA receptors. *Prog. Neurobiol.* 71, 67–80.

Lambert, J. J., Cooper, M. A., Simmons, R. D., Weir, C. J., and Belelli, D. (2009). Neurosteroids: endogenous allosteric modulators of GABA(A) receptors. *Psychoneuroendocrinology* 34(Suppl. 1), S48–S58.

Leonelli, E., Bianchi, R., Cavaletti, G., Caruso, D., Crippa, D., Garcia-Segura, L. M., et al. (2007). Progesterone and its derivatives are neuroprotective agents in experimental diabetic neuropathy: a multimodal analysis. *Neuroscience* 144, 1293–1304. doi: 10.1016/j.neuroscience.2006.11.014

Lerch, J. K., Alexander, J. K., Madalena, K. M., Motti, D., Quach, T., Dhamija, A., et al. (2017). Stress increases peripheral axon growth and regeneration through glucocorticoid receptor-dependent transcriptional programs. *eNeuro* 4:ENEURO.0246-17.2017.

Levin, E. R. (2011). Minireview: extranuclear steroid receptors: roles in modulation of cell functions. *Mol. Endocrinol.* 25, 377–384. doi: 10.1210/me.2010-0284

Li, H., and Papadopoulos, V. (1998). Peripheral-type benzodiazepine receptor function in cholesterol transport. Identification of a putative cholesterol recognition/interaction amino acid sequence and consensus pattern. *Endocrinology* 139, 4991–4997. doi: 10.1210/endo.139.12.6390

Lopez-Verrilli, M. A., and Court, F. A. (2012). Transfer of vesicles from schwann cells to axons: a novel mechanism of communication in the peripheral nervous system. *Front. Physiol.* 3:205. doi: 10.3389/fnins.2013.00205

Luo, H., Liu, J., Kang, D., and Cui, S. (2008). Ontogeny of estrogen receptor alpha, estrogen receptor beta and androgen receptor, and their co-localization with Islet-1 in the dorsal root ganglia of sheep fetuses during gestation. *Histochem. Cell Biol.* 129, 525–533. doi: 10.1007/s00418-008-0380-4

Magnaghi, V. (2007). GABA and neuroactive steroid interactions in glia: new roles for old players? *Curr. Neuropharmacol.* 5, 47–64. doi: 10.2174/157015907780077132

Magnaghi, V., Ballabio, M., Cavarretta, I. T., Froestl, W., Lambert, J. J., Zucchi, I., et al. (2004). GABAB receptors in schwann cells influence proliferation and myelin protein expression. *Eur. J. Neurosci.* 19, 2641–2649. doi: 10.1111/j.0953-816x.2004.03368.x

Magnaghi, V., Ballabio, M., Consoli, A., Lambert, J. J., Roglio, I., and Melcangi, R. C. (2006a). GABA receptor-mediated effects in the peripheral nervous system: a cross-interaction with neuroactive steroids. *J. Mol. Neurosci.* 28, 89–102. doi: 10.1385/jmn:28:1:89

Magnaghi, V., Veiga, S., Ballabio, M., Gonzalez, L. C., Garcia-Segura, L. M., and Melcangi, R. C. (2006b). Sex-dimorphic effects of progesterone and its reduced metabolites on gene expression of myelin proteins by rat schwann cells. *J. Peripher. Nerv. Syst.* 11, 111–118. doi: 10.1111/j.1085-9489.2006.00075.x

Magnaghi, V., Ballabio, M., Roglio, I., and Melcangi, R. C. (2007). Progesterone derivatives increase expression of Krox-20 and Sox-10 in rat Schwann cells. *J. Mol. Neurosci.* 31, 149–157.

Magnaghi, V., Castelnovo, L. F., Faroni, A., Cavalli, E., Caffino, L., Colciago, A., et al. (2014). Nerve regenerative effects of GABA-B ligands in a model of neuropathic pain. *Biomed. Res. Int.* 2014:368678.

Magnaghi, V., Cavarretta, I., Galbiati, M., Martini, L., and Melcangi, R. C. (2001). Neuroactive steroids and peripheral myelin proteins. *Brain Res. Brain Res. Rev.* 37, 360–371. doi: 10.1016/s0165-0173(01)00140-0

Magnaghi, V., Cavarretta, I., Zucchi, I., Susani, L., Rupprecht, R., Hermann, B., et al. (1999). Po gene expression is modulated by androgens in the sciatic nerve of adult male rats. *Brain Res. Mol. Brain Res.* 70, 36–44. doi: 10.1016/s0169-328x(99)00124-2

Magnaghi, V., Parducz, A., Frasca, A., Ballabio, M., Procacci, P., Racagni, G., et al. (2010). GABA synthesis in schwann cells is induced by the neuroactive steroid allopregnanolone. *J. Neurochem.* 112, 980–990. doi: 10.1111/j.1471-4159.2009.06512.x

Malet, M., and Brumovsky, P. R. (2015). VGLUTs and glutamate synthesis-focus on DRG neurons and pain. *Biomolecules* 5, 3416–3437. doi: 10.3390/biom5043416

Mantelas, A., Stamatakis, A., Kazanis, I., Philippidis, H., and Stylianopoulou, F. (2003). Control of neuronal nitric oxide synthase and brain-derived neurotrophic factor levels by GABA-A receptors in the developing rat cortex. *Brain Res. Dev. Brain Res.* 145, 185–195. doi: 10.1016/j.devbrainres.2003.08.001

Maurice, T., Phan, V. L., Urani, A., Kamei, H., Noda, Y., and Nabeshima, T. (1999). Neuroactive neurosteroids as endogenous effectors for the sigma1 (sigma1) receptor: pharmacological evidence and therapeutic opportunities. *Jpn. J. Pharmacol.* 81, 125–155. doi: 10.1254/jjp.81.125

Meffre, D., Labombarda, F., Delespierre, B., Chastre, A., De Nicola, A. F., Stein, D. G., et al. (2013). Distribution of membrane progesterone receptor alpha in the male mouse and rat brain and its regulation after traumatic brain injury. *Neuroscience* 231, 111–124. doi: 10.1016/j.neuroscience.2012.11.039

Melcangi, R. C., Celotti, F., Ballabio, M., Poletti, A., and Martini, L. (1990). Testosterone metabolism in peripheral nerves: presence of the 5 alpha-reductase-3 alpha-hydroxysteroid-dehydrogenase enzymatic system in the sciatic nerve of adult and aged rats. *J. Steroid Biochem.* 35, 145–148. doi: 10.1016/0022-4731(90)90159-p

Melcangi, R. C., Celotti, F., Castano, P., and Martini, L. (1992). Is the 5 alpha-reductase-3 alpha-hydroxysteroid dehydrogenase complex associated with the myelin in the peripheral nervous system of young and old male rats? *Endocr. Regul.* 26, 119–125.

Melcangi, R. C., Giatti, S., and Garcia-Segura, L. M. (2016). Levels and actions of neuroactive steroids in the nervous system under physiological and pathological conditions: sex-specific features. *Neurosci. Biobehav. Rev.* 67, 25–40. doi: 10.1016/j.neubiorev.2015.09.023

Melcangi, R. C., Leonelli, E., Magnaghi, V., Gherardi, G., Nobbio, L., and Schenone, A. (2003). Mifepristone (RU 38486) influences expression of glycoprotein Po and morphological parameters at the level of rat sciatic nerve: in vivo observations. *Exp. Neurol.* 184, 930–938. doi: 10.1016/s0014-4886(03)00338-8

Melcangi, R. C., Magnaghi, V., Cavarretta, I., Martini, L., and Piva, F. (1998). Age-induced decrease of glycoprotein Po and myelin basic protein gene expression in the rat sciatic nerve. Repair by steroid derivatives. *Neuroscience* 85, 569–578. doi: 10.1016/s0306-4522(97)00628-3

Melcangi, R. C., Magnaghi, V., Cavarretta, I., Zucchi, I., Bovolin, P., D'urso, D., et al. (1999a). Progesterone derivatives are able to influence peripheral myelin protein 22 and P0 gene expression: possible mechanisms of action. *J. Neurosci. Res.* 56, 349–357. doi: 10.1002/(sici)1097-4547(19990515)56:4<349::aid-jnr3>3.0.co;2-h

Melcangi, R. C., Magnaghi, V., and Martini, L. (1999b). Steroid metabolism and effects in central and peripheral glial cells. *J. Neurobiol.* 40, 471–483. doi: 10.1002/(sici)1097-4695(19990915)40:4<471::aid-neu5>3.0.co;2-p

Melcangi, R. C., Magnaghi, V., Galbiati, M., Ghelarducci, B., Sebastiani, L., and Martini, L. (2000a). The action of steroid hormones on peripheral myelin proteins: a possible new tool for the rebuilding of myelin? *J. Neurocytol.* 29, 327–339.

Melcangi, R. C., Magnaghi, V., and Martini, L. (2000b). Aging in peripheral nerves: regulation of myelin protein genes by steroid hormones. *Prog. Neurobiol.* 60, 291–308. doi: 10.1016/s0301-0082(99)00028-3

Melcangi, R. C., Magnaghi, V., Galbiati, M., and Martini, L. (2001). Formation and effects of neuroactive steroids in the central and peripheral nervous system. *Int. Rev. Neurobiol.* 46, 145–176. doi: 10.1016/s0074-7742(01)46062-4

Melfi, S., Montt Guevara, M. M., Bonalume, V., Ruscica, M., Colciago, A., Simoncini, T., et al. (2017). Src and phospho-FAK kinases are activated by allopregnanolone promoting Schwann cell motility, morphology and myelination. *J. Neurochem.* 141, 165–178. doi: 10.1111/jnc.13951

Mercier, G., Turque, N., and Schumacher, M. (2001). Early activation of transcription factor expression in schwann cells by progesterone. *Brain Res. Mol. Brain Res.* 97, 137–148. doi: 10.1016/s0169-328x(01)00311-4

Mihalek, R. M., Banerjee, P. K., Korpi, E. R., Quinlan, J. J., Firestone, L. L., Mi, Z. P., et al. (1999). Attenuated sensitivity to neuroactive steroids in gamma-aminobutyrate type A receptor delta subunit knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* 96, 12905–12910. doi: 10.1073/pnas.96.22.12905

Miller, K. E., Richards, B. A., and Kriebel, R. M. (2002). Glutamine-, glutamine synthetase-, glutamate dehydrogenase- and pyruvate carboxylase-immunoreactivities in the rat dorsal root ganglion and peripheral nerve. *Brain Res.* 945, 202–211. doi: 10.1016/s0006-8993(02)02802-0

Mitro, N., Cermenati, G., Brioschi, E., Abbiati, F., Audano, M., Giatti, S., et al. (2014). Neuroactive steroid treatment modulates myelin lipid profile in diabetic peripheral neuropathy. *J. Steroid Biochem. Mol. Biol.* 143, 115–121. doi: 10.1016/j.jsbmb.2014.02.015

Monnet, F. P., and Maurice, T. (2006). The sigma1 protein as a target for the non-genomic effects of neuro(active)steroids: molecular, physiological, and behavioral aspects. *J. Pharmacol. Sci.* 100, 93–118. doi: 10.1254/jphs.cr0050032

Morris, M. E., Di Costanzo, G. A., Fox, S., and Werman, R. (1983). Depolarizing action of GABA (gamma-aminobutyric acid) on myelinated fibers of peripheral nerves. *Brain Res.* 278, 117–126. doi: 10.1016/0006-8993(83)90230-5

Moss, S. J., and Smart, T. G. (1996). Modulation of amino acid-gated ion channels by protein phosphorylation. *Int. Rev. Neurobiol.* 39, 1–52. doi: 10.1016/s0074-7742(08)60662-5

Neuberger, T. J., Kalimi, O., Regelson, W., Kalimi, M., and De Vries, G. H. (1994). Glucocorticoids enhance the potency of schwann cell mitogens. *J. Neurosci. Res.* 38, 300–313. doi: 10.1002/jnr.490380308

Olsen, R. W., Snowhill, E. W., and Wamsley, J. K. (1984). Autoradiographic localization of low affinity GABA receptors with [3H]bicuculline methochloride. *Eur. J. Pharmacol.* 99, 247–248. doi: 10.1016/0014-2999(84)90249-8

Pabba, M., and Sibille, E. (2015). Sigma-1 and N-Methyl-d-Aspartate receptors: a partnership with beneficial outcomes. *Mol. Neuropsychiat.* 1, 47–51. doi: 10.1159/000376549

Park-Chung, M., Malayev, A., Purdy, R. H., Gibbs, T. T., and Farb, D. H. (1999). Sulfated and unsulfated steroids modulate gamma-aminobutyric acidA receptor function through distinct sites. *Brain Res.* 830, 72–87. doi: 10.1016/s0006-8993(99)01381-5

Patte-Mensah, C., Meyer, L., Taleb, O., and Mensah-Nyagan, A. G. (2014). Potential role of allopregnanolone for a safe and effective therapy of neuropathic pain. *Prog. Neurobiol.* 113, 70–78. doi: 10.1016/j.pneurobio.2013.07.004

Peluso, J. J., Romak, J., and Liu, X. (2008). Progesterone receptor membrane component-1 (PGRMC1) is the mediator of progesterone's antiapoptotic action in spontaneously immortalized granulosa cells as revealed by PGRMC1 small interfering ribonucleic acid treatment and functional analysis of PGRMC1 mutations. *Endocrinology* 149, 534–543. doi: 10.1210/en.2007-1050

Perego, C., Cairano, E. S., Ballabio, M., and Magnaghi, V. (2011). Neurosteroid allopregnanolone regulates EAAC1-mediated glutamate uptake and triggers actin changes in schwann cells. *J. Cell Physiol.* 227, 1740–1751.

Perego, C., Di Cairano, E. S., Ballabio, M., and Magnaghi, V. (2012). Neurosteroid allopregnanolone regulates EAAC1-mediated glutamate uptake and triggers actin changes in schwann cells. *J. Cell. Physiol.* 227, 1740–1751. doi: 10.1002/jcp.22898

Pesaresi, M., Giatti, S., Cavaletti, G., Abbiati, F., Calabrese, D., Bianchi, R., et al. (2011a). Sex differences in the manifestation of peripheral diabetic neuropathy in gonadectomized rats: a correlation with the levels of neuroactive steroids in the sciatic nerve. *Exp. Neurol.* 228, 215–221. doi: 10.1016/j.expneurol.2011.01.005

Pesaresi, M., Giatti, S., Cavaletti, G., Abbiati, F., Calabrese, D., Lombardi, R., et al. (2011b). Sex-dimorphic effects of dehydroepiandrosterone in diabetic neuropathy. *Neuroscience* 199, 401–409. doi: 10.1016/j.neuroscience.2011.09.013

Pesaresi, M., Maschi, O., Giatti, S., Garcia-Segura, L. M., Caruso, D., and Melcangi, R. C. (2010). Sex differences in neuroactive steroid levels in the nervous system of diabetic and non-diabetic rats. *Horm. Behav.* 57, 46–55. doi: 10.1016/j.yhbeh.2009.04.008

Prough, R. A., Clark, B. J., and Klinge, C. M. (2016). Novel mechanisms for DHEA action. *J. Mol. Endocrinol.* 56, R139–R155.

Puia, G., Ravazzini, F., Castelnovo, L. F., and Magnaghi, V. (2015). PKCepsilon and allopregnanolone: functional cross-talk at the GABAA receptor level. *Front. Cell. Neurosci.* 9:83. doi: 10.3389/fnins.2013.0083

Reddy, D. S., and Rogawski, M. A. (2002). Stress-induced deoxycorticosterone-derived neurosteroids modulate GABA(A) receptor function and seizure

susceptibility. *J. Neurosci.* 22, 3795–3805. doi: 10.1523/jneurosci.22-09-03795.2002

Robert, F., Guennoun, R., Desarnaud, F., Do-Thi, A., Benmessahel, Y., Baulieu, E. E., et al. (2001). Synthesis of progesterone in schwann cells: regulation by sensory neurons. *Eur. J. Neurosci.* 13, 916–924. doi: 10.1046/j.0953-816x.2001.01463.x

Roglio, I., Bianchi, R., Giatti, S., Cavaletti, G., Caruso, D., Scurati, S., et al. (2007). Testosterone derivatives are neuroprotective agents in experimental diabetic neuropathy. *Cell. Mol. Life Sci.* 64, 1158–1168. doi: 10.1007/s00018-007-7002-5

Roglio, I., Bianchi, R., Gotti, S., Scurati, S., Giatti, S., Pesaresi, M., et al. (2008). Neuroprotective effects of dihydroprogesterone and progesterone in an experimental model of nerve crush injury. *Neuroscience* 155, 673–685. doi: 10.1016/j.neuroscience.2008.06.034

Rupprecht, R., Di Michele, F., Hermann, B., Strohle, A., Lancel, M., Romeo, E., et al. (2001). Neuroactive steroids: molecular mechanisms of action and implications for neuropsychopharmacology. *Brain Res. Brain Res. Rev.* 37, 59–67. doi: 10.1016/s0165-0173(01)00123-0

Schaeffer, V., Meyer, L., Patte-Mensah, C., and Mensah-Nyagan, A. G. (2010). Progress in dorsal root ganglion neurosteroidogenic activity: basic evidence and pathophysiological correlation. *Prog. Neurobiol.* 92, 33–41. doi: 10.1016/j.pneurobio.2010.04.009

Schumacher, M., Guennoun, R., Mercier, G., Desarnaud, F., Lacor, P., Benavides, J., et al. (2001). Progesterone synthesis and myelin formation in peripheral nerves. *Brain Res. Brain Res. Rev.* 37, 343–359. doi: 10.1016/s0165-0173(01)00139-4

Sedlacek, M., Korinek, M., Petrovic, M., Cais, O., Adamusova, E., Chodounska, H., et al. (2008). Neurosteroid modulation of ionotropic glutamate receptors and excitatory synaptic transmission. *Physiol. Res.* 57(Suppl. 3), S49–S57.

Shaqura, M., Li, X., Al-Khrasani, M., Shakibaei, M., Tafelski, S., Furst, S., et al. (2016). Membrane-bound glucocorticoid receptors on distinct nociceptive neurons as potential targets for pain control through rapid non-genomic effects. *Neuropharmacology* 111, 1–13. doi: 10.1016/j.neuropharm.2016.08.019

Shu, H. J., Eisenman, L. N., Jinadasa, D., Covey, D. F., Zorumski, C. F., and Mennerick, S. (2004). Slow actions of neuroactive steroids at GABAA receptors. *J. Neurosci.* 24, 6667–6675. doi: 10.1523/jneurosci.1399-04.2004

Siriphorn, A., Chompoopong, S., and Floyd, C. L. (2010). 17beta-estradiol protects Schwann cells against H2O2-induced cytotoxicity and increases transplanted Schwann cell survival in a cervical hemicontusion spinal cord injury model. *J. Neurochem.* 115, 864–872. doi: 10.1111/j.1471-4159.2010.06770.x

Slater, E. P., Hesse, H., and Beato, M. (1994). Regulation of transcription by steroid hormones. *Ann. N. Y. Acad. Sci.* 733, 103–112.

Thomas, P., and Pang, Y. (2012). Membrane progesterone receptors: evidence for neuroprotective, neurosteroid signaling and neuroendocrine functions in neuronal cells. *Neuroendocrinology* 96, 162–171. doi: 10.1159/000339822

Thomas, P., Pang, Y., Filardo, E. J., and Dong, J. (2005). Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* 146, 624–632. doi: 10.1210/en.2004-1064

Veiga, S., Leonelli, E., Beelke, M., Garcia-Segura, L. M., and Melcangi, R. C. (2006). Neuroactive steroids prevent peripheral myelin alterations induced by diabetes. *Neurosci. Lett.* 402, 150–153. doi: 10.1016/j.neulet.2006.03.058

Vergnano, A. M., Schlichter, R., and Poisbeau, P. (2007). PKC activation sets an upper limit to the functional plasticity of GABAergic transmission induced by endogenous neurosteroids. *Eur. J. Neurosci.* 26, 1173–1182. doi: 10.1111/j.1460-9568.2007.05746.x

Wang, M., He, Y., Eisenman, L. N., Fields, C., Zeng, C. M., Mathews, J., et al. (2002). 3beta -hydroxypregnane steroids are pregnenolone sulfate-like GABA(A) receptor antagonists. *J. Neurosci.* 22, 3366–3375. doi: 10.1523/jneurosci.22-09-03366.2002

Whiting, P. J., Mcallister, G., Vassilatis, D., Bonnert, T. P., Heavens, R. P., Smith, D. W., et al. (1997). Neuronally restricted RNA splicing regulates the expression of a novel GABAA receptor subunit conferring atypical functional properties [corrected; erratum to be published]. *J. Neurosci.* 17, 5027–5037. doi: 10.1523/jneurosci.17-13-05027.1997

Whiting, P. J., Mckernan, R. M., and Wafford, K. A. (1995). Structure and pharmacology of vertebrate GABAA receptor subtypes. *Int. Rev. Neurobiol.* 38, 95–138. doi: 10.1016/s0074-7742(08)60525-5

Wu, F. S., Gibbs, T. T., and Farb, D. H. (1991). Pregnenolone sulfate: a positive allosteric modulator at the N-methyl-D-aspartate receptor. *Mol. Pharmacol.* 40, 333–336.

Zhang, X. L., Lee, K. Y., Priest, B. T., Belfer, I., and Gold, M. S. (2015). Inflammatory mediator-induced modulation of GABAA currents in human sensory neurons. *Neuroscience* 310, 401–409. doi: 10.1016/j.neuroscience.2015.09.048

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.

frontiers in Endocrinology

MINI REVIEW
published: 11 August 2020
doi: 10.3389/fendo.2020.00549

Pineal Neurosteroids: Biosynthesis and Physiological Functions

Shogo Haraguchi[1]* *and Kazuyoshi Tsutsui*[2]

[1] *Department of Biochemistry, Showa University School of Medicine, Tokyo, Japan,* [2] *Graduate School of Integrated Sciences for Life, Hiroshima University, Hiroshima, Japan*

Similar to the adrenal glands, gonads, and placenta, vertebrate brains also produce various steroids, which are known as "neurosteroids." Neurosteroids are mainly synthesized in the hippocampus, hypothalamus, and cerebellum; however, it has recently been discovered that in birds, the pineal gland, a photosensitive region in the brain, produces more neurosteroids than other brain regions. A series of experiments using molecular and biochemical techniques have found that the pineal gland produces various neurosteroids, including sex steroids, *de novo* from cholesterol. For instance, allopregnanolone and 7α-hydroxypregnenolone are actively produced in the pineal gland, unlike in other brain regions. Pineal 7α-hydroxypregnenolone, an up-regulator of locomotion, enhances locomotor activity in response to light stimuli in birds. Additionally, pineal allopregnanolone acts on Purkinje cells in the cerebellum and prevents neuronal apoptosis within the developing cerebellum in juvenile birds. Furthermore, exposure to light during nighttime hours can cause loss of diurnal variations of pineal allopregnanolone synthesis during early posthatch life, eventually leading to cerebellar Purkinje cell death in juvenile birds. In light of these new findings, this review summarizes the biosynthesis and physiological functions of pineal neurosteroids. Given that the circadian rhythms of individuals in modern societies are constantly interrupted by artificial light exposure, these findings in birds, which are excellent model diurnal animals, may have direct implications for addressing problems regarding the mental health and brain development of humans.

Keywords: allopregnanolone, 7α-hydroxypregnenolone, neurosteroid, pineal gland, cerebellum, light

OPEN ACCESS

Edited by:
Vance L. Trudeau, University of Ottawa, Canada

Reviewed by:
Vincent M. Cassone, University of Kentucky, United States
Maria Claudia Gonzalez Deniselle, CONICET Instituto de Biología y Medicina Experimental (IBYME), Argentina

***Correspondence:**
Shogo Haraguchi
shogo.haraguchi@gmail.com

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

Received: *30 April 2020*
Accepted: *06 July 2020*
Published: *11 August 2020*

Citation:
Haraguchi S and Tsutsui K (2020) Pineal Neurosteroids: Biosynthesis and Physiological Functions. Front. Endocrinol. 11:549. doi: 10.3389/fendo.2020.00549

INTRODUCTION

Similar to the gonads and placenta, vertebrate brains actively also produce various steroid hormones. These steroid hormones produced in the brain are named "neurosteroids." The production of neurosteroids was demonstrated firstly in mammals, and then in other vertebrates (1–5). Thus, neurosteroid production appears to be a universal feature of the brain in vertebrates.

It is known that neurosteroids are produced in glial cells and neurons of the central and peripheral nervous systems (1, 5). However, we have demonstrated that the pineal gland produces neurosteroids from cholesterol in birds during early posthatch period (6–8). Notably, allopregnanolone (also known as 3α,5α-tetrahydroprogesterone; 3α,5α-THP) and 7α-hydroxypregnenolone are the two major neurosteroids produced in the pineal gland (6, 7). Of these two, pineal allopregnanolone prevents the death of developing Purkinje cells (7, 8), and pineal 7α-hydroxypregnenolone functions as an up-regulator of locomotion, regulating locomotor activity in response to light stimuli in birds (6).

BIOSYNTHESIS OF PINEAL NEUROSTEROIDS

The pineal glands of vertebrates respond to light stimuli and fulfill important functions in the organization of circadian rhythms. The secretion of melatonin, a major hormone produced by the pineal gland, shows a clear daily rhythm with its peak concentration occurring at night (7, 9). However, it was not known whether the pineal gland produces neurosteroids until recently. We have recently demonstrated that the pineal gland is a newly found neurosteroidogenic organ producing a variety of neurosteroids from cholesterol (6, 7) (**Figure 1**).

Pregnenolone is an anabolic intermediate of most endogenous steroid hormones and is produced from cholesterol through the mitochondrial cholesterol side chain cleavage enzyme cytochrome P450scc (P450scc; encoded by the *Cyp11a* gene). We have demonstrated by transcription-polymerase chain reaction (RT-PCR) that the pineal gland in juvenile birds expresses *P450scc* mRNA (6, 7) (**Figure 1**). The protein product of this mRNA is localized in the cells that form the follicular structures in the pineal glands of birds (7). We have demonstrated by high-performance liquid chromatography (HPLC) with radioactive flow detector analysis that ^{3}H-cholesterol is converted to radioactive pregnenolone when incubated with pineal gland extract from juvenile birds (6, 7). This observation has confirmed the presence of functional P450scc in the pineal gland (**Figure 1**), which has also been detected by gas chromatography-mass spectrometry (GC/MS) (7). Subsequent RT-PCR–based assessment has revealed that key steroidogenic enzymes, cytochrome P450 7α-hydroxylase (P4507α; encoded by the *Cyp7b* gene), 3α-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3α-HSD; encoded by the *Hsd3a* gene), 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD; encoded by the *Hsd3b* gene), 5α-reductase (encoded by the *Srd5a* gene), 5β-reductase (encoded by the *Srd5b* gene), cytochrome P450 17α-hydroxylase/c17,20-lyase (P45017α,lyase; encoded by the *Cyp17* gene), 17β-hydroxysteroid dehydrogenase (17β-HSD; encoded by the *Hsd17b* gene), and cytochrome P450 aromatase (P450arom; encoded by the *Cyp19* gene), are expressed in the pineal gland of birds (6, 7) (**Figure 1**).

We further demonstrated that steroid hormones are indeed present in the pineal gland. Incubation of ^{3}H-pregnenolone with pineal glands from posthatch birds generates 7α- and/or 7β-hydroxypregnenolone by the action of P4507α found in the pineal glands (7) (**Figure 1**). In addition to these neurosteroid isomers, progesterone, allopregnanolone (3α, 5α-THP) and/or epipregnanolone (3β, 5β-THP), androstenedione, testosterone, 5α- and/or 5β-dihydrotestosterone, and estradiol-17β are also produced (7) (**Figure 1**). These *ex vivo* observations have confirmed that the pineal glands in juvenile birds have the biosynthetic machinery for major steroid hormones, which have also been verified to be produced as neurosteroids *in vivo* (7) (**Figure 1**). Although HPLC analysis has failed to resolve the isomers of these hormones, such as 7α-/β-hydroxypregnenolone, allo/epipregnanolone, and 5α-/β-dihydrotestosterone, several sets of isomers have been successfully isolated by GC/MS analysis (7). Especially, 7α-hydroxypregnenolone and allopregnanolone are actively released (6, 7).

Taken together, these findings indicate that the pineal gland in juvenile birds produces various neurosteroids from cholesterol. Accordingly, this is the first demonstration of neurosteroid synthesis in the pineal gland in a vertebrate.

PHYSIOLOGICAL FUNCTION OF PINEAL 7α-HYDROXYPREGNENOLONE IN LIGHT-DEPENDENT LOCOMOTION

The chick pineal gland is used as a model for studies on the light-dependent phase-shifting mechanism of the circadian clock (10). To search for genes involved in this mechanism, a differential GeneChip analysis has been performed. This transcriptomics analysis has identified the light-induced transcriptional activation of the full set of genes in the pineal gland involved in cholesterol biosynthesis (6). When the pineal gland was exposed to light, it produced cholesterol and 7α-hydroxypregnenolone *ex vivo*. Interestingly, this light-induced production of 7α-hydroxypregnenolone occurred only when the gland was exposed to light at early night but not at late night or during the daytime. During early night time, the circadian clock is sensitive to light, which causes phase-delay of the clock (10). Thus, the light-sensitive pineal production of 7α-hydroxypregnenolone appears to be regulated by the circadian clock.

In vertebrates, an intracerebroventricular injection of 7α-hydroxypregnenolone activates locomotor activities (11–15). Thus, the intracerebroventricular injection of 7α-hydroxypregnenolone was administered in a dose-dependent manner at early night in chicks (6). After the injection, chicks were placed individually for locomotor activity measurement in an open field apparatus for 20 min. Spontaneous locomotor activities of chicks were stimulated by the intracerebroventricular injection of 7α-hydroxypregnenolone in a dose-dependent manner (6). Furthermore, when chicks are exposed to light during early night time, their locomotor activities reach the daytime level (6). These results suggest that pineal 7α-hydroxypregnenolone reaches the target sites within the brain by volume transmission (16) upon light exposure at early night.

PHYSIOLOGICAL FUNCTION OF PINEAL ALLOPREGNANOLONE IN PURKINJE CELL SURVIVAL DURING DEVELOPMENT

7α-Hydroxypregnenolone and allopregnanolone are actively released during early posthatch period compared with adulthood (7). Therefore, 7α-hydroxypregnenolone and allopregnanolone may play key roles in birds during early posthatch period. In vertebrates, pinealectomy decreases cell number in the developing brain (17, 18). These findings suggest that these major neurosteroids secreted from the pineal gland are involved in the development of brain cells.

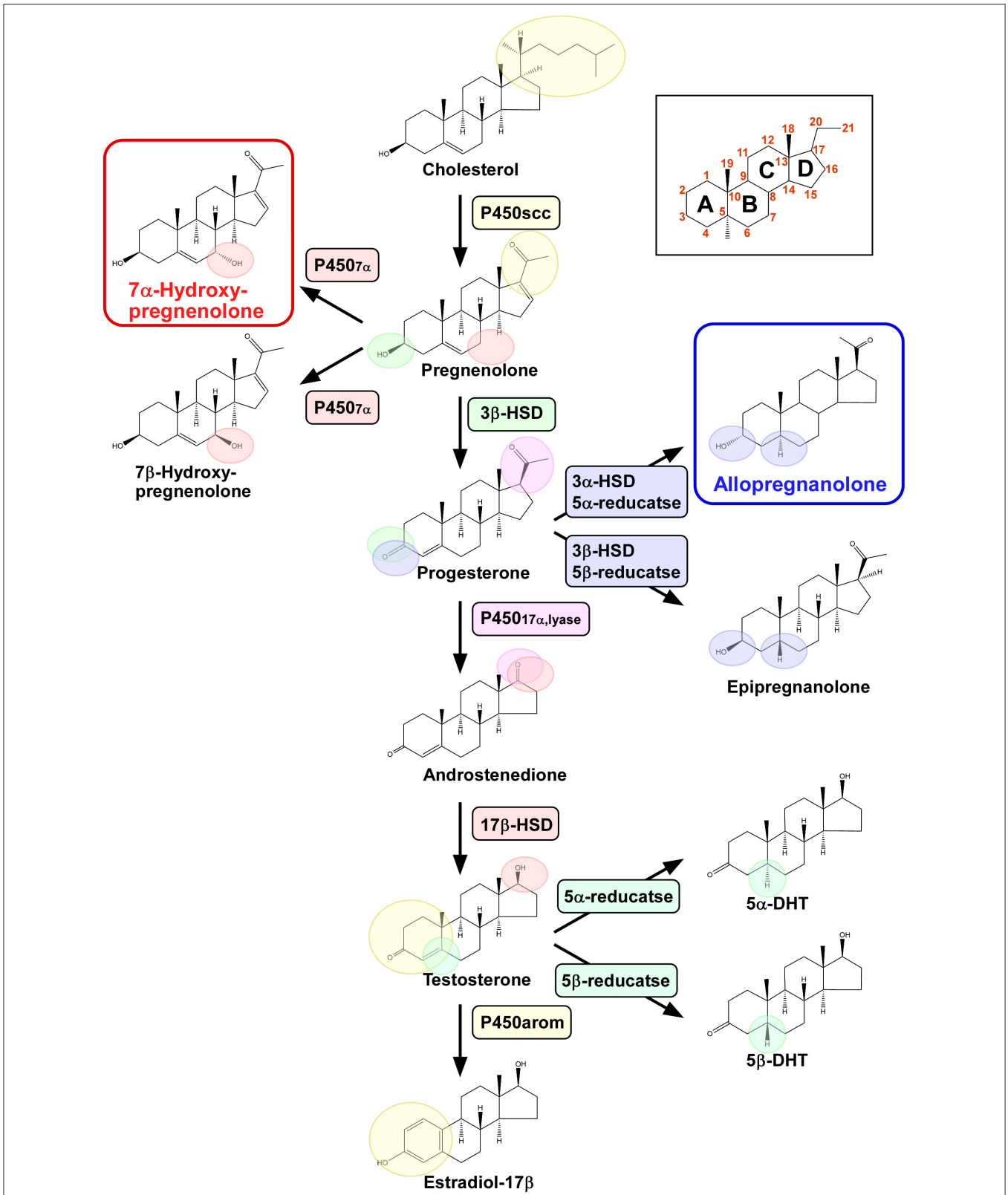

FIGURE 1 | Biosynthetic pathways of pineal neurosteroids. Allopregnanolone and 7α-hydroxypregnenolone are the major neurosteroids produced in the pineal gland of birds. P450scc, cytochrome P450 side-chain cleavage enzyme; P4507α, cytochrome P450 7α-hydroxylase; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase; 3α-HSD, 3α-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase; 5α-reductase; 5β-reductase; P45017α,lyase, cytochrome P450 17α-hydroxylase/c17,20-lyase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; and P450arom, cytochrome P450 aromatase.

In chicks, pinealectomy decreases the concentration of allopregnanolone and the number of cerebellar Purkinje cells, whereas the supplementation of allopregnanolone to pinealectomized birds increases the concentration of allopregnanolone and recovers the number of Purkinje cells (7). Thus, pineal allopregnanolone is considered to be an essential factor for the normal development of cerebellar Purkinje cells. It thus appears that pineal allopregnanolone functions as an essential factor for Purkinje cells during posthatch period.

In addition, pinealectomy in juvenile birds increases the expression of active caspase-3 in Purkinje cells, whereas allopregnanolone supplementation decreases the expression of active caspase-3 during posthatch period (7). Thus, the neuroprotective action of pineal allopregnanolone on cerebellar Purkinje cells is exerted by suppressing the activation of caspase-3 (**Figure 2**).

Allopregnanolone acts mainly as a ligand of the γ-aminobutyric acid type A ($GABA_A$) receptor and may also act as an agonist of the membrane progesterone receptors α (mPRα), as well as the mPRβ and mPRγ (19–21). Therefore, either mPR siRNA or isoallopregnanolone, an antagonist of allopregnanolone, was delivered into the cerebellum of posthatched chicks. It was found that the silencing of mPRα increases the number of Purkinje cells that express active caspase-3 in the cerebellum of chicks (8). Furthermore, to uncover the mechanism of neuroprotective action of allopregnanolone

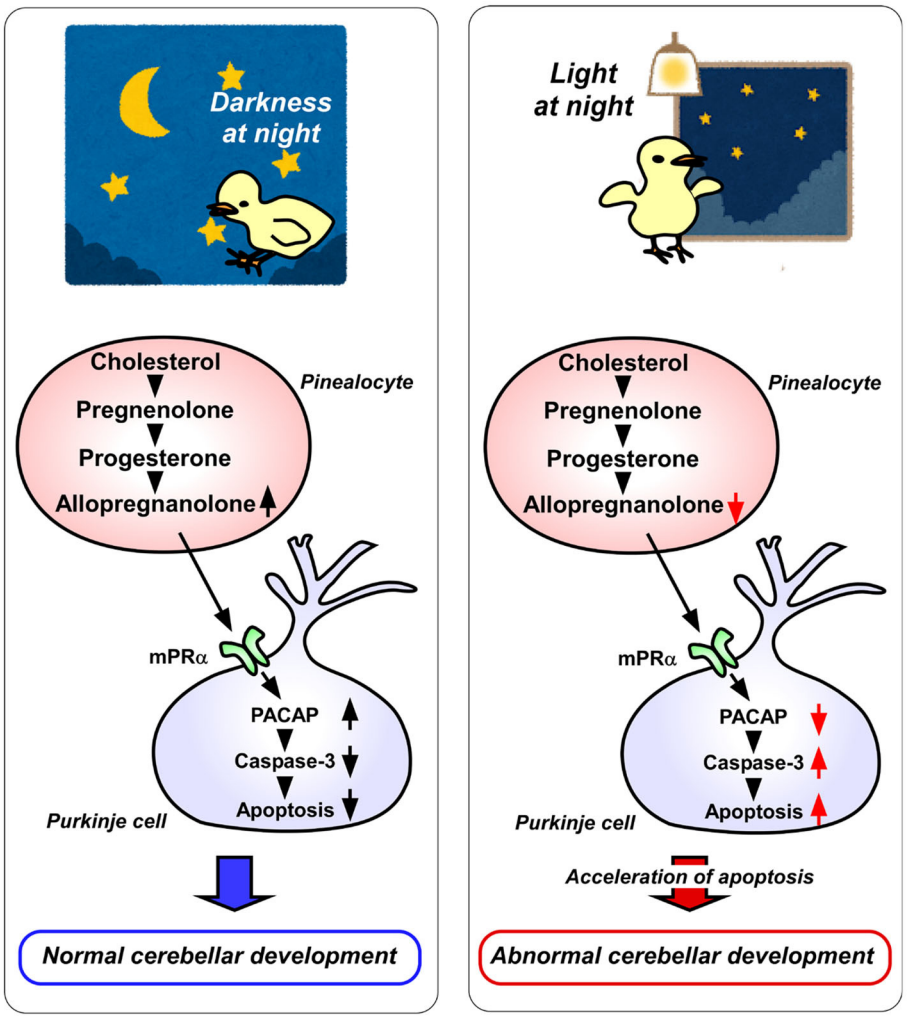

FIGURE 2 | A schematic model of the effect of pineal allopregnanolone on Purkinje cell survival immediately after hatching under a 12/12 h light/dark cycle or with 1 h light exposure during the dark period (light-at-night condition). **(Left)** panel The normal cerebellar development under a 12/12 h light/dark cycle during the first week after hatching. Pineal allopregnanolone induces the expression of pituitary adenylate cyclase-activating polypeptide (PACAP), a neuroprotective factor, through the membrane progestin receptor α (mPRα) receptor binding mechanism in Purkinje cells. Subsequently, PACAP inhibits the activation of caspase-3 that facilitates the apoptosis of cerebellar Purkinje cells. **(Right)** panel The abnormal cerebellar development under the light-at-night condition during the first week after hatching. The light-at-night condition disrupts the diurnal rhythm in pineal allopregnanolone synthesis. Decreased pineal allopregnanolone synthesis leads to decreased expression of PACAP in Purkinje cells. Consequently, the active caspase-3 level increases, inducing the apoptosis of Purkinje cells in the cerebellum.

in cerebellar Purkinje cells, allopregnanolone action on the expression of neuroprotective/neurotoxic factors (22–26) has been investigated. Pinealectomy decreases the mRNA levels of pituitary adenylate cyclase-activating polypeptide (PACAP), a neuroprotective factor, in the cerebellum of juvenile birds (8). It has been found that a daily injection of allopregnanolone in pinealectomized juvenile birds upregulates PACAP relative to the levels in control birds (8). These findings show that PACAP mediates the neuroprotective action of pineal allopregnanolone through mPRα receptor binding during cerebellar development (**Figure 2**).

LIGHT-AT-NIGHT AFFECTS THE DEVELOPMENT OF CEREBELLUM THROUGH A MECHANISM MEDIATED BY PINEAL ALLOPREGNANOLONE ACTION

It is known that environmental stimuli affect the development of animals including humans. In vertebrate brain development, a natural light-dark cycle promotes better brain development than constant conditions, such as constant light or constant darkness (27–31). However, the molecular mechanisms that control how environmental light conditions affect brain development remain unclear. The pineal gland is a photosensitive organ. To investigate whether light conditions are involved in the synthesis of allopregnanolone in the pineal gland, the birds have been incubated under either a 12/12 h light/dark (LD) cycle or LD cycle with 1 h light exposure during the dark period (light-at-night). Consequently, it has been found that the allopregnanolone concentration and synthesis during the dark period are higher in the pineal glands of LD birds than in those of light-at-night birds (8) (**Figure 2**). Furthermore, the number of cerebellar Purkinje cells is decreased by the light-at-night condition (8) (**Figure 2**). It is therefore considered that pineal allopregnanolone is a critical metabolite that affects cerebellar development in vertebrates, depending on the environmental light conditions.

CONCLUSIONS

This review summarized the recent data on pineal neurosteroids. Studies have indicated that the pineal gland produces neurosteroids from cholesterol in birds. Pineal 7α-hydroxypregnenolone regulates locomotion in response to light stimuli in birds. Pineal allopregnanolone prevents the death of developing Purkinje cells by suppressing neuronal apoptosis during development. In addition, circadian disruption by light exposure during nighttime leads to cell death of developing Purkinje cells through pineal allopregnanolone-dependent mechanisms in juvenile birds. These observations suggest that nighttime artificial light exposure in modern societies may also perturb the development of the human brain.

Almost all animals have circadian rhythms. However, modern life conditions chronically disrupt circadian rhythm through artificial light exposure. The disruption of circadian rhythm is associated with a decline in mental and physical health (32–34). The most potent circadian rhythm disruption is inappropriately timed bright light exposure (e.g., light-at-night). To investigate the effects of chronic circadian disruption in modern societies on mental and physical health, which is efficiently modeled by the light-at-night condition presented here, many studies have been conducted on mice. However, it is important for us to bear in mind that laboratory mice are mainly nocturnal animals, whereas humans are diurnal. Thus, birds are excellent animal models to uncover the effect of light-at-night on diurnal animals, including humans.

AUTHOR CONTRIBUTIONS

SH and KT wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by JSPS Grants-in-Aid for Scientific Research (KAKENHI) Grant Numbers JP15K18571 and JP19K09033.

REFERENCES

1. Baulieu EE. Neurosteroids: of the nervous system, by the nervous system, for the nervous system. *Rec Prog Hormone Res.* (1997) 52:1–32.
2. Tsutsui K, Ukena K, Takase M, Kohchi C, Lea. RW. Neurosteroid biosynthesis in vertebrate brains. *Comp Biochem Physiol C.* (1999) 124:121–9. doi: 10.1016/S0742-8413(99)00065-1
3. Compagnone NA, Mellon SH. Neurosteroids: biosynthesis and function of these novel neuromodulators. *Front Neuroendocrinol.* (2000) 21:1–56. doi: 10.1006/frne.1999.0188
4. Tsutsui K, Matsunaga M, Miyabara H, Ukena K. Neurosteroid biosynthesis in the quail brain. *J Exp Zool.* (2006) 305A:733–42. doi: 10.1002/jez.a.302
5. Do-Rego JL, Seong JY, Burel D, Leprince J, Luu-The V, Tsutsui K, et al. Neurosteroid biosynthesis: enzymatic pathways and neuroendocrine regulation by neurotransmitters and neuropeptides. *Front Neuroendocrinol.* (2009) 30:259–301. doi: 10.1016/j.yfrne.2009.05.006
6. Hatori M, Hirota T, Iitsuka M, Kurabayashi N, Haraguchi S, Kokame K, et al. Light-dependent and circadian clock-regulated activation of sterol regulatory element-binding protein, X-box-binding protein 1, and heat shock factor pathways. *Proc Natl Acad Sci USA.* (2011) 108:4864–9. doi: 10.1073/pnas.1015959108
7. Haraguchi, S Hara, S Ubuka T, Mita M, Tsutsui K. Possible role of pineal allopregnanolone in Purkinje cell survival. *Proc Natl Acad Sci USA.* (2012) 109:21110–5. doi: 10.1073/pnas.1210804109
8. Haraguchi S, Kamata M, Tokita T, Tashiro KI, Sato M, Nozaki M, et al. Light-at-night exposure affects brain development through pineal allopregnanolone-dependent mechanisms. *eLife.* (2019) 8:e45306. doi: 10.7554/eLife.45306.037
9. Reiter RJ. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. *Endocr Rev.* (1991) 12:151–180. doi: 10.1210/edrv-12-2-151
10. Fukada Y, Okano T. Circadian clock system in the pineal gland. *Mol Neurobiol.* (2002) 25:19–30. doi: 10.1385/MN:25:1:019
11. Matsunaga M, Ukena K, Baulieu EE, Tsutsui K. 7α-Hydroxypregnenolone acts as a neuronal activator to stimulate locomotor activity of breeding newts by means of the dopaminergic system. *Proc Natl Acad Sci USA.* (2004) 101:17282–7. doi: 10.1073/pnas.0407176101

12. Tsutsui K, Inoue K, Miyabara H, Suzuki S, Ogura Y, Haraguchi S. 7α-Hydroxypregnenolone mediates melatonin action underlying diurnal locomotor rhythms. *J Neurosci*. (2008) 28:2158–67. doi: 10.1523/JNEUROSCI.3562-07.2008
13. Haraguchi S, Koyama T, Hasunuma I, Vaudry H, Tsutsui K. Prolactin increases the synthesis of 7α-hydroxypregnenolone, a key factor for induction of locomotor activity, in breeding male newts. *Endocrinology*. (2010) 151:2211–22. doi: 10.1210/en.2009-1229
14. Haraguchi S, Koyama T, Hasunuma I, Okuyama S, Ubuka T, Kikuyama S, et al. Acute stress increases the synthesis of 7α-hydroxypregnenolone, a new key neurosteroid stimulating locomotor activity, through corticosterone action in newts. *Endocrinology*. (2012) 153:794–805. doi: 10.1210/en.2011-1422
15. Haraguchi S, Yamamoto Y, Suzuki Y, Hyung Chang J, Koyama T, Sato M, et al. 7α-Hydroxypregnenolone, a key neuronal modulator of locomotion, stimulates upstream migration by means of the dopaminergic system in salmon. *Sci Rep*. (2015) 5:12546. doi: 10.1038/srep12546
16. Fillenz M. Volume transmission in the brain: Novel mechanisms for neuronal transmission. In: Fuxe K, Agnati LF, editors. *Advances in Neurosciences*, Vol. 1. New York, NY: Raven Press (1991). p. 193–4.
17. Kilic E, Hermann DM, Isenmann S, Bähr M. Effects of pinealectomy and melatonin on the retrograde degeneration of retinal ganglion cells in a novel model of intraorbital optic nerve transection in mice. *J Pineal Res*. (2002) 32:106–11. doi: 10.1034/j.1600-079x.2002.1823.x
18. Tunç AT, Turgut M, Aslan H, Sahin B, Yurtseven ME, Kaplan S. Neonatal pinealectomy induces Purkinje cell loss in the cerebellum of the chick: a stereological study. *Brain Res*. (2006) 1067:95–102. doi: 10.1016/j.brainres.2005.10.011
19. Pang Y, Dong J, Thomas P. Characterization, neurosteroid binding and brain distribution of human membrane progesterone receptors δ and ε (mPRδ and mPRε) and mPRδ involvement in neurosteroid inhibition of apoptosis. *Endocrinology*. (2013) 154:283–95. doi: 10.1210/en.2012-1772
20. Schumacher M, Mattern C, Ghoumari A, Oudinet JP, Liere P, Labombarda F, et al. Revisiting the roles of progesterone and allopregnanolone in the nervous system: resurgence of the progesterone receptors. *Prog Neurobiol*. (2014) 113:6–39. doi: 10.1016/j.pneurobio.2013.09.004
21. Belelli D, Lambert JJ. Neurosteroids: endogenous regulators of the $GABA_A$ receptor. *Nat Rev Neurosci*. (2005) 6:565–75. doi: 10.1038/nrn1703
22. Bernal J. Thyroid hormone receptors in brain development and function. *Nat Clin Pract Endocrinol Metabol*. (2007) 3:249–59. doi: 10.1038/ncpendmet0424
23. Falluel-Morel A, Vaudry D, Aubert N, Galas L, Benard M, Basille M, et al. Pituitary adenylate cyclase-activating polypeptide prevents the effects of ceramides on migration, neurite outgrowth, and cytoskeleton remodeling. *Proc Natl Acad Sci USA*. (2005) 102:2637–42. doi: 10.1073/pnas.0409681102
24. Koibuchi N, Chin WW. Thyroid hormone action and brain development. *Trends Endocrinol Metab*. (2000) 11:123–8. doi: 10.1016/S1043-2760(00)00238-1
25. Vaudry D, Falluel-Morel A, Leuillet S, Vaudry H, Gonzalez BJ. Regulators of cerebellar granule cell development act through specific signaling pathways. *Science*. (2003) 300:1532–4. doi: 10.1126/science.1085260
26. Sasahara K, Shikimi H, Haraguchi S, Sakamoto H, Honda S, Harada N, et al. Mode of action and functional significance of estrogen-inducing dendritic growth, Spinogenesis, and synaptogenesis in the developing Purkinje cell. *J Neurosci*. (2007) 27:7408–17. doi: 10.1523/JNEUROSCI.0710-07.2007
27. Bakkum BW, Benevento LA, Cohen RS. Effects of light/dark- and dark-rearing on synaptic morphology in the superior colliculus and visual cortex of the postnatal and adult rat. *J Neurosci Res*. (1991) 28:65–80. doi: 10.1002/jnr.490280107
28. Brooks E, Waters E, Farrington L, Canal MM. Differential hypothalamic tyrosine hydroxylase distribution and activation by light in adult mice reared under different light conditions during the suckling period. *Brain Struct Funct*. (2011) 216:357–70. doi: 10.1007/s00429-011-0318-9
29. Dulcis D, Spitzer NC. Illumination controls differentiation of dopamine neurons regulating behaviour. *Nature*. (2008) 456:195–201. doi: 10.1038/nature07569
30. Li Y, Komuro Y, Fahrion JK, Hu T, Ohno N, Fenner KB, et al. Light stimuli control neuronal migration by altering of insulin-like growth factor 1 (IGF-1) signaling. *Proc Natl Acad Sci USA*. (2012) 109:2630–5. doi: 10.1073/pnas.1111326109
31. Ohta H, Mitchell AC, McMahon DG. Constant light disrupts the developing mouse biological clock. *Pediatr Res*. (2006) 60:304–8. doi: 10.1203/01.pdr.0000233114.18403.66
32. Kantermann T, Roenneberg T. Is light-at-night a health risk factor or a health risk predictor? *Chronobiol Int*. (2009) 26:1069–74. doi: 10.1080/07420520903223984
33. Wu J, Dauchy RT, Tirrell PC, Wu SS, Lynch DT, Jitawatanarat P, et al. Light at night activates IGF-1R/PDK1 signaling and accelerates tumor growth in human breast cancer xenografts. *Cancer Res*. (2011) 71:2622–31. doi: 10.1158/0008-5472.CAN-10-3837
34. Smarr BL, Grant AD, Perez L, Zucker I, Kriegsfeld LJ. Maternal and early-life circadian disruption have long-lasting negative consequences on offspring development and adult behavior in mice. *Sci Rep*. (2017) 7:3326. doi: 10.1038/s41598-017-03406-4

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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 30 September 2020
doi: 10.3389/fendo.2020.545973

Roles of Testosterone and Estradiol in Mediation of Acute Neuroendocrine and Electroencephalographic Effects of Sevoflurane During the Sensitive Period in Rats

Ningtao Li[1,2], Ning Xu[2], Yunan Lin[2], Lei Lei[2], Ling-Sha Ju[2], Timothy E. Morey[2], Nikolaus Gravenstein[2], Jiaqiang Zhang[1] and Anatoly E. Martynyuk[2,3]**

[1] Department of Anesthesiology and Perioperative Medicine, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, Zhengzhou, China, [2] Department of Anesthesiology, University of Florida College of Medicine, Gainesville, FL, United States, [3] McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL, United States

OPEN ACCESS

Edited by:
Sonoko Ogawa,
University of Tsukuba, Japan

Reviewed by:
Mumeko C. Tsuda,
Uniformed Services University of the Health Sciences, United States
Anders Agmo,
Arctic University of Norway, Norway

***Correspondence:**
Jiaqiang Zhang
hnmzxh@163.com
Anatoly E. Martynyuk
amartynyuk@anest.ufl.edu

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

Received: 09 April 2020
Accepted: 10 September 2020
Published: 30 September 2020

Citation:
Li N, Xu N, Lin Y, Lei L, Ju L-S, Morey TE, Gravenstein N, Zhang J and Martynyuk AE (2020) Roles of Testosterone and Estradiol in Mediation of Acute Neuroendocrine and Electroencephalographic Effects of Sevoflurane During the Sensitive Period in Rats. Front. Endocrinol. 11:545973. doi: 10.3389/fendo.2020.545973

Testosterone (T), predominantly acting through its derivative 17β-estradiol (E2), regulates the brain's sexual differentiation in rodents during the perinatal sensitive period, which mirrors the window of vulnerability to the adverse effects of general anesthetics. The mechanisms of anesthesia's adverse effects are poorly understood. We investigated whether sevoflurane alters T and E2 levels and whether they contribute to sevoflurane's acute adverse effects in postnatal day 5 Sprague-Dawley rats. The rats underwent electroencephalography recordings for 2 h of baseline activity or for 1 h before and another hour during 2.1% sevoflurane exposure, followed by collection of trunk blood and brain tissue. Pharmacological agents, including the GABA type A receptor inhibitor bicuculline and the aromatase inhibitor formestane, were administered 30 min before sevoflurane anesthesia. Sevoflurane increased serum T levels in males only. All other effects of sevoflurane were similar in both sexes, including increases in serum levels of E2, hypothalamic mRNA levels of aromatase, estrogen receptor α (*Erα*) [not estrogen receptor β (*Erβ*)], Na^+-K^+-Cl^- cotransporter (*Nkcc1*)/K^+-Cl^- cotransporter (*Kcc2*) mRNA ratio, electroencephalography-detectable seizures, and stress-like corticosterone secretion. Bicuculline and formestane alleviated these effects, except the T level increases. The ERα antagonist MPP, but not the ERβ antagonist PHTPP, reduced electroencephalography-detectable seizures and normalized the *Nkcc1/Kcc2* mRNA ratio. Collectively, sevoflurane exacerbates levels of T in males and E2 in both sexes during the period of their organizational effects in rodents. Sevoflurane acts through $GABA_A$R-mediated, systemic T-independent elevation of E2 to cause electroencephalography-detectable seizures, stress-like corticosterone secretion, and changes in the expression of genes critical for brain development.

Keywords: estradiol, testosterone, neonatal anesthesia, sevoflurane, corticosterone

INTRODUCTION

According to 2016 World Health Organization estimates, the number of surgeries performed globally rose from 226.4 million in 2004 to 312.9 million in 2012 (1). This progress would not be possible without modern general anesthesia, which can be viewed as a state of pharmacologically induced "reversible brain coma" (2). Despite advancements in refining anesthesia approaches, multiple studies support the idea that the effects of general anesthetics, which may also act as endocrine disruptors and stressors, are not completely reversible upon anesthesia withdrawal. The adverse effects of general anesthesia at the beginning and the end of the lifespan are an increasingly recognized health concern and the subject of extensive clinical and laboratory research (3–7). Experimental evidence indicates that GABAergic anesthetic agents, such as propofol, isoflurane, or sevoflurane, administered to neonatal rats, acutely induce electroencephalography (EEG)-detectable seizures and increased systemic levels of the main stress hormone corticosterone (8–10). The long-term effects of these anesthetics comprise an abnormal (increased) hypothalamic and hippocampal Na^+-K^+-Cl^- cotransporter (*Nkcc1*)/K^+-Cl^- cotransporter (*Kcc2*) mRNA ratio, an exacerbated hypothalamic-pituitary-adrenal (HPA) axis responses to stress, and behavioral deficiencies (8, 9, 11–13).

Intracellular concentrations of Cl^-, the main charge carriers through GABA type A receptor ($GABA_AR$) channels, are elevated in many neurons in the neonatal brain because of the relatively high and low levels of the NKCC1 and KCC2 Cl^- co-transporters, respectively (14, 15). Activation of $GABA_AR$ in these neurons causes Cl^- efflux, a strong membrane depolarization or excitation, and Ca^{++} influxes through the voltage-gated Ca^{++} channels and Ca^{++}-permeable N-methyl-D-aspartate receptor channels (14). $GABA_AR$-initiated depolarization and related Ca^{++} influxes regulate a wide spectrum of biological processes (14). The magnitude of $GABA_AR$ excitatory signaling and the proper timing of its transition from excitatory to inhibitory are key for normal brain development and functioning (14). Delays/impairments in the transition to inhibitory $GABA_AR$ signaling have been linked in humans and animal models to several cognitive neuropsychiatric disorders, such as autism spectrum disorder, schizophrenia, and Rett syndrome (16–18). Inhibition of the NKCC1 activity at the time of neonatal anesthesia ameliorates anesthetic-caused EEG-detectable seizures and many of the long-term developmental effects of GABAergic anesthetics in rats, suggesting that anesthetic-exacerbated excitatory $GABA_AR$ signaling at the time of anesthesia can be an initial step in the pathways that mediate the developmental effects of GABAergic anesthetics (8–12).

We have recently presented indirect evidence that the primary female sex steroid hormone 17β-estradiol (E2) may play a crucial role in mediating sevoflurane-caused EEG-detectable seizures by enhancing excitatory $GABA_AR$ signaling in neonatal rats (10). E2, however, is known to modulate GABAergic signaling not only by affecting the $GABA_AR$-based neurotransmission machinery (10, 19–22), but also by altering the expression and activity of KCC2 and NKCC1, respectively (23, 24). In the neonatal rodent brain, E2 exerts the brain's sexual differentiation (masculinization) through organizational/persistent actions during the sensitive period, which mirrors the window of vulnerability to the adverse effects of general anesthetics. In rats, the sensitive period starts on embryonic days 18.5 to 19.5, with the onset of testosterone (T) production in the testis and ends during the second postnatal week when exogenous sex hormones can no longer induce brain masculinization in females (25). E2 in the brain is produced by converting testis-derived T in males and *via de novo* synthesis in the brain in both sexes. In both pathways, the final step in E2 synthesis is T aromatization by the enzyme aromatase, whose activity is regulated by Ca^{++} (26). E2 acts primarily through its receptors α (ERα) and β (ERβ) (27–29). It is widely accepted that neonatal rodents become less susceptible to the neurodevelopmental effects of general anesthetics during the second postnatal week (13, 30–32).

Here, we studied the roles of T and E2 in the mediation of sevoflurane's acute (initial) adverse effects in the postnatal day (P) 5 rats. We did so by measuring sevoflurane-caused EEG-detectable seizures, changes in systemic levels of T, E2, and corticosterone, and changes in the expression of hypothalamic *aromatase*, *Erα*, *Erβ*, *Nkcc1*, and *Kcc2* under different treatment conditions to modulate $GABA_AR$ and T/E2 signaling pathways. T/E2 may affect sexual differentiation in the rodent brain during this age period through lasting organizational effects (25) and sevoflurane may induce long-term developmental abnormalities (8, 9, 11, 12). For that reason, understanding the involvement of sevoflurane-altered levels of T and E2 in mediating the acute adverse effects of sevoflurane in neonatal rats at the time of anesthesia may help to explain the mechanistic basis of more complex long-term deficiencies induced by the anesthetic.

MATERIALS AND METHODS

Animals

The University of Florida Institutional Animal Care and Use Committee approved all experimental procedures. Sprague-Dawley rats were bred at the University of Florida animal care facility. We housed the rats under controlled illumination (12-h light/dark cycle, lights on at 7:00 a.m.) and temperature (23–24°C) with free access to food and water. Within 24 h of delivery, litters were culled to 12 pups. Pups from each litter were used for different treatment conditions. Multiple sets of animals were used in the experiments.

Electroencephalography, Anesthesia Regimen, and Treatment Groups

The P5 rats were instrumented for EEG recording during a minor 12- to 15-min surgical procedure performed under isoflurane anesthesia (2.0%–2.5%) as we previously described (10, 30, 33). The four EEG electrodes were implanted bilaterally in the occipital and frontal regions of the rat's skull, with the left frontal electrode serving as the reference electrode. The EEG recordings were started after the rats recovered from isoflurane anesthesia for electrode implantation; they lasted for 2 h of baseline activity (group 1, the Control group) or for 1 h before the initiation of sevoflurane anesthesia (baseline activity) and

continued for another hour during sevoflurane anesthesia: 6% sevoflurane for 3 min for anesthesia induction and 2.1% sevoflurane for 57 min for anesthesia maintenance (groups 2–7; **Figure 1**). We have previously verified that no obvious differences in the effects of sevoflurane to cause EEG-detectable seizures were detected when EEG electrode implantation was done either prior or 1 to 2 days before the start of EEG recording (34). During the EEG recordings, the rats were in a thermostated chamber to maintain body temperature at ~37°C with a continuous supply of 30% oxygen in air (1.5 L min^{-1}). A rectal temperature probe was placed in some animals to monitor body temperature. Gas monitoring was performed using a calibrated Datex side stream analyzer (Datex-Ohmeda, Helsinki, Finland), which sampled from the animal chamber interior. According to Orliaguet et al. (35), 2.1% sevoflurane lies near the 0.6 minimum alveolar concentration for P5 rats. At 2.1% sevoflurane, the pups did not exhibit a righting reflex but responded to a noxious stimulation. None of the animals exhibited cyanosis and all were breathing regularly during anesthesia exposure. Previously, we have shown that blood glucose and gas levels after 2.1% sevoflurane anesthesia were in the normal range (30). The EEG recordings were performed using an EEG/electromyogram system (Pinnacle Technology, Lawrence, KS, United States). Acquisition of the EEG was performed using Sirenia software (Pinnacle Technology). The sampling interval per signal was 200 μs (5 kHz). Sirenia Score (Pinnacle Technology), Clampfit 9.2 (Axon Instruments, Union City, CA, United States), and Mini Analysis (Synaptosoft Inc., Fort Lee, NJ, United States) programs were used for EEG data analysis. Data were filtered offline using a bandpass Bessel (8-pole) 0.04- to 56-Hz filter. EEG patterns characterized by an amplitude of at least three times higher than baseline and rhythmic activity (>2 Hz) that lasted for at least 3 s and abruptly reverted to the baseline were defined as seizure-like EEG patterns. In most cases, these patterns started as high frequency-low amplitude activity that developed to increased amplitude and decreased frequency and then abruptly reverted to baseline activity. All parameters for EEG seizures such as the total duration, number of episodes, and average episode duration were calculated for the entire 60-min period of sevoflurane exposure. All EEG records were analyzed by three independent investigators. The investigators who analyzed the EEG records were blinded to the experimental conditions. Animals that exhibited episode(s) of seizure-like EEG patterns before the start of anesthesia were removed from the data analysis. Typically, in our studies about 5% of animals exhibit episode(s) of seizure-like EEG patterns before the start of anesthesia and are not included in the data analysis (10, 30, 33). Histopathological analysis links most of these seizures to brain injuries during surgery for the EEG electrode implantation.

Rats were randomized for treatment groups using a randomization plan generator (http://www.randomization.com/) and the investigators were blind to group assignments. Treatments or vehicle were administered to the P5 rat pups 30 min prior to sevoflurane anesthesia. The following treatment groups were investigated:

1. (Group 1) EEG recording for 2 h without exposure to sevoflurane (the Control group);
2. (Group 2) EEG recording for 1 h without sevoflurane and for 1 h with sevoflurane; vehicle [subcutaneous (SC) or intraperitoneal injection (IP)] 30 min prior to the start of anesthesia with sevoflurane (the Vehicle + Sevo group);
3. (Group 3) E2 synthesis inhibitor formestane (2 mg/kg, SC) 30 min prior to sevoflurane anesthesia (the Formestane + Sevo group);
4. (Group 4) $GABA_AR$ antagonist bicuculline methiodide (0.01 mg/kg, IP) 30 min prior to sevoflurane anesthesia (the Bicuculline + Sevo group);
5. (Group 5) ERα antagonist MPP (0.2 mg/kg, IP) 30 min prior to sevoflurane anesthesia (the MPP + Sevo group);
6. (Group 6) ERβ antagonist PHTPP (4 mg/kg, IP) 30 min prior to sevoflurane anesthesia (the PHTPP + Sevo group);
7. (Group 7) G-protein-coupled estrogen receptor (GPER) antagonist G-15 (5 μg/kg, IP) 30 min prior to sevoflurane anesthesia (the G-15 + Sevo group).

All study agents were dissolved in 33% DMSO plus 67% saline, which was the vehicle noted in Group 2. Therefore, equal volumes of DMSO and saline were used as vehicle. DMSO at these doses

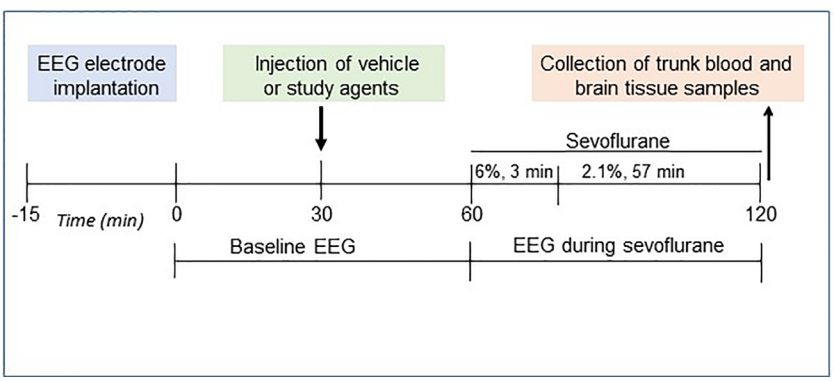

FIGURE 1 | Study design.

does not cause any detectable effects on hyperexcitatory EEG or serum corticosterone levels in neonatal rats (33). These concentrations/doses were chosen based on our published and preliminary findings and data in the literature (10, 30, 33, 36). Immediately after we completed EEG recordings, rats were sacrificed by decapitation while being anesthetized with sevoflurane and trunk blood and brain tissue samples were isolated.

Tissue Collection

After decapitation, the trunk blood samples were collected and centrifuged at 4°C, 1,000 g for 15 min, and kept at −80°C for hormone assays. The brains were removed from the skull onto ice pads. The hypothalamus was isolated by making an anterior cut at the level of the optic chiasm, a posterior coronal section anterior to the mammillary bodies, two sagittal cuts parallel to the lateral ventricles, and a dorsal horizontal cut at the level of the anterior commissure, as described previously (37). All tissue samples were placed in vials filled with RNAlater solution (Invitrogen, Carlsbad, CA, United States) and stored at −80°C.

Measurements of Serum T, E2, and Corticosterone Levels

Using commercially available kits and following the manufacturer's instructions, we measured serum levels of hormones in trunk blood samples isolated from P5 rats immediately after we completed EEG recording. Serum T, E2, and corticosterone concentrations were measured using ELISA kits (582701, Cayman Chemical Company, Ann Arbor, MI, United States; EA100859, Origene Technologies Inc., Rockville, MD, United States; and 501320, Cayman Chemical Company, respectively).

Quantitative mRNA Measurements

We analyzed levels of mRNA for *aromatase*, *Erα*, *Erβ*, *Nkcc1*, and *Kcc2* in the hypothalamus *via* reverse transcription-PCR (qRT-PCR) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) as previously described by our laboratory (12). We extracted RNA from the samples using an RNeasy Plus Kit (Qiagen, Valencia, CA, United States), reverse transcribed with a high-capacity cDNA reverse transcription kit (Bio-Rad Laboratories, Hercules, CA, United States), and analyzed *via* qRT-PCR. Taqman probes specific for the above genes were obtained from Applied Biosystems (Carlsbad, CA): *aromatase* (Rn00567222_m1), *Erα* (Rn01430446_m1), *Erβ* (Rn00562610_m1), *Nkcc1* (Rn00582505_m1), and *Kcc2* (Rn00592624_m1). Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA (Rn01775763_g1). Gene expression was calculated using the ΔΔCT method and data are presented as relative fold change from control animals.

Drugs

Sevoflurane (NDC: 0074-4456-04) was manufactured by AbbVie Inc. (North Chicago, IL, United States). The aromatase inhibitor formestane (CAS Number: 566-48-3) and the $GABA_AR$ inhibitor bicuculline methiodide (CAS Number: 40709-69-1) were purchased from Sigma-Aldrich (St. Louis, MO, United States). GPER antagonist G-15 [(3aS,4R,9bR)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c] quinolone] (CAS Number: 1161002-05-6) was purchased from Cayman Chemical Company (Ann Arbor, MI, United States). The ERα antagonist MPP (1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride) (CAS Number: 289726-02-9) and the ERβ antagonist PHTPP (4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]-pyrimidin-3-yl]phenol) (CAS Number: 805239-56-9) were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, United States).

Statistical Analysis

We conducted statistical analyses on raw data using SigmaPlot 14.0 software (Systat Software Inc., San Jose, CA, United States), which automatically checks if a data set meets test criteria (Shapiro-Wilk for normality test and Brown-Forsythe for equal variance test). Values are reported as mean ± SEM. Animal numbers in each experiment are presented in the respective figure legends. An independent t-test was used to compare sevoflurane-induced EEG seizures in male and female rat pups and the effects of G-15 on sevoflurane-induced EEG seizures and serum levels of corticosterone. Two-way ANOVA with treatment and sex as independent variables was used to analyze EEG seizures, levels of T, E2, and corticosterone, and mRNA levels for *aromatase*, *Erα*, *Erβ*, *Nkcc1*, and *Kcc2* in the control and sevoflurane-exposed male and female rat pups. One-way ANOVA was used to analyze the effects of pretreatments with bicuculline, formestane, MPP, and PHTPP on sevoflurane-induced changes in EEG seizures, serum levels of T, E2, and corticosterone, and mRNA levels of *aromatase*, *Erα*, *Erβ*, *Nkcc1*, and *Kcc2* in each sex. All multiple pairwise comparisons were done with the Holm-Sidak method. $P < 0.05$ was considered statistically significant. Statistical details are presented in the text and in figure legends. The sample sizes in this study were based on previous experience with the same animal model, experimental techniques, and measured variables (8–12, 30, 33).

RESULTS

Effects of Sevoflurane in Male and Female Rat Pups

Episodes of epileptic seizures were found in the electroencephalograms of male and female P5 rats recorded during 1 h of sevoflurane anesthesia (**Figures 2A, B**). The differences in the number of episodes of EEG-detectable seizures ($t_{(17)} = 0.764$; $P = 0.455$; **Figure 2B**), the total duration of seizures during 1 h of anesthesia ($t_{(17)} = 1.205$; $p = 0.245$; **Figure 2B**), and the duration of a single episode ($t_{(17)} = 1.165$; $P = 0.260$; **Figure 2B**) were not significant between the sexes. Immediately after we completed EEG recordings, the blood and brain tissue samples were collected to analyze serum levels of T, E2 and corticosterone and mRNA levels of *aromatase*, *Erα*, *Erβ*, *Nkcc1*, and *Kcc2* in the hypothalamus. Two-way ANOVA revealed significant effects of treatment and significant treatment x sex interaction on serum levels of T. Anesthesia with sevoflurane increased serum levels

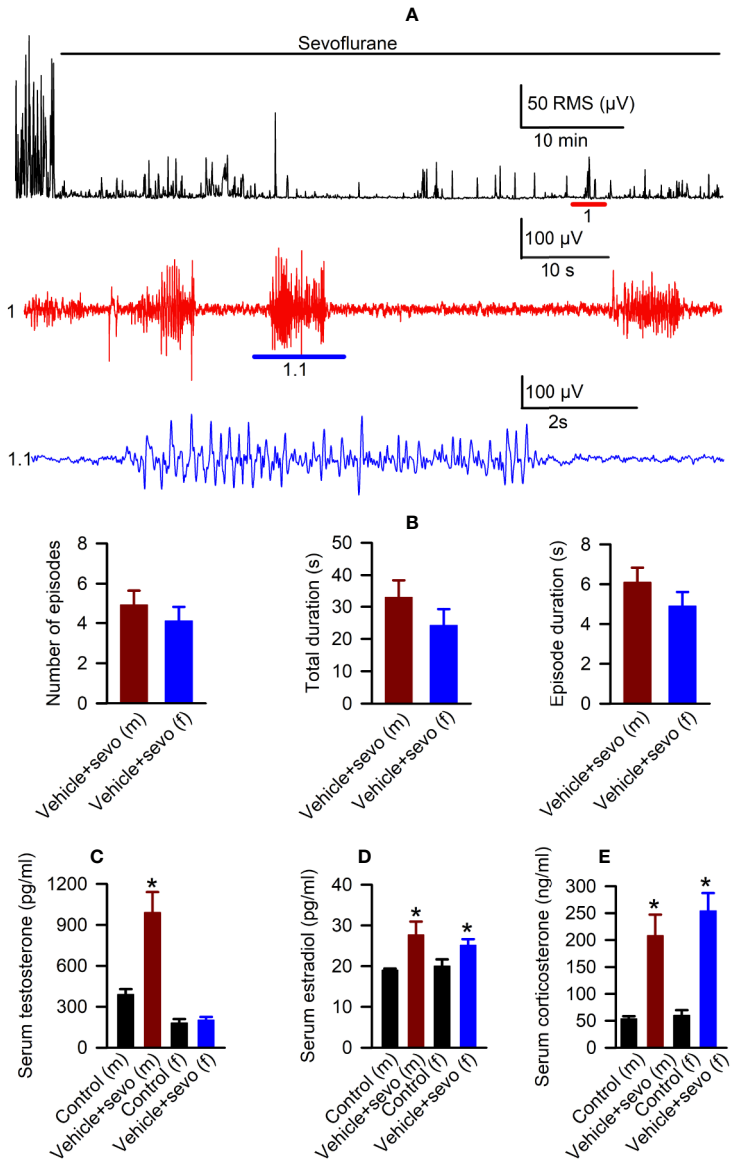

FIGURE 2 | Effects of sevoflurane in postnatal day 5 rats at the systemic level. **(A)** Illustration of the electroencephalogram (EEG)-detectable seizures in a male rat pup during sevoflurane anesthesia. Top trace: root mean square (RMS) of the EEG of a male rat before and during exposure to sevoflurane. The red horizontal line marks the occurrence of EEG-detectable seizures (1); corresponding section of the EEG is shown at expanded time scale in 1 (red trace); 1.1 (blue trace) shows at expanded scale the section of EEG recording marked by blue horizontal line in 1. **(B)** Histograms showing parameters of EEG-detectable seizures during 60-min exposure to sevoflurane of male (n = 10) and female (n = 9) rats. Plots showing the levels of serum testosterone **(C)**, estradiol **(D)**, and corticosterone **(E)** in trunk blood samples collected from male and female rats immediately after completion of EEG recordings during 2 h of baseline activity (the Control group) or 1 h of baseline activity followed by another hour during exposure to sevoflurane (the Vehicle + Sevo group). Data are means ± SEM from six rats/group. *P < 0.05 vs. the Control group.

of T in male pups only (**Table 1**, **Figure 2C**). In contrast to serum levels of T, both male and female pups anesthetized with sevoflurane had similarly increased serum levels of E2 (**Table 1**, **Figure 2D**), serum levels of corticosterone (**Table 1**, **Figure 2E**), and hypothalamic mRNA levels of *aromatase*, *Erα*, *Nkcc1*, and the *Nkcc1*/*Kcc2* mRNA ratio, while hypothalamic mRNA levels of *Kcc2* were decreased in both sexes. Anesthesia with sevoflurane did not affect hypothalamic mRNA levels of *Erβ* (**Table 1**, **Figures 3A–F**).

Effects of Pretreatments With Formestane and Bicuculline

Next, we studied sevoflurane-induced changes in EEG-detectable seizures, serum levels of T, E2 and corticosterone and hypothalamic expressions of *aromatase*, *Erα*, *Erβ*, *Nkcc1*, and *Kcc2* in rats that were pretreated with the $GABA_AR$ antagonist bicuculline or the inhibitor of E2 synthesis formestane prior to the initiation of sevoflurane anesthesia. The number of episodes

TABLE 1 | Acute effects of sevoflurane in male and female P5 rat pups.

Measured variables	The results of the statistical analyses	
	Treatment	Treatment/sex interaction
Serum corticosterone	$F_{(1,20)} = 44.290$, $p < 0.001$	$F_{(1,20)} = 0.575$, $p = 0.457$
Serum testosterone	$F_{(1,20)} = 15.366$, $p < 0.001$	$F_{(1,20)} = 13.348$, $p = 0.002$
Serum estradiol	$F_{(1,17)} = 10.318$, $p = 0.005$	$F_{(1,17)} = 0.689$, $p = 0.418$
Aromatase mRNA	$F_{(1,20)} = 13.083$, $p = 0.002$	$F_{(1,20)} = 0.006$, $p = 0.938$
Erα mRNA	$F_{(1,19)} = 18.886$, $p < 0.001$	$F_{(1,19)} = 0.098$, $p = 0.757$
Erβ mRNA	$F_{(1,19)} = 0.0859$, $p = 0.773$	$F_{(1,19)} = 0.942$, $p = 0.344$
Nkcc1 mRNA	$F_{(1,20)} = 19.093$, $p < 0.001$	$F_{(1,20)} = 0.563$, $p = 0.462$
Kcc2 mRNA	$F_{(1,20)} = 11.595$, $p = 0.003$	$F_{(1,20)} = 0.740$, $p = 0.400$
Nkcc1/Kcc2 mRNA	$F_{(1,20)} = 19.435$, $p < 0.001$	$F_{(1,20)} = 2.790$, $p = 0.110$

Aromatase, aromatase gene; Erα, estrogen receptor α gene; Erβ, estrogen receptor β gene; Nkcc1, Na^+-K^+-Cl^- cotransporter gene; Kcc2, K^+-Cl^- cotransporter gene.

($F_{(2,23)} = 18.788$, $P < 0.001$), the total duration ($F_{(2,23)} = 22.505$, $P < 0.001$), and the duration of a single episode ($F_{(2,23)} = 12.103$, $P < 0.001$) of EEG-detectable seizures caused by sevoflurane were significantly diminished in the pretreated male rats (**Figures 4A, B**). Specifically, compared with male rats pretreated with the vehicle (the Vehicle + Sevo group), the total duration ($P < 0.001$, both the Bicuculline + Sevo and Formestane + Sevo groups), the number of episodes ($P < 0.001$, both the Bicuculline + Sevo and Formestane + Sevo groups), and the duration of a single episode ($P < 0.001$, the Formestane + Sevo group; $P = 0.004$, the Bicuculline + Sevo group) were reduced in rats pretreated with either agent. Also, there were between-subjects effects of pretreatments prior to exposure to sevoflurane on the total duration ($F_{(2,20)} = 9.496$, $P = 0.001$) and the number of episodes ($F_{(2,20)} = 8.107$, $P = 0.003$), but not on the duration of a single episode ($F_{(2,20)} = 1.930$, $P = 0.171$) of EEG-detectable seizures caused by sevoflurane in female rat pups (**Figure 4B**). Again, significant reductions of the total duration ($P = 0.004$, both the Bicuculline + Sevo and Formestane + Sevo groups) and the number of episodes ($P = 0.007$, the Formestane + Sevo group; $P = 0.006$, the Bicuculline + Sevo group) were observed in female rats pretreated with bicuculline or formestane.

There were no between-subjects effects of bicuculline or formestane on the serum levels of T in males ($F_{(2,15)} = 0.459$, $P = 0.641$; **Figure 4C**) and females ($F_{(2,15)} = 0.706$, $P = 0.509$; **Figure 4C**). In contrast to serum levels of T, there were between-subjects effects of the pretreatments on serum levels of E2 in males ($F_{(2,13)} = 6.349$, $P = 0.012$; **Figure 4D**). Pairwise multiple comparison analysis showed that bicuculline ($P = 0.020$ vs. the Vehicle + Sevo group) and formestane ($P = 0.029$ vs. the Vehicle + Sevo group) reduced serum levels of E2 in male rat

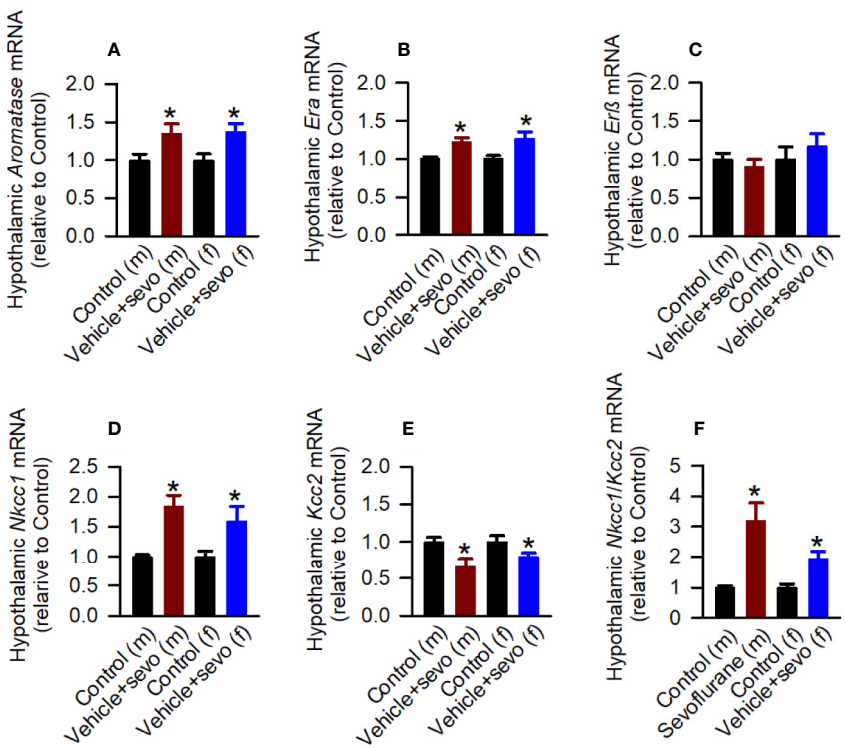

FIGURE 3 | Effects of sevoflurane in postnatal day 5 rats at the molecular level. Brain tissue samples were collected from male and female rats immediately after completion of electroencephalography (EEG) recordings during 2 h of baseline activity (the Control group) or 1 h of baseline activity followed by another hour during exposure to sevoflurane (the Vehicle + Sevo group). **(A–F)** The respective levels of *aromatase* mRNA, estrogen receptor α (*Erα*) mRNA, estrogen receptor β (*Erβ*) mRNA, Na^+-K^+-Cl^- cotransporter *(Nkcc1)* mRNA, K^+-Cl^- cotransporter (*Kcc2*) mRNA, and *Nkcc1/Kcc2* mRNA ratio in the hypothalamus of male and female rats. Data normalized against control are means ± SEM from six rats/group. *$P < 0.05$ vs. the Control group.

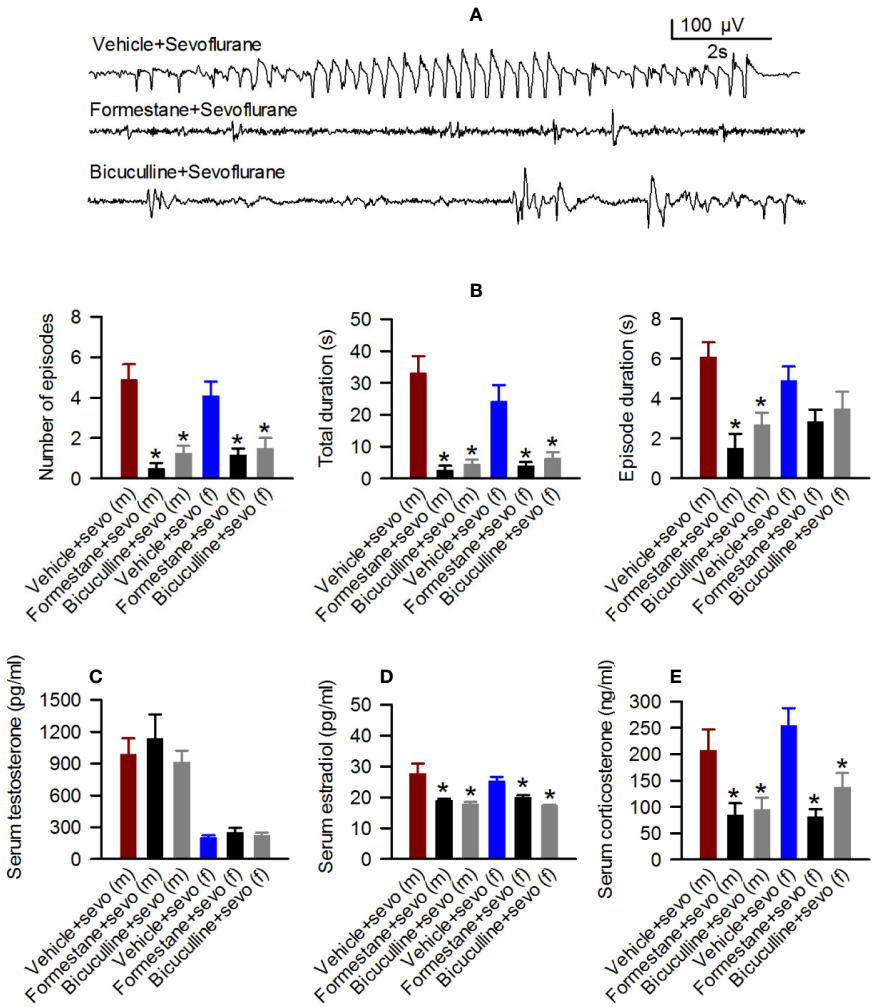

FIGURE 4 | Effects of bicuculline and formestane on sevoflurane-caused electroencephalogram (EEG)-detectable seizures and increases in serum levels of testosterone, estradiol and corticosterone in postnatal day (P) 5 rats. **(A, B)** Effect of bicuculline and formestane on sevoflurane-caused seizures in male and female rats. **(A)** examples of EEGs in sevoflurane-anesthetized P5 male rats that received vehicle, formestane, or bicuculline 30 min prior to initiation of sevoflurane anesthesia. **(B)** Histograms showing parameters of EEG-detectable seizures in male and female rats exposed to sevoflurane that received as pretreatment vehicle, bicuculline, or formestane. Data are means ± SEM from 10 males in the Vehicle + Sevo group, 8 males in the Bicuculline + Sevo group and in the Formestane + Sevo group, 9 females in the Vehicle + Sevo o group, 8 females in the Bicuculline + Sevo group, and 6 females in the Formestane + Sevo group. Animals in the Vehicle + Sevo groups are the same as in **Figure 2**. *$P < 0.05$ vs. the Vehicle + Sevo group in the respective sex. **(C–E)** Plots showing serum levels of testosterone, estradiol, and corticosterone in blood samples collected after completion of EEG recordings as in **(B)** Data are means ± SEM from six rats/group, five rats/group for estradiol measurements, except six males group in the Vehicle + Sevo group. *$P < 0.05$ vs. the Vehicle + Sevo group in the respective sex. The tissue samples in the Vehicle + Sevo groups are from the same animals as in **Figures 2** and **3**.

pups. Similarly, effects of bicuculline and formestane on serum levels of E2 were found in females ($F_{(2,12)} = 17.842$, $P < 0.001$; **Figure 4D**). Both bicuculline ($P < 0.001$ vs. the Vehicle + Sevo group) and formestane ($P = 0.005$ vs. the Vehicle + Sevo) reduced serum levels of E2 in female rat pups. Pretreatments with bicuculline or formestane had significant effects on serum levels of corticosterone increased by sevoflurane anesthesia in males ($F_{(2,15)} = 5.543$, $P = 0.016$) and in females ($F_{(2,15)} = 11.780$, $P < 0.001$; **Figure 4E**). Compared to the Vehicle + Sevo group, both bicuculline ($P = 0.029$, males; $P = 0.012$, females) and formestane ($P = 0.027$, males; $P < 0.001$, females) reduced serum levels of corticosterone (**Figure 4E**).

In the hypothalamic gene expression measurements, there was a statistically significant between-subjects effect of pretreatments on the hypothalamic levels of *aromatase* mRNA in males ($F_{(2,15)} = 3.893$, $P = 0.043$; **Figure 5A**) and females ($F_{(2,15)} = 4.949$, $P = 0.022$; **Figure 5A**). Pairwise multiple comparison analysis showed that compared to the Vehicle + Sevo group, formestane ($P = 0.015$, males; $P = 0.009$, females) but not bicuculline ($P = 0.317$, males, $P = 0.557$, females) ameliorated

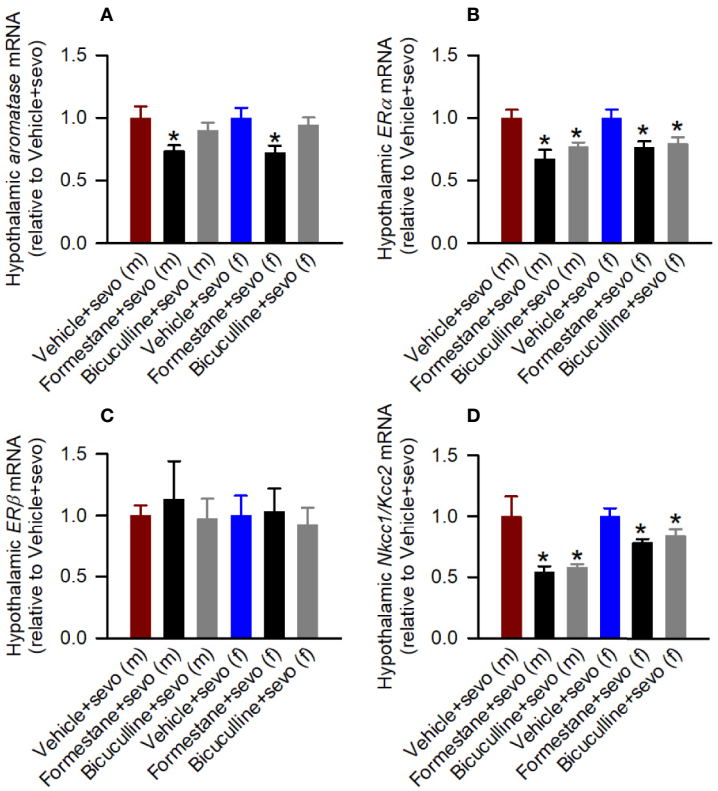

FIGURE 5 | Effects of bicuculline and formestane on sevoflurane-induced changes in gene expressions in the hypothalamus of postnatal day (P) 5 rats. Brain tissue samples were collected from male and female rats immediately after completion of electroencephalography recordings in sevoflurane-anesthetized P5 rats that received vehicle (the Vehicle + Sevo group), formestane (the Formestane + Sevo group), or bicuculline (the Bicuculline + Sevo group) 30 min prior to initiation of sevoflurane anesthesia. **(A–D)** The respective levels of *aromatase* mRNA, estrogen receptor α (*Erα*) mRNA, estrogen receptor β (*Erβ*) mRNA, Na^+-K^+-Cl^- cotransporter *(Nkcc1)*/K^+-Cl^- cotransporter (*Kcc2*) mRNA ratios in the hypothalamus of male and female rats. Data are means ± SEM from six rats/group. *$P < 0.05$ vs. the sevoflurane group in the respective sex. The tissue samples in the Vehicle + Sevo groups are from the same animals as in **Figure 3**.

sevoflurane-induced increase in *aromatase* expression. Similarly, there was a statistically significant between-subjects effect of pretreatments on the hypothalamic levels of *Erα* mRNA in males ($F_{(2,15)} = 8.176$, $P = 0.004$; **Figure 5B**), and females ($F_{(2,15)} = 5.208$, $P = 0.019$; **Figure 5B**), but not on the hypothalamic levels of *Erβ* mRNA in males ($F_{(2,15)} = 0.168$, $P = 0.847$; **Figure 5C**) and females ($F_{(2,15)} = 0.107$, $P = 0.899$; **Figure 5C**). Pairwise multiple comparison analysis showed that compared to the Vehicle + Sevo group, formestane ($P = 0.004$, males; $P = 0.030$, females) and bicuculline ($P = 0.028$, males, $P = 0.039$, females) ameliorated the sevoflurane-induced increase in *Erα* expression.

There was a statistically significant between-subjects effect of pretreatments with formestane and bicuculline on the hypothalamic *Nkcc1*/*Kcc2* mRNA ratios in males ($F_{(2,15)} = 6.487$, $P = 0.009$; **Figure 5D**) and in females ($F_{(2,15)} = 4.578$, $P = 0.028$; **Figure 5D**). The *Nkcc1*/*Kcc2* mRNA ratios compared with the Vehicle + Sevo group were decreased by pretreatments with bicuculline or formestane in males ($P = 0.019$, the Bicuculline + Sevo group; $P = 0.016$, the Formestane + Sevo group) and in females ($P = 0.049$, the Bicuculline + Sevo group; and $P = 0.001$, the Formestane + Sevo group).

Effects of Pretreatments With Estrogen Receptor Antagonists

In males, there were statistically significant effects of pretreatments with estrogen receptor antagonists on the total durations of sevoflurane-induced seizures ($F_{(3,22)} = 12.824$, $P < 0.001$; **Figures 6A, B**), the number of seizure episodes ($F_{(3,22)} = 9.614$, $P < 0.001$; **Figure 6B**), and the episode's duration ($F_{(3,22)} = 4.577$, $P = 0.022$; **Figure 6B**). Pairwise multiple comparison analysis showed that compared with the Vehicle + Sevo group, only the ERα antagonist MPP reduced the total durations of seizures ($P < 0.001$, the MPP + Sevo group; and $P = 0.097$, the PHTPP + Sevo group), the number of seizure episodes ($P = 0.002$, the MPP + Sevo group; $P = 0.859$, the PHTPP + Sevo group) and the episode's duration ($P = 0.019$, the MPP + Sevo group; $P = 0.244$, the PHTPP + Sevo group). Similarly, in females there were statistically significant effects of pretreatments with estrogen receptor antagonists on the total durations of sevoflurane-induced seizures ($F_{(3,22)} = 6.918$, $P = 0.005$; **Figure 6B**) and on the number of seizure episodes ($F_{(3,22)} = 47.277$, $P = 0.004$; **Figure 6B**), but not on the episode's duration ($F_{(3,22)} = 2.291$, $P = 0.125$; **Figure 6B**). Again, pairwise multiple comparison analysis showed

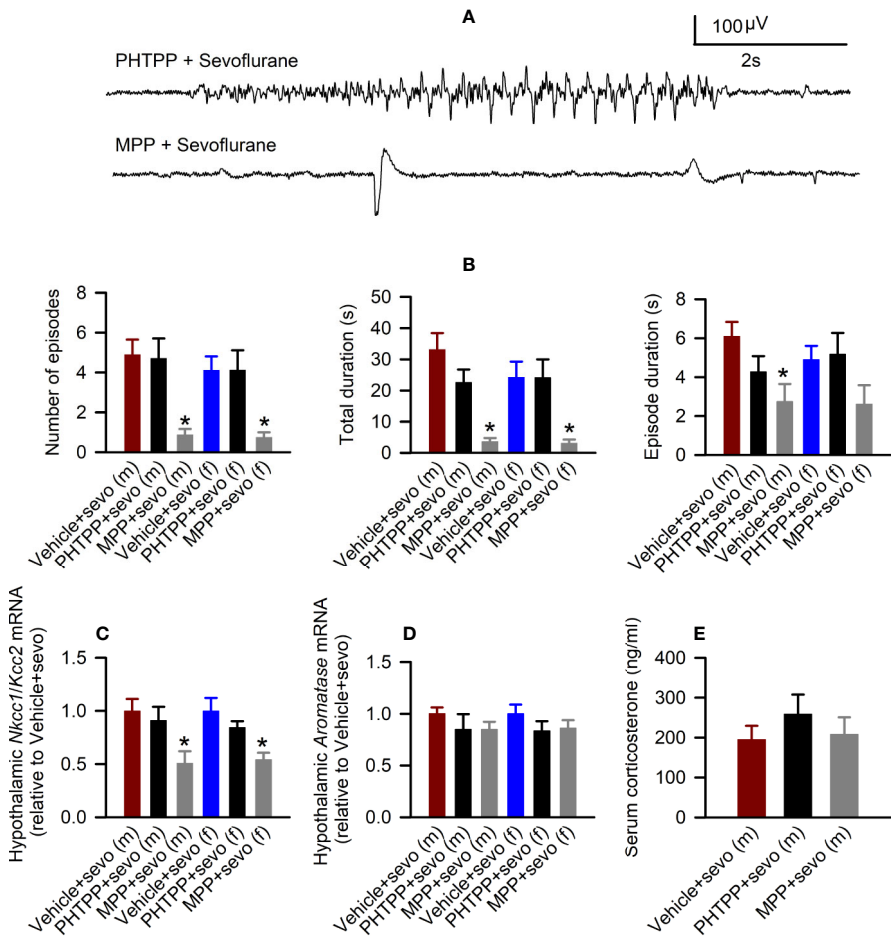

FIGURE 6 | Effects of the estrogen receptor α (ERα) and β (ERβ) antagonists on sevoflurane-induced abnormalities in postnatal day (P) 5 rats. **(A, B)** Effects of the ERα antagonist MPP and ERβ antagonist PHTPP on sevoflurane-caused electroencephalogram (EEG)-detectable seizures in male and female rats. **(A)** examples of EEGs in sevoflurane-anesthetized P5 male rats that received MPP (the MPP + Sevo group), or PHTPP (the PHTPP + Sevo group) 30 min prior to the initiation of sevoflurane anesthesia. **(B)** Histograms showing parameters of EEG-detectable seizures in male and female rats exposed to sevoflurane that received as pretreatment vehicle (the Vehicle + Sevo group), MPP (the MPP + Sevo group), or PHTPP (the PHTPP + Sevo group). Data are means ± SEM from 10 males in the Vehicle + Sevo group, 8 males in the MPP + Sevo group, 7 males in the PHTPP + Sevo group, 9 females in the Vehicle + Sevo group, 8 females in the MPP + Sevo group, and 8 females in the PHTPP + Sevo group. Animals in the Vehicle + Sevo groups are the same as in **Figure 2**. *$P < 0.05$ vs. the Vehicle + Sevo group in the respective sex. **(C)** The Na^+-K^+-Cl^- cotransporter *(Nkcc1)*/K^+-Cl^- cotransporter (*Kcc2*) mRNA ratios in the hypothalamus of male and female rats. Data are means ± SEM from 6 rats/group. *$P < 0.05$ vs. the Vehicle + Sevo group in the respective sex. The mRNA levels of aromatase in the male and female hypothalamus **(D)** and serum levels of corticosterone in males **(E)**. Data are means ± SEM from six rats/group. *$P < 0.05$ vs. the Vehicle + Sevo group in the respective sex. The tissue samples in the Vehicle + Sevo groups are from the same animals as in **Figures 2** and **3** .

that compared with the Vehicle + Sevo group only the ERα antagonist MPP reduced the total durations of seizures ($P = 0.010$, the MPP + Sevo group; and $P = 0.988$, the PHTPP + Sevo group) and the number of episodes ($P = 0.008$, the MPP + Sevo group; and $P = 0.989$, the PHTPP + Sevo group).

Also, there was a statistically significant between-subjects effect of pretreatments with estrogen receptor antagonists on the hypothalamic *Nkcc1*/*Kcc2* mRNA ratios in males ($F_{(2,15)} = 4.909$, $P = 0.023$; **Figure 6C**) or in females ($F_{(2,15)} = 7.188$, $P = 0.006$; **Figure 6C**). Pairwise multiple comparison analysis showed that compared with the sevoflurane group, only the ERα antagonist MPP ameliorated the effects of sevoflurane on the hypothalamic *Nkcc1*/*Kcc2* mRNA ratios in males ($P = 0.030$, the MPP + Sevo group; and $P = 0.600$, the PHTPP + Sevo group, **Figure 6C**) and females ($P = 0.006$, the MPP + Sevo group; and $P = 0.220$, the PHTPP + Sevo group; **Figure 6C**).

Despite the ameliorating effects of pretreatments with formestane on sevoflurane-induced increases in the serum levels of corticosterone (**Figure 4E**) and hypothalamic expression of aromatase (**Figure 5A**), pretreatments with either MPP or PHTP did not alter these effects of sevoflurane (**Figures 6D, E**). To test whether the GPER, which is expressed in the hypothalamus, hippocampus, and cortex (25) can be involved in the mediation of these effects of sevoflurane, we have studied the

effects of pretreatments with the GPER antagonist G-15 on sevoflurane-caused seizures and increases in serum levels of corticosterone in male rats. Compared with the Vehicle + Sevo group, pretreatments with G-15 had no effect on the total durations of sevoflurane-induced EEG-detectable seizures (33.1 ± 5.3 s, the Vehicle + Sevo group (n = 10), and 31.0 ± 10.1 s (n = 7), the G15 + Sevo group, $t_{(15)} = 0.200$; $P = 0.844$), as well as on the sevoflurane-caused increase in the serum levels of corticosterone (192.232 ± 24.225 ng/ml, the Vehicle + Sevo group, and 192.205 ± 27.253 ng/ml, the G15 + Sevo group, n = 6/group, $t_{(10)} = 0.001$; $P = 0.999$). All experimental findings are summarized in **Table 2**.

DISCUSSION

The novel findings of this study are that during the sensitive period, when T/E2 are known to program permanent changes in the rodent brain (organizational effects), sevoflurane administered to neonatal rats increases the systemic levels of T in males, while it elevates the levels of E2 and upregulates crucial components of the E2 and $GABA_AR$ signaling pathways in both sexes. The findings of this study indicate that the sevoflurane-increased E2 levels, which are independent of the sevoflurane-increased systemic levels of T, are essential to mediate sevoflurane's acute adverse effects, at least as they relate to measured gene expressions, EEG-detectable seizures, and stress-like corticosterone responses. Thus, despite the sevoflurane-caused increase in serum T levels in males, all other acute adverse effects of sevoflurane were similar in males and females. All of these acute adverse effects except the sevoflurane-caused increase in the systemic levels of T in males were deterred by pretreatments with the E2 synthesis inhibitor formestane.

Our findings, along with data in the literature, suggest that the brain is the source of heightened systemic levels of E2 in male and female pups anesthetized with sevoflurane. This possibility is consistent with the notion that the ovary, the main source of systemic E2 in females, is quiescent at this age (25, 26) and that both male and female rat pups have similar serum levels of E2 during this age period (38). Also, data in the literature show that removal at birth of the peripheral steroidogenic organs does not reduce the systemic levels of E2 and does not change the brain levels of E2 in rat pups (38). Our finding that the sevoflurane-induced increases in serum levels of E2 in both sexes, but not the sevoflurane-induced increases in serum levels of T in males, were reduced by pretreatments with bicuculline further supports the idea that there are different sources of the sevoflurane-induced increases in serum levels of E2 and T. Although bicuculline is considered poorly permeable through the blood-brain barrier, data in the literature (36) and our findings of the alleviating effects of bicuculline on sevoflurane-caused EEG-detectable seizures and changes in the hypothalamic expressions of estrogen receptors and Cl^- co-transporters suggest that bicuculline methiodide induces central effects in neonatal rats. The latter may include the bicuculline methiodide-caused reduction in the systemic levels of E2 in sevoflurane-exposed rat pups. Finally, the sevoflurane-induced saturating levels of E2 synthesized *de novo* in the brain may be a reason for the lack of additive effects of E2 that can be synthesized through the

TABLE 2 | Summary of the findings of the effects of sevoflurane and the studied agents (pretreatments) on the effects of sevoflurane in postnatal day 5 male and female rats.

Target	Effects of Sevoflurane on targets shown in the left column	
	Males	**Females**
Testosterone	Increased (↑)	No effect (0)
Estradiol	Increased (↑)	Increased (↑)
Corticosterone	Increased (↑)	Increased (↑)
Seizures	Increased (↑)	Increased (↑)
Nkcc1 mRNA	Increased (↑)	Increased (↑)
Kcc2 mRNA	Decreased (↓)	Decreased (↓)
Nkcc1/Kcc2 mRNA	Increased (↑)	Increased (↑)
Aromatase mRNA	Increased (↑)	Increased (↑)
Erα mRNA	Increased (↑)	Increased (↑)
Erβ mRNA	No effect (0)	No effect (0)

Target	Effects of pretreatments on the effects of Sevoflurane shown above									
	Pretreatments in males					Pretreatments in females				
	Bicuculline	Formestane	MPP	PHTPP	G-15	Bicuculline	Formestane	MPP	PHTPP	G-15
Testosterone	0	0				0	0			
Estradiol	↓	↓				↓	↓			
Corticosterone	↓	↓	0	0	0	↓	↓			
Seizures	↓	↓	↓	0	0	↓	↓	↓	0	0
Nkcc1/Kcc2 mRNA	↓	↓	↓	0	0	↓	↓	↓	0	0
Aromatase mRNA	0	↓	0	0	0	0	↓	0	0	0
Erα mRNA	↓	↓				↓	↓			
Erβ mRNA	0	0				0	0			

↑ and ↓ show a significant increase and decrease, respectively, caused by sevoflurane when compared to the Control group and by a pretreatment + sevoflurane compared with the Vehicle + Sevoflurane group. 0 means no significant effect and empty cells mean no measure was conducted. Testosterone, estradiol, and corticosterone were measured in serum. The mRNA levels of aromatase, aromatase gene; Erα, estrogen receptor α gene; Erβ, estrogen receptor β gene; Nkcc1, Na^+-K^+-Cl^- cotransporter gene; Kcc2, K^+-Cl^- cotransporter gene, were measured in the hypothalamus. See text for details.

aromatization of systemic T in the brain in male pups. All of these findings suggest that sevoflurane-increased levels of E2, which can be detected as elevated levels in the serum, are formed by E2 that is synthesized in the brain *de novo*. In future studies, it will be important to confirm or refute this possibility by measuring E2 and T levels in different regions of the brain in male and female pups anesthetized with sevoflurane.

If it is further confirmed that E2 synthesized in the brain *de novo*, but not E2 synthesized from systemic T, is involved in the mediation of the acute adverse effects of sevoflurane, this finding may be important for understanding the mechanisms of the developmental effects of sevoflurane. Moreover, it may be important for investigating the fundamental mechanisms of brain development and brain sexual differentiation in particular. It is known that T exerts lasting organizational effects in developing rodent brains (brain masculinization) primarily through its derivative E2 (23, 26). However, the relative roles of E2 synthesized from testis-derived T and E2 synthesized from T in the brain *de novo* are not fully understood (26, 38). Our findings suggest that at least in sevoflurane-anesthetized neonatal rats, the levels of E2 synthesized in the brain *de novo* may be high enough that the brain's E2 synthesized from testis-derived T does not induce additional acute effects.

The sex hormone-regulated perinatally organized neurocircuitry is activated by the adult steroid hormone environment to express sex-appropriate behavior and physiology, including stress responsivity (39, 40). We have previously demonstrated that adult rats neonatally exposed to sevoflurane or other anesthetics that act through $GABA_AR$s not only have an elevated *Nkcc1*/*Kcc2* mRNA ratio in the hypothalamus and hippocampus, but also exhibit behavioral deficiencies and exacerbated HPA axis responses to stress (8–12). These effects are more pronounced in males. The sevoflurane-induced changes in the systemic levels of T during the early postnatal period may contribute to the anesthetic-induced long-term sex-dependent abnormalities. Notably, although testis-produced T initiates organizational effects in male brain primarily through its aromatized metabolite E2 and subsequent activation of ERα, the androgen receptor-mediated effects of T are also required for T's organizational effects (41, 42). The organizational T effects in perinatal brain emerge later. The relatively modest short-term effects of T in perinatal brain are followed by dramatic changes in gene expression and behavior in adulthood (43, 44). Of potential relevance, adult male rats, neonatally treated with exogenous T, had significantly decreased circulating T levels and increased aromatase and E2 levels (45). These levels are typically associated with exacerbated HPA axis responses to stress. In future studies, it will be important to investigate in detail the long-term effects of alterations in levels of T and E2 induced by neonatal anesthesia with sevoflurane and other GABAergic anesthetics.

The sevoflurane-induced increases in E2 levels may contribute to acute functional abnormalities induced by sevoflurane in neonatal rats (e.g., EEG-detectable seizures and heightened corticosterone levels) by enhancing excitatory $GABA_AR$ signaling in the cortex and in the HPA axis, respectively. The $GABA_AR$-mediated acute effects of E2 are supported by our previously published findings that exogenous E2 potentiated sevoflurane-caused EEG-detectable seizures in rat pups and increased $GABA_AR$-mediated currents in hippocampal slices from these animals (10). The alleviating effects of the pretreatments with bicuculline and formestane on sevoflurane-caused, EEG-detectable seizures and heightened serum levels of corticosterone found in this study further support the $GABA_AR$-mediated effects of sevoflurane/E2. The findings in this study, however, suggest a much more complex involvement of E2 in the mediation of sevoflurane-induced adverse effects than just positive modulation of the $GABA_AR$-based neurotransmission machinery. Sevoflurane increased not only the levels of E2, but also the mRNA levels of hypothalamic *Erα* and the *Nkcc1*/*Kcc2* ratio. These effects were ameliorated by pretreatments with the inhibitors of $GABA_AR$ and E2 synthesis. The latter suggests a mediating role of $GABA_AR$-dependent E2 synthesis in the transcriptional effects of sevoflurane. Interestingly, the mechanisms mediating sevoflurane-induced changes in gene expression of different components of the E2 pathway may be different. Thus, formestane, but not bicuculline, alleviated the sevoflurane-caused increase in the hypothalamic levels of aromatase. Future studies will be needed to uncover the mediating mechanisms of these effects. This study was not designed to elucidate how changes in gene expressions in each specific experimental group translate to changes in respective protein levels. For that reason, future studies will be needed to determine the relative role of sevoflurane-induced changes in the E2 pathway gene transcriptions in the acute and long-term developmental effects of the anesthetic.

Notably, ERα predominantly, but not exclusively, localizes to the brain regions involved in regulating sexual behavior such as the hypothalamus, whereas ERβ has a broader distribution in the neurons of the hippocampus, cerebral cortex, and amygdala, as well as in microglia and oligodendrocytes (26–29). Nevertheless, the antagonist of ERα, but not ERβ, not only reduced the sevoflurane-increased hypothalamic *Nkcc1*/*Kcc2* mRNA ratio, but also depressed the sevoflurane-induced EEG-detectable seizures, which should predominantly reflect the brain's cortical activity. In addition, sevoflurane induced an increase in the expression of ERα but not ERβ in the hypothalamus of male and female rat pups, pointing to ERα-specific adverse effects of neonatal sevoflurane. An unexpected and unexplained finding was that the antagonist of ERα, MPP, despite having alleviating effects on sevoflurane-induced EEG-detectable seizures, and the increase in hypothalamic *Nkcc1*/*Kcc2* mRNA ratios did not affect sevoflurane-induced changes in serum levels of corticosterone and hypothalamic expression of *aromatase*. Although the aromatase inhibitor formestane alleviated these effects of sevoflurane, they were not sensitive to pretreatments with the antagonists of ERα, ERβ, and GPER. The latter is consistent with our previously reported observation that the nonselective estrogen receptor antagonist ICI182780 was also ineffective in preventing sevoflurane-caused increases in serum levels of corticosterone in neonatal rats (10). We studied the effects of the ERα, ERβ, or GPER selective antagonists using a single dose for each compound. Therefore, the possibility that treatment with higher doses of the ERα selective antagonist may still be effective against sevoflurane-induced changes in serum corticosterone levels and *aromatase* expression cannot be ruled out. However, the alleviating effects of

MPP at this dose on sevoflurane-caused seizures and increases in the *Nkcc1*/*Kcc2* mRNA ratio reduces the probability that higher doses of MPP will be effective against sevoflurane-caused increases in serum corticosterone levels and hypothalamic expression of aromatase.

In conclusion, this study provides evidence that sevoflurane increases systemic levels of T in male rat pups and E2 levels in males and females during the sensitive period of the organizational effects of T and E2. Our findings demonstrate that sevoflurane-increased levels of E2, which are independent of sevoflurane-increased systemic levels of T, are sufficient to mediate sevoflurane-induced corticosterone secretion, EEG-detectable seizures, and complex alterations in expressions of genes that are crucial for the E2 and $GABA_AR$ signaling pathways. In this study, we provided evidence that a specific estrogen receptor, ERα, is involved in the mediation of E2-dependent acute adverse effects of sevoflurane in neonatal rats.

DATA AVAILABILITY STATEMENT

All relevant data is contained within the article: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by: All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AM and JZ conceptualized and designed the study. NL, YL, NX, and LL acquired data and performed data analysis. Analyses and interpretation of the data and writing of the article were performed by NL, AM, JZ, LJ, TM, and NG. All authors contributed to the article and approved the submitted version.

FUNDING

Supported by the Natural Science Foundation of China (Nos. 81771149 and U1704165 to JZ), the National Institutes of Health, Bethesda, MD, USA (Nos. R01NS091542, R01NS091542-S and R56HD102898 to AM), the I. Heermann Anesthesia Foundation (LJ), and the Jerome H. Modell, M.D., F.A.H.A. Endowed Professorship (NG).

REFERENCES

1. Weiser TG, Haynes AB, Molina G, Lipsitz SR, Esquivel MM, Uribe-Leitz T, et al. Size and distribution of the global volume of surgery in 2012. *Bull World Health Organ* (2016) 94(3):201–9f. doi: 10.2471/blt.15.159293
2. Brown EN, Lydic R, Schiff ND. General anesthesia, sleep, and coma. *N Engl J Med* (2010) 363(27):2638–50. doi: 10.1056/NEJMra0808281
3. Hsu AA, von Elten K, Chan D, Flynn T, Walker K, Barnhill J, et al. Characterization of the cortisol stress response to sedation and anesthesia in children. *J Clin Endocrinol Metab* (2012) 97(10):E1830–5. doi: 10.1210/jc.2012-1499
4. Lin EP, Lee JR, Lee CS, Deng M, Loepke AW. Do anesthetics harm the developing human brain? An integrative analysis of animal and human studies. *Neurotoxicol Teratol* (2017) 60:117–28. doi: 10.1016/j.ntt.2016.10.008
5. Rains PC, Rampersad N, De Lima J, Murrell D, Kinchington D, Lee JW, et al. Cortisol response to general anaesthesia for medical imaging in children. *Clin Endocrinol* (2009) 71(6):834–9. doi: 10.1111/j.1365-2265.2009.03591.x
6. Vutskits L, Xie Z. Lasting impact of general anaesthesia on the brain: mechanisms and relevance. *Nat Rev Neurosci* (2016) 17(11):705–17. doi: 10.1038/nrn.2016.128
7. Banerjee P, Rossi MG, Anghelescu DL, Liu W, Breazeale AM, Reddick WE, et al. Association Between Anesthesia Exposure and Neurocognitive and Neuroimaging Outcomes in Long-term Survivors of Childhood Acute Lymphoblastic Leukemia. *JAMA Oncol* (2019) 5(10):1456–63. doi: 10.1001/jamaoncol.2019.1094
8. Tan S, Xu C, Zhu W, Willis J, Seubert CN, Gravenstein N, et al. Endocrine and neurobehavioral abnormalities induced by propofol administered to neonatal rats. *Anesthesiology* (2014) 121(5):1010–7. doi: 10.1097/aln.0000000000000366
9. Xu C, Tan S, Zhang J, Seubert CN, Gravenstein N, Sumners C, et al. Anesthesia with sevoflurane in neonatal rats: Developmental neuroendocrine abnormalities and alleviating effects of the corticosteroid and Cl(-) importer antagonists. *Psychoneuroendocrinology* (2015) 60:173–81. doi: 10.1016/j.psyneuen.2015.06.016
10. Zhang J, Xu C, Puentes DL, Seubert CN, Gravenstein N, Martynyuk AE. Role of Steroids in Hyperexcitatory Adverse and Anesthetic Effects of Sevoflurane in Neonatal Rats. *Neuroendocrinology* (2016) 103(5):440–51. doi: 10.1159/000437267
11. Ju LS, Yang JJ, Gravenstein N, Seubert CN, Morey TE, Sumners C, et al. Role of environmental stressors in determining the developmental outcome of neonatal anesthesia. *Psychoneuroendocrinology* (2017) 81:96–104. doi: 10.1016/j.psyneuen.2017.04.001
12. Ju LS, Yang JJ, Morey TE, Gravenstein N, Seubert CN, Resnick JL, et al. Role of epigenetic mechanisms in transmitting the effects of neonatal sevoflurane exposure to the next generation of male, but not female, rats. *Br J Anaesth* (2018) 121(2):406–16. doi: 10.1016/j.bja.2018.04.034
13. Sasaki Russell JM, Chinn GA, Maharjan D, Eichbaum Y, Sall JW. Female rats are more vulnerable to lasting cognitive impairment after isoflurane exposure on postnatal day 4 than 7. *Br J Anaesth* (2019) 122(4):490–9. doi: 10.1016/j.bja.2018.12.008
14. Ben-Ari Y. The GABA excitatory/inhibitory developmental sequence: a personal journey. *Neuroscience* (2014) 279:187–219. doi: 10.1016/j.neuroscience.2014.08.001
15. Khazipov R, Valeeva G, Khalilov I. Depolarizing GABA and developmental epilepsies. *CNS Neurosci Ther* (2015) 21(2):83–91. doi: 10.1111/cns.12353
16. Lemonnier E, Villeneuve N, Sonie S, Serret S, Rosier A, Roue M, et al. Effects of bumetanide on neurobehavioral function in children and adolescents with autism spectrum disorders. *Transl Psychiatry* (2017) 7(3):e1056. doi: 10.1038/tp.2017.10
17. Luscher B, Fuchs T. GABAergic control of depression-related brain states. *Adv Pharmacol* (2015) 73:97–144. doi: 10.1016/bs.apha.2014.11.003
18. Tang X, Kim J, Zhou L, Wengert E, Zhang L, Wu Z, et al. KCC2 rescues functional deficits in human neurons derived from patients with Rett syndrome. *Proc Natl Acad Sci U S A* (2016) 113(3):751–6. doi: 10.1073/pnas.1524013113
19. Guerra-Araiza C, Miranda-Martinez A, Neri-Gómez T, Camacho-Arroyo I. Sex steroids effects on the content of GAD, TH, GABA(A), and glutamate receptors in the olfactory bulb of the male rat. *Neurochem Res* (2008) 33(8):1568–73. doi: 10.1007/s11064-008-9665-1
20. Nuñez JL, McCarthy MM. Estradiol exacerbates hippocampal damage in a model of preterm infant brain injury. *Endocrinology* (2003) 144(6):2350–9. doi: 10.1210/en.2002-220840
21. Perrot-Sinal TS, Davis AM, Gregerson KA, Kao JP, McCarthy MM. Estradiol enhances excitatory gamma-aminobutyric [corrected] acid-mediated calcium signaling in neonatal hypothalamic neurons. *Endocrinology* (2001) 142(6):2238–43. doi: 10.1210/endo.142.6.8180

22. Wójtowicz T, Lebida K, Mozrzymas JW. 17beta-estradiol affects GABAergic transmission in developing hippocampus. *Brain Res* (2008) 1241:7–17. doi: 10.1016/j.brainres.2008.09.005
23. Nugent BM, Valenzuela CV, Simons TJ, McCarthy MM. Kinases SPAK and OSR1 are upregulated by estradiol and activate NKCC1 in the developing hypothalamus. *J Neurosci* (2012) 32(2):593–8. doi: 10.1523/jneurosci.5415-11.2012
24. Perrot-Sinal TS, Sinal CJ, Reader JC, Speert DB, McCarthy MM. Sex differences in the chloride cotransporters, NKCC1 and KCC2, in the developing hypothalamus. *J Neuroendocrinol* (2007) 19(4):302–8. doi: 10.1111/j.1365-2826.2007.01530.x
25. McCarthy MM, Nugent BM, Lenz KM. Neuroimmunology and neuroepigenetics in the establishment of sex differences in the brain. *Nat Rev Neurosci* (2017) 18(8):471–84. doi: 10.1038/nrn.2017.61
26. McCarthy MM. The two faces of estradiol: effects on the developing brain. *Neuroscientist* (2009) 15(6):599–610. doi: 10.1177/1073858409340924
27. Brock O, De Mees C, Bakker J. Hypothalamic expression of oestrogen receptor α and androgen receptor is sex-, age- and region-dependent in mice. *J Neuroendocrinol* (2015) 27(4):264–76. doi: 10.1111/jne.12258
28. Fan X, Xu H, Warner M, Gustafsson JA. ERbeta in CNS: new roles in development and function. *Prog Brain Res* (2010) 181:233–50. doi: 10.1016/s0079-6123(08)81013-8
29. Hara Y, Waters EM, McEwen BS, Morrison JH. Estrogen Effects on Cognitive and Synaptic Health Over the Lifecourse. *Physiol Rev* (2015) 95(3):785–807. doi: 10.1152/physrev.00036.2014
30. Edwards DA, Shah HP, Cao W, Gravenstein N, Seubert CN, Martynyuk AE. Bumetanide alleviates epileptogenic and neurotoxic effects of sevoflurane in neonatal rat brain. *Anesthesiology* (2010) 112(3):567–75. doi: 10.1097/ALN.0b013e3181cf9138
31. Sanders RD, Hassell J, Davidson AJ, Robertson NJ, Ma D. Impact of anaesthetics and surgery on neurodevelopment: an update. *Br J Anaesth* (2013) 110 Suppl 1(Suppl 1):i53–72. doi: 10.1093/bja/aet054
32. Stratmann G. Review article: Neurotoxicity of anesthetic drugs in the developing brain. *Anesth Analg* (2011) 113(5):1170–9. doi: 10.1213/ANE.0b013e318232066c
33. Willis J, Zhu W, Perez-Downes J, Tan S, Xu C, Seubert C, et al. Propofol-induced electroencephalographic seizures in neonatal rats: the role of corticosteroids and γ-aminobutyric acid type A receptor-mediated excitation. *Anesth Analg* (2015) 120 (2):433–9. doi: 10.1213/ane.0000000000000529
34. Cao W, Pavlinec C, Gravenstein N, Seubert CN, Martynyuk AE. Roles of aldosterone and oxytocin in abnormalities caused by sevoflurane anesthesia in neonatal rats. *Anesthesiology* (2012) 117(4):791–800. doi: 10.1097/ALN.0b013e318266c62d
35. Orliaguet G, Vivien B, Langeron O, Bouhemad B, Coriat P, Riou B. Minimum alveolar concentration of volatile anesthetics in rats during postnatal maturation. *Anesthesiology* (2001) 95(3):734–9. doi: 10.1097/00000542-200109000-00028
36. Mares P, Chino M, Kubová H, Mathern P, Veliký M. Convulsant action of systemically administered glutamate and bicuculline methiodide in immature rats. *Epilepsy Res* (2000) 42(2-3):183–9. doi: 10.1016/s0920-1211(00)00179-0
37. Quennell JH, Howell CS, Roa J, Augustine RA, Grattan DR, Anderson GM. Leptin deficiency and diet-induced obesity reduce hypothalamic kisspeptin expression in mice. *Endocrinology* (2011) 152(4):1541–50. doi: 10.1210/en.2010-1100
38. Konkle AT, McCarthy MM. Developmental time course of estradiol, testosterone, and dihydrotestosterone levels in discrete regions of male and female rat brain. *Endocrinology* (2011) 152(1):223–35. doi: 10.1210/en.2010-0607
39. Clarkson J, Herbison AE. Hypothalamic control of the male neonatal testosterone surge. *Philos Trans R Soc Lond B Biol Sci* (1688) 2016) 371:20150115. doi: 10.1098/rstb.2015.0115
40. Seale JV, Wood SA, Atkinson HC, Lightman SL, Harbuz MS. Organizational role for testosterone and estrogen on adult hypothalamic-pituitary-adrenal axis activity in the male rat. *Endocrinology* (2005) 146(4):1973–82. doi: 10.1210/en.2004-1201
41. Sato T, Matsumoto T, Kawano H, Watanabe T, Uematsu Y, Sekine K, et al. Brain masculinization requires androgen receptor function. *Proc Natl Acad Sci U S A* (2004) 101(6):1673–8. doi: 10.1073/pnas.0305303101
42. Zhang JM, Tonelli L, Regenold WT, McCarthy MM. Effects of neonatal flutamide treatment on hippocampal neurogenesis and synaptogenesis correlate with depression-like behaviors in preadolescent male rats. *Neuroscience* (2010) 169(1):544–54. doi: 10.1016/j.neuroscience.2010.03.029
43. Forgie ML, Kolb B. Manipulation of gonadal hormones in neonatal rats alters the morphological response of cortical neurons to brain injury in adulthood. *Behav Neurosci* (2003) 117(2):257–62. doi: 10.1037/0735-7044.117.2.257
44. Ghahramani NM, Ngun TC, Chen PY, Tian Y, Krishnan S, Muir S, et al. The effects of perinatal testosterone exposure on the DNA methylome of the mouse brain are late-emerging. *Biol Sex Differ* (2014) 5:8. doi: 10.1186/2042-6410-5-8
45. Persky RW, Liu F, Xu Y, Weston G, Levy S, Roselli CE, et al. Neonatal testosterone exposure protects adult male rats from stroke. *Neuroendocrinology* (2013) 97 (3):271–82. doi: 10.1159/000343804

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.

frontiers in Endocrinology

SYSTEMATIC REVIEW
published: 30 September 2020
doi: 10.3389/fendo.2020.541802

Neurosteroids and Seizure Activity

Barbara Miziak[1], *Magdalena Chrościńska-Krawczyk*[2] *and Stanisław J. Czuczwar*[1]*

[1] Department of Pathophysiology, Medical University of Lublin, Lublin, Poland, [2] Department of Child Neurology, Medical University of Lublin, Lublin, Poland

Still circa 25% to 30% of patients with epilepsy cannot be efficiently controlled with available antiepileptic drugs so newer pharmacological treatment options have been continuously searched for. In this context, a group of endogenous or exogenous neurosteroids allosterically positively modulating GABA-A receptors may offer a promising approach. Among endogenous neurosteroids synthesized in the brain, allopregnanolone or allotetrahydrodeoxycorticosterone have been documented to exert anticonvulsant activity in a number of experimental models of seizures—pentylenetetrazol-, bicuculline- pilocarpine-, or 6 Hz-induced convulsions in rodents. Neurosteroids can also inhibit fully kindled seizures and some of them have been reported to counteract maximal electroshock-induced convulsions. An exogenous neurosteroid, alphaxalone, significantly elevated the threshold for maximal electroconvulsions in mice but it did not potentiate the anticonvulsive action of a number of conventional antiepileptic drugs against maximal electroshock-induced seizures. Androsterone not only elevated the threshold but significantly enhanced the protective action of carbamazepine, gabapentin and phenobarbital against maximal electroshock in mice, as well. Ganaxolone (a 3beta-methylated analog of allopregnanolone) needs special consideration for two reasons. First, it performed better than conventional antiepileptic drugs, diazepam or valproate, in suppressing convulsive and lethal effects of pentylenetetrazol in pentylenetetrazol-kindled mice. Second, ganaxolone has been evaluated in the randomized, double-blind, placebo-controlled phase 2 trial in patients with intractable partial seizures, taking maximally 3 antiepileptic drugs. The initial results indicate that add-on therapy with ganaxolone resulted in reduced seizure frequency with adverse effect being mainly mild to moderate. Possibly, ganaxolone may be also considered against catamenial seizures. Some positive effects of ganaxolone as an adjuvant were also observed in children with refractory seizures and its use may also prove efficient for the management of neonatal seizures associated with hypoxic injury. Neurosteroids positively modulating GABA-A receptor complex exert anticonvulsive activity in many experimental models of seizures. Their interactions with antiepileptic drugs seem ambiguous in mice. Initial clinical data indicate that ganaxolone may provide a better seizure control in patients with drug-resistant epilepsy.

Keywords: neurosteroids, ganaxolone, seizures, epilepsy, catamenial epilepsy

OPEN ACCESS

Edited by:
Takayoshi Ubuka, Waseda University, Japan

Reviewed by:
Giovambattista De Sarro, University of Catanzaro, Italy
Ashish Dhir, University of California, Davis, United States
Giuseppe Biagini, University of Modena and Reggio Emilia, Italy

***Correspondence:**
Stanisław J. Czuczwar
czuczwarsj@yahoo.com

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

Received: *16 March 2020*
Accepted: *09 September 2020*
Published: *30 September 2020*

Citation:
Miziak B, Chrościńska-Krawczyk M and Czuczwar SJ (2020) Neurosteroids and Seizure Activity. Front. Endocrinol. 11:541802. doi: 10.3389/fendo.2020.541802

INTRODUCTION

Epilepsy is a neurological disease which affects more than 70 million of the global population, 30% of whom are patients with refractory epilepsy (drug-resistant epilepsy) (1–3). Research conducted globally has invariably shown that, in terms of risk factors, epilepsy is hardly ever caused by a single determinant. A number of underlying causes are usually present, together with a genetic predisposition to the disease. Huge progress in terms of medical and pharmacological studies has facilitated the discovery of antiepileptic drugs (AEDs), which in one third of cases can suppress epileptic seizures, yet they do not affect in any way the root cause of the disease, so one cannot expect any improvements in the long-term prognosis of patients. Other solutions include surgical treatments, which might seem the most effective therapy. However, also in this case it has to be borne in mind that not every patient can undergo such a procedure (1, 4). In recent years other strategies in epilepsy treatment have emerged; however, there is still a great necessity for other options to be pursued, among which the most effective would be treatment impacting the etiopathogenesis of the disease, which would help to manage the condition, particularly in patients with refractory epilepsy (1, 2). Refractory seizures still affect about one third of patients with epilepsy in spite of a number of newer AEDs which appeared on the market over the last two decades (5). Consequently, there is a continuous search for more effective strategies and possibly neurosteroids could reduce the number of refractory epilepsy patients. When a newer or potential AED is evaluated, it is used in the form of an add-on therapy to the already existing antiepileptic treatment (6) and this is also the case with neurosteroids tried in patients with drug-resistant epilepsy (see below).

METHODS

Literature search for this review was generally based on English language articles with a few exceptions. PUBMED databases were the main source of relevant papers and the search areas included: neurosteroids, neurosteroids and seizure activity, neurosteroids and AEDs, neurosteroids and epilepsy. Some references of particular importance were searched for in the most relevant publications extracted from PUBMED.

NEUROSTEROIDS

Among the endogenous and exogenous steroid compounds, it is possible to distinguish neurosteroids—compounds possessing the ability to modulate neuronal activity and affect the physiology of the central nervous system (CNS). There is a distinction between steroid hormones which are secreted outside the nervous system (by endocrine glands) and neurosteroids (7). The term neurosteroids was introduced by Baulieu in 1981. This name is usually given to steroids which are synthesised *de novo* from cholesterol in the CNS, mainly by the glial cells and by neuronal mitochondria through pathways which are independent of the endocrine system. At first, only the elevation of dehydroepiandrosterone sulfate (DHEAS) was observed that was found in high concentrations in the brain long after gonadectomy and adrenalectomy. Only at a later stage did it occur that DHEAS could be also synthesised in the brain. Other neurosteroids were discovered with time, including androstenedione, pregnenolone (with their sulfates and lipid derivatives) as well as tetrahydrometabolites of progesterone and deoxycorticosterone (DOC) (7). There are two groups of neurosteroids—natural (endogenous—produced in the brain) and exogenous (**Figure 1**).

Mechanism of neurosteroid action is connected with allosteric regulation of gamma-aminobutyric acid (GABA)-A receptors, *N*-methyl-D-aspartate (NMDA) receptors, alpha1 glycine receptors, sigma receptors and voltage-dependent calcium channels (8–11). Neurosteroids potentiate synaptic GABA-A receptor function and also activate δ-subunit-containing extrasynaptic GABA-A receptors that mediate tonic currents and thus may play an important role in neuronal network excitability and seizure susceptibility (12).

Depending on their concentration, neurosteroids can directly activate receptors (in the case of high concentrations) or act as potent positive allosteric agonists (in the case of low concentrations) (12–15). In addition, these compounds can have a positive or a negative impact on the modulation of GABA-A receptor activity. The mechanism based on positive modulation was observed in the case of such neurosteroids as allopregnanolone (5alpha-pregnan-3alpha-ol-20-one, ALLO), androsterone, progesterone, DOC, and tetrahydrodeoxy-corticosterone (THDOC). Through their binding to a specific place of the receptor, the above compounds bring about changes to its conformation, which results in the influx of Cl^- ions into a neuron. Local hyperpolarization of the neuron which occurs in such situations leads to the inhibition of the cell's activity (12, 16). Neurosteroids in fact allosterically modulate synaptic and extrasynaptic GABA-A receptors, exhibiting a greater potency for the latter, containing delta subunits. Positive modulators, acting on extrasynaptic GABA-A receptors, located in the dentate gyrus of the hippocampus, may reduce seizure susceptibility (17). The second group includes compounds (pregnenolone sulfate and DHEAS), which suppress the GABAergic response through the mechanism of negative GABA-A receptor activity modulation. The above mechanism is based on the inhibition of chloride current, resulting in cell depolarization and activation (18). Further studies have shown that intracerebroventricular pregnenolone sulfate enhanced the convulsive activity of peripherally administered NMDA in mice and this effect was possibly dependent on the elevated concentration of hippocampal alanine, a precursor of glutamate and a probable co-agonist at the NMDA receptors (19).

The existing evidence also points to sigma1 receptor as a possible target for neurosteroids. In fact, some neurosteroids were demonstrated to displace sigma1 receptor radioligands *in vivo* and *in vitro* (20). Moreover, steroid sulfate esters (DHEAS or pregnenolone sulfate) differentially affected NMDA-induced noradrenaline release from preloaded hippocampal slices (DHEAS was an enhancer whilst pregnenolone sulfate—an

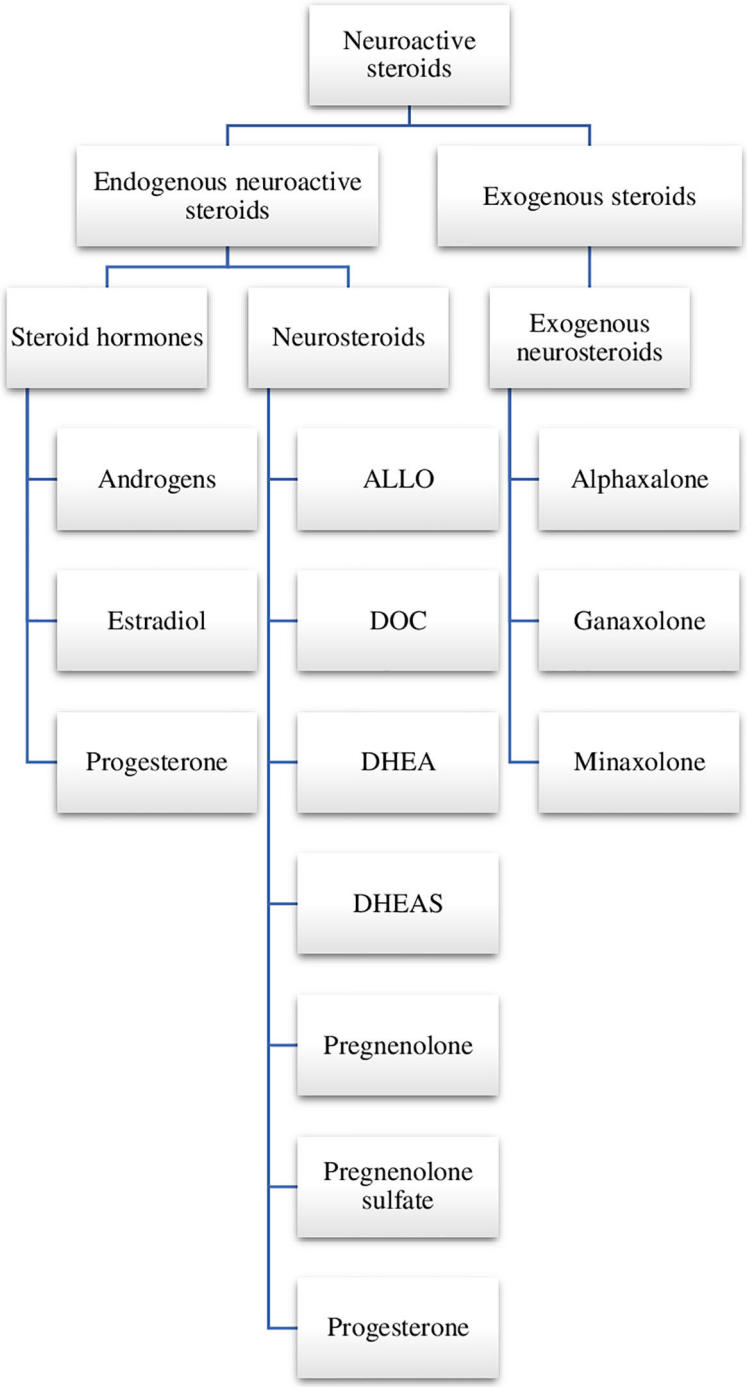

FIGURE 1 | Neuroactive steroids. ALLO, allopregnanolone; DOC, deoxycorticosterone; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; SGE-516, 1-((3R,5R,8R,9R,10S,13S,14S,17S)-3-hydroxy-3,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-(2H-1,2,3-triazol-2-yl)ethan-1-one.

inhibitor). The inhibitory effect upon this parameter was shared by the high-affinity inverse sigma agonist and the sigma antagonist, haloperidol, attenuated the effect of steroid sulfate esters (21). The authors of this study conclude that DHEAS behaves as a sigma agonist whilst pregnenolone sulfate—as a sigma inverse agonist. Interestingly, progesterone behaved identically to haloperidol so it seems to be a sigma receptor antagonist (21).

Literature data draw attention to the effect of progesterone on calcium signaling—progesterone-calcium signaling hypothesis,

which may explain therapeutic benefits of neurosteroids—a reduction of inflammation and edema, prevention of demyelination or inhibition of excitotoxic neuronal death. These detrimental effects are associated with significant elevations of the intracellular calcium concentration. Consequently, neuroprotection provided by progesterone seems dependent on the direct inhibition of voltage-gated calcium channels (22).

NEUROSTEROIDS AND SEIZURE ACTIVITY

Progesterone and Its Metabolite

There are studies which indicate that neurosteroids might also be important when it comes to the epileptogenesis described as the latent period. The latent period, which is a seizure-free period, has been defined as interval between the effect of the primary brain insult and the onset of the first spontaneous seizure. During this time, pathological changes occur in the brain, converting the normal brain into one that generates seizures (23).

In studies conducted on rats in the pilocarpine model of status epilepticus, it was found that during the latent period of epilepsy a higher expression of cytochrome P450 cholesterol side-chain cleavage (P450scc) enzyme was noted. This enzyme takes part in the brain synthesis of neurosteroids (24). Cholesterol is delivered by translocator protein (TSPO) into mitochondria in which P450scc is located (in the inner mitochondrial membrane). Next, cholesterol is cleaved to produce pregnenolone and is metabolized to tissue-specific steroids by enzymes, which operate in the endoplasmic reticulum (24, 25). Blocking neurosteroid synthesis by finasteride (a 5alpha-reductase and neurosteroid synthesis inhibitor) in rats after status epilepticus induced by pilocarpine, resulted in terminating the latent period and moreover, finasteride was evidently proconvulsant in rats with spontaneous seizure activity occurring after the latent period (24). Finasteride was also tested upon the anti-epileptogenic activity of progesterone in the mouse hippocampus kindling model of epileptogenesis (26). The neurosteroid synthesis inhibitor completely inhibited the progesterone anti-epileptogenic effect (reflected by the retardation of the kindled seizures) which could suggest that this particular action of progesterone may be dependent upon ALLO (26). Seyle et al. (27) were probably the first to conduct this type of study, in 1942, in which they evaluated the progesterone activity in pentylenetetrazol animal model, using immature male rats, demonstrating the hormone's anticonvulsant properties. In the subsequent years, other researchers also confirmed these results in studies conducted on both male and female rodents, using various progesterone doses and convulsive animal models, including the amygdala kindling model (28, 29), hippocampal kindling model (30), WAG/Rij rats, the genetic absence model (in this case, an increase in the number and duration of spike-wave discharges was demonstrated) (31) or kainate model (32). Billiar et al. (33), in turn, evaluated the distribution and metabolism of progesterone, the products of its metabolism, and estradiol, but this time they administered a constant infusion of [3H]- or [14C] progesterone and estradiol [3H] to female rhesus monkeys. The experiments showed that the hormone levels were significantly higher in the cerebral tissues than in the carotid arterial blood, with the estradiol concentration being highest in the anterior pituitary (20 times). In the case of progesterone, its lowered concentrations were observed in the "*central gray (P less than 0.05); the concentration levels were the same for the amygdala, hippocampus, preoptic-anterior hypothalamus, cerebellum, hypothalamus, thalamus, and anterior pituitary; and were higher in the cervical spinal cord, optic chiasm, mesencephalon, medulla oblongata, and pons*" (33), when compared with the control group. Many years of research have shown that the administration of exogenous progesterone results in its cerebral-tissue concentration's being tripled when compared to peripheral tissue levels. In the case of the administration of both progesterone and its metabolite—5alpha-pregnan-3,20-dione (5alpha-DHP) to rats (*iv* administration), it was demonstrated that the compounds accumulated in the brain with the highest concentration in the hypothalamus and anterior pituitary regions, but in the cerebral cortex the concentration was very low (34). Whilst its high concentration in the anterior pituitary and hypothalamus is associated with gonadotropin release (34), the progesterone metabolite may possess an anticonvulsive potential. Actually, intravenous 5alpha-DHP reduced generalized and focal seizures in female fully kindled rats (35) and this effect was related to its interaction with GABA-A receptors (36).

Akula et al. (37) compared the anticonvulsant effect of progesterone (at doses of 20-80 mg/kg s.c). and AEDs on the intravenous pentylenetetrazol-induced seizure threshold in mice. All the studied compounds proved to have anticonvulsant dose-dependent effects. Moreover, it was shown that progesterone's effect was stronger than that of tiagabine, GABA, adenosine, gabapentin, but weaker than that of clonazepam, diazepam, chlordiazepoxide, phenobarbital, carbamazepine, pentobarbital, pregabalin, and phenytoin (37).

Clinical data indicate that status epilepticus may be associated with a profound reduction in cerebrospinal fluid concentration of progesterone which was even 64% lower than in matched controls (38).

Allopregnanolone

Among the known neurosteroids, ALLO is the potent and the most thoroughly examined natural endogenous positive GABA-A receptor modulator (8). This compound is responsible for the maturing of the central nervous system, and a range of behaviour in adult life, which was proved by Mòdol et al. (39). It was found that ALLO stimulated myelinisation, synaptogenesis and displayed protective and trophic properties in relation to neurons, both during the development period and in the case of disorders (40–42). Extensive research has shown that disturbances in its levels play an important role in the pathomechanism of many diseases, including neurological and psychiatric disorders (43). By modulating the level of neurosteroids in rat neonates, it was revealed that ALLO

modified exploratory and anxiety-like behaviour and disrupted aversive learning in the adult animals. (39).

Lévesque et al. (44) evaluated the effect of ALLO (at doses of 9.6-12.8 mg/kg/day) on interictal jumps and high frequency oscillations (ripples: 80-200 Hz, rapid ripples: 250-500 Hz) in the pilocarpine model of mesial temporal lobe epilepsy. It was determined that the neurosteroid significantly decreased the frequency of interictal spikes and fast ripples in the hippocampal CA3 field when compared to the control group. Others showed the anticonvulsant activity of the neurosteroid which attenuated behavioral and electrographic seizures in a model of status epilepticus despite benzodiazepine resistance (45). Interestingly, hippocampal ALLO concentration was significantly lowered in rats surviving kainate-induced status epilepticus when measured 9 weeks after (46). Human data seem in line with the results observed in rodents. Meletti et al. (38, 47) analyzed ALLO concentrations in samples of cerebrospinal fluid of patients with status epilepticus. There was a significant around 30% decrease in ALLO levels. In 2017, two cases of adults with super-refractory status epilepticus were reported, in whom the introduction of treatment with ALLO (at doses of 5.6 mg/h for 5 days in the form of 120 h continuous infusion) brought positive results (48).

Deoxycorticosterone

The next neurosteroid—DOC is a mineralocorticoid precursor whose anticonvulsant properties are connected with its enzymatic conversion to THDOC. The converted neurosteroid acts as a positive allosteric modulator of GABA-A receptors. It was found that DOC level can change under stress, because its synthesis is controlled by an adrenocorticotropic hormone. The above relationship can be explained by stress-dependent changes in the susceptibility to seizures, which are especially observed in children (49).

Anticonvulsant activity of DOC has been shown below (in the section devoted to the paediatric population). Interestingly, when evaluated in adult rats challenged with gamma-hydroxybutyric acid (a model of generalized absence seizures), THDOC potentiated seizure activity when given systemically or focally into thalamic ventrobasal nucleus (50).

Pregnenolone Sulphate

In the case of pregnenolone sulphate, the available results point to its proconvulsive activity. Maciejak et al. (19) revealed that an increase in the level of pregnenolone sulfate resulted in increased alanine concentration—a precursor of glutamate, which contributes to the development of seizures. A similar effect was obtained by Reddy and Kulkarni (18), who studied the impact of long-term pregnenolone sulphate and DHEAS (both at a dose of 10 mg/kg/day for 4 week) administration on convulsive activity in the mouse pentylenetetrazol model. A clear cut decrease in the seizure threshold was observed. In addition, the authors drew attention to the fact that the long-term administration of DHEAS resulting in the proconvulsive activity was prevented by long-term pretreatment with progesterone (at a dose of 5 mg/kg) or ALLO (at a dose of 0.1 mg/kg) (18).

Ganaxolone

Ganaxolone (3alpha-hydroxy-3beta-methyl-5alpha-pregnan-20-one; GNX) belongs to exogenous neurosteroids, and is the 3beta-methylated exogenous analogue of ALLO. GNX has a mechanism similar to its natural analogue, i.e. it is an allosteric modulator of GABA-A receptors (binds to both synaptic and extrasynaptic GABA-A receptors) (51, 52). In the case of the activation of synaptic GABA-A receptors, whereas the activation of extrasynaptic GABA-A receptors is connected with persistent or tonic inhibition (53). Additionally, it was confirmed that the neurosteroid did not activate classic nuclear progesterone receptors (54).

GNX displays anticonvulsive properties, which was found in many animal seizure models, including limbic seizures in the 6-Hz model, clonic seizures induced by pentylenetetrazol and bicuculline or amygdala-kindled seizures (54). Gasior et al. (55) in their experiments compared the activity of GNX with diazepam and valproate, in which they showed that GNX (effective doses of GNX predicted to protect 50% of the mice in this model [ED_{50}]—3.45 mg/kg against the clonic phase), was the most effective anticonvulsant, because it decreased the convulsive activity (clonic and tonic seizures) and lethal effects of pentylenetetrazol. By comparison, diazepam displayed anticonvulsant activity involving tonic seizures and lethality, and valproate only suppressed the tonic attacks (55). These results were confirmed in behavioral and electrographic seizures in fully amygdala-kindled mice, representing a model of mesial temporal lobe epilepsy (56). Mares and Stehlíková (57) studied the activity of GNX (in 5–40 mg/kg doses) in another model—cortical epileptic afterdischarges. Rats (12, 18, and 25-day-old) were used for this study. The administration of GNX (at 40 mg/kg) inhibited progressive prolongation of cortical epileptic afterdischarges in 25-day-old rats and postponed it in 12-day-old rats. In 18-day-old rats, no significant protective effects of the drug were observed (56). GNX was also evaluated in WAG/Rij rats, a genetic model of absence epilepsy, following intracerebral injections (57). When administered bilaterally into nucleus ventralis posteromedialis, nucleus reticularis thalami, nucleus ventralis posterolateralis (thalamic nuclei), the occurrence of epileptic spike-wave discharges was worsened. However, when microinjected into the peri-oral region of the primary somatosensory cortex, GNX (at doses of 100, 200, 400 pmol and 1 nmol/side) suppressed spike-wave discharges. Practically, ALLO (at doses of 200, 400 pmol and 1 nmol/side) produced a very similar response. The effects of pregenenolone sulphate (at doses of 100, 200, 400 pmol and 1 nmol/side) were dose-dependent—the drug was generally proconvulsant at low doses and anticonvulsant at higher doses when microinjected into thalamic nuclei or somatosensory cortex (58).

GNX (at a dose of 1500 mg/day) has been also studied as an add-on therapy in randomized, placebo-controlled trials involving adult patients with partial onset epilepsy with or without secondary generalization. A Phase II trial was conducted in 147 refractory adults (100 females and 47 males in the age range of 18-69 years) (59). Out of 131 patients who completed the trial, 124 subjects were enrolled into the open-

label extension study. The results of the trial were quite encouraging—GNX reduced by 18% mean weekly seizure frequency (vs a 2% enhancement in placebo group). Responder rates were evaluated as a percentage of patients in whom reduction of seizures reached at least 50%. The rates were 26 and 13% in GNX and placebo groups, respectively. A possibility that the observed beneficial results in patients on GNX could be dependent on gender or concomitant medication was excluded. Remarkably, GNX positive efficacy seems to be long-term which can be inferred from the open-label extension (59). Because of the adverse effects (mainly dizziness, fatigue and somnolence), 7% of GNX and 6% of placebo subjects had to discontinue treatment. Thirty six patients in the open-label extension continued GNX treatment for a longer period than one year and the observed adverse effects did not differ from those reported in the Phase II trial (58). Some more results from the open-label extension were published in 2013 (60). Among adverse effects observed in more than 10% of patients, were: headache (21%), convulsion (16%), fatigue (16%), fall (14%), nasopharyngitis (14%), dizziness (13%), contusion (12%), and nasal congestion (10%) (60). Detailed analysis of the results was published in 2017 (61). The authors indicate that generally, the adverse effects in the GNX group were mild to moderate. Among the untoward events leading to discontinuation in patients receiving GNX were: headache, lethargy and in placebo group: postictal psychosis, headache and convulsion. Clinical laboratory tests revealed no major disturbances—only in one patient on GNX mild thrombocytopenia was found which, however, did not result in treatment discontinuation (61).

Minaxolone and Alphaxalone

Other exogenous neurosteroids include minaxolone (2β,3α,5α,11α)-11-(dimethylamino)-2-(ethoxy-3-hydroxypregnan-20-one), and alphaxalone (5α-pregnan-3α-ol-11,20-dione)—compounds whose mechanisms of action are also based on positive interactions at the α1 glycine receptor. However, the respective EC50 values for minaxolone or alphaxalone were roughly 10 times higher compared to GABA-A receptors (62). Both minaxolone and alphaxalone proved to be effective anticonvulsant agents against pentylenetetrazol - and bicuculline-induced convulsions in rodents. In addition, moderate anticonvulsive activity was displayed by the latter in other models, including kindling and electroconvulsions in mice (63). Also, alphaxalone exerted distinct anticonvulsant effects against NMDA-induced convulsions in mice and reduced NMDA-produced mortality (64). This protective activity was shared by other positive modulators of GABA-A receptors—ALLO and androsterone. Interestingly, the ALLO precursor, 5α-pregnane-3,20-dione, was completely ineffective in this respect. However, this neurosteroid has nothing to do with GABA-A receptors (64). Another study, however, found alphaxalone ineffective against pentylenetetrazol-induced seizure activity in mice or amygdala-kindled seizures in rats (65). Aminophylline-induced convulsions in mice were also not affected by the neurosteroid (65). Apart from the evaluation of the anticonvulsant activity of alphaxalone per se, this compound was also combined with a number of AEDs in the maximal electroshock (MES)- and pentylenetetrazol-induced convulsions in mice. First, alphaxalone (at 2.5 mg/kg) was demonstrated to raise the threshold for electronvulsions but in this effective dose it surprisingly reduced the protective activity of valproate against MES and at 2.5-5 mg/kg, alphaxalone also negatively interacted with this AED in pentylenetetrazol-induced seizures. As regards other AEDs, the anticonvulsant activity of carbamazepine, phenobarbital, phenytoin and clonazepam was not modified by the neurosteroid against MES. Similarly, the protective action of clonazepam, ethosuximide and phenobarbital remained unchanged in the presence of alphaxalone. In aminophylline-induced convulsions and amygdala-kindled seizures the protective activity of conventional AEDs (including valproate) was not affected by alphaxalone (65).

Alphaxalone was also tried in a rat model of generalized absence seizures produced by gamma-hydroxybutyric acid (50). Following its systemic or focal administration into thalamic ventrobasal nucleus, an exacerbation of seizures was noted. However, no effect was observed following its administration into thalamic reticular nucleus (50).

Combination of Neurosteroids (Allopregnanolone and Ganaxolone) With Tiagabine or Midazolam

The examined combination of neurosteroids included ALLO and GNX with the GABA-reuptake inhibitor tiagabine or the benzodiazepine derivative - midazolam against tonic inhibition in dentate gyrus granule cells (DGGCs) or in the hippocampal kindling and 6-Hz seizure models (66). The authors provided evidence that combining individual neurosteroids with tiagabine in three standard proportions (1:1, 3:1 and 1:3) showed considerable synergism in their anticonvulsant action, and the pharmacological studies consistently pointed to the combinations' anticonvulsant effect. Similar results were obtained in the case of neurosteroids combined with midazolam. The combination of tiagabine with GNX at a 1:1 dose ratio exerted the strongest effect. As noted by the authors, the possible mechanism behind this action may result from both the effects on extrasynaptic GABA-A receptors and tiagabine -induced increase in synaptic GABA concentration. In turn, when considering the combination of the neurosteroid with midazolam, such positive effects may be possibly related to their actions at both synaptic and extrasynaptic GABA-A receptors (66). Apart from assessing pharmacokinetic parameters (see below), Zolkowska et al. (67) also evaluated the efficacy of intramuscular ALLO and GNX (each at a dose of 3 mg/kg) in the treatment of status epilepticus induced by tetramethylenedisulfotetramine in mice. The experiments showed that both neurosteroids were effective, however, ALLO displayed slightly greater effectiveness and speed of action, which was probably connected with its greater GABA-ergic potency (67).

There are data on the pharmacokinetic properties of ALLO and GNX (67). Plasma and brain levels of ALLO and GNX were determined in naïve mice at various time points following intramuscular dosing (in both cases at a dose of 3 mg/kg).

Maximum concentration values for ALLO and GNX (plasma Cmax) were 645 and 550 ng/mL, respectively. Brain levels rose more slowly and peaked at 10 min in both cases, the respective Cmax values being 845 ng/mL for ALLO and 1239 ng/mL for GNX. On the basis of all pharmacokinetic parameters, it was found that the peak brain concentrations and brain exposure (AUC) for both steroids, was approximately 3-fold the plasma exposure (additionally, GNX was shown to be higher than ALLO). In the first case, the probable cause is the slightly higher hydrophobicity of GNX than ALLO (logP values—5.423 and 5.042, respectively). In the second case, the authors indicate a higher lipophilicity of GNX as a probable cause (log P with 5.3 vs 4.9 for ALLO). Therefore, GNX initially concentrates in the brain to higher levels, being subsequently redistributed to fat tissue. Maintaining a more flatter distribution in the brain is another factor that may be responsible for the higher effectiveness of ALLO. The common feature of both neurosteroids is that both ALLO and GNX were highly bioavailable, indicating that they were almost completely absorbed following intramuscular injection (67).

NEUROSTEROIDS AND CATAMENIAL EPILEPSY

One of the possible causes of refractory epileptic seizures in women might be disturbances in the levels of progesterone and estrogen. These hormones may affect the electrical excitability of neurons and thus the seizure threshold. It was found that in the case of concentration fluctuations during the menstrual cycle, these hormones contributed to seizure exacerbation, called catamenial epilepsy. It is a dominant a type of drug-refractory epilepsy found in women of reproductive age. The fact that the characteristic feature of catamenial epilepsy is increased frequency of seizures at specific and repetitive times in the menstrual cycle serves as confirmation of how important a role is played here by the hormones (68, 69).

It was confirmed that the menstrual cycle had 3 sensitive periods in which increased seizure activity could be observed. Such increased activity usually occurs perimenstrually (C1 pattern), at ovulation (C2 pattern), and during the luteal phase (C3 pattern). The C1 and C3 phases see a drop in progesterone concentration, and the C2 period—a pre-ovulatory surge in estrogen. A decrease in progesterone concentration reduces sensitivity to the inhibitory neurotransmiter—GABA (70). It is suggested that the fluctuations in GABA-A receptor subtypes (especially in extrasynaptic δ-GABA-A receptors) could be of importance as revealed in animal experiments (71, 72). It is suspected that the occurrence of this type of seizures in female patients is usually correlated with the discontinuation of neurosteroids, hence the idea to administer exogenous progesterone during the luteal phase, which is expected to eliminate the risk of a sudden drop in the hormone levels (68). However, the results of various research efforts, including randomised studies, appear to be discordant on that matter (70).

Progesterone is synthesised in the mitochondrial membranes of body cells, and in the cerebral tissue, in several stages, the first of which is homogenous, while the remaining stages can develop in various ways, which are different in the peripheral and central compartments. There is evidence that in the peripheral compartment, the 5 beta reduction pathway predominates, in turn in the central compartment—the 5 alpha pathway which is predominant in rats, monkeys, and humans (73).

At first it was believed that progesterone itself, in its basic form, possessed anticonvulsant properties, yet subsequent studies supported a notion that its anticonvulsant activity was most likely possible through its metabolism to other compounds. In addition, it is a known fact that progesterone and its secondary metabolites ALLO and 5alpha-DHP show anticonvulsant activity (73). Furthermore, this is supported by the fact that progesterone, through its neurosteroid derivative, ALLO, increases GABA-A receptor density in the brain, thus controlling possible seizures.

Animal studies were conducted in which the progesterone metabolism was blocked by finasteride which resulted in suppressing the anticonvulsant properties of this hormone (26).

It is very likely that the progesterone nuclear receptor does not participate in the anticonvulsant mechanism which is supported by double blind, placebo controlled and randomized studies by Dan-Haeri and Richens (74), conducted in a group of patients with catamenial exacerbation. The authors examined norethisterone—a compound which is similar to progesterone but has a stronger affinity to the progesterone nuclear receptor when compared to progesterone. However, these studies did not provide the primarily expected results, as it turned out that norethisterone did not possess anticonvulsant effects (74).

There is also a case of a woman with catamenial epilepsy, involving intractable complex partial and secondary generalized seizures and accompanying hormonal disorders, in whom seizure control could not be achieved, despite the administering of various AEDs, including barbiturates, carbamazepine, phenytoin and valproate. It was progesterone therapy which brought the expected therapeutic results, but only to the point at which the dermatologist ordered the treatment with finasteride (in view of the rapidly progressive male-pattern baldness). The additional therapy resulted in the recurrence of seizures, despite continued progesterone treatment, which again might suggest it is not the hormone which has anticonvulsant properties, but its metabolites are in fact anticonvulsant (75).

Herzog et al. (76, 77) conducted a randomized, double-blind, placebo-controlled, phase III, multicenter, NIH Progesterone Trial in which they examined the efficacy of adjunctive cyclic natural progesterone therapy in a group of almost 300 women who had been diagnosed with intractable partial seizures, with or without catamenial exacerbation. The patients in the study group were given progesterone at a dose of 200 mg 3 times a day for 12 months (from the 14^{th} to the 25^{th} day of each menstrual cycle). The dose was gradually reduced in the subsequent 3 days. The experiments showed that progesterone could be a very beneficial solution for women with perimenstrually exacerbated seizures; however, in the case of women with intractable partial epilepsy, cyclic progesterone is ineffective (76, 77).

A smaller study was conducted in 36 women with catamenial epilepsy—the patients experienced seizure activity throughout

the second half of the menstrual cycle, accompanied by low serum concentrations of progesterone (78). Progesterone was administered at a daily dose of 50 mg starting from the day 16th and ending on day 25th of each cycle. The results were encouraging. There was a 55.9% decline in the seizure frequency (primary and secondary generalized seizures) and a 63.1% decline in the partial seizure frequency—no improvement was evident in 5 patients (78).

The case is more difficult to prove with estrogen, because this hormone displays both pro- (79–81) and anticonvulsant properties (82), its mechanisms of actions being not fully understood. It is suspected that estrogen works through intracellular estrogen receptors, ER-alpha and ER-beta, which are found in nuclei of some neurons, e.g. in the hippocampus. Despite the fact that they are not very numerous when it comes to this structure, they seem to have a strong influence on the formation of synapses by neurons that do not have high levels of nuclear estrogen receptors. It was observed that non-nuclear estrogen receptors can occur outside of the cellular nuclei in dendrites, presynaptic terminals, and glial cells, where estrogen receptors can connect to second messenger systems to regulate various cellular events and signals to the nucleus through transcriptional regulators such as CREB (83). A different research group suggested that estradiol affected the hippocampal dendritic spine density through the activation of specific NMDA receptors (80). Smejkalova and Woolley (81), in turn, demonstrated that the hormone potentiated excitatory neurotransmission through a presynaptic mechanism *via* increased glutamate release. Studies are available in which the authors point to antiepileptic, and even neuroprotective, effects of estrogen. The hormone decreases neuronal death during seizures through up-regulation of the prosurvival molecule—Bcl-2, anti-oxidant potential as well as protection of NPY interneurons (82). In addition, it was demonstrated that the examined steroids caused *"the induction of dendritic spine proliferation on serotonin neurons thus thawing a profound effect on serotonergic transmission"* by the activation of 5-HT3 and 5-HT1A receptors (82).

It is possible that androgens also display a bimodal character. Animal studies have shown that testosterone can cause convulsive episodes to be aggravated, but, on the other hand, it was demonstrated to possess anticonvulsant properties which was connected with its transformation to various metabolites. The first case is a testosterone metabolism to 5α-DHT by 5α-reductase, which is then reduced by 3α-hydroxysteroid oxidoreductase enzyme resulting in the synthesis of anticonvulsant metabolite 3α-androstanediol, a potent GABA-A receptor modulating neurosteroid (84). In the second case, reduction of testosterone by aromatase generates proconvulsant 17-β estradiol (84, 85). Tutka et al. (86), in their experiments, assessed the effects of androsterone on the anticonvulsant properties of AEDs against MES-induced convulsions in mice. It was shown that androsterone, when administered alone (80 mg/kg), elevated the seizure threshold. This was not observed at lower doses (5-40 mg/kg). When combined with AEDs, androsterone (at 40 mg/kg) significantly enhanced the anticonvulsant activity of phenobarbital, gabapentin and carbamazepine, but it did not affect the protective activity of phenytoin, lamotrigine, oxcarbazepine, topiramate, or valproate. The observed lack of androsterone's effect on the brain total concentration of AEDs suggests that the positive effect of this neurosteroid was not connected with pharmacokinetic interactions (86). Effects of neurosteroids on seizure susceptibility have been summarized in **Table 1**.

NEUROSTEROIDS AND SEIZURE ACTIVITY IN THE PEDIATRIC POPULATION (HUMAN AND ANIMAL DATA)

Epilepsy in children is a crucial problem, because its prevalence is greater than epilepsy in the adult population. This is associated with the increased susceptibility of immature cerebral tissue to spontaneous neuronal discharges. Due to the high risk of irreversible pathological changes, it is recommended to commence treatment as soon as possible. In addition, it has been shown that not only is an early start important, but also the appropriate type of treatment administered. The authors have proved that the introduction of the wrong AED therapy in the first line of treatment has long-term effects, including the reduction of the effectiveness of subsequent treatment courses with the appropriately selected AEDs (89). The treatment of neonates has proved to be extremely challenging, because epileptic seizures occurring in this group of patients are usually refractory to standard pharmacological treatment. There are cases in which AED administration can even aggravate the seizures (90). Consequently, there is a huge need to introduce new therapies which can be used in the youngest epilepsy patients.

Although ALLO possesses anticonvulsant properties, it is uncertain what effects it would display in children with epilepsy. With paediatric patients it should be borne in mind that GABA may become a depolarizing neurotransmitter in the brain, and GABAergic inhibition can be a result of both membrane hyperpolarization and a stimulus. In such a case GABA can act as both a stimulatory and an inhibitory neurotransmitter in an immature brain (91, 92).

The stimulating nature of GABAergic neurotransmission in the neonatal period can stem from, on the one hand, an increase in the level of Na^+–K^+–$2Cl^-$ co-transporter (importing Cl^- inside the cell—NKCC1), and, on the other, a decrease in the level of K^+–Cl^- co-transporter 2 (mediating Cl^- transport out of the cell, KCC2). As a result, a high level of chloride ions is noted inside a cell (93). The researchers are of opinion that the activity of NKCC1 was high in the hippocampal and cortical neurons, especially in the first week of life, in both rats and humans, and it gradually decreased with time which was particularly apparent from the 14th day of life (94, 95). It is believed that this is connected with the depolarizing to hyperpolarizing shift of GABA receptors, which starts around the 8-10th day of life and ends on the 14th day. This was proven in experiments on rat

TABLE 1 | Neurosteroids—mechanisms of action and effects on seizure activity.

	Neurosteroid	Mechanism of neurosteroid action	Anticonvulsant action	Proconvulsant action	Experiments on animal models
Endogenous neurosteroids	Allopregnanolone	positive allosteric modulator of GABA-A receptors (38)	+	–	Kainite, PTZ, 4-aminopyridine model (13)
	Androsterone	positive allosteric modulator of GABA-A receptors	+	–	MES model (86)
	Deoxycorticosterone	positive allosteric modulator of GABA-A receptors (46)	+	-	MES and PTZ model in juvenile rats (87, 88)
					hippocampal kindling in juvenile rats (88)
			–	+	Model of generalized absence seizures in rats (50)
	Dehydroepiandrosterone sulfate	negative modulator of GABA-A receptors (18)	–	+	PTZ model (18)
		modulates the NMDA receptor (19)			
	Pregnenolone sulfate	negative modulator of GABA-A receptors (18)	–	+	seizures induced by picrotoxin, bicuculline and NMDA (19)
		modulates the NMDA receptor (19)			
	Progesterone	positive allosteric modulator of GABA-A receptors (59)	+	–	Amygdala kindling model in rats (19, 29), hippocampal kindling model (30), WAG/Rij rats, the genetic absence model (31)
					kainate model in rats (32)
Exogenous neurosteroids	Alphaxalone	positive allosteric modulator of GABA-A and α1 glycine receptor (59)	+	–	PTZ- and bicuculline-induced convulsion (63)
			–	+	Model of generalized absence seizures in rats (50)
	Ganaxolone	positive allosteric modulator of GABA-A receptors (47, 48)	+	–	6 Hz model, PTZ, bicuculline seizures, amygdala-kindled seizures (54, 55). cortical epileptic afterdischarges in rats (57)
	Minaxolone	positive allosteric modulator of GABA-A, α1 glycine receptor (59)	+	–	PTZ- and bicuculline-induced convulsions (62)

Experiments were carried out in mice unless stated otherwise. MES, maximal electroshock; NMDA, N-methyl-D-aspartate; PTZ, pentylenetetrazol; +, present; –, absent.

CA3 hippocampal pyramidal cells (94, 96). Kolbaev et al. (97) in their *in vivo* studies on the CA3 region of hippocampal slices from immature (postnatal day 4-7) rats, showed that synaptic GABAergic neurotransmission suppressed epileptic discharges, and, in turn, strengthened extrasynaptic GABAergic drive caused epileptiform activity. It is also important to note that δ-subunit expressing GABA-A receptors which participates in extrasynaptic GABAergic transmission, can be a potential target of neurosteroids, which was confirmed in a mouse model (98). Therefore, it is important to study the effects of ALLO on neuronal excitability in an immature brain. There are studies which show the anticonvulsant effect of ALLO in an immature brain. Sharopov et al. (99) examined the impact of ALLO on epileptiform activity in an in-toto hippocampus preparation of early postnatal mice (postnatal days 4-7). It was found that the neurosteroid, through a positive modulation of GABA-A receptors, did not show any impact on ictal-like epileptiform activity, however, an increase in interictal epileptiform events was observed. Additionally, based on studies using a patch-clamp, it was determined that ALLO prolonged the decay of GABAergic postsynaptic currents, but did not result in changes in the case of tonic GABAergic currents, which could result in an increase in the neuronal excitability of an immature brain (99).On the other hand, in the case of *in vivo* studies, results by Dhir and Chopra (13) should be taken into consideration. They examined the effects of a ALLO in 9-day-old rat neonates against seizures induced by kainic acid, pentylenetetrazol or 4-aminopyridine. Treatment with ALLO (5 and 10 mg/kg) delayed the occurrence of status epilepticus, but did not impact on the myoclonic jerks or the mortality rate in the kainic acid group. In the case of convulsions evoked by 4-aminopyridine, neurosteroid treatment—only at a higher dose of 10 mg/kg—contributed only to a delay in seizure activity. ALLO treatment (at 5 mg/kg) in the pentylenetetrazol group, resulted in the protection of rat neonates against seizures and death (13). Another research group evaluated the impact of neurosteroids (ALLO—at doses of 20, 30 and 40 mg/kg, pregnanolone and triethylammonium 3alpha-hydroxy-20-oxo-5alpha-pregnan-21-yl hydrogensuccinate (THDOC-conjugate—at 20 and 40 mg/kg) on rat neonates in 3 age groups: 12-, 18- and 25-day-old rats (in the case of ALLO treatment) and in two age groups: 12- and 25-day-old rats (in the case of other neurosteroids). Convulsions were induced through electrical stimulation using intracerebral electrodes. The experiments showed that all neurosteroids displayed anticonvulsant properties (the strongest effect was observed with pregnanolone) in 12-day-old rats, but only a tendency in 25-day-old ones. In turn, in the case of ALLO, no such tendency was observed in 18-day-old rats (100). Subsequent studies also assessed the effects of ALLO (at doses of 5 – 40 mg/kg) on convulsive activity in the pentylenetetrazol model, but this

time the treatment involved 7-, 12-, 18-, 25- or 90-day-old rats (100). Similarly to the previous experiments, the neurosteroid supressed generalized tonic-clonic and minimal clonic seizures, in particular in the 12-day-old rat study group. The weakest anticonvulsant effect was, in turn, observed in the 90-day-old rat study group. The above results can be explained by the fact that the effects of ALLO lasted longer in young, than in adult rats (101). Other studies, also conducted by the same research team, evaluated the anticonvulsant properties of an analogue of ALLO —3alpha-hydroxy-21xi,22-oxido-21-homo-5alpha-pregnan-20-on (at 40 mg/kg) and GNX (at 60 mg/kg) in a group of 12- and 25-day-old rat neonates against pentylenetetrazol-induced convulsions. A similar activity by both neurosteroids was found, but a more-favourable result was observed in younger rats (102). Based on the above studies it can be concluded that neurosteroids are the most effective when used in 12-day-old rat neonates, which the authors explain by the increased sensitivity towards the anticonvulsant potential of neurosteroids in younger rats (100). It is worth noting that, despite the positive effects of neurosteroids in rat neonates, one-week-old animals were excluded from the above studies while their brains roughly resemble those of pre-term children (103). In the case of preterms, it is worth stressing that they are particularly prone to CNS disorders. The high concentration of neurosteroids in advanced pregnancy protects the fetal brain against hypoxia and supports the normal development of CNS. When the ALLO level decreases, it brings about overexcitability in the neurons and increases the risk of brain damage secondary to hypoxia. Following birth (both normal and preterm) the neurosteroid level is found to be lower, which is particularly unfavourable for a preterm (104).

Age-dependent effect was also evident for DOC which exhibited clear cut anticonvulsant effects in neonatal, infant, weanling and juvenile rats against PTZ-induced convulsions (87). DOC at low dose (10 mg/kg) lost its protective effect and its anticonvulsant activity after a high sedating dose (40 mg/kg) was significantly reduced after puberty (87). Further experiments provided evidence that this neurosteroid was effective against MES (87, 88) and hippocampal kindling in 15-day-old rats, too. Much higher doses of DOC were required for adult rodents (88). Interestingly, the anticonvulsant efficacy of DOC against PTZ was significantly reduced by finasteride, indicating that the neurosteroid acts *via* its metabolites, dehydrodeoxycorticosterone and THDOC (105). Actually, these metabolites were found effective against PTZ-induced convulsions in infant (15-day-old) rats (105).

Kaciński et al. (43) assessed the effects of ALLO on pseudoseizures in children. The children were divided into 3 groups: (I) children with pseudoseizures without treatment with AEDs; (II) children with pseudoseizures and treated with AEDs; (III) children without pseudoseizure attacks and no treatment with AEDs. The results showed no significant changes in ALLO levels, both before and after provoking pseudoseizures by placebo. It might point to the fact that during pseudoseizure attacks, in contrast to epileptic seizures, endogenous anticonvulsant and anxiolytic neurosteroid levels do not increase. In addition, the authors noted that the low ALLO level can intensify the stress response and contribute to the occurrence of pseudoseizures (43). Broomall et al. (106) were the first to describe two children with super-refractory status epilepticus. This disorder is characterized by resistance to benzodiazepine and barbiturate treatment, which is most probably connected to the internalization of synaptic GABA-A receptors. ALLO was used, and it made it possible to discontinue general anesthetic infusions (106). In their studies, Grosso et al. (107), suggest that circulating ALLO significantly increases in the post-ictal phase. They found no significant differences in the post ictal serum ALLO between patients with partial seizures and those with generalized seizures. They included three groups of subjects in the study. Group 1 consisted of 18 children affected by complex partial seizures. Group 2 consisted of 11 children presenting with generalized epilepsy. Group 3 consisted of 20 healthy age-matched subjects. Serum ALLO levels were assayed in the inter-ictal phase and within 30 min after an epileptic event (107). A possibility exists that a reduced blood ALLO concentration may be causally related to pathophysiology of protocadherin 19 female limited epilepsy (PCDH19-FE) which is actually a clear cut infantile onset syndrome with autism and intellectual disability in some cases. Genes, encoding enzymes involved in the metabolism of steroid hormones, were evaluated in transcriptomes of primary skin fibroblasts. Out of the AKR1C1-3 genes, significant changes were found in AKR1C3 in terms of reduced mRNA and protein concentrations in PCDH19-FE patients (108). Obviously, the reduced blood concentration of ALLO followed in these patients which could be responsible for the development of PCDH19-FE (108). Further studies on PCDH19-FE patients confirmed the earlier findings of Tan et al. (108), showing that the serum concentration of ALLO was significantly reduced not only in baseline but after stimulation with ACTH as well (109). Strikingly, the synthesis of pregnenolone sulphate was even more reduced than that of ALLO so a hypothesis that seizures could be generated by an assumed imbalance in the ALLO/pregnenolone sulphate ratio proved unlikely (109).

One of the first studies to evaluate the effect of GNX on convulsive activity in the paediatric population was conducted approximately 20 years ago. Kerrigan et al. (110) examined this neurosteroid in the population of children with refractory infantile spasms, or with continuing seizures following a prior history of infantile spasms (aged 7 months to 7 years). It was determined that GNX reduced the number and frequency of seizures from 25% to > 50%. In turn, Pieribone et al. (111) evaluated the anticonvulsive effects of GNX in children (aged 5-15) with highly refractory focal and generalized crypto-symptomatic epilepsy. The results confirmed that the neurosteroid produced anticonvulsive effects. In some patients, adverse events were observed; however, all of them were described as mild to moderate. Yawno et al. (112) suggested that GNX should also be administered in the case of convulsions in neonates and preterms. The author draws attention to the fact that the drug, on one hand, is very safe, because most probably it produces no negative effects on the neonates' brains, and, on the other, it can prevent or considerably reduce the prevalence of permanent damage resulting from hypoxia, preterm birth, or epilepsy (112). Clinical research has been presented in **Table 2**.

TABLE 2 | Neurosteroids—clinical research.

Neurosteroids	Type of seizures	Trial group	Doses applied	References
Allopregnanolone	generalized convulsions and myoclonus	Adults	670.8 mg (5.6 mg/h for 5 days), intravenous solution, containing 6% hydroxypropyl-β-cyclodextrin in 0.9% sodium chloride injection	(48)
	super-refractory status epilepticus	Children	iv solution (0.5 mg/ml in 0.9% NaCl with 6% Captisol for 5 days	(106)
	complex partial seizures generalized epilepsy	Children (aged 11 months to 7.8 years)	no data	(107)
Progesterone	catamenial epilepsy	Adults (women)	200 mg three times daily on days 14–25, followed by a 3-day taper) of each cycle	(75)
	intractable partial seizures, with or without catamenial exacerbation.	Adults (women)	200 mg 3 times a day for 12 months (from the 14th to the 25th day of each menstrual cycle)	(76, 79)
	catamenial epilepsy	Adults (women)	50 mg starting from the day 16th and ending on day 25th of each cycle.	(78)
Ganaxolone	partial onset epilepsy with or without secondary generalization	Adults (aged 18-69 years)	1,500 mg/day	(59)
	refractory infantile spasms, or with continuing seizures following a prior history of infantile spasms	Children (aged 7 months to 7 years).	the dose of ganaxolone was progressively increased to 36 mg/kg/d (or to the maximum tolerable dose) over a period of 4 weeks and then maintained for 8 weeks prior to tapering and discontinuation of the attack	(110)
	highly refractory focal and generalized crypto-symptomatic epilepsy	Children (aged 5-15 years)	ganaxolone in a 1:1 complex with β-cyclodextrin in a dose escalation (1 mg/kg, b.i.d. to 12 mg/kg t.i.d.) schedule over 16 days	(111)

CONCLUSIONS

It is evident that endogenous and exogenous neurosteroids may exert anti- or proconvulsant activity. As already indicated above, the anticonvulsant activity is associated with the positive modulation of GABA-A receptors and by the way, some anticonvulsant neurosteroids were documented to inhibit aspartate release from rat hippocampal slices (64). The anticonvulsant neurosteroids comprise for instance: androsterone, progesterone, ALLO, alphaxalone, GNX. In contrast, proconvulsant neurosteroids (pregnenolone sulfate or DHEAS) negatively modulate the function of GABA-A receptor complex. Modulation of seizure activity may be also associated with sigma receptors as a sigma receptor antagonist, rimcazole, lowered the convulsive threshold on one hand, but on the other, it potentiated the anticonvulsive activity of phenobarbital and valproate against MES in mice (113). When considering anticonvulsant effects of neurosteroids, progesterone block of voltage-operated calcium channels may be of importance as many agents expressing this mechanism of action express anticonvulsant activity (114). Modulation of seizures *via* glycine 1 receptors seems rather unlikely *in vivo* due to the relatively weak binding of neurosteroids to these receptors. A completely different situation may be encountered in absence seizures as revealed from WAG/Rij rats. The observed worsening of absence seizures in 6-month-old WAG/Rij rats may be associated with the up-regulation of thalamic α-4 and δ GABA-A receptor subunits which probably leads to an enhanced GABA-ergic inhibition of thalamic relay neurons (115).

Because intractable seizures generally require adjuvant treatments, interactions of neurosteroids with AEDs are of particular importance. Preclinical data point to the beneficial effects of androsterone when combined with carbamazepine, gabapentin and phenobarbital (86). Also, combinations of ALLO and GNX with tiagabine or midazolam were found highly effective against hippocampal kindling and 6 Hz-induced convulsions in mice (66). The results concerning alphaxalone are not that encouraging as this neurosteroid diminished the anticonvulsant activity of valproate against MES or pentylenetetrazol in mice. Unexpectedly, the total brain concentration of valproate was elevated by alphaxalone (65).

Clinical data are generally positive, pointing to progesterone as an effective drug against catamenial epilepsy (76–78). Nevertheless, the hormone is ineffective as regards intractable partial seizures in women (76). A very promising AED, GNX, has entered phase III study (116). It has shown a considerable efficacy as an adjuvant against infantile spasms (110), highly refractory epilepsy in children (111) and possibly ganaxolone will prove effective for the management of neonatal seizures following hypoxic injury (112). Positive results are also available from a trial conducted on adult patients with partial epilepsy (59–61).

Using neurosteroids in the paediatric population deserves special attention, especially in the period of the last semester of gestation up till the first several years after birth. This is actually the period of the intensive synaptogenesis (117). Experimental data obtained from immature animals clearly indicate the drugs enhancing GABA-mediated inhibition can induce massive neuronal apoptosis similarly to alcohol which is known to cause fetal alcohol syndrome (117).Whether neurosteroids *via* GABA-mediated events may cause remote clinical problems in the paediatric population due to the enhanced apoptosis is at present not known.

AUTHOR CONTRIBUTIONS

BM was involved in writing most parts of the first draft and designing the table and schemes. MC-K dealt with some aspects of the treatment of pediatric patients with neurosteroids. SC prepared conclusions and critically read the whole manuscript, performing necessary additions (with relevant references) and revisions.

FUNDING

This study received no external funding. The authors greatly acknowledge a statutory grant (DS 475/20) from Medical University of Lublin. Medical University of Lublin had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. Thijs RD, Surges R, O'Brien TJ, Sander JW. Epilepsy in adults. *Lancet* (2019) 16:393(10172):689–701. doi: 10.1016/S0140-6736(18)32596-0
2. Amin U, Benbadis SR. Avoiding complacency when treating uncontrolled seizures: why and how? *Expert Rev Neurother* (2020) 15:1–9. doi: 10.1080/14737175.2020.1713100
3. Chouchi M, Klaa H, Ben-Youssef Turki I, Hila L. ABCB1 Polymorphisms and drug-resistant epilepsy in a Tunisian population. *Dis Markers* (2019) 2019:1343650. doi: 10.1155/2019/1343650
4. Ma BB, Fields MC, Knowlton RC, Chang EF, Szaflarski JP, Marcuse LV, et al. Responsive neurostimulation for regional neocortical epilepsy. *Epilepsia* (2020) 61(1):96–106. doi: 10.1111/epi.16409
5. Łukawski K, Gryta P, Łuszczki J, Czuczwar SJ. Exploring the latest avenues for antiepileptic drug discovery and development. *Expert Opin Drug Discovery* (2016) 11(4):369–82. doi: 10.1517/17460441.2016.1154840
6. Błaszczyk B, Miziak B, Czuczwar P, Wierzchowska-Cioch E, Pluta R, Czuczwar SJ. A viewpoint on rational and irrational fixed-drug combinations. *Expert Rev Clin Pharmacol* (2018) 11(8):761–71. doi: 10.1080/17512433.2018.1500895
7. Dubrovsky BO. Steroids, neuroactive steroids and neurosteroids in psychopathology. *Prog Neuropsychopharmacol Biol Psychiatry* (2005) 29 (2):169–92. doi: 10.1016/j.pnpbp.2004.11.001
8. Basta-Kaim A, Leskiewicz M, Budziszewska B, Lason W. The role of neurosteroids in the central nervous system function. [Article in Polish]. *Przegl Lek* (2005) 62:1287–92.
9. Compagnone NA, Mellon SH. Neurosteroids: biosynthesis and function of these novel neuromodulators. *Front Neuroendocrinol* (2000) 21:1–56. doi: 10.1006/frne.1999.0188
10. Leśkiewicz M, Budziszewska B, Basta-Kaim A, Zając A, Kacinski M, Lason W. Effects of neurosteroids on neuronal survival: molecular basis and clinical perspectives. *Acta Neurobiol Exp (Wars)* (2006) 66:359–67.
11. Borowicz KK, Czuczwar SJ. [Perspectives of neurosteroid derivative application in antiepileptic therapy]. [Article in Polish]. *Przegl Lek* (2005) 62(11):1293–5.
12. Reddy DS. Role of hormones and neurosteroids in epileptogenesis. *Front Cell Neurosci* (2013) 7:115:115. doi: 10.3389/fncel.2013.00115
13. Dhir A, Chopra K. On the anticonvulsant effect of allopregnanolone (a neurosteroid) in neonatal rats. *Life Sci* (2015) 143:202–8. doi: 10.1016/j.lfs.2015.09.008
14. Amengual-Gual M, Sánchez Fernández I, Wainwright MS. Novel drugs and early polypharmacotherapy in status epilepticus. *Seizure* (2019) 68:79–88. doi: 10.1016/j.seizure.2018.08.004
15. Ratner MH, Kumaresan V, Farb DH. Neurosteroid actions in memory and neurologic/neuropsychiatric disorders. *Front Endocrinol (Lausanne)* (2019) 10:169:169. doi: 10.3389/fendo.2019.00169
16. Gibbs TT, Russek SJ, Farb DH. Sulfated steroids as endogenous neuromodulators. *Pharmacol Biochem Behav* (2006) 84(4):555–67. doi: 10.1016/j.pbb.2006.07.031
17. Reddy DS. GABA-A receptors mediate tonic inhibition and neurosteroid sensitivity in the brain. *Vitam Horm* (2018) 107:177–91. doi: 10.1016/bs.vh.2017.12.001
18. Reddy DS, Kulkarni SK. Proconvulsant effects of neurosteroids pregnenolone sulfate and dehydroepiandrosterone sulfate in mice. *Eur J Pharmacol* (1998) 345(1):55–9. doi: 10.1016/S0014-2999(98)00034-X
19. Maciejak P, Członkowska AI, Bidziński A, Walkowiak J, Szyndler J, Lehner M, et al. Pregnenolone sulfate potentiates the effects of NMDA on hippocampal alanine and dopamine. *Pharmacol Biochem Behav* (2004) 78 (4):781–6. doi: 10.1016/j.pbb.2004.05.009
20. Maurice T, Phan VL, Urani A, Kamei H, Noda Y, Nabeshima T. Neuroactive neurosteroids as endogenous effectors for the sigma1 (sigma1) receptor: pharmacological evidence and therapeutic opportunities. *Jpn J Pharmacol* (1999) 81(2):125–55. doi: 10.1254/jjp.81.125
21. Monnet FP, Mahé V, Robel P, Baulieu EE. Neurosteroids, via sigma receptors, modulate the [3H]norepinephrine release evoked by N-methyl-D-aspartate in the rat hippocampus. *Proc Natl Acad Sci U S A* (1995) 92 (9):3774–8. doi: 10.1073/pnas.92.9.3774
22. Luoma JI, Stern CM, Mermelstein PG. Progesterone inhibition of neuronal calcium signaling underlies aspects of progesterone-mediated neuroprotection. *J Steroid Biochem Mol Biol* (2012) 131(1-2):30–6. doi: 10.1016/j.jsbmb.2011.11.002
23. Maguire J. Epileptogenesis: more than just the latent period. *Epilepsy Curr* (2016) 16(1):31–3. doi: 10.5698/1535-7597-16.1.31
24. Biagini G, Rustichelli C, Curia G, Vinet J, Lucchi C, Pugnaghi M, et al. Neurosteroids and epileptogenesis. *J Neuroendocrinol* (2013) 25(11):980–90. doi: 10.1111/jne.12063
25. Papadopoulos V, Baraldi M, Guilarte TR, Knudsen TB, Lacapère JJ, Lindemann P, et al. Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. *Trends Pharmacol Sci* (2006) 27(8):402–9. doi: 10.1016/j.tips.2006.06.005
26. Samba Reddy D, Ramanathan G. Finasteride inhibits the disease-modifying activity of progesterone in the hippocampus kindling model of epileptogenesis. *Epilepsy Behav* (2012) 25(1):92–7. doi: 10.1016/j.yebeh.2012.05.024
27. Selye H. The antagonism between anesthetic steroid hormones and pentamethylenetetrazol (Metrazol). *J Lab Clin Med* (1942) 27:3.
28. Mohammad S, Abolhassan A, Pourgholami MH. Evaluation of the anticonvulsant profile of progesterone in male amygdala-kindled rats. *Epilepsy Res* (1998) 30:195–202. doi: 10.1016/S0920-1211(98)00004-7
29. Lonsdale D, Nylen K, McIntyre Burnham W. The anticonvulsant effects of progesterone and its metabolites on amygdala-kindled seizures in male rats. *Brain Res* (2006) 1101:110–6. doi: 10.1016/j.brainres.2006.05.005
30. Jeffrey M, Lang M, Gane J, Chow E, Wu C, Zhang L. Novel anticonvulsive effects of progesterone in a mouse model of hippocampal electrical kindling. *Neuroscience* (2014) 257:65–75. doi: 10.1016/j.neuroscience.2013.10.074
31. van Luijtelaar G, Budziszewska B, Jaworska-Feil L, Ellis J, Coenen A, Lasoń W. The ovarian hormones and absence epilepsy: a long-term EEG study and pharmacological effects in a genetic absence epilepsy model. *Epilepsy Res* (2001) 46:225–39. doi: 10.1016/s0920-1211(01)00277-7
32. Frye CA, Scalise TJ. Anti-seizure effects of progesterone and 3alpha, 5alpha-THP in kainic acid and perforant pathway models of epilepsy. *Psychoneuroendocrinology* (2000) 25:407–20. doi: 10.1016/s0306-4530(99)00068-2
33. Billiar RB, Little B, Kline I, Reier P, Takaoka Y, White RJ. The metabolic clearance rate, head and brain extractions, and brain distribution and metabolism of progesterone in the anesthetized, female monkey (Macaca mulatta). *Brain Res* (1975) 94:99–113. doi: 10.1016/0006-8993(75)90880-x
34. Karavolas HJ, Hodges D, O'Brien D. Uptake of (3H)progesterone and (3H) 5alphadihydroprogesterone by rat tissues in vivo and analysis of accumulated radioactivity: accumulation of 5alpha-dihydroprogesterone by pituitary and hypothalamic tissues. *Endocrinology* (1976) 98:164–75. doi: 10.1210/endo-98-1-164
35. Wu YV, Burnham WM. The anti-seizure effects of IV 5α-dihydroprogesterone on amygdala-kindled seizures in rats. *Epilepsy Res* (2018) 146:132–6. doi: 10.1016/j.eplepsyres.2018.07.022

36. Zorumski CF, Wittmer LL, Isenberg KE, Hu Y, Covey DF. Effects of neurosteroid and benz[e]indene enantiomers on GABAA receptors in cultured hippocampal neurons and transfected HEK-293 cells. *Neuropharmacology* (1996) 35(9-10):1161–8. doi: 10.1016/S0028-3908(96)00035-4
37. Akula KK, Dhir A, Kulkarni SK. Effect of various antiepileptic drugs in a pentylenetetrazol-induced seizure model in mice. *Methods Find Exp Clin Pharmacol* (2009) 31(7):423–32. doi: 10.1358/mf.2009.31.7.1415891
38. Meletti S, Lucchi C, Monti G, Giovannini G, Bedin R, Trenti T, et al. Low levels of progesterone and derivatives in cerebrospinal fluid of patients affected by status epilepticus. *J Neurochem* (2018) 147(2):275–84. doi: 10.1111/jnc.14550
39. Mòdol L, Darbra S, Vallèe M, Pallarès M. Alteration of neonatal allopregnanolone levels affects exploration, anxiety, aversive learning and adult behavioural response to intrahippocampal neurosteroids. *Behav Brain Res* (2013) 241:96–104. doi: 10.1016/j.bbr.2012.11.043
40. Noorbakhsh F, Ellestad KK, Maingat F, Warren KG, Han MH, Steinman L, et al. Impaired neurosteroid synthesis in multiple sclerosis. *Brain* (2011) 134 (Pt 9):2703–21. doi: 10.1093/brain/awr200
41. Djebaili M, Guo Q, Pettus EH, Hoffman SW, Stein DG. The neurosteroids progesterone and allopregnanolone reduce cell death, gliosis, and functional deficits after traumatic brain injury in rats. *J Neurotrauma* (2005) 22(1):106–18. doi: 10.1089/neu.2005.22.106
42. Wojtal K, Trojnar MK, Czuczwar SJ. Endogenous neuroprotective factors: neurosteroids. *Pharmacol Rep* (2006) 58(3):335–40.
43. Kaciński M, Leśkiewicz M, Jaworska-Feil L, Zajac A, Kubik A, Budziszewska B, et al. Pseudo-epileptic seizures in children are not associated with enhanced plasma level of allopregnanolone. *Pharmacol Rep* (2007) 59 (6):683–90.
44. Lévesque M, Herrington R, Leclerc L, Rogawski MA, Avoli M. Allopregnanolone decreases interictal spiking and fast ripples in an animal model of mesial temporal lobe epilepsy. *Neuropharmacology* (2017) 121:12–9. doi: 10.1016/j.neuropharm.2017.04.020
45. Rogawski MA, Loya CM, Reddy K, Zolkowska D, Lossin C. Neuroactive steroids for the treatment of status epilepticus. *Epilepsia* (2013) 54 Suppl 6:93–8. doi: 10.1111/epi.12289
46. Lucchi C, Costa AM, Rustichelli C, Biagini G. Allopregnanolone and pregnanolone are reduced in the hippocampus of epileptic rats, but only allopregnanolone correlates with the seizure frequency. *Neuroendocrinology* (2020). doi: 10.1159/000509093
47. Meletti S, Lucchi C, Monti G, Giovannini G, Bedin R, Trenti T, et al. Decreased allopregnanolone levels in cerebrospinal fluid obtained during status epilepticus. *Epilepsia* (2017) 58(2):e16–20. doi: 10.1111/epi.13625
48. Vaitkevicius H, Husain AM, Rosenthal ES, Rosand J, Bobb W, Reddy K, et al. First-in-man allopregnanolone use in super-refractory status epilepticus. *Ann Clin Transl Neurol* (2017) 4(6):411–14. doi: 10.1002/acn3.408
49. Rogawski MA, Reddy DS. Neurosteroids and infantile spasms: the deoxycorticosterone hypothesis. *Int Rev Neurobiol* (2002) 49:199–219. doi: 10.1016/S0074-7742(02)49014-9
50. Banerjee PK, Snead OC3. Neuroactive steroids exacerbate gamma-hydroxybutyric acid-induced absence in rats. *Eur J Pharmacol* (1998) 359 (1):41–8. doi: 10.1016/S0014-2999(98)00629-3
51. Nohria V, Giller E. Ganaxolone. *Neurotherapeutics* (2007) 4(1):102–5. doi: 10.1016/j.nurt.2006.11.003
52. Carver CM, Reddy DS. Neurosteroid interactions with synaptic and extrasynaptic GABA(A) receptors: regulation of subunit plasticity, phasic and tonic inhibition, and neuronal network excitability. *Psychopharmacol (Berl)* (2013) 230(2):151–88. doi: 10.1007/s00213-013-3276-5
53. Zaccara G, Schmidt D. Do traditional anti-seizure drugs have a future? A review of potential antiseizure drugs in clinical development. *Pharmacol Res* (2016) 104:38–48. doi: 10.1016/j.phrs.2015.12.011
54. Reddy DS, Woodward R. Ganaxolone: a prospective overview. *Drugs Future* (2004) 29:227–42. doi: 10.1358/dof.2004.029.03.793135
55. Gasior M, Ungard JT, Beekman M, Carter RB, Witkin JM. Acute and chronic effects of the synthetic neuroactive steroid, ganaxolone, against the convulsive and lethal effects of pentylenetetrazol in seizure-kindled mice: comparison with diazepam and valproate. *Neuropharmacology* (2000) 39 (7):1184–96. doi: 10.1016/s0028-3908(99)00190-2
56. Reddy DS, Rogawski MA. Ganaxolone suppression of behavioral and electrographic seizures in the mouse amygdala kindling model. *Epilepsy Res* (2010) 89(2-3):254–60. doi: 10.1016/j.eplepsyres.2010.01.009
57. Mares P, Stehlíková M. Anticonvulsant doses of ganaxolone do not compromise motor performance in immature rats. *Neurosci Lett* (2010) 469(3):396–9. doi: 10.1016/j.neulet.2009.12.037
58. Citraro R, Russo E, Di Paola ED, Ibbadu GF, Gratteri S, Marra R, et al. Effects of some neurosteroids injected into some brain areas of WAG/Rij rats, an animal model of generalized absence epilepsy. *Neuropharmacology* (2006) 50 (8):1059–71. doi: 10.1016/j.neuropharm.2006.02.011
59. Bialer M, Johannessen SI, Levy RH, Perucca E, Tomson T, White HS. Progress report on new antiepileptic drugs: a summary of the Tenth Eilat Conference (EILAT X). *Epilepsy Res* (2010) 92(2-3):89–124. doi: 10.1016/j.eplepsyres.2010.09.001
60. Bialer M, Johannessen SI, Levy RH, Perucca E, Tomson T, White HS. Progress report on new antiepileptic drugs: a summary of the Eleventh Eilat Conference (EILAT XI). *Epilepsy Res* (2013) 103(1):2–30. doi: 10.1016/j.eplepsyres.2012.10.001
61. Sperling MR, Klein P, Tsai J. Randomized, double-blind, placebo-controlled phase 2 study of ganaxolone as add-on therapy in adults with uncontrolled partial-onset seizures. *Epilepsia* (2017) 58(4):558–64. doi: 10.1111/epi.13705
62. Weir CJ, Ling AT, Belelli D, Wildsmith JA, Peters JA, Lambert JJ. The interaction of anaesthetic steroids with recombinant glycine and GABAA receptors. *Br J Anaesth* (2004) 92(5):704–11. doi: 10.1093/bja/aeh125
63. Rogawski MA, Reddy DS. *Neurosteroids: endogenous modulators of seizures susceptibility: in Epilepsy: Scientific Foundations of Clinical Practive*. JM Rho, R Sankar and J Cavazos, editors. New York: Marcel Dekker (2004) p. 319–55.
64. Budziszewska B, Siwanowicz J, Leśkiewicz M, Jaworska-Feil L, Lasoń W. Protective effects of neurosteroids against NMDA-induced seizures and lethality in mice. *Eur J Neuropsychopharmacol* (1998) 8(1):7–12. doi: 10.1016/S0924-977X(97)00037-0
65. Borowicz KK, Zadrozniak M, Swiader M, Kowalska A, Kleinrok Z, Czuczwar SJ. Interaction of the neurosteroid alphaxalone with conventional antiepileptic drugs in different types of experimental seizures. *Eur J Pharmacol* (2002) 449(1-2):85–90. doi: 10.1016/S0014-2999(02)01975-1
66. Chuang SH, Reddy DS. Isobolographic analysis of antiseizure activity of the GABA-A receptor-modulating synthetic neurosteroids brexanolone and ganaxolone with tiagabine and midazolam. *J Pharmacol Exp Ther* (2020) 372(3):285–98. doi: 10.1124/jpet.119.261735
67. Zolkowska D, Wu CY, Rogawski MA. Intramuscular allopregnanolone and ganaxolone in a mouse model of treatment-resistant status epilepticus. *Epilepsia* (2018) 59 Suppl 2:220–7. doi: 10.1111/epi.13999
68. Joshi S, Kapur J. Neurosteroid regulation of GABAA receptors: A role in catamenial epilepsy. *Brain Res* (2019) 1703:31–40. doi: 10.1016/j.brainres.2018.02.031
69. Herzog AG. Catamenial epilepsy: Update on prevalence, pathophysiology and treatment from the findings of the NIH Progesterone Treatment Trial. *Seizure* (2015) 28:18–25. doi: 10.1016/j.seizure.2015.02.024
70. Maguire MJ, Nevitt SJ. Treatments for seizures in catamenial (menstrual-related) epilepsy. *Cochrane Database Syst Rev* (2019) 10:CD013225. doi: 10.1002/14651858.CD013225
71. Maguire JL, Stell BM, Rafizadeh M, Mody I. Ovarian cycle-linked changes in GABA(A) receptors mediating tonic inhibition alter seizure susceptibility and anxiety. *Nat Neurosci* (2005) 8(6):797–804. doi: 10.1038/nn1469
72. Reddy DS. Neurosteroids and their role in sex-specific epilepsies. *Neurobiol Dis* (2014) 72 PB:198–209. doi: 10.1016/j.nbd.2014.06.010
73. Wu YV, Burnham WM. Progesterone, 5a-dihydropogesterone and allopregnanolone's effects on seizures: A review of animal and clinical studies. *Seizure* (2018) 63:26–36. doi: 10.1016/j.seizure.2018.10.012
74. Dana-Haeri J, Richens A. Effect of norethisterone on seizures associated with menstruation. *Epilepsia* (1983) 24(3):377–81. doi: 10.1111/j.1528-1157.1983.tb04901.x
75. Herzog AG, Frye CA. Seizure exacerbation associated with inhibition of progesterone metabolism. *Ann Neurol* (2003) 53(3):390–1. doi: 10.1002/ana.10508
76. Herzog AG, Fowler KM, Smithson SD, Kalayjian LA, Heck CN, Sperling MR, et al. Progesterone vs placebo therapy for women with epilepsy: A

randomized clinical trial. *Neurology* (2012) 78(24):1959–66. doi: 10.1212/WNL.0b013e318259e1f9
77. Herzog AG, Frye CAProgesterone Trial Study Group. Allopregnanolone levels and seizure frequency in progesterone-treated women with epilepsy. *Neurology* (2014) 83(4):345–8. doi: 10.1212/WNL.0000000000000623
78. Motta E, Golba A, Ostrowska Z, Steposz A, Huc M, Kotas-Rusnak J, et al. Progesterone therapy in women with epilepsy. *Pharmacol Rep* (2013) 65 (1):89–98. doi: 10.1016/s1734-1140(13)70967-8
79. Mostacci B, Esposto R, Lello S, Bisulli F, Licchetta L, Tinuper P. Estrogen-related seizure exacerbation following hormone therapy for assisted reproduction in women with epilepsy. *Seizure* (2018) 61:200–2. doi: 10.1016/j.seizure.2018.08.024
80. Woolley CS, McEwen BS. Estradiol regulates hippocampal dendritic spine density via an N-methyl-D-aspartate receptor-dependent mechanism. *J Neurosci* (1994) 14(12):7680–7. doi: 10.1523/JNEUROSCI.14-12-07680.1994
81. Smejkalova T, Woolley CS. Estradiol acutely potentiates hippocampal excitatory synaptic transmission through a presynaptic mechanism. *J Neurosci* (2010) 30(48):16137–48. doi: 10.1523/JNEUROSCI.4161-10.2010
82. Pottoo FH, Javed MN, Barkat MA, Alam MS, Nowshehri JA, Alshayban DM, et al. Estrogen and serotonin: Complexity of interactions and implications for epileptic seizures and epileptogenesis. *Curr Neuropharmacol* (2019) 17(3):214–31. doi: 10.2174/1570159X16666180628164432
83. McEwen BS. Invited review: Estrogens effects on the brain: multiple sites and molecular mechanisms. *J Appl Physiol* (2001) 91(6):2785–801. doi: 10.1152/jappl.2001.91.6.2785
84. Iqbal R, Jain GK, Siraj F, Vohora D. Aromatase inhibition by letrozole attenuates kainic acid-induced seizures but not neurotoxicity in mice. *Epilepsy Res* (2018) 143:60–9. doi: 10.1016/j.eplepsyres.2018.04.004
85. Rashid D, Panda BP, Vohora D. Reduced estradiol synthesis by letrozole, an aromatase inhibitor, is protective against development of pentylenetetrazole-induced kindling in mice. *Neurochem Int* (2015) 90:271–4. doi: 10.1016/j.neuint.2015.10.001
86. Tutka P, Mróz K, Mróz T, Buszewicz G, Aebisher D, Bartusik-Aebisher D, et al. Effects of androsterone on the protective action of various antiepileptic drugs against maximal electroshock-induced seizures in mice. *Psychoneuroendocrinology* (2019) 101:27–34. doi: 10.1016/j.psyneuen.2018.10.017
87. Edwards HE, Vimal S, Burnham WM. Dose-, time-, age, and sex-response profiles for the anticonvulsant effects of deoxycorticosterone in 15-day-old-rats. *Exp Neurol* (2002) 176(2):364–70. doi: 10.1006/exnr.2002.7931
88. Perez-Cruz C, Lonsdale D, Burnham WM. Anticonvulsant actions of deoxycorticosterone. *Brain Res* (2007) 1145:81–9. doi: 10.1016/j.brainres.2007.01.123
89. Pawluski JL, Kuchenbuch M, Hadjadj S, Dieuset G, Costet N, Vercueil L, et al. Long-term negative impact of an inappropriate first antiepileptic medication on the efficacy of a second antiepileptic medication in mice. *Epilepsia* (2018) 59(7):e109–e13. doi: 10.1111/epi.14454
90. Löscher W, Puskarjov M, Kaila K. Cation-chloride cotransporters NKCC1 and KCC2 as potential targets for novel antiepileptic and antiepileptogenic treatments. *Neuropharmacology* (2013) 69:62–74. doi: 10.1016/j.neuropharm.2012.05.045
91. Flossmann T, Kaas T, Rahmati V, Kiebel SJ, Witte OW, Holthoff K, et al. Somatostatin interneurons promote neuronal synchrony in the neonatal hippocampus. *Cell Rep* (2019) 26(12):3173–82. doi: 10.1016/j.celrep.2019.02.061
92. Valeeva G, Tressard T, Mukhtarov M, Baude A, Khazipov R. An optogenetic approach for investigation of excitatory and inhibitory network GABA actions in mice expressing channelrhodopsin-2 in GABAergic neurons. *J Neurosci* (2016) 36(22):5961–73. doi: 10.1523/JNEUROSCI.3482-15.2016
93. Ben-Ari Y, Khalilov I, Kahle KT, Cherubini E. The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. *Neuroscientist* (2012) 18(5):467–86. doi: 10.1177/1073858412438697
94. Plotkin MD, Snyder EY, Hebert SC, Delpire E. Expression of the Na-K-2Cl cotransporter is developmentally regulated in postnatal rat brains: a possible mechanism underlying GABA's excitatory role in immature brain. *J Neurobiol* (1997) 33(6):781–95. doi: 10.1002/(SICI)1097-4695(19971120)33:6<781::AID-NEU6>3.0.CO;2-5
95. Dzhala VI, Talos DM, Sdrulla DA, Brumback AC, Mathews GC, Benke TA, et al. NKCC1 transporter facilitates seizures in the developing brain. *Nat Med* (2005) 11(11):1205–13. doi: 10.1038/nm1301
96. Tyzio R, Holmes GL, Ben-Ari Y, Khazipov R. Timing of the developmental switch in GABA(A) mediated signaling from excitation to inhibition in CA3 rat hippocampus using gramicidin perforated patch and extracellular recordings. *Epilepsia* (2007) 48 Suppl 5:96–105. doi: 10.1111/j.1528-1167.2007.01295.x
97. Kolbaev SN, Sharopov S, Dierkes PW, Luhmann HJ, Kilb W. Phasic GABAA-receptor activation is required to suppress epileptiform activity in the CA3 region of the immature rat hippocampus. *Epilepsia* (2012) 53 (5):888–96. doi: 10.1111/j.1528-1167.2012.03442.x
98. Spigelman I, Li Z, Liang J, Cagetti E, Samzadeh S, Mihalek RM, et al. Reduced inhibition and sensitivity to neurosteroids in hippocampus of mice lacking the GABA(A) receptor delta subunit. *J Neurophysiol* (2003) 90 (2):903–10. doi: 10.1152/jn.01022.2002
99. Sharopov S, Winkler P, Uehara R, Lombardi A, Halbhuber L, Okabe A, et al. Allopregnanolone augments epileptiform activity of an in-vitro mouse hippocampal preparation in the first postnatal week. *Epilepsy Res* (2019) 157:106196. doi: 10.1016/j.eplepsyres.2019.106196
100. Mares P. Anticonvulsant action of three neurosteroids against cortical epileptic afterdischarges in immature rats. *Brain Res Bull* (2005) 68 (3):179–84. doi: 10.1016/j.brainresbull.2005.08.008
101. Mares P, Mikulecká A, Haugvicová R, Kasal A. Anticonvulsant action of allopregnanolone in immature rats. *Epilepsy Res* (2006) 70(2-3):110–7. doi: 10.1016/j.eplepsyres.2006.03.009
102. Mares P, Kubová H, Kasal A. Anticonvulsant action of a new analogue of allopregnanolone in immature rats. *Physiol Res* (2010) 59(2):305–8.
103. Clancy B, Finlay BL, Darlington RB, Anand KJ. Extrapolating brain development from experimental species to humans. *Neurotoxicology* (2007) 28(5):931–7. doi: 10.1016/j.neuro.2007.01.014
104. Hirst JJ, Cumberland AL, Shaw JC, Bennett GA, Kelleher MA, Walker DW, et al. Loss of neurosteroid-mediated protection following stress during fetal life. *J Steroid Biochem Mol Biol* (2016) 160:181–8. doi: 10.1016/j.jsbmb.2015.09.012
105. Perez-Cruz C, Likhodii S, Burnham WM. Deoxycorticosterone,s anticonvulsant effects in infant rats are blocked by finasteride, but not by indomethacin. *Exp Neurol* (2006) 200(2):283–9. doi: 10.1016/j.expneurol.2006.02.012
106. Broomall E, Natale JE, Grimason M, Goldstein J, Smith CM, Chang C, et al. Pediatric super-refractory status epilepticus treated with allopregnanolone. *Ann Neurol* (2014) 76(6):911–5. doi: 10.1002/ana.24295
107. Grosso S, Luisi S, Mostardini R, Farnetani M, Cobellis L, Morgese G, et al. Inter-ictal and post-ictal circulating levels of allopregnanolone, an anticonvulsant metabolite of progesterone, in epileptic children. *Epilepsy Res* (2003) 54(1):29–34. doi: 10.1016/S0920-1211(03)00042-1
108. Tan C, Shard C, Ranieri E, Hynes K, Pham DH, Leach D, et al. Mutations of protocadherin 19 in female epilepsy (PCDH19-FE) lead to allopregnanolone deficiency. *J Hum Mol Genet* (2015) 24(18):5250–9. doi: 10.1093/hmg/ddv245
109. Trivisano M, Lucchi C, Rustichelli C, Terracciano A, Cusmai R, Ubertini GM, et al. Reduced steroidogenesis in patients with PCDH19-female limited epilepsy. *Epilepsia* (2017) 58(6):e91–5. doi: 10.1111/epi.13772
110. Kerrigan JF, Shields WD, Nelson TY, Bluestone DL, Dodson WE, Bourgeois BF, et al. Ganaxolone for treating intractable infantile spasms: a multicenter, open-label, add-on trial. *Epilepsy Res* (2000) 42(2-3):133–9. doi: 10.1016/S0920-1211(00)00170-4
111. Pieribone VA, Tsai J, Soufflet C, Rey E, Shaw K, Giller E, et al. Clinical evaluation of ganaxolone in pediatric and adolescent patients with refractory epilepsy. *Epilepsia* (2007) 48(10):1870–4. doi: 10.1111/j.1528-1167.2007.01182.x
112. Yawno T, Miller SL, Bennet L, Wong F, Hirst JJ, Fahey M, et al. Ganaxolone: a new treatment for neonatal seizures. *Front Cell Neurosci* (2017) 11:246:246. doi: 10.3389/fncel.2017.00246
113. Borowicz KK, Wilczyński PM, Kleinrok Z, Czuczwar SJ. Rimcazole, a sigma receptor ligand, and the anticonvulsive action of conventional antiepileptic drugs. *J Neural Transm* (1998) 105(6-7):601–12. doi: 10.1007/s007020050082
114. Kułak W, Sobaniec W, Wojtal K, Czuczwar SJ. Calcium modulation in epilepsy. *Pol J Pharmacol* (2004) 56(1):29–41.
115. Pisu MG, Mostallino MC, Dore R, Mura ML, Maciocco E, Russo E, et al. Neuroactive steroids and GABAA receptor plasticity in the brain of the WAG/Rij rat, a model of absence epilepsy. *J Neurochem* (2008) 106(6):2502–14. doi: 10.1111/j.1471-4159.2008.05538.x

116. Perry MS. New and emerging medications for treatment of pediatric epilepsy. *Pediatr Neurol* (2019) 107:24–7. doi: 10.1016/j.pediatrneurol.2019.11.008
117. Olney JW, Young C, Wozniak DF, Jevtovic-Todorovic V, Ikonomidou C. Do pediatric drugs cause developing neurons to commit suicide? *Trends Pharmacol Sci* (2004) 25(3):135–9. doi: 10.1016/j.tips.2004.01.002

Conflict of Interest: SC was granted financial support from Bayer, GlaxoSmithKline, Janssen, Novartis, Sanofi-Aventis for lecturing (until 2014). He also received an unrestricted grant from GlaxoSmithKline for the period of 2006-2007.

The remaining 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: 07 October 2020
doi: 10.3389/fendo.2020.570560

The Role of Estrogen in Anxiety-Like Behavior and Memory of Middle-Aged Female Rats

*Emese Renczés[1], Veronika Borbélyová[1], Manuel Steinhardt[1], Tim Höpfner[1], Thomas Stehle[1], Daniela Ostatníková[2] and Peter Celec[1,3,4]**

[1] Institute of Molecular Biomedicine, Faculty of Medicine, Comenius University in Bratislava, Bratislava, Slovakia, [2] Institute of Physiology, Faculty of Medicine, Comenius University in Bratislava, Bratislava, Slovakia, [3] Institute of Pathophysiology, Faculty of Medicine, Comenius University in Bratislava, Bratislava, Slovakia, [4] Department of Molecular Biology, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, Slovakia

Aging in women is associated with low estrogen, but also with cognitive decline and affective disorders. Whether low estrogen is causally responsible for these behavioral symptoms is not clear. Thus, we aimed to examine the role of estradiol in anxiety-like behavior and memory in rats at middle age. Twelve-month old female rats underwent ovariectomy (OVX) or were treated with 1 mg/kg of letrozole—an aromatase inhibitor. In half of the OVX females, 10 μg/kg of 17β-estradiol was supplemented daily for 4 weeks. Vehicle-treated sham-operated and OVX females served as controls. For behavioral assessment open field, elevated plus maze and novel object recognition tests were performed. Interaction between ovarian condition and additional treatment had the main effect on anxiety-like behavior of rats in the open field test. In comparison to control females, OVX females entered less frequently into the center zone of the open field ($p < 0.01$) and showed lower novel object discrimination ($p = 0.05$). However, estradiol-supplemented OVX rats had higher number of center-zone entries ($p < 0.01$), spent more time in the center zone ($p < 0.05$), and showed lower thigmotaxis ($p < 0.01$) when compared to OVX group. None of the hormonal manipulations affected anxiety-like behavior in the elevated plus maze test significantly, but a mild effect of interaction between ovarian condition and treatment was shown ($p = 0.05$). In conclusion, ovariectomy had slight negative effect on open-field ambulation and short-term recognition memory in middle-aged rats. In addition, a test-specific anxiolytic effect of estradiol supplementation was found. In contrast, letrozole treatment neither affected anxiety-like behavior nor memory.

Keywords: exploration, gonadectomy, masculinization, mood disorders, senescence

OPEN ACCESS

Edited by:
Kiran K. Soma,
University of British Columbia, Canada

Reviewed by:
Aaron M. Jasnow,
Kent State University, United States
Tamara S. Bodnar,
University of British Columbia, Canada

***Correspondence:**
Peter Celec
petercelec@gmail.com

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

Received: *08 June 2020*
Accepted: *02 September 2020*
Published: *07 October 2020*

Citation:
Renczés E, Borbélyová V, Steinhardt M, Höpfner T, Stehle T, Ostatníková D and Celec P (2020) The Role of Estrogen in Anxiety-Like Behavior and Memory of Middle-Aged Female Rats. Front. Endocrinol. 11:570560. doi: 10.3389/fendo.2020.570560

INTRODUCTION

Post-menopause is a life period in women accompanied by reproductive senescence, including decline in ovarian sex hormones (1). Besides physical changes (2), there are many mental and psychological disorders related to post-menopausal syndrome, such as depression, anxiety or dementia (3–6). The underlying mechanism of age-related cognitive decline and anxiety is unclear, but the role of estrogen loss is considered (7–10). However, most of the post-menopausal symptoms

may result from the aging process as well as from loss in ovarian endocrine function (11). To analyze the causal effect of menopause-related decline in sex hormone production, further experimental studies are needed.

In animal experiments, the bilateral removal of ovaries—ovariectomy—is the most used tool to mirror the post-menopausal state of women (12). It has been shown that ovariectomy causes impaired learning and memory indicated by longer latency time to find a platform and less time spent in the target zone in the Morris water maze, and by lower recognition index in the novel object recognition and object placement tasks (13–16). Similarly, there are numerous data showing anxiogenic effect of ovariectomy in several tests, such as open field, elevated plus maze or light-dark box (16–20). On the other hand, estradiol replacement in the surgical model of menopause may improve cognitive functions and decrease anxiety-like behavior in rodents (14, 21–31).

Inhibition of aromatase leads to decreased estrogen production as well, while it increases gonadotropin releasing hormone or luteinizing hormone, and causes hyperandrogenism (32–34). Animal studies using pharmacological aromatase inhibitors - e.g., letrozole, and aromatase knock-out mice have suggested that aromatase has a role in cognitive functions (35). Neurobehavioral studies have found that aromatase inhibition may result in decreased spine and synaptic density in forebrain, impaired spatial memory, recognition memory, and contextual fear memory (36, 37). On the contrary, letrozole treatment in middle-aged females may increase neurogenesis in the hippocampus (38). Although letrozole might be efficient for treatment of infertility at reproductive age, e.g., in women with polycystic ovary syndrome (39), its most common clinical application is in postmenopausal women suffering from breast cancer (40).

Natural menopause in women occurs typically at midlife, i.e., 50 years (41). Therefore, studies performed in middle-aged rats are valuable and important for understanding the causal relationship between estrogen deficiency and psychological features of post-menopausal syndrome. Although there are published studies on the effect of ovariectomy (29, 30, 42–44) and estradiol treatment (21–23, 25, 29, 31) on memory and anxiety-like behavior in middle-aged (∼12-month old) and old rodents (>18-month old), studies on such behavioral effects of letrozole treatment are rare (45).

In this study, we consider that ovariectomy is accompanied by decreased production of ovarian sex hormones including androgens, while inhibited aromatization of testosterone may lead to its excess. Thus, our main goal was to compare and explore the causal effect of both, surgical and pharmacological inhibition of estrogen production on anxiety-like behavior and memory in middle-aged rats. We hypothesized that if estrogen deficiency is the main cause of cognitive dysfunction and anxiety in middle-aged females, both ovariectomy and letrozole-treatment will impair memory and induce higher anxiety-like behavior in comparison to controls. In addition, we aimed to examine whether estradiol supplementation may improve memory and attenuate anxiety in middle-aged ovariectomized female rats.

METHODS

Animals

Female Wistar rats ($n = 42$, 12-month old, weighing 362 ± 52 g) were used in the experiment. Twelve-weeks old animals were purchased from Velaz (Prague, Czech Republic), and maintained under standard conditions (temperature 25 ± 2°C and humidity 55 ± 10%) with a 12:12 light-dark cycle, and housed in groups, 4-5 per cage (w:38 × l:60 × h:20 cm). Except the duration of behavioral testing, animals had *ad libitum* access to food and water. The experiment was approved by the local Ethics committee and performed according to the Slovak legislation.

Ovariectomy

Twelve-months old female rats underwent either ovariectomy (OVX, $n = 21$) or sham surgery (F, $n = 21$). The procedures were performed under general anesthesia using intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). A single ventral transverse incision of 1–1.5 cm was made at the middle abdominal region. The uterine horns and vessels were ligated on both sides, the ovaries were cut, and the remaining tissue was placed back into the abdominal cavity. The muscle and skin layers were sutured with absorbable silk (size 4-0).

Hormonal Treatment

Five weeks after surgery, females were randomly divided into several treatment groups. For 4 weeks, sham-operated females were treated with an aromatase inhibitor, letrozole, in a dose of 1 mg/kg (Sigma-Aldrich, Darmstadt, Germany; F+LET, $n = 11$), and OVX females with 17β-estradiol in a dose of 10 μg/kg (Sigma-Aldrich, Darmstadt, Germany; OVX+E, $n = 11$). Vehicle-treated F and OVX groups received olive oil in a volume of 1 ml/kg (Olivae Oleum Raffinatum, Galvex, Banská Bystrica, Slovak Republic). The treatments were administered once daily, between 3 and 5 p.m., by subcutaneous injection.

Behavioral Testing

During the last week of treatment, the animals were tested for locomotor activity, anxiety-like behavior and memory using a battery of behavioral tests. The behavioral phenotyping of rats was carried out in three consecutive days (one test per day), between 8 and 12 a.m., in the fixed order from the least to the most stressful, i.e., open field, novel object recognition, and elevated plus maze test. Each test was recorded using a camcorder placed above the apparatus in the middle of the testing room. All observed parameters were analyzed using the image and video processing system EthoVision XT 10.0 (Noldus Information Technology, Wageningen, Netherlands).

Open Field Test

Open field test was performed to assess locomotor activity and anxiety-like behavior of animals. A square shaped (100 × 100 cm) apparatus was used, virtually divided into a center (40 × 40 cm) and border zone, which was slightly illuminated with white light (25 lx). Animals were placed individually into the center zone, and were allowed to freely explore the arena for 5 min. Time spent in and number of entries into the center zone, as well as distance

to the wall indicating thigmotaxis was monitored as indexes of anxiety-like behavior.

Novel Object Recognition Test

The novel object recognition test was conducted in the familiar apparatus and under the same condition as the open field test. During the training phase, the animals were exposed to two identical objects (two green plastic or two transparent glass bottles) for 5 min. Total time of interactions with any of the objects was analyzed to assess explorative behavior. One hour later, one of the two objects was swapped for a novel object with a different shape, material and color as the familiar one, and the animals were returned into the arena for another 5 min. To avoid any preference of side or features, the objects (green plastic/transparent glass bottle) were randomly selected as familiar or novel, as well as the position of the novel object in left or right side was systematically altered between the trials. To assess memory, novel-object discrimination was calculated as following: interaction with novel object/(interaction with novel + familiar object)*100. The animals were excluded from analysis, if the object exploration during the training and/or during the testing was below 5% (<15 s from 5 min).

Elevated Plus Maze Test

A plus-shape apparatus elevated to a height of 60 cm above the floor was used to assess anxiety-like behavior. The arena consisted of two opposite open with 100–110 lx illumination and two opposite closed arms with 3–5 lx illumination, extending from central platform. Animals were placed onto the central platform facing to an open arm, and allowed to explore the maze for 5 min. The open-arm preference was evaluated as the number of entries into [open-arm entries/total entries] and time spent in the open arms [time on open arms/(time in open + closed arms)] relative to entries and time in any of the arms.

Uterus Weight and Testosterone Concentration

To verify the effect of surgery and hormonal replacement, uterus weight and testosterone concentration were assessed. At sacrifice of the animals, blood was taken from abdominal aorta and the uterus was dissected. Uterus weight was measured on an analytic scale, and adjusted to body weight (46). Blood samples were centrifuged at 2,400g for 5 min, and plasma samples were stored at −20°C until analysis. The concentration of testosterone in plasma was measured using a commercial ELISA kit (DRG Diagnostic, Marburg, Germany) with 0.029 nmol/L analytical sensitivity, and <5% inter- and intra-assay coefficients of variations.

Statistical Analysis

For statistical analysis GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA) was used. Data were analyzed using two-way ANOVA – one factor being ovarian condition (sham, OVX) and the other factor being additional treatment (control, letrozole in sham groups or estradiol in OVX group). Bonferroni multiple comparison *post hoc* test was used to compare mean of each group with means of every other groups. *P*-values have been adjusted to account for multiple comparison bias. Differences were considered statistically significant when $p < 0.05$. Data are shown as mean plus standard deviation (SD).

RESULTS

To confirm the endocrine changes induced by ovariectomy, estradiol supplementation and letrozole treatment, uterus weight and plasma testosterone were measured. There was a main effect of ovarian condition [$F_{(1, 37)} = 17.0$, $p < 0.001$], a main effect of treatment [$F_{(1, 37)} = 40.7$, $p < 0.001$] and a significant interaction between these two factors [$F_{(1, 37)} = 153$, $p < 0.001$] on uterus weight (**Figure 1A**). In comparison to the F group, the weight of the uterus was significantly lower in OVX ($p < 0.001$) as well as in F + LET group ($p < 0.001$). OVX + E females had a higher uterus weight when compared to OVX group ($p < 0.001$), F group ($p < 0.001$) or F + LET group ($p < 0.001$).

A main effect of ovarian condition [$F_{(1, 36)} = 98.1$, $p < 0.001$], a main effect of treatment [$F_{(1, 36)} = 11.1$, $p < 0.01$], and a significant interaction between ovarian and treatment condition [$F_{(1, 36)} = 10.2$, $p < 0.01$] was observed also on concentration of testosterone (**Figure 1B**). Both, the OVX ($p < 0.001$) and OVX + E groups ($p < 0.001$) had lower, while F + LET group had higher ($p < 0.001$) concentration of testosterone in comparison to control F group. The F + LET group had higher testosterone than OVX ($p < 0.001$) and OVX + E group ($p < 0.001$). OVX + E did not differ from OVX in testosterone concentration ($p > 0.99$).

A significant interaction between ovarian condition and treatment was found [$F_{(1, 37)} = 18.5$, $p < 0.001$], but neither ovarian condition [$F_{1, 37} = 1.03$, $p = 0.32$] nor treatment [$F_{(1, 37)} = 1.41$, $p = 0.24$] had a main effect on the number of entries into the center zone in the open field test (**Figure 2A**). OVX group entered less frequently into the center zone than F group ($p < 0.01$). The number of entries was higher in OVX + E when compared to OVX ($p < 0.01$). F + LET ($p = 0.19$) and OVX + E ($p > 0.99$) groups were comparable to F. In addition, the F + LET group neither differed from OVX group ($p = 0.80$) in center-zone entries.

On anxiety-like behavior assessed by time spent in the center zone in the open field test (**Figure 2B**), a significant main effect of treatment [$F_{(1, 37)} = 5.02$, $p < 0.05$], and a significant interaction between ovarian condition and treatment [$F_{(1, 37)} = 4.60$, $p < 0.05$] was found, while no effect of ovarian condition alone was observed [$F_{(1, 37)} = 2.60$, $p = 0.12$]. OVX + E group spent more time in the center zone in comparison to the OVX group ($p < 0.05$), F group ($p = 0.05$) and F + LET group ($p = 0.06$). Neither OVX ($p > 0.99$) nor F+LET ($p > 0.99$) groups differ from F. In addition, the OVX group was comparable to F + LET group ($p > 0.99$).

There was a main effect of treatment [$F_{(1, 37)} = 5.37$, $p < 0.05$], and a significant interaction between ovarian condition and treatment [$F_{(1, 37)} = 7.02$, $p < 0.05$], but no effect of ovarian condition [$F_{(1, 37)} = 0.04$, $p = 0.84$] on thigmotaxis assessed by distance to the walls during the open-field exploration (**Figure 2C**). Animals in OVX + E group explored the arena

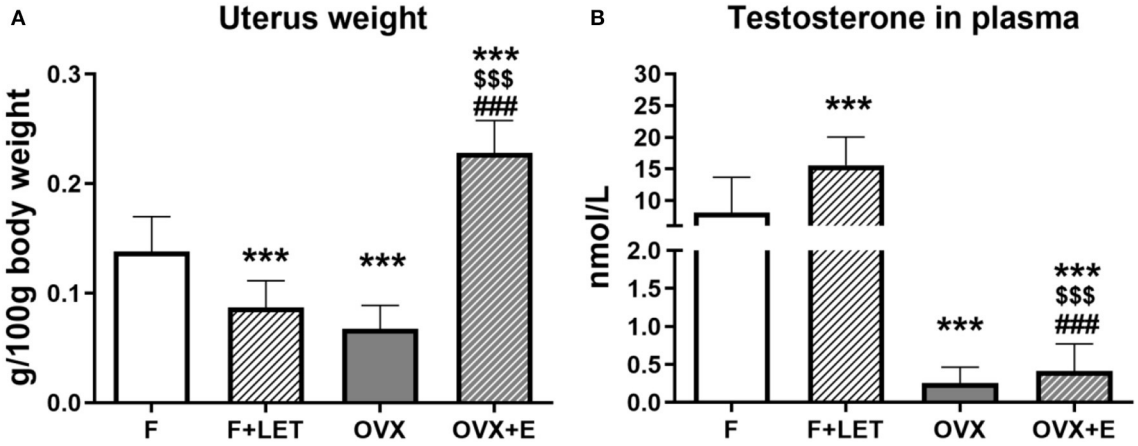

FIGURE 1 | Uterus weight adjusted to body weight **(A)** and testosterone concentration in plasma **(B)**. Data are presented as mean + SD. $^{***}p < 0.001$ in comparison to F group, $^{\$\$\$}p < 0.001$ in comparison to F + LET group, $^{\#\#\#}p < 0.001$ in comparison to OVX group; $n = 9$–11/group; F, females; F + LET, letrozole-treated females; OVX, ovariectomized females; OVX + E, estradiol-treated ovariectomized females.

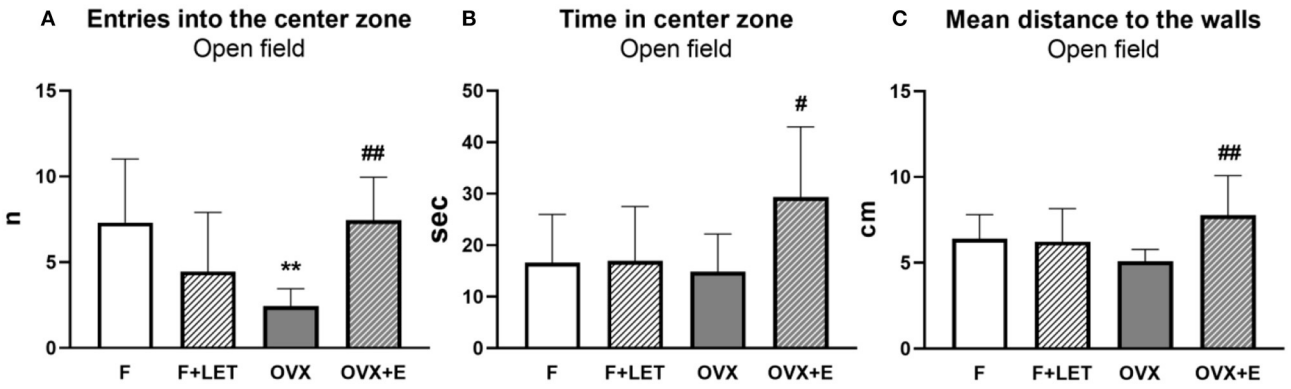

FIGURE 2 | Anxiety-like behavior assessed in the open field test by number of entries into the center zone **(A)**, time spent in the center zone **(B)**, and mean distance to the walls **(C)**. Data are presented as mean + SD. $^{**}p < 0.01$ in comparison to F group, $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ in comparison to OVX group; $n = 9$–11/group; F, females; F + LET, letrozole-treated females; OVX, ovariectomized females; OVX + E, estradiol-treated ovariectomized females.

farther from the walls in comparison to the animals in OVX group ($p < 0.01$). No other differences were found between the groups (OVX vs. F: $p = 0.62$; F + LET vs. F: $p > 0.99$; OVX + E vs. F: $p = 0.47$; F + LET vs. OVX: $p = 0.90$; OVX + E vs. F + LET: $p = 0.26$).

In the elevated plus maze test, no significant main effect of ovarian condition [$F_{(1, 37)} = 2.41$, $p = 0.13$] or treatment [$F_{(1, 37)} = 0.87$, $p = 0.36$], and a statistically non-significant interaction between ovarian condition and treatment [$F_{(1, 37)} = 3.94$, $p = 0.05$] was found on the number of entries onto the open arms (**Figure 3A**). Similarly, neither ovarian condition [$F_{(1, 38)} = 2.13$, $p = 0.15$], nor treatment [$F_{(1, 38)} = 0.52$, $p = 0.47$], nor interaction between these two factors [$F_{(1, 38)} = 1.27$, $p = 0.27$] affected anxiety-like behavior assessed by time spent on the open arms (**Figure 3B**).

In the training phase of the novel object recognition test (**Figure 4A**), no effect of ovarian condition [$F_{(1, 29)} = 2.69$, $p = 0.11$] or treatment [$F_{(1, 29)} = 2.67$, $p = 0.11$], and neither a significant interaction between these two factors [$F_{(1, 29)} = 0.07$, $p = 0.80$] was shown, while some animals had to been excluded from analysis ($n = 5$ in F + LET group and $n = 4$ in OVX group) due to a low object-exploration time. To assess short-term recognition memory, we analyzed novel-object discrimination in the testing phase of the novel object recognition test (**Figure 4B**). Animals exploring the objects for <15 s during the training and/or in testing phase have been excluded from analysis ($n = 1$ in F, $n = 5$ in F + LET, $n = 4$ OVX, $n = 1$ OVX + E). There was a significant main effect of ovarian condition [$F_{(1, 27)} = 5.40$, $p < 0.05$], but no significant effect of treatment [$F_{(1, 27)} = 0.24$, $p = 0.63$] or an interaction between ovarian condition and treatment [$F_{1, 27} = 1.09$, $p = 0.30$] on novel object discrimination.

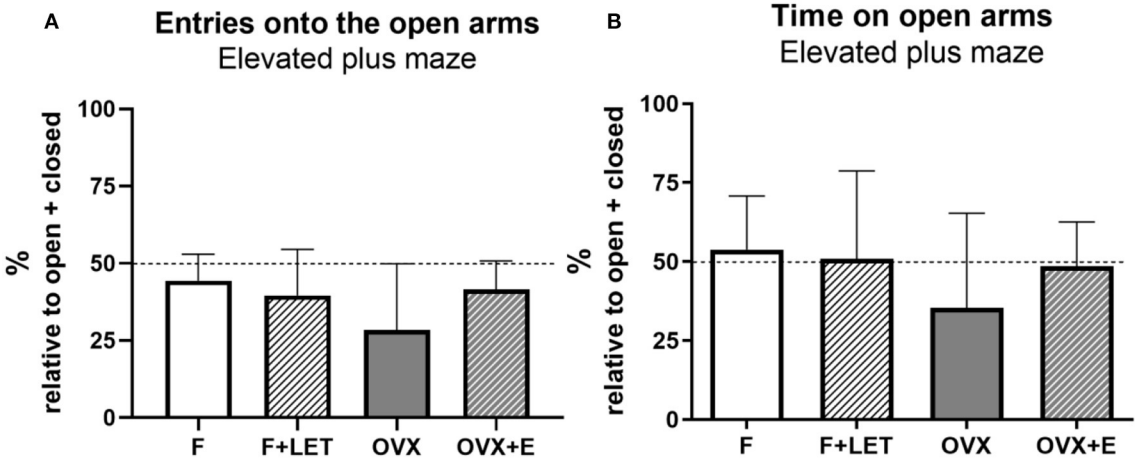

FIGURE 3 | Anxiety-like behavior assessed in the elevated plus maze test by number of entries onto the open arms **(A)** and time spent on the open arms **(B)**. These parameters are expressed as percentage of total entries made into or time spent in the closed + open arms, respectively. Dashed line indicates equal frequency and time exploring open and closed arms. Data are presented as mean + SD. $n = 10–11$/group; F, females; F + LET, letrozole-treated females; OVX, ovariectomized females; OVX + E, estradiol-treated ovariectomized females.

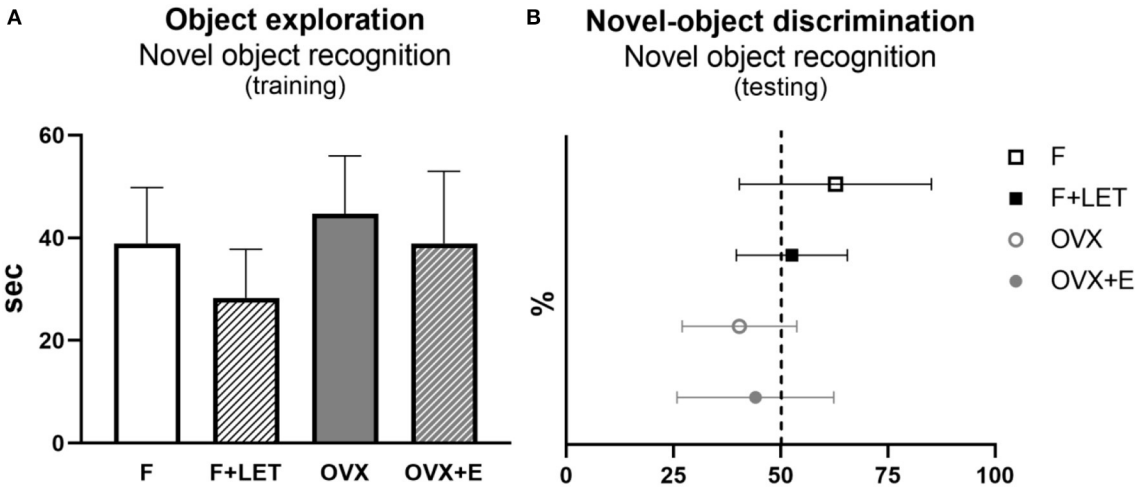

FIGURE 4 | Exploratory behavior assessed by total time spent exploring the objects during the training period **(A)** and recognition memory assessed by novel-object discrimination during the testing period in the novel object recognition test **(B)**. Novel-object discrimination is expressed as percentage of total time spent exploring the novel and familiar objects. Dashed line indicates equal time spent exploring novel and familiar objects. Data are presented as mean + SD. $n = 6–11$/group; F, females; F + LET, letrozole-treated females; OVX, ovariectomized females; OVX + E, estradiol-treated ovariectomized females.

Based on the *post-hoc* test, the OVX group ($p = 0.05$) exhibited lower discrimination of the novel object when compared to F group.

DISCUSSION

In this experiment, ovariectomy in middle-aged females had a slight effect on open-field ambulation and on recognition memory. On the contrary, letrozole treatment had no effect either on anxiety-like behavior or memory in females. Estradiol supplementation in OVX females had test-specific anxiolytic effect in the open field test, but no effect on memory in the novel object recognition task.

There is some evidence indicating that ovariectomy in young-adult females has anxiogenic effect (16–20). However, there is little data on the effect of ovariectomy on anxiety in middle-aged rats (44). Extensive research has been dedicated to examine anxiolytic effect of estradiol in young-adult OVX females (27, 47–52), while only few studies are available on the effect of estradiol on anxiety-like behavior in aged OVX (21) or gonadally intact females (53). Recently, lower anxiety-like behavior was found in 16–18-months old OVX females when compared to the intact counterparts 14 weeks after ovariectomy in elevated plus maze

and light-dark box test (44). On the contrary, we found only a mild effect of ovariectomy on anxiety-like behavior in 12-month-old females in open field or in elevated plus maze test assessed 8 weeks after surgery. The differences might be caused by different age and/or length of ovarian hormone deficiency (54, 55). In our experiment, lower number of entries into the center zone was observed in OVX females when compared to the sham-operated F group, while these two groups were comparable in time spent in the center zone or in distance to walls during the open-field ambulation. However, OVX + E group entered more frequently and also spent more time in the center zone, as well as showed lower thigmotaxis in comparison to OVX females. It is possible that ovariectomy decreases exploratory behavior (13) without any effect on anxiety-like behavior in the open field, while estradiol treatment in OVX females may rescue this impairment in exploration and may decrease anxiety-like behavior as well (21). These results indicate differences in molecular mechanisms of endogenous and exogenous estrogens (28). On the other hand, estradiol treatment did not affect anxiety-like behavior of OVX rats in the elevated plus maze test. The time period between ovariectomy and hormone replacement (21), as well as the length of estradiol treatment (56) are crucial factors modulating anxiolytic effect of estradiol. Hormonal changes may down- or up-regulate the expression of specific estrogen receptor subtypes, which mediate either anxiogenic or anxiolytic effect (57). Thus, the age- and ovariectomy-related changes in expression pattern of estrogen receptors, and the sensitive time-window for estrogen action in different brain regions underlying anxiety should be examined to better understand the test-specific effect of estradiol in aging OVX rats (58).

A large number of studies have shown that ovariectomy may induce cognitive dysfunction in young (13–16) and also in middle-aged rats (29, 30, 43), while estrogens may improve learning and memory performance following ovariectomy in both, young (15, 24, 59–61) and middle-aged females (21–23, 25, 29, 31). However, OVX females in our experiment exhibited mild memory impairment only, and the estradiol-treated group did not outperform vehicle-treated OVX rats. Discrepancies between our and previously published results may arise again from differences in length of exposure to endogenous ovarian hormones (30), in delay period between ovariectomy and estradiol supplementation (21, 62–64), and in dose and frequency of treatment (65). Furthermore, the effect of age should be considered. Age-dependent changes have been shown in estrogen signaling in memory-related brain regions (66, 67), indicating an age-dependent effect of ovariectomy and estrogen supplementation on cognitive functions in rats (68–70). On the contrary, there is some evidence indicating that estradiol treatment in young adult (5-month old) and old (24-month old) ovariectomized mice may improve memory in object recognition task in the same manner (71). The type of memory assessed by different tasks should also be taken into account. Most of the published experiments in middle-aged rats use tasks for hippocampal-dependent spatial memory (29, 30, 30, 43), while in our experiment the non-spatial recognition memory was examined. Moreover, the published studies examining the effect of estradiol on cognition in females are mostly focused on long-term consequences, particularly on memory consolidation (64), but rarely on memory perception or acquisition as we did in our experiment. In contrast to spatial and fear memory tested in the Morris water maze, radial arm maze, T-maze or Pavlovian learning tasks, recognition memory assessed in the novel object recognition test does not include stress stimuli, such as shock, food deprivation or swimming (72, 73). As shown, ovariectomy in middle-aged rats may induce several alterations in hippocampal gene expression influencing neurogenesis, synaptic plasticity and immune modulation (74). However, recognition memory may involve other crucial circuits besides hippocampus, e.g., medial prefrontal cortex (75, 76). To better understand the mechanisms for impaired spatial and recognition memory, further studies are required examining the effect of ovariectomy on expression pattern of specific genes in cognition-associated brain regions in middle-aged female rats.

It should be noted that aging (77) as well as letrozole-treatment (78) and ovariectomy (79) increase luteinizing hormone (LH) production. Many age-related disorders, including the Alzheimer's disease, have been attributed to this LH excess (77). However, it has been shown that the LH rise is smaller if ovariectomy is performed at old age in comparison to the effect of ovariectomy performed in young adulthood (79). This may explain differences between our results obtained from middle-aged rats and previously published data from young-adult ovariectomized females, although LH was not assessed in our study.

Ovariectomy induces loss of ovarian hormones, including estrogens, androgens as well as progesterone (80). On the contrary, inhibition of testosterone aromatization decreases peripheral estrogen production, while the concentrations of other sex hormones, such as testosterone and progesterone, might be maintained, increased and decreased, depending on the dose of the inhibitor - e.g. letrozole (78). To distinguish between the effect of estrogen- and androgen deficiency, we examined the consequences of both ovariectomy and letrozole-treatment in middle-aged rats. While ovariectomy had some mild effect on behavior of females in the open field and novel object tests, treatment with letrozole did not affect either anxiety-like behavior or memory of female rats. Similarly, Chaiton et al. (38) did not found any effect of chronic letrozole treatment on depressive-like behavior in middle-aged female mice (38). In addition, our findings are in line also with our previous results showing that letrozole may induce anxiety in males but not in female aging rats (45). The differences between behavioral consequences of ovariectomy and letrozole treatment indicate that other ovariectomy-induced endocrine changes besides estrogen deficiency, such as altered concentration of progesterone (81), androgen (82) or gonadotropins (18) may underly the observed effect of ovariectomy. The strength of this study is the use of both, surgical and pharmacological tools to induce estrogen deficiency. More importantly, the treatments were initiated at middle-age of rats, which may be more relevant to mirror estrogen loss associated with aging than ovariectomy in adult females. The main limitation is that young-adult animals were not included in the study to investigate the age-dependent effect. Furthermore, only testosterone was assessed in

plasma. Immunoassays are not applicable to measure estradiol concentration in rodents, so, it was estimated indirectly via uterus weight (46). Nevertheless, as previously shown, hippocampal and not circulating estradiol is associated with age-related memory functions in female rats (83). Last but not least, there is a need to examine age-associated alterations in estrogen and androgen signaling pathways in relevant brain regions.

CONCLUSION

In this experiment, we found that ovariectomy, but not letrozole, may attenuate open-field ambulation and slightly impair recognition memory in middle-aged females. Our findings indicate different neurobehavioral consequences of surgically induced estrogen deficiency and pharmacological inhibition of estrogen production. Although we failed to show the anxiogenic effect of ovariectomy, we found that exogenous estradiol may reduce anxiety-like behavior in middle-aged ovariectomized rats, at least in the open field. On the contrary, it seems that ovariectomy may impair short-term memory, but estradiol treatment does not improve it. Thus, we conclude that endocrine changes induced by ovariectomy and aromatase inhibition affect the brain differently, and the molecular mechanisms activated by exogenous estradiol may differ from the signaling pathways of endogenous estrogens. For future experiments elucidating the role of estrogen in postmenopausal syndrome, we suggest to consider some methodological issues, such as age-related changes in the neuroendocrine system or type of manipulation used to induce estrogen deficiency.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of the Institute of Molecular Biomedicine.

AUTHOR CONTRIBUTIONS

ER and PC drafted the manuscript. ER, VB, MS, TH, and TS performed the experiment. DO and PC corrected the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Funding for this study was provided by the Slovak Research and Development Agency (APVV-15-0045) and the Ministry of Education of Slovak Republic (VEGA 1/0635/20).

REFERENCES

1. Dalal PK, Agarwal M. Postmenopausal syndrome. *Indian J Psychiatry*. (2015) 57:S222–32. doi: 10.4103/0019-5545.161483
2. Dennerstein L. A prospective population-based study of menopausal symptoms. *Obstet Gynecol*. (2000) 96:351–8. doi: 10.1016/S0029-7844(00)00930-3
3. Rice D, Barone S Jr. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ Health Perspect*. (2000) 108 (Suppl.):511–533. doi: 10.1289/ehp.00108s3511
4. Kang IH, Kim HS, Shin JH, Kim TS, Moon HJ, Kim IY, et al. Comparison of anti-androgenic activity of flutamide, vinclozolin, procymidone, linuron, and p, p′-DDE in rodent 10-day Hershberger assay. *Toxicology*. (2004) 199:145–59. doi: 10.1016/j.tox.2004.02.019
5. Roselli CE, Resko JA, Stormshak F. Expression of steroid hormone receptors in the fetal sheep brain during the critical period for sexual differentiation. *Brain Res*. (2006) 1110:76–80. doi: 10.1016/j.brainres.2006.06.070
6. Dotlic J, Radovanovic S, Rancic B, Milosevic B, Nicevic S, Kurtagic I, et al. Mental health aspect of quality of life in the menopausal transition. *J Psychosom Obstet Gynecol*. (2020) 4:1—10. doi: 10.1080/0167482X.2020.1734789
7. Altemus M. Sex differences in depression and anxiety disorders : potential biological determinants. *Horm Behav*. (2006) 50:534–8. doi: 10.1016/j.yhbeh.2006.06.031
8. Solomon MB, Herman JP. Sex differences in psychopathology: of gonads, adrenals and mental illness. *Physiol Behav*. (2009) 97:250–8. doi: 10.1016/j.physbeh.2009.02.033
9. McHenry J, Carrier N, Hull E, Kabbaj M. Sex differences in anxiety and depression: role of testosterone. *Front Neuroendocrinol*. (2014) 35:42–57. doi: 10.1016/j.yfrne.2013.09.001
10. Maeng LY, Milad MR. Sex differences in anxiety disorders: Interactions between fear, stress, and gonadal hormones. *Horm Behav*. (2015) 76:106–17. doi: 10.1016/j.yhbeh.2015.04.002
11. Koebele SV, Bimonte-Nelson HA. The endocrine-brain-aging triad where many paths meet: female reproductive hormone changes at midlife and their influence on circuits important for learning and memory. *Exp Gerontol*. (2017) 94:14–23. doi: 10.1016/j.exger.2016.12.011
12. Diaz Brinton R. Minireview: translational animal models of human menopause: challenges and emerging opportunities. *Endocrinology*. (2012) 153:3571–8. doi: 10.1210/en.2012-1340
13. Djiogue S, Djeuda ABD, Etet PFS, Wanda GJMK, Tadah RND, Njamen D. Memory and exploratory behavior impairment in ovariectomized Wistar rats. *Behav Brain Funct*. (2018) 14:14. doi: 10.1186/s12993-018-0146-7
14. Zhang YY, Liu MY, Liu Z, Zhao JK, Zhao YG, He L, et al. GPR30-mediated estrogenic regulation of actin polymerization and spatial memory involves SRC-1 and PI3K-mTORC2 in the hippocampus of female mice. *CNS Neurosci Ther*. (2019) 25:714–33. doi: 10.1111/cns.13108
15. Rashidy-Pour A, Bavarsad K, Miladi-Gorji H, Seraj Z, Vafaei AA. Voluntary exercise and estradiol reverse ovariectomy-induced spatial learning and memory deficits and reduction in hippocampal brain-derived neurotrophic factor in rats. *Pharmacol Biochem Behav*. (2019) 187:172819. doi: 10.1016/j.pbb.2019.172819
16. Campos GV, de Souza AM, Ji H, West CA, Wu X, Lee DL, et al. The angiotensin type 1 receptor antagonist losartan prevents ovariectomy-induced cognitive dysfunction and anxiety-like behavior in long evans rats. *Cell Mol Neurobiol*. (2020) 40:407–20. doi: 10.1007/s10571-019-00744-x
17. Fedotova J, Pivina S, Sushko A. Effects of chronic vitamin D3 hormone administration on anxiety-like behavior in adult female rats after long-term ovariectomy. *Nutrients*. (2017) 9:28. doi: 10.3390/nu9010028
18. Arfa-Fatollahkhani P, Nahavandi A, Abtahi H, Anjidani S, Borhani S, Jameie SB, et al. The effect of luteinizing hormone reducing agent on anxiety and novel object recognition memory in gonadectomized rats. *Basic Clin Neurosci J*. (2017) 8:113–20. doi: 10.18869/nirp.bcn.8.2.113
19. Dornellas AP, Boldarine VT, Pedroso AP, Carvalho LO, de Andrade IS, Vulcani-Freitas TM, et al. High-fat feeding improves anxiety-type

behavior induced by ovariectomy in rats. *Front Neurosci*. (2018) 12:1–13. doi: 10.3389/fnins.2018.00738
20. Puga-Olguín A, Rodríguez-Landa JF, de Jesús Rovirosa-Hernández M, Germán-Ponciano LJ, Caba M, Meza E, et al. Long-term ovariectomy increases anxiety- and despair-like behaviors associated with lower Fos immunoreactivity in the lateral septal nucleus in rats. *Behav Brain Res*. (2019) 360:185–95. doi: 10.1016/j.bbr.2018.12.017
21. Walf AA, Paris JJ, Frye CA. Chronic estradiol replacement to aged female rats reduces anxiety-like and depression-like behavior and enhances cognitive performance. *Psychoneuroendocrinology*. (2009) 34:909–16. doi: 10.1016/j.psyneuen.2009.01.004
22. Rodgers SP, Bohacek J, Daniel JM. Transient estradiol exposure during middle age in ovariectomized rats exerts lasting effects on cognitive function and the hippocampus. *Endocrinology*. (2010) 151:1194–203. doi: 10.1210/en.2009-1245
23. Witty CF, Gardella LP, Perez MC, Daniel JM. Short-term estradiol administration in aging ovariectomized rats provides lasting benefits for memory and the hippocampus: A role for insulin-like growth factor-I. *Endocrinology*. (2013) 154:842–52. doi: 10.1210/en.2012-1698
24. Kim J, Szinte JS, Boulware MI, Frick KM. 17 -Estradiol and agonism of g-protein-coupled estrogen receptor enhance hippocampal memory via different cell-signaling mechanisms. *J Neurosci*. (2016) 36:3309–21. doi: 10.1523/JNEUROSCI.0257-15.2016
25. Luo Y, Xiao Q, Chao F, He Q, Lv F, Zhang L, et al. 17β-estradiol replacement therapy protects myelin sheaths in the white matter of middle-aged female ovariectomized rats: a stereological study. *Neurobiol Aging*. (2016) 47:139–48. doi: 10.1016/j.neurobiolaging.2016.07.023
26. Mitra S, Bastos CP, Bates K, Pereira GS, Bult-Ito A. Ovarian sex hormones modulate compulsive, affective and cognitive functions in a non-induced mouse model of obsessive-compulsive disorder. *Front Behav Neurosci*. (2016) 10:1–14. doi: 10.3389/fnbeh.2016.00215
27. Rodríguez-Landa JF, Cueto-Escobedo J, Puga-Olguín A, Rivadeneyra-Domínguez E, Bernal-Morales B, Herrera-Huerta EV, et al. The phytoestrogen genistein produces similar effects as 17 β -estradiol on anxiety-like behavior in rats at 12 weeks after ovariectomy. *Biomed Res Int*. (2017) 2017:1–10. doi: 10.1155/2017/9073816
28. Frick KM, Tuscher JJ, Koss WA, Kim J, Taxier LR. Estrogenic regulation of memory consolidation: a look beyond the hippocampus, ovaries, and females. *Physiol Behav*. (2018) 187:57–66. doi: 10.1016/j.physbeh.2017.07.028
29. Prakapenka AV, Hiroi R, Quihuis AM, Carson C, Patel S, Berns-Leone C, et al. Contrasting effects of individual versus combined estrogen and progestogen regimens as working memory load increases in middle-aged ovariectomized rats: one plus one does not equal two. *Neurobiol Aging*. (2018) 64:1–14. doi: 10.1016/j.neurobiolaging.2017.11.015
30. Black KL, Baumgartner NE, Daniel JM. Lasting impact on memory of midlife exposure to exogenous and endogenous estrogens. *Behav Neurosci*. (2018) 132:547–51. doi: 10.1037/bne0000270
31. Koebele SV, Nishimura KJ, Bimonte-Nelson HA, Kemmou S, Ortiz JB, Judd JM, et al. A long-term cyclic plus tonic regimen of 17β-estradiol improves the ability to handle a high spatial working memory load in ovariectomized middle-aged female rats. *Horm Behav*. (2020) 118:104656. doi: 10.1016/j.yhbeh.2019.104656
32. Rezvanfar MA, Shojaei Saadi HA, Gooshe M, Abdolghaffari AH, Baeeri M, Abdollahi M. Ovarian aging-like phenotype in the hyperandrogenism-induced murine model of polycystic ovary. *Oxid Med Cell Longev*. (2014) 2014:1–10. doi: 10.1155/2014/948951
33. Kauffman AS, Thackray VG, Ryan GE, Tolson KP, Glidewell-Kenney CA, Semaan SJ, et al. A novel letrozole model recapitulates both the reproductive and metabolic phenotypes of polycystic ovary syndrome in female mice1. *Biol Reprod*. (2015) 93:1–12. doi: 10.1095/biolreprod.115.131631
34. Chaudhari N, Dawalbhakta M, Nampoothiri L. GnRH dysregulation in polycystic ovarian syndrome (PCOS) is a manifestation of an altered neurotransmitter profile. *Reprod Biol Endocrinol*. (2018) 16:37. doi: 10.1186/s12958-018-0354-x
35. Rosenfeld CS, Shay DA, Vieira-Potter VJ. Cognitive effects of aromatase and possible role in memory disorders. *Front Endocrinol*. (2018) 9:610. doi: 10.3389/fendo.2018.00610
36. Tuscher JJ, Szinte JS, Starrett JR, Krentzel AA, Fortress AM, Remage-Healey L, et al. Inhibition of local estrogen synthesis in the hippocampus impairs hippocampal memory consolidation in ovariectomized female mice. *Horm Behav*. (2016) 83:60–7. doi: 10.1016/j.yhbeh.2016.05.001
37. Lu Y, Sareddy GR, Wang J, Wang R, Li Y, Dong Y, et al. Neuron-derived estrogen regulates synaptic plasticity and memory. *J Neurosci*. (2019) 39:2792–809. doi: 10.1523/JNEUROSCI.1970-18.2019
38. Chaiton JA, Wong SJ, Galea LA. Chronic aromatase inhibition increases ventral hippocampal neurogenesis in middle-aged female mice. *Psychoneuroendocrinology*. (2019) 106:111–6. doi: 10.1016/j.psyneuen.2019.04.003
39. Ghahiri A, Mogharehabed N, Mamourian M. Letrozole as the first-line treatment of infertile women with poly cystic ovarian syndrome (PCOS) compared with clomiphene citrate: A clinical trial. *Adv Biomed Res*. (2016) 5:6. doi: 10.4103/2277-9175.175237
40. Barnadas A, Estévez LG, Lluch-Hernández A, Rodriguez-Lescure Á, Rodriguez-Sanchez C, Sanchez-Rovira P. An overview of letrozole in postmenopausal women with hormone-responsive breast cancer. *Adv Ther*. (2011) 28:1045–58. doi: 10.1007/s12325-011-0075-4
41. Gold EB. The timing of the age at which natural menopause occurs. *Obstet Gynecol Clin North Am*. (2011) 38:425–40. doi: 10.1016/j.ogc.2011.05.002
42. Braden BB, Kingston ML, Koenig EN, Lavery CN, Tsang CW, Bimonte-Nelson HA. The GABAA antagonist bicuculline attenuates progesterone-induced memory impairments in middle-aged ovariectomized rats. *Front Aging Neurosci*. (2015) 7:149. doi: 10.3389/fnagi.2015.00149
43. Black KL, Witty CF, Daniel JM. Previous midlife oestradiol treatment results in long-term maintenance of hippocampal oestrogen receptor α levels in ovariectomised rats: mechanisms and implications for memory. *J Neuroendocrinol*. (2016) 28:77–85. doi: 10.1111/jne.12429
44. Fedotova JO. Vitamin D3 treatment differentially affects anxiety-like behavior in the old ovariectomized female rats and old ovariectomized female rats treated with low dose of 17β-estradiol. *BMC Med Genet*. (2019) 20:49. doi: 10.1186/s12881-019-0774-2
45. Borbélyová V, Domonkos E, Csongová M, Kačmárová M, Ostatníková D, Celec P, et al. Sex-dependent effects of letrozole on anxiety in middle-aged rats. *Clin Exp Pharmacol Physiol*. (2017) 44:93–8. doi: 10.1111/1440-1681.12731
46. Espinosa E, Curtis KS. Increased locomotor activity in estrogen-treated ovariectomized rats is associated with nucleus accumbens dopamine and is not reduced by dietary sodium deprivation. *Integr Zool*. (2018) 13:783–94. doi: 10.1111/1749-4877.12333
47. Koss WA, Gehlert DR, Shekhar A. Different effects of subchronic doses of 17-β estradiol in two ethologically based models of anxiety utilizing female rats. *Horm Behav*. (2004) 46:158–64. doi: 10.1016/j.yhbeh.2004.02.011
48. Walf AA, Frye CA. Antianxiety and antidepressive behavior produced by physiological estradiol regimen may be modulated by hypothalamic–pituitary–adrenal axis activity. *Neuropsychopharmacology*. (2005) 30:1288–301. doi: 10.1038/sj.npp.1300708
49. Walf AA, Frye CA. Estradiol decreases anxiety behavior and enhances inhibitory avoidance and gestational stress produces opposite effects. *Stress*. (2007) 10:251–60. doi: 10.1080/00958970701220416
50. Olivera-Lopez JI, Molina-Hernández M, Tellez-Alcántara NP, Jaramillo MT. Estradiol and neuropeptide, Y (intra-lateral septal) reduce anxiety-like behavior in two animal models of anxiety. *Peptides*. (2008) 29:1396–403. doi: 10.1016/j.peptides.2008.04.002
51. Walf AA, Koonce CJ, Frye CA. Estradiol or diarylpropionitrile decrease anxiety-like behavior of wildtype, but not estrogen receptor beta knockout, mice. *Behav Neurosci*. (2008) 122:974–81. doi: 10.1037/a0012749
52. Fedotova J. Anxiolytic-like effect of quinpirole in combination with a low dose of 17β-estradiol in ovariectomized rats. *Acta Physiol Hung*. (2013) 100:211–23. doi: 10.1556/APhysiol.100.2013.2.8
53. Walf AA, Frye CA. Estradiol reduces anxiety- and depression-like behavior of aged female mice. *Physiol Behav*. (2010) 99:169–74. doi: 10.1016/j.physbeh.2009.09.017
54. Ferguson SA, Gray EP. Aging effects on elevated plus maze behavior in spontaneously hypertensive, Wistar–Kyoto and Sprague–Dawley male and female rats. *Physiol Behav*. (2005) 85:621–8. doi: 10.1016/j.physbeh.2005.06.009

55. Renczés E, Borbélyová V, Keresztesová L, Ostatníková D, Celec P, Hodosy J. The age-dependent effect of pre-pubertal castration on anxiety-like behaviour in male rats. *Andrologia*. (2020) 52:e13649. doi: 10.1111/and.13649
56. Diz-Chaves Y, Kwiatkowska-Naqvi A, Von Hülst H, Pernía O, Carrero P, Garcia-Segura LM. Behavioral effects of estradiol therapy in ovariectomized rats depend on the age when the treatment is initiated. *Exp Gerontol*. (2012) 47:93–9. doi: 10.1016/j.exger.2011.10.008
57. Borrow AP, Handa RJ. Estrogen receptors modulation of anxiety-like behavior. *Vitam Horm*. (2017) 103:27–52. doi: 10.1016/bs.vh.2016.08.004
58. Adhikari A. Distributed circuits underlying anxiety. *Front Behav Neurosci*. (2014) 8:1–6. doi: 10.3389/fnbeh.2014.00112
59. Jacome LF, Gautreaux C, Inagaki T, Mohan G, Alves S, Lubbers LS, et al. Estradiol and ERβ agonists enhance recognition memory, and DPN, an ERβ agonist, alters brain monoamines. *Neurobiol Learn Mem*. (2010) 94:488–98. doi: 10.1016/j.nlm.2010.08.016
60. Uzum G, Bahcekapili N, Baltaci AK, Mogulkoc R, Ziylan YZ. Chronic (3-Weeks) treatment of estrogen (17β-Estradiol) enhances working and reference memory in ovariectomized rats: role of acetylcholine. *Neurochem Res*. (2016) 41:1468–74. doi: 10.1007/s11064-016-1858-4
61. Koss WA, Haertel JM, Philippi SM, Frick KM. Sex differences in the rapid cell signaling mechanisms underlying the memory-enhancing effects of 17β-Estradiol. *eNeuro*. (2018). 5:ENEURO.0267-18.2018. doi: 10.1523/ENEURO.0267-18.2018
62. Daniel JM, Hulst JL, Berbling JL. Estradiol replacement enhances working memory in middle-aged rats when initiated immediately after ovariectomy but not after a long-term period of ovarian hormone deprivation. *Endocrinology*. (2006) 147:607–14. doi: 10.1210/en.2005-0998
63. McLaughlin KJ, Bimonte-Nelson H, Neisewander JL, Conrad CD. Assessment of estradiol influence on spatial tasks and hippocampal CA1 spines: Evidence that the duration of hormone deprivation after ovariectomy compromises 17β-estradiol effectiveness in altering CA1 spines. *Horm Behav*. (2008) 54:386–95. doi: 10.1016/j.yhbeh.2008.04.010
64. Tuscher JJ, Fortress AM, Kim J, Frick KM. Regulation of object recognition and object placement by ovarian sex steroid hormones. *Behav Brain Res*. (2015) 285:140–57. doi: 10.1016/j.bbr.2014.08.001
65. Gresack JE, Frick KM. Effects of continuous and intermittent estrogen treatments on memory in aging female mice. *Brain Res*. (2006) 1115:135–47. doi: 10.1016/j.brainres.2006.07.067
66. Waters EM, Yildirim M, Janssen WG, Lou WW, McEwen BS, Morrison JH, et al. Estrogen and aging affect the synaptic distribution of estrogen receptor beta-immunoreactivity in the CA1 region of female rat hippocampus. *Brain Res*. (2011) 1379:86–97. doi: 10.1016/j.brainres.2010.09.069
67. Evola CM, Hudson TL, Huang L, Corbett AM, Mayes DA. Gender- and region-specific changes in estrogen signaling in aging rat brain mitochondria. *Aging*. (2018) 10:2148–69. doi: 10.18632/aging.101538
68. Foster TC, Sharrow KM, Kumar A, Masse J. Interaction of age and chronic estradiol replacement on memory and markers of brain aging. *Neurobiol Aging*. (2003) 24:839–52. doi: 10.1016/S0197-4580(03)00014-9
69. Savonenko AV, Markowska AL. The cognitive effects of ovariectomy and estrogen replacement are modulated by aging. *Neuroscience*. (2003) 119:821–30. doi: 10.1016/S0306-4522(03)00213-6
70. Chisholm NC, Juraska JM. Factors influencing the cognitive and neural effects of hormone treatment during aging in a rodent model. *Brain Res*. (2013) 1514:40–9. doi: 10.1016/j.brainres.2013.02.020
71. Vaucher E, Reymond I, Najaffe R, Kar S, Quirion R, Miller MM, et al. Estrogen effects on object memory and cholinergic receptors in young and old female mice. *Neurobiol Aging*. (2002) 23:87–95. doi: 10.1016/S0197-4580(01)00250-0
72. Luine VN. Estradiol and cognitive function: past, present and future. *Horm Behav*. (2014) 66:602–18. doi: 10.1016/j.yhbeh.2014.08.011
73. Luine V. Recognition memory tasks in neuroendocrine research. *Behav Brain Res*. (2015) 285:158–64. doi: 10.1016/j.bbr.2014.04.032
74. Sárvári M, Kalló I, Hrabovszky E, Solymosi N, Liposits Z. Ovariectomy alters gene expression of the hippocampal formation in middle-aged rats. *Endocrinology*. (2016) 158:en.2016-1516. doi: 10.1210/en.2016-1516
75. Morici JF, Bekinschtein P, Weisstaub NV. Medial prefrontal cortex role in recognition memory in rodents. *Behav Brain Res*. (2015) 292:241–51. doi: 10.1016/j.bbr.2015.06.030
76. Tuscher JJ, Taxier LR, Schalk JC, Haerte JM, Frick KM. Chemogenetic suppression of medial prefrontal-dorsal hippocampal interactions prevents estrogenic enhancement of memory consolidation in female mice. *eNeuro*. (2019) 6:ENEURO.0451-18.2019. doi: 10.1523/ENEURO.0451-18.2019
77. Bhatta S, Blair JA, Casadesus G. Luteinizing hormone involvement in aging female cognition: not all is estrogen loss. *Front Endocrinol*. (2018) 9:1–8. doi: 10.3389/fendo.2018.00544
78. Kafali H, Iriadam M, Ozardali I, Demir N. Letrozole-induced polycystic ovaries in the rat: a new model for cystic ovarian disease. *Arch Med Res*. (2004) 35:103–8. doi: 10.1016/j.arcmed.2003.10.005
79. Wise PM, Ratner A. Effect of ovariectomy on plasma LH, FSH, estradiol, and progesterone and medial basal hypothalamic LHRH concentrations in old and young rats. *Neuroendocrinology*. (1980) 30:15–9. doi: 10.1159/000122968
80. Flores A, Gallegos AI, Velasco J, Mendoza FD, Montiel C, Everardo PM, et al. The acute effects of bilateral ovariectomy or adrenalectomy on progesterone, testosterone and estradiol serum levels depend on the surgical approach and the day of the estrous cycle when they are performed. *Reprod Biol Endocrinol*. (2008) 6:48. doi: 10.1186/1477-7827-6-48
81. Koonce CJ, Frye CA. Progesterone facilitates exploration, affective and social behaviors among wildtype, but not 5α-reductase Type 1 mutant, mice. *Behav Brain Res*. (2013) 253:232–9. doi: 10.1016/j.bbr.2013.07.025
82. Feng Y, Shao R, Weijdegård B, Wang T, Johansson J, Sun S, et al. Effects of androgen and leptin on behavioral and cellular responses in female rats. *Horm Behav*. (2011) 60:427–38. doi: 10.1016/j.yhbeh.2011.07.012
83. Chamniansawat S, Sawatdiyaphanon C. Age-related memory impairment associated with decreased endogenous estradiol in the hippocampus of female rats. *Int J Toxicol*. (2018) 37:207–15. doi: 10.1177/1091581818761653

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.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 19 October 2020
doi: 10.3389/fnins.2020.565668

Human Cognitive Ability Is Modulated by Aromatase Availability in the Brain in a Sex-Specific Manner

*Nelly Alia-Klein[1], Rebecca N. Preston-Campbell[2], Sung Won Kim[3], Deborah Pareto[4], Jean Logan[5], Gene-Jack Wang[3], Scott J. Moeller[6], Joanna S. Fowler[7] and Anat Biegon[6]**

[1] Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [2] Missouri Institute of Mental Health, St. Louis, MO, United States, [3] National Institute on Alcohol and Alcohol Abuse, Bethesda, MD, United States, [4] Neuroradiology Unit, Vall d'Hebron University Hospital, Barcelona, Spain, [5] New York University, Langone Medical Center, New York, NY, United States, [6] Stony Brook University School of Medicine, Stony Brook, NY, United States, [7] Brookhaven National Laboratory, Upton, NY, United States

The enzyme aromatase catalyzes the final step in estrogen biosynthesis, converting testosterone to estradiol, and is expressed in the brain of all mammals. Estrogens are thought to be important for maintenance of cognitive function in women, whereas testosterone is thought to modulate cognitive abilities in men. Here, we compare differences in cognitive performance in relation to brain aromatase availability in healthy men and women. Twenty-seven healthy participants were administered tests of verbal learning and memory and perceptual/abstract reasoning. *In vivo* images of brain aromatase availability were acquired in this sample using positron emission tomography (PET) with the validated aromatase radiotracer [^{11}C]vorozole. Regions of interest were placed bilaterally on the amygdala and thalamus where aromatase availability is highest in the human brain. Though cognitive performance and aromatase availability did not differ as a function of sex, higher availability of aromatase in the amygdala was associated with lower cognitive performance in men. No such relationship was found in women; and the corresponding regression slopes were significantly different between the sexes. Thalamic aromatase availability was not significantly correlated with cognitive performance in either sex. These findings suggest that the effects of brain aromatase on cognitive performance are both region- and sex-specific and may explain some of the normal variance seen in verbal and nonverbal cognitive abilities in men and women as well as sex differences in the trajectory of cognitive decline associated with Alzheimer's disease.

Keywords: aromatase, [^{11}C]vorozole, estrogen, testosterone, cognition, PET, amygdala, human brain

OPEN ACCESS

Edited by:
Vance L. Trudeau, University of Ottawa, Canada

Reviewed by:
Thierry D. Charlier, University of Rennes 1, France
Neil James MacLusky, University of Guelph, Canada

***Correspondence:**
Anat Biegon
anat.biegon@stonybrookmedicine.edu

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

Received: *03 July 2020*
Accepted: *14 September 2020*
Published: *19 October 2020*

Citation:
Alia-Klein N, Preston-Campbell RN, Kim SW, Pareto D, Logan J, Wang G-J, Moeller SJ, Fowler JS and Biegon A (2020) Human Cognitive Ability Is Modulated by Aromatase Availability in the Brain in a Sex-Specific Manner. Front. Neurosci. 14:565668. doi: 10.3389/fnins.2020.565668

INTRODUCTION

The last and obligatory step in estrogen biosynthesis in all organs and species is catalyzed by the enzyme aromatase (estrogen synthase, Cyp19 gene product), which converts the androgens androstenedione and testosterone, to the estrogens, estrone, and estradiol (Simpson et al., 2002). In reproductively competent women, the ovary is the primary source of circulating estrogens (Simpson, 2003). In both sexes, a major site of extra-gonadal estrogen synthesis is the brain, and it

is characterized by widespread but heterogeneous aromatase availability (Biegon et al., 2010a, 2015; Azcoitia et al., 2011; Takahashi et al., 2018). A recent study revealed some region- and sex-specific associations between aromatase availability in the human brain and personality characteristics (Takahashi et al., 2018). To date, however, there are virtually no *in vivo* human studies testing the relationship between brain aromatase *availability* in the human brain and basic cognitive functions. The potential role of aromatase in cognition is supported by animal studies and studies in women with breast cancer reporting that administration of aromatase inhibitors (AIs) is linked to cognitive dysfunction (Rosenfeld et al., 2018). The most common deficits in women were seen in executive function and verbal episodic memory performance, although the effects of aromatase manipulation on neurobehavioral function in both animals and humans appeared to be sexually dimorphic (Shay et al., 2018). Further support of the role of aromatase in human cognition comes from postmortem studies in humans, which demonstrate region-specific changes in aromatase levels in Alzheimer's disease (Ishunina et al., 2005; Prange-Kiel et al., 2016), suggesting that aromatase may be implicated in normal as well as pathological variations in learning and memory.

The development and application of positron emission tomography (PET) tracers for aromatase have afforded the ability to measure its availability in different brain regions noninvasively in the living human brain (Biegon et al., 2010a, 2015; Takahashi et al., 2018). With the use of this technology, it has been demonstrated that the regional distribution pattern of [^{11}C]vorozole is heterogeneous with the highest levels of aromatase availability found in the thalamus and amygdala. In the present study, PET with [^{11}C]vorozole was used to measure aromatase availability in the bilateral amygdala and thalamus of healthy men and women. Blood levels of testosterone and estrogen were also obtained. Participants completed tests of verbal learning and memory and perceptual reasoning in order to explore differing domains in cognitive functioning that utilize both verbal and nonverbal abilities, exploring a sex-specific aromatase–cognition association.

MATERIALS AND METHODS

Participants

The study population comprised 27 healthy adult participants (men, n = 12; women, n = 15), age 21–67 years. All individuals provided written informed consent prior to study participation in accordance with the Institutional Review Board and the Radioactive Drug Research Committee of Stony Brook University/Brookhaven National Laboratory. Participants were excluded for (1) recent or current use of gonadal steroids (including hormonal contraceptives); (2) cigarette smoking (Biegon et al., 2010b, 2012, 2015), recreational drug use, and medications affecting brain function; (3) neurological, psychiatric, or metabolic disorders; and (4) pregnancy in women. During the screening visit, premenopausal women reported the date of their last menstrual period, and PET scans were scheduled to coincide with the early follicular stage.

Verification of Hormonal Status on Study Day: On the day of the PET study, blood samples were obtained and sent to an outside laboratory (ARUP) for measurement of hormone levels. In men, free testosterone and estradiol levels were obtained to exclude hypogonadism. Serum estradiol (E2) concentration was determined by tandem mass spectrometry (TMS). In order to calculate free testosterone (fT), total testosterone and sex hormone binding globulin (SHBG) were measured by quantitative electrochemiluminescent immunoassay. Adult male reference intervals for fT (47–244 pg/ml) and E2 (10.0–42.0 pg/ml) were provided by ARUP. In women, progesterone and luteinizing hormone (LH) were additionally measured to verify the stage of the menstrual cycle as well as menopausal status. Five of the 15 women were postmenopausal, defined as age above 50 and more than 12 months since the last menstrual period by self-report and confirmed by high levels of LH, and low estradiol and progesterone in the postmenopausal range. Reference values for the various hormones supplied by ARUP included the following: LH females: follicular: 2.4–12.6 IU/L; mid-cycle: 14.0–95.6 IU/L; luteal: 1.0–11.4 IU/L; postmenopausal: 7.7–58.5. Estradiol: follicular phase, 27–122 pg/ml; mid-cycle phase, 95–433 pg/ml; luteal phase, 49–291 pg/ml; postmenopausal, <41 pg/ml. Progesterone: cycle days reference interval (ng/ml) 1–6, ≤0.17; 7–12, <1.35; 13–15, ≤15.63; 16–28, ≤25.55; postmenopausal: ≤0.10 IU/L).

Cognitive Tests

Participants completed the California Verbal Learning Test-Second Edition (CVLT-II) (Kramer et al., 2000) by the standard method. The CVLT-II is an individually administered test assessing episodic verbal learning and memory. It measures recall and recognition of two word-lists containing 16 words each recalled over immediate and delayed memory trials. There are five presentation trials followed by an immediate recall of the first list (A), followed by a one-time presentation and immediate recall of the interference list (B). Measures of free and semantically cued recall are obtained after the trial with List B (Short Delay free or cued recall), followed by a 20-min delay (Long Delay Free or Cued Recall) during which the participant cannot engage in verbal tasks. After the delay, a recognition trial is completed during which the participant is asked to identify the items from List A from a larger list that contains distractor words. Following another 10-min delay, a forced-choice trial is administered. We chose select outcome variables aimed at indexing learning through memory [total recall over the five learning trials of list A (Trials 1–5), Short and Delayed Cued Recall, and Short and Delayed Free Recall] as the primary measures of learning on the CVLT-II (Elwood, 1995).

Participants also completed the Matrix Reasoning subtest of the Wechsler Abbreviated Scale of Intelligence (Wechsler, 1999), which is designed to assess nonverbal abstract problem solving, spatial, and inductive reasoning and is considered a general estimate of nonverbal intelligence. In addition, the word reading subtest of the Wide Range Achievement Test-3 (WRAT-3) (Wilkinson, 1993), an estimate of verbal intelligence also considered to be a valid measure of education level, was

administered to ascertain letter and word decoding abilities (Wilkinson, 1993; Manly et al., 2002).

Positron Emission Tomography Scans

The PET images were acquired over a 90-min period using a whole-body positron emission tomograph (Siemen's HR1, spatial resolution 4.5 mm × 4.5 mm × 4.8 mm, at the center of field of view). Radiotracer synthesis, image acquisition, and PET data analysis were carried out as previously described (Kim et al., 2009; Biegon et al., 2010a). Briefly, subjects were administered [^{11}C]vorozole (3–8 mCi; specific activity >0.1 mCi/nmol at the time of injection) intravenously. A metabolite-corrected arterial plasma input function for [^{11}C]vorozole was obtained from arterial blood samples withdrawn every 2.5 s for the first 2 min (Ole Dich automatic blood sampler) and then at 3, 4, 5, 6, 8, 10, 15, 20, 30, 45, 60, and up to 90 min (end of study).

Image Analysis

Time frames were summed over the 90-min scanning period. The summed PET images were co-registered with structural three-dimensional magnetic resonance images of the same subject when available, using PMOD software (PMOD Technologies, Zurich, Switzerland) to confirm the anatomical location of tracer accumulation (**Figure 1**, right panel). Regions of interest (ROIs) were placed bilaterally on the summed image and then projected onto the dynamic images to obtain regional time activity curves. Regions occurring bilaterally (i.e., at a distance from the midline) were averaged. Carbon-11 concentration in each ROI was divided by the injected dose to obtain the % dose/cm^3. A two-compartment model was used to estimate the total tissue distribution volume, V_T, which includes free and nonspecifically bound tracer as well as specifically bound tracer (Innis et al., 2007). The four model parameters of the two-compartment model were optimized to obtain the best fit to the ROI data for each participant (Flannery et al., 1990; Innis et al., 2007; Biegon et al., 2010a; Pareto et al., 2013; Logan et al., 2014).

Statistical Analysis

Statistical analyses were conducted using SPSS Statistics software (Version 25, IBM). Independent *t*-tests were used to determine if men and women differed on age, education estimates, cognitive performance, and aromatase availability. The scores on cognitive performance, as well as aromatase availability in the ROIs, were normally distributed; thus, our primary analyses were five general linear models such that sex (categorical), aromatase availability in the amygdala or thalamus (continuous), and their interaction were entered to predict each of five memory outcomes (CVLT-II outcome: Trials 1–5, Long and Short Delay Free and Cued Recall). The same approach was taken for the Matrix Reasoning subtest. A general linear model was used such that sex (categorical), amygdala or thalamus aromatase availability (continuous), and their interaction were entered to predict the estimate of perceptual reasoning. Next, we performed two-tailed Pearson correlations between aromatase availability in the amygdala or thalamus and cognitive performance both across the whole sample and as a function of sex. We further tested the difference in regression slopes between the separate test-by-sex correlations. The cognitive tests use normative data that have been stratified based on age and/or education level(s) as these factors have been found to be consistently correlated with performance, supporting our approach of separate sex by test correlations. Age and education were used as covariates in these analyses, as increasing age and lower education are associated with poorer performance on tasks of verbal learning and memory (Wilkinson, 1993). For education, we used the raw score on the Reading subtest of the WRAT (Manly et al., 2002) as a valid indicator of grade level (Wilkinson, 1993). Finally, we tested whether plasma testosterone and estradiol levels in our sample affected the aforementioned analyses. For this purpose, first, we conducted partial correlations between the cognitive test scores, aromatase availability, and blood measures of estradiol and testosterone. Second, we entered estradiol and testosterone levels in our general linear models to test whether their presence changes the models. All analyses were considered significant at the $p < 0.05$ threshold.

RESULTS

There were no significant differences between men and women in age, education estimates, and aromatase availability in the amygdala and thalamus (**Table 1**). Likewise, there were no differences between men and women on their performance on the verbal or nonverbal cognitive tasks (**Table 2**). However, amygdala aromatase availability correlated with cognitive performance scores (**Table 3**). Analyses showed that in men only, lower amygdala aromatase availability was associated with better performance on the CVLT-II—recall following a short or long delay (r = -0.57 to -0.66, $p < 0.05$). Indeed, the interaction of correlation trend lines, sex × aromatase availability in the amygdala predicted free recall performance following a short delay (free: $F_{1,22} = 5.76$, $p = 0.025$; cued: $F_{1,22} = 6.51$ $p = 0.018$, **Figure 1** top left). Similar results were found for recall after a long delay (free: $F_{1,22} = 4.85$, $p = 0.038$) with a trend for Long Delay Cued Recall ($F_{1,22} = 3.16$, $p = 0.089$). The same pattern was revealed on the nonverbal test, Matrix Reasoning ($F_{1,21} = 5.32$, $p = 0.035$) (**Figure 1**, bottom left). In the thalamus, there were no correlations between aromatase availability and cognitive performance in men or women (men, r = -0.08 to 0.30, $p > 0.35$; women, r = -0.46–0.25, $p > 0.09$). Plasma levels of estradiol and testosterone did not correlate with aromatase availability in either the amygdala or thalamus, nor with any of the cognitive test scores ($p > 0.05$). Furthermore, adding blood measures of estrogen and/or testosterone to the aforementioned general linear models did not change the results.

DISCUSSION

Here, we have used [^{11}C]vorozole, a thoroughly validated radiotracer for brain aromatase (3; 6; 18, 21; 22) in conjunction with PET to examine the relationship between aromatase availability in high-density regions (amygdala and thalamus) and

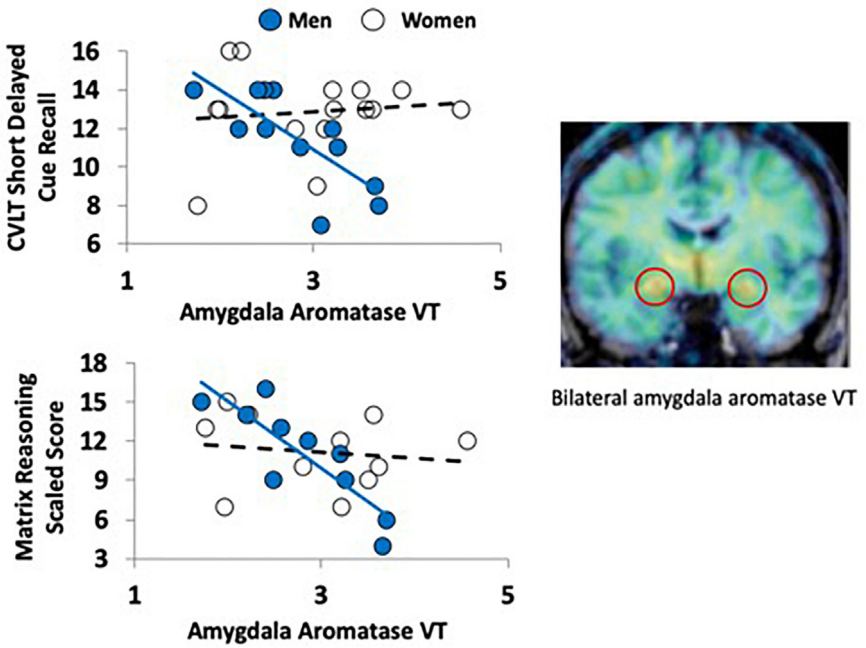

FIGURE 1 | Aromatase availability in the amygdala and cognitive performance in men and women. The PET image on the right (coronal plane, overlaid on MRI) shows the regional distribution of tracer uptake at the level of the amygdala (red circles). The image was pseudocolored using the rainbow scale, with purple/blue at the low end and orange/red at the high end. The graphs on the left show the regression lines between a verbal (top left) and nonverbal (bottom left) task performance and aromatase availability in the amygdala (VT) in men (blue circles) and women (empty circles).

cognitive abilities in healthy subjects. Our data show that brain aromatase availability predicted individual differences in verbal and nonverbal cognitive performance in men but not in women. Men with lower amygdala levels of aromatase had better recall for a list of words (Short Delay Free and Cued Recall and Long Delay Free Recall), on the CVLT-II. Similarly, men with lower aromatase in the amygdala also performed better on the Matrix Reasoning test. These effects were not dependent on plasma levels of estradiol and testosterone.

Animal studies suggest that brain aromatase availability is higher in males than in females and is modulated by changes in testosterone levels (Abdelgadir et al., 1994). As in our previous studies on this cohort (Biegon et al., 2010a, 2015), there were no statistically significant differences in aromatase availability in the amygdala as a function of sex, in line with previous human studies that reported comparable levels of brain aromatase and gene expression in men and women (Steckelbroeck et al., 1999; Stoffel-Wagner et al., 1999). There were also no significant effects of age or hormonal status on aromatase in the brain or any other organ beside the ovary, confirming the organ- and tissue-specific

TABLE 1 | Age, reading ability, and aromatase availability in the brains of men and women.

	Test	*p*-value	Male	Female
			(*N* = 12)	(*N* = 15)
Age (years)	t_{25} = -0.56	p = 0.58	41.17 ± 16.44	37.53 ± 16.81
WRAT-3 reading subtest	t_{20} = -0.23	p = 0.82	101.50 ± 14.01	100.00 ± 16.41
Aromatase VT				
Amygdala	t_{25} = 0.60	p = 0.55	2.80 ± 0.60	2.97 ± 0.82
Thalamus	t_{25} = 0.63	p = 0.54	4.68 ± 0.86	5.04 ± 1.82

Values are means ± SD.

TABLE 2 | Comparisons between male and female performance on tests of perceptual reasoning and verbal learning and memory.

	Test	*p*-value	Male	Female
			(*N* = 12)	(*N* = 15)
Matrix reasoning[a]	$F_{1,8}$ = 0.077	p = 0.78	10.90 ± 3.90	11.18 ± 2.79
CVLT-II[b]				
Trials 1–5	$F_{2,4}$ = 2.15	p = 0.15	50.75 ± 5.08	54.79 ± 8.09
Short Delay Free Recall	$F_{2,4}$ = 2.20	p = 0.15	10.50 ± 3.00	11.73 ± 2.22
Short Delay Cued Recall	$F_{2,4}$ = 3.22	p = 0.09	11.50 ± 2.43	12.87 ± 2.13
Long Delay Free Recall	$F_{2,4}$ = 838	p = 0.37	11.33 ± 2.64	12.20 ± 2.34
Long Delay Cued Recall	$F_{2,4}$ = 400	p = 0.53	12.17 ± 2.08	12.60 ± 2.35

Values are means ± SD. [a]*Values for Matrix Reasoning were calculated using Wide Range Achievement Test-3 (WRAT-3) Standard Score as a covariate.* [b]*Values for California Verbal Learning Test-Second Edition (CVLT-II) are calculated using age as a covariate.*

TABLE 3 | Results of Pearson's correlations between aromatase amygdala availability and measures of abstract reasoning and verbal learning and memory.

	All participants (*N* = 27) *r*	Males only (*N* = 12) *r*	Females only (*N* = 15) *r*
Matrix reasoning[a]	−0.34	**−0.84****	−0.08
CVLT-II[b]			
word recall 1–5	−0.17	**−0.66***	−0.04
short delay free recall	−0.06	**−0.59#**	0.26
short delay cued recall	−0.10	**−0.68***	0.15
long delay free recall	−0.11	**−0.60***	0.14
long delay cued recall	−0.06	**−0.57#**	0.14

*r = Pearson's correlation coefficient. #trend (0.1 > p > 0.05). *Correlation is significant at the 0.05 level. **Correlation is significant at the 0.01 level. [a]Values for Matrix Reasoning are calculated using Wide Range Achievement Test-3 (WRAT-3) Standard Score as a covariate. [b]Values for California Verbal Learning Test-Second Edition (CVLT-II) are calculated using age as a covariate. Bolded values indicate correlations are significant or approaching signifiance.*

regulation of aromatase expression (Bulun and Simpson, 1994; Biegon et al., 2010a, 2015). Imaging studies suggest that even in the absence of behavioral sex differences, there are clear sex-dependent activations in regions of the brain associated with memory tasks (De Vries, 2004; Gillies and McArthur, 2010), which could be attributed to sex-linked levels of estrogens, androgens, and their receptors in the brain (Cahill, 2006). Therefore, it is not unexpected that despite the absence of sex-based differences in the amygdala aromatase availability and in cognitive performance, the relationship between the two is nonetheless sexually dimorphic.

In this regard, it is important to note that aromatase activity, while giving rise to estrogen, also decreases testosterone levels. In humans, testosterone has been shown to enhance spatial performance in men, whereas estradiol has been shown to enhance verbal memory in women (Matousek and Sherwin, 2010). Furthermore, postmortem studies in brains of men who died with Alzheimer's disease consistently show large declines in testosterone levels, which correlated with levels of amyloid, a disease pathological marker (Rosario et al., 2004, 2011).

An additional compelling reason to support the sex-specific relationship between aromatase in the amygdala and cognitive function in our cohort, composed of mostly premenopausal women, is that in men, in whom circulating estrogen levels are low, aromatase-dependent production of estrogens from androgens is the main source of estrogens in the brain. This is not true in reproductively competent women, in whom brain levels of estrogen derive from local production as well as peripheral estrogens produced in the ovary, which diffuse freely into the brain. Since ovarian and brain aromatase expression are regulated independently (Rosario et al., 2004) via organ-specific promoter control (Golovine et al., 2003; Rosario et al., 2011), it is to be expected that androgenic and estrogenic modulation of brain function will be regional as well as more tightly correlated with local aromatase availability in men relative to women (Bulun et al., 2003; Golovine et al., 2003).

Our results further suggest that extra-gonadal (i.e., brain) estrogen synthesis and testosterone metabolism, mediated by aromatase, is implicated in verbal and nonverbal cognitive processes and therefore reveals a previously unappreciated sex-dependent relationship between aromatase and cognitive function in humans. Men with lower amygdala levels of aromatase, expected to result in lower estrogen and higher testosterone levels, had better recall for a list of words on the CVLT during short and long delay following encoding. These findings also resonate with a report showing that aromatase inhibition before and during a learning task improved working memory in male rats (Alejandre-Gomez et al., 2007). To date, there have been very few *in vivo* studies of brain aromatase and behavior in humans, yet recently published studies show that individual differences in brain aromatase availability are associated with individual differences in personality traits, with some sex-specific findings (Takahashi et al., 2018; Biegon et al., 2020). This is the first *in vivo* study to show that individual differences in aromatase availability correspond to cognitive performance, including memory. While the amygdala is a brain region best known for modulation of emotion, it is also thought to play a major role in higher cognition (Schaefer and Gray, 2007; Janak and Tye, 2015), and some of these effects are sex-dependent (Cahill, 2010; Carre et al., 2013; Shvil et al., 2014). Based on several studies and theories in the last decades, it is asserted in the literature that amygdala function is implicated in long- and short-term memory, abstract reasoning, and attention vigilance during mentally demanding cognitive tasks such as used in this paper. Even in the absence of emotion triggers during neutral cognitive tasks, attention vigilance and suppression of emotion are needed, implicating the amygdala in the output of every cognitive demand [(for good reviews, see Schaefer and Gray (2007) and Janak and Tye (2015)]. Lastly, robust sex differences have been reported in the functional connectivity of the human amygdala, specifically cortical connections, (Kilpatrick et al., 2006) further suggesting that the effects of varying levels of aromatase in the human amygdala on cognitive function are also likely to be sex-dependent. Notably, we have not observed significant sex difference in verbal learning and memory in our relatively small cohort, although better performance on CVLT in women is a consistent finding across age groups (Kramer et al., 2003; McCarrey et al., 2016; Graves et al., 2017). However, the absolute difference is also consistently small (∼10%, ibid), and studies reporting this difference as significant need a much larger sample (more than 400 subjects/sex, ibid).

CONCLUSION

This study used PET with [^{11}C]vorozole to document for the first time the association between availability of aromatase in the amygdala and individual differences in cognition in healthy men and women. We demonstrate a clear sex-specific relationship between aromatase levels in the amygdala and cognitive performance, showing that men with lower aromatase availability in the amygdala had better verbal memory and spatial reasoning performance than men having higher amygdala aromatase availability. These findings also suggest that the

cognitive impact of brain aromatase is both region- and sex-specific, potentially contributing to the normal variation of cognitive performance in healthy men and women.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board and the Radioactive Drug Research Committee of Stony Brook University/Brookhaven National Laboratory. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AB and NA-K conceived the idea and wrote the first draft of the manuscript. All other authors read and edited the final manuscript. In addition, SK was responsible for tracer production. DP and JL performed PET image data analysis and modeling. RP-C conducted statistical analyses and writing. SM assisted in statistical analyses and writing. G-JW was the study physician. JF secured the funding.

FUNDING

This study was supported by NIH 1R21EB012707 (AB is the Principal Investigator). In addition, RP-C was supported by a training grant from the National Institute on Drug Abuse (5T32DA007135-32 to RP). SM was supported by 1K01DA037452.

REFERENCES

Abdelgadir, S. E., Resko, J. A., Ojeda, S. R., Lephart, E. D., McPhaul, M. J., and Roselli, C. E. (1994). Androgens regulate aromatase cytochrome P450 messenger ribonucleic acid in rat brain. *Endocrinology* 135, 395–401. doi: 10.1210/endo.135.1.8013375

Alejandre-Gomez, M., Garcia-Segura, L. M., and Gonzalez-Burgos, I. (2007). Administration of an inhibitor of estrogen biosynthesis facilitates working memory acquisition in male rats. *Neurosci. Res.* 58, 272–277. doi: 10.1016/j.neures.2007.03.011

Azcoitia, I., Yague, J. G., and Garcia-Segura, L. M. (2011). Estradiol synthesis within the human brain. *Neuroscience* 191, 139–147.

Biegon, A., Alexoff, D. L., Kim, S. W., Logan, J., Pareto, D., and Schlyer, D. (2015). Aromatase imaging with [N-methyl-11C]vorozole PET in healthy men and women. *J. Nucl. Med.* 56, 580–585. doi: 10.2967/jnumed.114.150383

Biegon, A., ALia-Klein, N., Alexoff, D. L., Fowler, J. S., Kim, S. W., Logan, J., et al. (2020). Relationship of estrogen synthesis capacity in the brain with obesity and self-control in men and women. *Proc. Natl. Acad. Sci. U.S.A.* 117(37):22962–22966 doi: 10.1073/pnas.2006117117

Biegon, A., Alia-Klein, N., and Fowler, J. S. (2012). Potential contribution of aromatase inhibition to the effects of nicotine and related compounds on the brain. *Front. Pharmacol.* 3:185. doi: 10.3389/fphar.2012.00185

Biegon, A., Kim, S. W., Alexoff, D. L., Jayne, M., Carter, P., Hubbard, B., et al. (2010a). Unique distribution of aromatase in the human brain: in vivo studies with PET and [N-methyl-11C]vorozole. *Synapse* 64, 801–807. doi: 10.1002/syn.20791

Biegon, A., Kim, S. W., Logan, J., Hooker, J. M., Muench, L., and Fowler, J. S. (2010b). Nicotine blocks brain estrogen synthase (aromatase): in vivo positron emission tomography studies in female baboons. *Biol. Psychiatry* 67, 774–777. doi: 10.1016/j.biopsych.2010.01.004

Bulun, S. E., and Simpson, E. R. (1994). Regulation of aromatase expression in human tissues. *Breast Cancer Res. Treat.* 30, 19–29. doi: 10.1007/bf00682738

Bulun, S. E., Sebastian, S., Takayama, K., Suzuki, T., Sasano, H., and Shozu, M. (2003). The human CYP19 (aromatase P450) gene: update on physiologic roles and genomic organization of promoters. *J. Steroid Biochem. Mol. Biol.* 86, 219–224. doi: 10.1016/s0960-0760(03)00359-5

Cahill, L. (2006). Why sex matters for neuroscience. *Nat. Rev. Neurosci.* 7, 477–484. doi: 10.1038/nrn1909

Cahill, L. (2010). Sex influences on brain and emotional memory: the burden of proof has shifted. *Prog. Brain Res.* 186, 29-40.

Carre, J. M., Murphy, K. R., and Hariri, A. R. (2013). What lies beneath the face of aggression? *Soc. Cogn. Affect. Neurosci.* 8, 224–229. doi: 10.1093/scan/nsr096

De Vries, G. J. (2004). Minireview: sex differences in adult and developing brains: compensation, compensation, compensation. *Endocrinology* 145, 1063–1068. doi: 10.1210/en.2003-1504

Elwood, R. W. (1995). the california verbal learning test: psychometric characteristics and clinical application. *Neuropsychol. Rev.* 5, 173–201. doi: 10.1007/bf02214761

Flannery, B. P., Press, W., and Teukolsky, S, Vetterline, W. T. (1990). *Numerical Recipes in C: the Art of Scientific Computing*. Cambridge: Cambridge Univeristy Press.

Gillies, G. E., and McArthur, S. (2010). Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines. *Pharmacol. Rev.* 62, 155–198. doi: 10.1124/pr.109.002071

Golovine, K., Schwerin, M., and Vanselow, J. (2003). Three different promoters control expression of the aromatase cytochrome p450 gene (cyp19) in mouse gonads and brain. *Biol. Reprod.* 68, 978–984. doi: 10.1095/biolreprod.102.008037

Graves, L. V., Moreno, C. C., Seewald, M., Holden, H. M., Uttarwar, V., and McDonald, C. R. (2017). Effects of age and gender on recall and recognition discriminability. *Arch. Clin. Neuropsychol.* 32, 972–979. doi: 10.1093/arclin/acx024

Innis, R. B., Cunningham, V. J., Delforge, J., Fujita, M., Gjedde, A., and Gunn, R. N. (2007). Consensus nomenclature for in vivo imaging of reversibly binding radioligands. *J. Cereb. Blood Flow Metab.* 27, 1533–1539. doi: 10.1038/sj.jcbfm.9600493

Ishunina, T. A., van Beurden, D., van der Meulen, G., Unmehopa, U. A., Hol, E. M., Huitinga, I., et al. (2005). Diminished aromatase immunoreactivity in the hypothalamus, but not in the basal forebrain nuclei in Alzheimer's disease. *Neurobiol. Aging* 26, 173–194. doi: 10.1016/j.neurobiolaging.2004.03.010

Janak, P. H., and Tye, K. M. (2015). From circuits to behaviour in the amygdala. *Nature* 517, 284–292. doi: 10.1038/nature14188

Kilpatrick, L. A., Zald, D. H., Pardo, J. V., and Cahill, L. F. (2006). Sex-related differences in amygdala functional connectivity during resting conditions. *NeuroImage* 30, 452–461. doi: 10.1016/j.neuroimage.2005.09.065

Kim, S. W., Biegon, A., Katsamanis, Z. E., Ehrlich, C. W., Hooker, J. M., Shea, C., et al. (2009). Reinvestigation of the synthesis and evaluation of [N-methyl-(11)C]vorozole, a radiotracer targeting cytochrome P450 aromatase. *Nucl. Med. Biol.* 36, 323–334. doi: 10.1016/j.nucmedbio.2008.12.013

Kramer, J. H., Delis, D. C., Kaplan, E., and Ober, B. A. (2000). *California Verbal Learning Test*—2nd Edition, San Antonio, TX: Psychological Corporation.

Kramer, J. H., Yaffe, K., Lengenfelder, J., and Delis, D. C. (2003). Age and gender interactions on verbal memory performance. *J. Int. Neuropsychol. Soc.* 9, 97–102 doi: 10.1017/s1355617703910113

Logan, J., Kim, S. W., Pareto, D., Telang, F., Wang, G. J., Fowler, J. S., et al. (2014). Kinetic analysis of [11C]vorozole binding in the human brain with positron emission tomography. *Mol. Imag.* 13, 1–12.

Manly, J. J., Jacobs, D. M., Touradji, P., Small, S. A., and Stern, Y. (2002). Reading level attenuates differences in neuropsychological test performance between African American and White elders. *J. Int. Neuropsychol. Soc.* 341–348. doi: 10.1017/s1355617702813157

Matousek, R. H., and Sherwin, B. B. (2010). Sex steroid hormones and cognitive functioning in healthy, older men. *Horm. Behav.* 57, 352–359. doi: 10.1016/j.yhbeh.2010.01.004

McCarrey, A., Yang, A., Kitner-Triolo, M., Ferrucci, L., and Resnick, S. (2016). Sex differences in cognitive trajectories in clinically normal older adults. *Psychol. Aging* 31, 166–175. doi: 10.1037/pag0000070

Pareto, D., Biegon, A., Alexoff, D., Carter, P., Shea, C., Muench, L., Xu, Y. (2013). In vivo imaging of brain aromatase in female baboons: [11C]vorozole kinetics and effect of the menstrual cycle. *Mol. Imag.* 12, 518–524.

Prange-Kiel, J., Dudzinski, D. A., Prols, F., Glatzel, M., Matschke, J., and Rune, G. M. (2016). Aromatase expression in the hippocampus of AD patients and 5xFAD Mice. *Neural. Plast.* 2016:9802086.

Rosario, E. R., Chang, L., Stanczyk, F. Z., and Pike, C. J. (2004). Age-related testosterone depletion and the development of Alzheimer disease. *JAMA* 292, 1431–1432. doi: 10.1001/jama.292.12.1431-b

Rosario, E. R., Chang, L., Head, E. H., Stanczyk, F. Z., and Pike, C. J. (2011). Brain levels of sex steroid hormones in men and women during normal aging and in Alzheimer's disease. *Neurobiol. Aging* 32, 604–613. doi: 10.1016/j.neurobiolaging.2009.04.008

Rosenfeld, C. S., Shay, D. A., and Vieira-Potter, V. J. (2018). Cognitive effects of aromatase and possible role in memory disorders. *Front. Endocrinol. (Lausanne)* 9:610. doi: 10.3389/fendo.2018.00610

Schaefer, A., and Gray, J. R. (2007). A role for the human amygdala in higher cognition. *Rev. Neurosci.* 18, 355–363.

Shay, D. A., Vieira-Potter, V. J, and Rosenfeld, C. S. (2018). Sexually dimorphic effects of aromatase on neurobehavioral responses. *Front. Mol. Neurosci.* 11:374. doi: 10.3389/fnmol.2018.00374

Shvil, E., Sullivan, G. M., Schafer, S., Markowitz, J. C., Campeas, M., Wager, T. D., et al. (2014). Sex differences in extinction recall in posttraumatic stress disorder: a pilot fMRI study. *Neurobiol. Learn. Mem.* 113, 101–108. doi: 10.1016/j.nlm.2014.02.003

Simpson, E. R. (2003). Sources of estrogen and their importance. *J. Steroid Biochem. Mol. Biol.* 86, 225–230. doi: 10.1016/s0960-0760(03)00360-1

Simpson, E. R., Clyne, C., Rubin, G., Robertson, K., Britt, K., Speed, C., et al. (2002). Aromatase–a brief overview. *Annu. Rev. Physiol.* 64, 93–127. doi: 10.1146/annurev.physiol.64.081601.142703

Steckelbroeck, S., Heidrich, D. D., Hans, V. H., Schramm, J., Bidlingmaier, F., and Klingmuller, D. (1999). Characterization of aromatase cytochrome P450 activity in the human temporal lobe. *J. Clin. Endocrinol. Metab.* 84, 2795–2801. doi: 10.1210/jc.84.8.2795

Stoffel-Wagner, B., Watzka, M., Schramm, J., Bidlingmaier, F., and Klingmuller, D. (1999). Expression of CYP19 (aromatase) mRNA in different areas of the human brain. *J. Steroid Biochem. Mol. Biol.* 70, 237–241. doi: 10.1016/s0960-0760(99)00114-4

Takahashi, K., Hosoya, T., Onoe, K., Takashima, T., Tanaka, M., Ishii, A., et al. (2018). Association between aromatase in human brains and personality traits. *Sci. Rep.* 8:16841.

Wechsler, D. (1999). *Wechsler Abbreviated Scale of Intelligence*., San Antonio, TX: Psychological Cooporation.

Wilkinson, G. (1993). *The Wide-Range Achievement Test 3: Administration Manual*. Wilminton, DE: Wide-Range Inc.

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.

frontiers in Neuroscience

MINI REVIEW
published: 27 October 2020
doi: 10.3389/fnins.2020.572511

Rapid Estrogenic and Androgenic Neurosteroids Effects in the Induction of Long-Term Synaptic Changes: Implication for Early Memory Formation

Alessandro Tozzi, Laura Bellingacci and Vito Enrico Pettorossi*

Department of Experimental Medicine, University of Perugia, Perugia, Italy

Mounting experimental evidence demonstrate that sex neuroactive steroids (neurosteroids) are essential for memory formation. Neurosteroids have a profound impact on the function and structure of neural circuits and their local synthesis is necessary for the induction of both long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission and for neural spine formation in different areas of the central nervous system (CNS). Several studies demonstrated that in the hippocampus, 17β-estradiol (E2) is necessary for inducing LTP, while 5α-dihydrotestosterone (DHT) is necessary for inducing LTD. This contribution has been proven by administering sex neurosteroids in rodent models and by using blocking agents of their synthesis or of their specific receptors. The general opposite role of sex neurosteroids in synaptic plasticity appears to be dependent on their different local availability in response to low or high frequency of synaptic stimulation, allowing the induction of bidirectional synaptic plasticity. The relevant contribution of these neurosteroids to synaptic plasticity has also been described in other brain regions involved in memory processes such as motor learning, as in the case of the vestibular nuclei, the cerebellum, and the basal ganglia, or as the emotional circuit of the amygdala. The rapid effects of sex neurosteroids on neural synaptic plasticity need the maintenance of a tonic or phasic local steroid synthesis determined by neural activity but might also be influenced by circulating hormones, age, and gender. To disclose the exact mechanisms how sex neurosteroids participate in finely tuning long-term synaptic changes and spine remodeling, further investigation is required.

Keywords: 17β-estradiol, testosterone, 5α-dihydrotestosterone, P450 aromatase, 5α-reductase, LTP, LTD, synaptic plasticity

OPEN ACCESS

Edited by:
Takayoshi Ubuka,
Waseda University, Japan

Reviewed by:
Yasushi Hojo,
Saitama Medical University, Japan
Tetsuya Kimoto,
The University of Tokyo, Japan

****Correspondence:***
Alessandro Tozzi
alessandro.tozzi@unipg.it

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

Received: *14 June 2020*
Accepted: *21 August 2020*
Published: *27 October 2020*

Citation:
Tozzi A, Bellingacci L and Pettorossi VE (2020) Rapid Estrogenic and Androgenic Neurosteroids Effects in the Induction of Long-Term Synaptic Changes: Implication for Early Memory Formation. Front. Neurosci. 14:572511. doi: 10.3389/fnins.2020.572511

INTRODUCTION

Sex neurosteroids are a group of cholesterol-derived molecules that are synthesized *de novo* within the central nervous system (CNS) where they are able to exert local effects (Baulieu, 1981; Baulieu and Robel, 1990). Among these, estrogens and androgens influence the general development of neural circuits, motor adaptation, learning, memory (Luine and Frankfurt, 2012), emotional

behavior (Altemus, 2017), and cognition (Luine, 2014), and they are implicated in some psychiatric disorders (Bangasser and Valentino, 2014) modulating the function of different areas of the brain such as the hippocampus, the amygdala, the vestibular and cerebellar systems, and the nucleus striatum (Beauchet, 2006; Andreescu et al., 2007; Colciago et al., 2015; Bender et al., 2017; Diotel et al., 2018; Mohamad et al., 2018; Wagner et al., 2018; Dieni et al., 2020; Luine and Frankfurt, 2020).

These actions have a major role in the induction and maintenance of neural connections during development and the entire life-span by directing synaptogenesis, dendritic spine formation, and functional long-term potentiation (LTP) or long-term depression (LTD) of synaptic transmission.

Enzymes responsible for the biosynthesis of neurosteroids from cholesterol are present in the nervous system (Mensah-Nyagan et al., 1999; Giatti et al., 2019). Testosterone is synthesized through conversion of cholesterol into pregnenolone, a neurosteroid that is subsequently metabolized, leading to formation of 5α-dihydrotestosterone (DHT) or 17β-estradiol (E2) by the action of the 5α-reductase or P450 aromatase enzymes, respectively. The local concentration of these neurosteroids was found to be significantly higher in some areas of the CNS (e.g., hippocampus) in respect to blood, where they bind to specific estrogen receptors (ERα, ERβ, and multiple isoforms) or to androgen receptors (AR) (Mukai et al., 2006; Hatanaka et al., 2015). These receptors are largely distributed in the CNS and their activation can elicit diverse genomic and non-genomic cell functions.

Besides the genomic effects that occur within hours, leading to up- or downregulation of gene transcription, E2 and DHT, the most active metabolite of testosterone, can also activate rapid intracellular signaling pathways, acting within seconds or minutes via extranuclear membrane-associated forms of receptors (Kawato, 2004). In neurons, membrane effects of E2 may lead to the stimulation of different enzymes such as phospholipase C (PLC) and protein kinase C (PKC) that in turn stimulate the production of IP3 and the elevation of intracellular calcium levels. These molecules may rapidly change the function of neurons by acting as second messengers leading to the activation of further enzymatic cascades including the src kinase and the MAPK/ERK pathway (Evinger and Levin, 2005; Srivastava, 2012; Murakami et al., 2018). As in the case of estrogens, evidence has been accumulated to involve rapid responses to androgens, dependent or independent on ARs (Foradori et al., 2008). Many cellular responses to androgens are transcription independent; in fact, activated ARs are able to associate with molecular substrates in the cytoplasm and the inner layer of the cell membrane to activate intracellular kinase cascades (Soma et al., 2018).

The activation of neural network within the CNS might produce rapid behavioral changes by stimulating estrogenic or androgenic sex neurosteroid signaling and rapidly modulating the enzyme function involved in the induction of long-term synaptic plasticity. Accordingly, the consolidation of hippocampal memory might be facilitated within minutes after treatments with estrogenic and androgenic neurosteroids or with agonists of their receptors, enhancing the performance on hippocampal memory tasks, as reported in experiments performed with rats and mice (Luine et al., 2003; Aubele et al., 2008; Benice and Raber, 2009; Inagaki et al., 2010, 2012; Babanejad et al., 2012; Phan et al., 2012, 2015; Jacome et al., 2016).

Here, we will review evidence supporting the immediate involvement of the most neuroactive estrogenic and androgenic neurosteroids, namely E2 and testosterone/DHT, in the induction of neuronal synaptic plasticity, the capability of neuronal circuitries to modify their function in response to environment-triggered electric signals. The electrophysiological actions of E2 and DHT on long-term synaptic plasticity will be presented.

NEURAL E2 INFLUENCES HIPPOCAMPAL LTP

The role of E2 acting as a neurosteroid in memory formation has been described since the late 1980s and has been recently confirmed by different research groups (Ooishi et al., 2012; Di Mauro et al., 2015; Fester and Rune, 2015; Hasegawa et al., 2015; Lu et al., 2019; Tozzi et al., 2019). These studies were guided by the major general evidence that inhibition of E2 synthase (P450 aromatase) activity produced hippocampal-related memory deficits both in women and in female rodents (Bayer et al., 2015; Tuscher et al., 2016). They also pointed out that E2, synthesized in the CNS, regulates cognition and behavior independently of gender, assigning to the neural E2 signaling system a general modulator role of CNS function (Moradpour et al., 2006; Saldanha et al., 2011; Bailey et al., 2013, 2017; Tuscher et al., 2016; Fester et al., 2017). In the hippocampus, E2-mediated synaptic activity was found to be related with memory formation and early and late influence of E2 on long-term electrophysiological synaptic effects and on dendritic spine formation has been described in animal models (Kramar et al., 2009).

The question how E2 can regulate the physiology of neurons involved in memory is still a matter of debate. E2 can directly influence the electric membrane properties of neurons or it can affect synaptic transmission, with synaptic changes suggested to be strongly correlated to functional modifications of nervous system networks. For example, E2 administration changed very rapidly the neuronal excitability and the synaptic responses of hippocampal pyramidal neurons, also triggering both short- and long-term effects on glutamate-mediated signaling (Wong and Moss, 1991, 1992). These rapid effects, occurring a few minutes after administration of this neurosteroid, strongly suggest major non-genomic mechanisms of action for E2-dependent facilitation of LTP (Warren et al., 1995; Cordoba Montoya and Carrer, 1997; Good et al., 1999). Electrophysiological recordings of hippocampal rat slices evidenced that E2 facilitation of LTP is mediated by NMDAR currents with effects both in males and females (Foy et al., 1999), as confirmed by pharmacological inhibition of the E2- and NMDA-dependent LTP using GluN2B-containing NMDA receptor antagonists (Smith and McMahon, 2005, 2006). The action of the E2-mediated modulation of NMDAR-dependent LTP was found

to involve several intracellular protein kinases and membrane-associated targets, as suggested by experiments where incubation of hippocampal slices with blockers of ERs, MAPK/ERK, PKA, PKC, PI3K, NR2B, or CaMKII did not allow the induction of LTP in the presence of known E2 concentrations (Hasegawa et al., 2015). However, most of the studies exploring the influence of hippocampal E2 on synaptic memory involved experimental protocols where E2 was applied exogenously, an approach that may have limited the possibility to determine the exact source of neural E2, leaving unsolved the question whether LTP induction in physiological conditions needs the presence of the E2 neurosteroid. An important advance in the knowledge on the E2 origin and the mechanism of E2-mediated LTP was provided by studies using an electrophysiological and pharmacological approach of investigation that involved the use of drugs inhibiting the E2 synthesis or blockers of the ERs in animal models. Electrophysiological experiments aimed at investigating local mechanisms of LTP induction by using blocking agents of E2 synthesis were first performed in brain slices of male rats containing the medial vestibular nuclei (Grassi et al., 2009). Here, it has been found that the acute pharmacological inhibition of P450 aromatase with letrozole or the acute blockade of ERs (Grassi et al., 2009) with ICI 182780 (Scarduzio et al., 2013) prevented the LTP induction. This approach was then extended to the hippocampus where the role of locally synthesized E2 was demonstrated in male rats by measuring the effect of blockade of the E2 synthesis or receptors on the synaptic field potentials evoked in the CA1 region (Grassi et al., 2011; Pettorossi et al., 2013a). In fact, the LTP amplitude was markedly reduced under block of E2 synthesis or receptors, suggesting a facilitatory role of E2 on LTP. Further experiments performed in single hippocampal pyramidal neurons evidenced that in most of the neurons of the CA1 region, E2 is necessary for inducing LTP because pharmacological ER blockade fully prevented this form of synaptic plasticity (Tozzi et al., 2019). These studies also allowed to explore the role of ER subtypes on LTP induction. The use of selective ERα and ERβ inhibitors demonstrated that these ERs are both involved, as the ERα blocker MPP and the ERβ blocker PHTPP were both individually able to reduce LTP and were able to fully prevent it when applied together (Tozzi et al., 2019). The role of E2 synthesis on hippocampal LTP was further explored in male rats with experiments inhibiting the P450 aromatase activity (Tanaka and Sokabe, 2012, 2013; Hojo et al., 2015) and more recently by proving that ERα stimulation is able to induce LTP (Clements and Harvey, 2020). The role of E2 in the LTP induction has also been confirmed in mice knock-out for the P450 aromatase of both sexes, providing direct evidence that in the hippocampus, neuron-derived E2 is able to rapidly regulate the P450 aromatase-dependent Akt-ERK and CREB-BDNF signaling, essential for normal expression of LTP and synaptic plasticity (Lu et al., 2019).

The influence of E2 on LTP induction was generally observed in the hippocampus of adult male rats; however, different research groups pointed to age-dependent effects, with E2-dependent LTP observed only in young animals (Foy et al., 1999; Bi et al., 2000; Hojo et al., 2008). The influence of E2 on LTP has been also hypothesized to be gender specific. When E2 synthesis was chronically inhibited with letrozole, the LTP induction was prevented in female rats but not in males. This gender-dependent effect was paralleled by reduction of spine formation (Vierk et al., 2012; Fester and Rune, 2015). However, in a subsequent paper, the same group provided evidence that P450 aromatase inhibition by letrozole, applied acutely, prevented LTP induction both in male and female animals, confirming a more general role of E2 in hippocampal LTP (Vierk et al., 2012).

MODULATION OF P450 AROMATASE AFFECTS LTP INDUCTION

The pharmacological prevention of hippocampal LTP by inhibition of the E2 synthesis or of the ERs activity suggests that E2 is required during the induction phase of LTP, when neuronal afferences are activated by electrical stimulation. P450 aromatase is expressed at hippocampal level and it has been shown that its activation depends on neuronal activity (Kimoto et al., 2001; Hojo et al., 2004, 2008; Balthazart et al., 2006). Whether the P450 aromatase activity is responsible for a tonic synthesis of local E2 or is dependent on neural phasic inputs has not been established yet. Experimental evidences show that different high- or low-frequency stimulations in the rat hippocampus may account for bidirectional synaptic plasticity, with the synthesis of estrogen and androgens directly implicated in synaptic potentiation or depression, respectively (Tozzi et al., 2019). For example, whereas the inhibition of P450 aromatase impedes LTP induced by high-frequency stimulation, the concomitant presence of exogenous E2 allows a full LTP expression in a dose-dependent manner (Di Mauro et al., 2015, 2017). Moreover, while low-frequency stimulation normally depresses synaptic transmission (LTD), in the presence of exogenous E2, it is able to induce LTP. These evidences confirm that in the rat hippocampus E2 is a key factor for eliciting LTP and suggest that different stimulating frequency patterns might modulate the P450 aromatase activity and the local E2 neo-synthesis to sustain LTP (Di Mauro et al., 2015, 2017).

Different research groups described a relation between changes of intracellular Ca^{2+} concentrations and P450 aromatase activity, implying the modulation of E2 synthesis (Balthazart et al., 2005; Fester et al., 2016). However, the precise mechanism by which neuronal electrical activity may lead to a Ca^{2+}-dependent increase or decrease of E2 synthesis is still unknown. Exogenous glutamate application and K^{+}-induced membrane depolarization have been suggested to trigger neuronal calcium-induced calcium release from intracellular Ca^{2+} stores, leading to enhancement of Ca^{2+} concentrations. This has been shown to lead to the activation of Ca^{2+}-dependent kinases, P450 aromatase phosphorylation, and subsequent inhibition of E2 synthesis (Balthazart et al., 2001, 2003, 2005; Balthazart and Ball, 2006; Charlier et al., 2015; Fester et al., 2016). Moreover, a mechanism involving the action of extracellular E2 on NMDAR and on voltage-activated Ca^{2+} channels has been proposed to regulate E2 synthesis by influencing P450 aromatase activity (Zhao et al., 2005; Fester et al., 2016; **Figure 1**). Other investigators suggested a different scenario, describing a direct role of the NMDAR

activation in the enhancement of intracellular Ca^{2+} with opposite effects on P450 aromatase activity. It has been reported that NMDAR activation triggers Ca^{2+}-dependent kinases activity and increases E2 synthesis by P450 aromatase stimulation (Hojo et al., 2004; **Figure 1**). One possible explanation that might account for these opposite findings is the different experimental modality employed to stimulate neurons. Different glutamate receptors' activation and velocity of Ca^{2+} entry into the cell may lead to opposite phosphorylation–dephosphorylation processes. The induction of LTP under the enhanced E2 availability was prevented by an inhibitor of calcium–calmodulin-dependent protein kinase II (CaMKII), suggesting that E2 is able to potentiate NMDA receptor function inducing an increase of postsynaptic Ca^{2+} concentration that in turn activates CaMKII leading to LTP induction (Hasegawa et al., 2015; **Figure 1**).

INVOLVEMENT OF NEURAL E2 ON HIPPOCAMPAL LTD

The involvement of neural E2 on the scaling down of synaptic transmission, as in the case of LTD, has also been explored because the ability of E2 to modify synaptic plasticity via long-term depression may be an additional mechanism by which

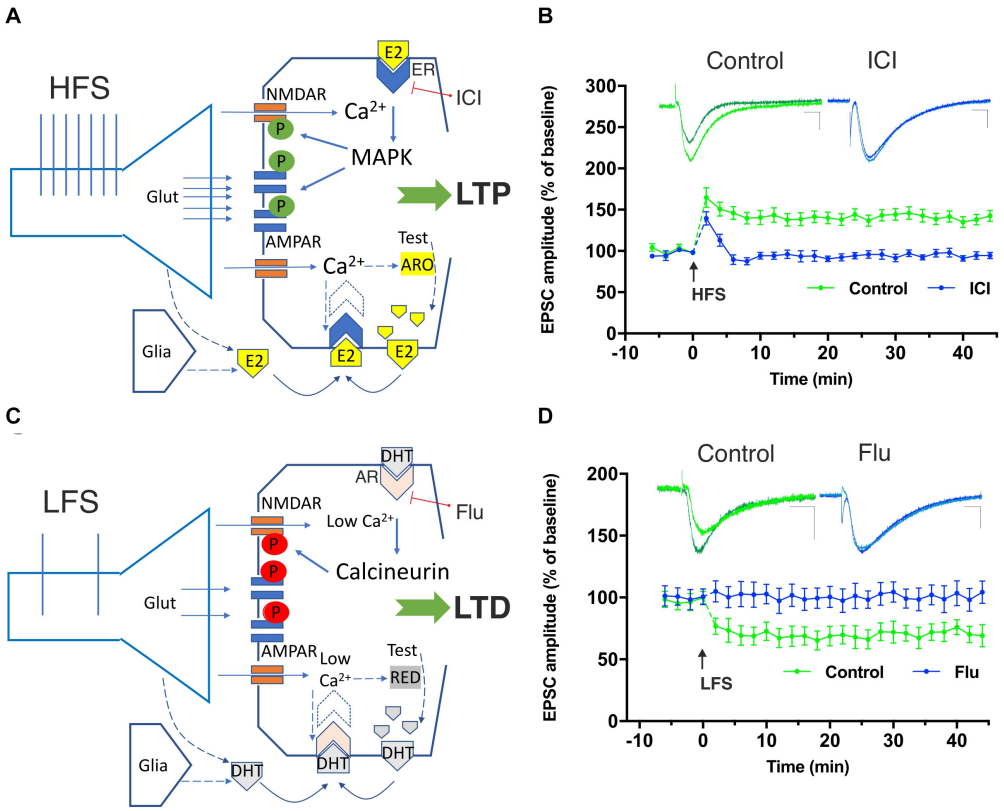

FIGURE 1 | Involvement of neural 17β-estradiol (E2) and 5α-dihydrotestosterone (DHT), produced by testosterone (Test), in the induction of hippocampal high-frequency stimulation (HFS)–dependent long-term potentiation (LTP) and low-frequency stimulation (LFS)–dependent long-term depression (LTD). **(A)** The HFS-mediated glutamate (Glut) release stimulates large Ca^{2+} influx into dendritic spines through NMDA receptors. Ca^{2+} can activate calmodulin, PKC, and MAPK leading to phosphorylation (green symbols) of AMPA and NMDA glutamate receptors triggering LTP. Ca^{2+} is suggested to also stimulate P450 aromatase (ARO) activity increasing or decreasing the synthesis of E2, according to specific inputs to NMDARs and the Ca^{2+}-dependent enzymes involved. After HFS, the synthesis of E2 may be locally enhanced and E2, released by presynaptic terminals and by the glia, may bind to membrane-gathered estrogen receptors (ERs) and to induce LTP through MAPK stimulation. **(B)** Graph showing the time course of the excitatory postsynaptic current (EPSC) amplitudes as percentage of the baseline, before and after an HFS is delivered to Schaffer collaterals in patch-clamp experiments from rat hippocampal CA1 pyramidal cells in control conditions (green time-course plot) and in the presence of the ER blocker ICI 182780 (10 μM, ICI) applied for the duration of the experiment (blue time-course plot). Note the LTP induced in control conditions but not in the presence of the ER blocking agent ICI. Superimposed representative traces showing an EPSC recorded before and 40 min after the HFS protocol in control conditions (green) and in the presence of ICI (blue). Scale bars: 10 ms; 50 pA. **(C)** LFS allows modest Ca^{2+} inflow into the postsynaptic neuron through NMDARs. Low Ca^{2+} levels are suggested to stimulate the 5α-reductase (RED)-dependent biosynthesis of DHT from testosterone (Lu et al., 2019). The locally synthesized DHT together with DHT released by presynaptic terminals and glia is suggested to activate membrane-gathered androgen receptors (AR) leading to the activation of calcineurin and to the dephosphorylation (red symbols) of different targets involved in LTD including the NMDAR. **(D)** Time-course graph of the EPSC amplitudes, before and after an LFS in control conditions (green time-course plot) and in the presence of the AR blocker flutamide (10 μM, Flu) applied for the duration of the experiment (blue time-course plot). Note the LTD induced in control conditions but not in the presence of the AR blocking agent Flu. Superimposed representative traces showing an EPSC recorded before and 40 min after the LFS protocol in control conditions (green) and in the presence of Flu (blue). Scale bars 10 ms; 50 pA. Modified by Tozzi et al. (2019).

E2 can enhance learning and memory. In fact, E2 might act to improve memory by suppressing forgetting via an LTD-based mechanism.

Brain aging is generally associated to a decreased ability of memory processing and, in aged rats, to a facilitated hippocampal LTD. Accordingly, LTD amplitude was found to be larger in aged rats than in adults, and interestingly, treatments with E2 was reported to prevent this enhanced LTD in these aged animals but not in adults (Foy, 2001). Hippocampal LTD can be elicited by modest Ca^{2+} inflows entering into neurons via NMDA or metabotropic group I glutamate (mGluI) receptors (Shiroma et al., 2005). Interestingly, it has been found that the threshold for the induction of the NMDAR-dependent LTD could be enhanced or lowered by the stimulation of ERα or ERβ, respectively, confirming the role of ERs on LTD induction (Ooishi et al., 2012). Moreover, experiments also using ERα- and ERβ-knock-out mice confirmed that E2 may be able to rapidly enhance hippocampal LTD, presumably by a NMDAR- and ERα-dependent mechanism of action (Mukai et al., 2007; Murakami et al., 2015). However, electrophysiological experiments conducted in the presence of inhibitors of the E2 synthesizing enzyme or of the ERs suggested a minor role of E2 in hippocampal LTD because in the presence of these drugs, hippocampal LTD of male adult rats remained unchanged (Pettorossi et al., 2013a; Di Mauro et al., 2015, 2017).

ANDROGENS AFFECT HIPPOCAMPAL LTP AND LTD

It is well known that testosterone and its more active metabolite DHT attenuate mild cognitive impairment in men, suggesting a role of androgens in sustaining synaptic memory (Hogervorst et al., 2004; Pike et al., 2006; Kang et al., 2014; Pan et al., 2016). The concentration of these neurosteroids appears to be positively related with hippocampal synaptic spine density, suggesting that androgens are required for hippocampus-related cognitive performances (Moffat et al., 2002; Ooishi et al., 2012). The effects of androgens on the induction of hippocampal LTP and LTD has been recently examined by using drugs able to inhibit ARs or the 5α-reductase, the enzyme catalyzing the conversion of testosterone into DHT. Experimental findings demonstrated that in the rat CA1 hippocampal area, LTD is fully prevented under pharmacological blockade of either the AR or the 5α-reductase. These pharmacological actions, however, were unable to influence LTP in the same brain region and suggest that in the hippocampus, androgens sustain LTD but not LTP induction (Grassi et al., 2011; Grassi et al., 2013; Pettorossi et al., 2013a; Di Mauro et al., 2015; Di Mauro et al., 2017; Tozzi et al., 2019; **Figure 1**). Accordingly, ARs were found to be expressed in the hippocampus at the postsynaptic level (Tabori et al., 2005; Hatanaka et al., 2009), implying that ARs may participate in androgen-induced LTD.

Because testosterone may be converted either into E2 or DHT, depending on the prevalence, respectively, of the P450 aromatase or 5α-reductase synthesizing activity, it is suggested that the modulation of these biosynthetic pathways might in turn promote LTP or LTD in neurons (Di Mauro et al., 2015, 2017). Moreover, it is possible that the conversion of testosterone into DHT might limit the E2 neo-synthesis and the consequent possibility to induce LTP. Moreover, it has been hypothesized that a specific frequency of neuronal stimulation might drive hippocampal metabolism of testosterone toward conversion into E2 or DHT to sustain, respectively, LTP or LTD induction (Di Mauro et al., 2015).

Long-term depression in neurons may be based on different mechanisms, most of them implying an NMDAR-, mGluR- or endocannabinoid-dependent signaling. Ca^{2+} is suggested to play a role in LTD entering into neurons by glutamate receptors such as NMDARs during synaptic electric stimulations. Low-frequency stimulations (LFS) would produce low Ca^{2+} increases through NMDARs in the postsynaptic dendritic spines (**Figure 1**). After Ca^{2+}-calmodulin formation, sequential activation of protein phosphatase 2B (calcineurin), dephosphorylation of inhibitor-1, activation of protein phosphatase 1 (PP1), and dephosphorylation of ser845 on the AMPAR subunit GluA1 would lead to internalization of AMPARs from the synapse, changes of the conductance properties of these receptors ultimately inducing LTD (Mulkey et al., 1994). However, how androgens are involved in LTD induction has been poorly investigated. It has been proposed that LFS-induced LTD is established in the hippocampus via DHT binding to synaptic ARs on delivery of LFS, leading to calcineurin activation and NMDAR suppression, resulting in a decreased presence or dephosphorylation of AMPARs (Hasegawa et al., 2015).

ESTROGENIC AND ANDROGENIC SEX NEUROSTEROIDS AFFECT VESTIBULAR LTP AND LTD

The vestibular system is responsible for stabilizing the eyes and the body in space and is crucial for self-motion perception. It is involved in several plastic phenomena like the visuo-vestibular calibration (Lisberger and Miles, 1980), the vestibular compensation (Smith and Curthoys, 1989; Dutia, 2010), and the responsiveness to intense stimulation (Massot et al., 2012; Pettorossi et al., 2013b). Because LTP and LTD expression have been demonstrated in the vestibular nuclei and are likely involved in these adaptive responses (Grassi et al., 1995), the contribution of sex neurosteroids has been explored in medial vestibular nucleus (MVN).

Medial vestibular nucleus neurons express the E2 synthesizing enzyme P450 aromatase and both estrogen and androgen receptors. Specifically, immunoreactivity for ERα, ERβ, and AR have been found in these neurons, most of them co-localizing ERβ and AR (Grassi et al., 2013). Electrophysiological studies in rat MVN neurons showed that E2 affects synaptic transmission and neuronal excitability facilitating both the LTP of the primary vestibular afferents and the intrinsic membrane excitability (Grassi et al., 2007, 2009, 2010). Specifically, during LTP, E2 depresses the spontaneous action potential discharge in both regular (A type) and irregular (B type) discharging neurons, while the synaptic response to vestibular nerve stimulation is

increased in B type neurons with a net effect of enhancement of the signal-to-noise ratio of synaptic response in these neurons, relative to resting activity of all MVN neurons. These combined effects may be necessary to specifically enhance the dynamic properties of neuronal activation of vestibular circuits. Interestingly, pharmacological inhibition of E2 synthesis by letrozole or antagonism of ERs by ICI 182780 was reported not only to prevent the induction of LTP but also to unmask LTD of synaptic plasticity, suggesting that the level of neural E2 is a key modulator of MVN neurons' synaptic plasticity, being able to shift LTP into LTD according to the local availability of this neurosteroid (Grassi et al., 2009, 2010).

Because MVN neurons have been reported to also express androgens, it is plausible that androgens play an important role in LTD induction of synaptic plasticity also in MVN, as demonstrated in other brain regions such as the hippocampus. In fact, it has been shown that the pharmacological antagonism of ARs abolishes this form of synaptic plasticity in MVN neurons, as observed by electrophysiological recordings (Scarduzio et al., 2012, 2013). However, differently from what was observed in the hippocampus, the reduction of DHT synthesis from testosterone by pharmacological inhibition of 5α-reductase did not affect LTD, suggesting a more direct effect of testosterone and/or a greater sensitivity of MVN neurons to this neurosteroid with respect to DHT (Scarduzio et al., 2012, 2013).

NEURAL E2 AFFECTS CEREBELLAR LTP AND LTD

The cerebellum participates with the vestibular system to most of the adaptation observed in the gaze stability with multiple synaptic plasticity mechanisms (Lisberger and Miles, 1980). In particular, together with the vestibular nuclei, it is responsible for the vestibulo-ocular reflex calibration (Hogervorst et al., 2004). In this encephalic structure, the encoding gain increase and decrease adaptation is thought to be mediated by LTP occurring at the parallel fiber–Purkinje cell synapses (PF-LTP) and by LTD (PF-LTD) at the same or different synapse subset, respectively (Hansel et al., 2001; Boyden and Raymond, 2003; Boyden et al., 2004; Coesmans et al., 2004; Broussard et al., 2011).

The cerebellum expresses ERs, ARs, and the synthesizing enzymes for E2 and androgens (Sakamoto et al., 2003; Tsutsui et al., 2011; Hedges et al., 2012). The first evidence of the influence of neural E2 in cerebellar learning has been provided by a study in which synaptic plasticity at the Purkinje cell and the VOR adaptation was examined in ovariectomized mice, in ERβ knock-out female mice, and in male mice, after the administration of E2 (Andreescu et al., 2007). These authors found that E2 had relevant impact on the expression of VOR gain-down adaptation and regulated cerebellar synaptic plasticity influencing PF-LTP. First, it was shown that E2 enhanced the LTP amplitude at the PF–Purkinje cell synapse, while leaving LTD unaffected. Second, in Purkinje cells, ERβ activation by E2 significantly improved the gain-decrease adaptation of the VOR (Andreescu et al., 2007). In a subsequent study, the impact of E2 in the cerebellar synaptic plasticity was examined by pharmacological inhibition of E2 synthesis in male rats both *in vitro* at the PF–Purkinje cell synapses and *in vivo* evaluating the VOR adaptation (Dieni et al., 2018a,b). The application of the P450 aromatase inhibitor letrozole in the flocculus of cerebellar slices prevented the PF-LTP without affecting the PF-LTD impeding the adaptive gain reduction of the VOR. Together with the sex neurosteroid-mediated bidirectional vestibular synaptic plasticity, the cerebellum participates in the visuo-vestibular calibration of the VOR. It is likely that E2 facilitates the gain-increase of VOR by acting at the level of the vestibular system and the gain-down regulation by acting at the level of the cerebellum (Dieni et al., 2018a,b).

NEURAL E2 AFFECTS STRIATAL LTP

Neural E2 exerts an important role in LTP induction in the nucleus striatum with E2 receptors diffusely expressed in the basal ganglia (Creutz and Kritzer, 2004; Krentzel et al., 2019). Electrophysiological experiments performed in neurons of the dorsal striatum of the male rat showed that E2 synthesis and ER activation are required for the induction of LTP in both spiny projection neurons (SPNs) and cholinergic interneurons because the pharmacological inhibition of P450 aromatase or antagonism of ERs completely prevented the LTP induction of SPNs with no effect on LTD or synaptic depotentiation (Tozzi et al., 2015). Because striatal dopamine (DA) release is critical for LTP induction in this brain structure (Calabresi et al., 2007) and based on the evidence that striatal LTP depends on E2 local synthesis, the interaction between E2 and DA in controlling SPNs' LTP was explored. Tozzi et al. (2015) suggested that the E2 and DA signaling systems converge on the stimulation of the cAMP–PKA intracellular pathway to facilitate LTP induction in striatal neurons via a cooperation between the D1 DA receptor and the ERs (Tozzi et al., 2015). These findings were also supported by experiments showing a possible facilitatory influence of E2 in the dorsal striatum where DA release has been demonstrated to be potentiated by E2 (Song et al., 2019).

NEURAL E2 AFFECTS LTP IN THE AMYGDALA

17β-estradiol is suggested to play a role in synaptic plasticity also in the amygdala, a brain structure where the presence of P450 aromatase has been reported (Zhao et al., 2007; Bender et al., 2017). The amygdala is considered a core nucleus of "emotional" memory and for the responses to emotion (Pape and Pare, 2010). Accordingly, dysfunction of its neuronal networks is implicated in pathological conditions such as depression and post-traumatic stress disorder (Tovote et al., 2015). Depression-like symptoms have been observed in women under treatment with P450 aromatase inhibitors providing evidence of the importance of the E2 signaling system in the amygdala-related physiology (Gallicchio et al., 2012).

In a recent study, Bender and colleagues (Bender et al., 2017) explored the possible influence of the E2 in the LTP induction of

neurons of the basolateral amygdala, a brain region characterized by important synaptic plasticity and by the presence of P450 aromatase both in male and female rodents. Here, the authors found that, beside the effect on the spine density, pharmacological inhibition of P450 aromatase prevented the LTP induction in amygdala slices of female rodents but not in males. The gender-specific role of E2 in LTP of amygdala neurons points to the importance of conducting further studies in the field to better understand the sex-related differences observed in mood disorders and to take into account the side effects of P450 aromatase inhibitors.

THE ROLE OF CIRCULATING HORMONES

Whether circulating sex hormones affect the long-term synaptic changes induced by electrical neuronal activity in the brain is an intriguing possibility. E2 synthesized in the CNS may be affected by the estrous cycle in rodent, as reported in the rat hippocampus where neural E2 levels changed according to different phases of the cycle (Kato et al., 2013). Neural E2 changes were suggested to result by indirect access of fluctuating hematic progesterone into the brain and its subsequent conversion into E2 (Kato et al., 2013) or by changes of the phosphorylation status of P450 aromatase depending on fluctuation of different kinases related to synaptic plasticity (Balthazart and Ball, 2006; Hojo and Kawato, 2018). The resulting effect of neural E2 fluctuations appeared to be correlated in the rat hippocampus with the induction of LTP or LTD together with changes of dendritic spine density. In fact, it has been shown that the LTP amplitude changes are depending on the estrum phase in the hippocampus (Warren et al., 1995). Subsequent comparative studies on the occurrence and amplitude of LTP were performed in vestibular neurons of male and female rats (Pettorossi et al., 2011). Specifically, while HFS protocol was able to consistently induce LTP in male rats within seconds (fast-developing LTP), in females HFS protocol produced fast and slow LTP or even LTD. The amplitude and occurrence of LTP depended on the estrous phases, with minor probability to induce fast LTP in proestrus (Pettorossi et al., 2011). This effect might depend on the marked structural changes of the neurons during the estrous cycle, but it might also be a result of the influence of circulating hormones in the synthesis of the neurosteroids. It has been found, in fact, that the acute administration of testosterone that induces LTP in male can induce LTD in female only during the proestrus phase (Grassi et al., 2012). This suggests that in the presence of high levels of circulating E2 (proestrus), the conversion of testosterone into E2 is inhibited, whereas the synthesis of DHT is facilitated.

The interesting data obtained by analyzing the long-term responses in vestibular nuclei prompt to extend this study to the hippocampus by using a similar approach for understanding how memory and learning could be correlated with the expression of the neural synaptic plasticity. Changes in the thresholds of the induction of long-term phenomena might vary with behavioral changes, as reported in EEG experiments in women (Sumner et al., 2018).

DENDRITIC SPINE REMODELING IS ASSOCIATED TO HIPPOCAMPAL E2-DEPENDENT LTP

Most of the studies on the role of sex neurosteroids in hippocampal synaptic plasticity demonstrated that E2 facilitates LTP induction and enhances dendritic spine formation. E2 has been reported to increase apical dendritic spine formation in projections that are closed to presynaptic terminals on hippocampal CA1 pyramidal cells. It has been reported that this action is achieved by activation of genomic ERs, most likely the ERα (Gould et al., 1990; Woolley et al., 1990, 1996; Woolley and McEwen, 1993; Murphy and Segal, 1996; Woolley, 1998; Hao et al., 2003; MacLusky et al., 2005). Accordingly, ERs activation is able to either decrease GABAergic inhibition (Murphy et al., 1998; Rudick and Woolley, 2001) and increase NMDAR expression and function (Weiland, 1992; Warren et al., 1995; Gazzaley et al., 1996; Cordoba Montoya and Carrer, 1997; Woolley et al., 1997; Good et al., 1999; Cyr et al., 2000, 2001; Bi et al., 2001; Rudick and Woolley, 2001), ultimately increasing the spine density of neurons. Hippocampal LTP have in fact been correlated to the simultaneous increase of both the spine density and the NMDAR-dependent synaptic transmission (Smith and McMahon, 2005, 2006), suggesting that E2 during LTP formation is able to trigger both morphological and functional changes. Synaptic changes induced by E2 during LTP were reported to affect the structure of neuronal circuitry by increasing the polymerization of filamentous actin proteins in the dendritic spines (Kramar et al., 2009) and by rapidly enhancing their head structure and density via activation of ERα, but not ERβ (Ooishi et al., 2012).

The morphological and electrophysiological changes triggered by E2 during LTP may be part of the same E2-dependent mechanism. Synergic effects consisting of formation of new spines and stabilization of synaptic strength in active mature synapses have in fact been reported to be necessary for long-term synaptic consolidation of neurotransmission (Toni et al., 1999; Yang et al., 2008). However, an alternative possibility considers the functional and structural events happening during E2-dependent LTP formation as two distinct phenomena, according to experiments of acute or chronic inhibition of E2 synthesis (Vierk et al., 2012; Fester and Rune, 2015).

Overall, even if it is possible to hypothesize that initial structural changes of dendritic spines induced by E2 precede a full LTP induction, the idea that neural E2 is able to trigger LTP more rapidly than any possible change of synaptic structure is likely to occur. In this scenario, the induction of LTP of synaptic transmission, involving the increased synthesis or availability of E2 during neuronal activity, might represent the early phase in the process that allows subsequent spine formation and consolidation (Yang et al., 2008; Kato et al., 2013; Dickens et al., 2014).

Interestingly, neural androgens may also increase spine structure suggesting that the machinery at the basis of synaptic plasticity needs structural improvement either for the induction of the E2-mediated LTP and for the induction of androgen-mediated LTD (Leranth et al., 2004; Hatanaka et al., 2009).

Thus, sex neurosteroids are suggested to influence long-term synaptic plasticity by complex mechanisms of action, with estrogens and androgens producing opposite effects on LTP and LTD induction, respectively, and both of them being responsible for the enhancement of dendritic spine formation. This reveals a multifaceted mechanism at the base of neurosteroid influence in synaptic plasticity. In fact, estrogens and androgens have opposite effects in the long-term synaptic events, being the LTP induced by E2 and the LTD by androgens, whereas both neurosteroids enhance dendritic spine formation.

Beside their contribution in synaptic plasticity, the evidence reported in this review of the literature underly the involvement of these molecules in the regulation of several behavioral aspects that are related to the motor system function, to emotional manifestations and cognition.

AUTHOR CONTRIBUTIONS

AT and VP conceived and wrote the manuscript. LB reviewed the manuscript. All authors contributed to the figure preparation and critically revised the manuscript.

ACKNOWLEDGMENTS

The support of a Telethon Italy grant (GGP16131) to AT was gratefully acknowledged.

REFERENCES

Altemus, M. (2017). Neuroendocrine networks and functionality. *Psychiatr. Clin. North Am.* 40, 189–200. doi: 10.1016/j.psc.2017.01.008

Andreescu, C. E., Milojkovic, B. A., Haasdijk, E. D., Kramer, P., De Jong, F. H., Krust, A., et al. (2007). Estradiol improves cerebellar memory formation by activating estrogen receptor beta. *J. Neurosci.* 27, 10832–10839. doi: 10.1523/JNEUROSCI.2588-07.2007

Aubele, T., Kaufman, R., Montalmant, F., and Kritzer, M. F. (2008). Effects of gonadectomy and hormone replacement on a spontaneous novel object recognition task in adult male rats. *Horm. Behav.* 54, 244–252. doi: 10.1016/j.yhbeh.2008.04.001

Babanejad, S., Naghdi, N., and Haeri Rohani, S. A. (2012). Microinjection of Dihydrotestosterone as a 5α-Reduced Metabolite of Testosterone into CA1 Region of Hippocampus Could Improve Spatial Learning in the Adult Male Rats. *Iran. J. Pharm. Res.* 11, 661–669.

Bailey, D. J., Ma, C., Soma, K. K., and Saldanha, C. J. (2013). Inhibition of hippocampal aromatization impairs spatial memory performance in a male songbird. *Endocrinology* 154, 4707–4714. doi: 10.1210/en.2013-1684

Bailey, D. J., Makeyeva, Y. V., Paitel, E. R., Pedersen, A. L., Hon, A. T., Gunderson, J. A., et al. (2017). Hippocampal aromatization modulates spatial memory and characteristics of the synaptic membrane in the male zebra finch. *Endocrinology* 158, 852–859. doi: 10.1210/en.2016-1692

Balthazart, J., Baillien, M., and Ball, G. F. (2001). Phosphorylation processes mediate rapid changes of brain aromatase activity. *J. Steroid Biochem. Mol. Biol.* 79, 261–277. doi: 10.1016/s0960-0760(01)00143-1

Balthazart, J., Baillien, M., and Ball, G. F. (2006). Rapid control of brain aromatase activity by glutamatergic inputs. *Endocrinology* 147, 359–366. doi: 10.1210/en.2005-0845

Balthazart, J., Baillien, M., Charlier, T. D., and Ball, G. F. (2003). Calcium-dependent phosphorylation processes control brain aromatase in quail. *Eur. J. Neurosci.* 17, 1591–1606. doi: 10.1046/j.1460-9568.2003.02598.x

Balthazart, J., Baillien, M., Charlier, T. D., and Ball, G. F. (2005). Effects of calmodulin on aromatase activity in the preoptic area. *J. Neuroendocrinol.* 17, 664–671. doi: 10.1111/j.1365-2826.2005.01355.x

Balthazart, J., and Ball, G. F. (2006). Is brain estradiol a hormone or a neurotransmitter? *Trends Neurosci.* 29, 241–249. doi: 10.1016/j.tins.2006.03.004

Bangasser, D. A., and Valentino, R. J. (2014). Sex differences in stress-related psychiatric disorders: neurobiological perspectives. *Front. Neuroendocrinol.* 35, 303–319. doi: 10.1016/j.yfrne.2014.03.008

Baulieu, E. E. (1981). "Steroid hormone in the brain: several mechanism?" in *Steroid Hormone Regulation of the Brain*, eds K. Fuxe, J. A. Gustafsson, and L. Wetterberg, (Oxford: Pergamon), 3–14.

Baulieu, E. E., and Robel, P. (1990). Neurosteroids: A new brain function? *J. Steroid Biochem. Mol. Biol.* 37, 395–403. doi: 10.1016/0960-0760(90)90490-c

Bayer, J., Rune, G., Schultz, H., Tobia, M. J., Mebes, I., Katzler, O., et al. (2015). The effect of estrogen synthesis inhibition on hippocampal memory. *Psychoneuroendocrinology* 56, 213–225. doi: 10.1016/j.psyneuen.2015.03.011

Beauchet, O. (2006). Testosterone and cognitive function: current clinical evidence of a relationship. *Eur. J. Endocrinol.* 155, 773–781. doi: 10.1530/eje.1.02306

Bender, R. A., Zhou, L., Vierk, R., Brandt, N., Keller, A., Gee, C. E., et al. (2017). Sex-dependent regulation of aromatase-mediated synaptic plasticity in the basolateral amygdala. *J. Neurosci.* 37, 1532–1545. doi: 10.1523/JNEUROSCI.1532-16.2016

Benice, T. S., and Raber, J. (2009). Dihydrotestosterone modulates spatial working-memory performance in male mice. *J. Neurochem.* 110, 902–911. doi: 10.1111/j.1471-4159.2009.06183.x

Bi, R., Broutman, G., Foy, M. R., Thompson, R. F., and Baudry, M. (2000). The tyrosine kinase and mitogen-activated protein kinase pathways mediate multiple effects of estrogen in hippocampus. *Proc. Natl. Acad. Sci. U.S.A.* 97, 3602–3607. doi: 10.1073/pnas.060034497

Bi, R., Foy, M. R., Vouimba, R. M., Thompson, R. F., and Baudry, M. (2001). Cyclic changes in estradiol regulate synaptic plasticity through the MAP kinase pathway. *Proc. Natl. Acad. Sci. U.S.A.* 98, 13391–13395. doi: 10.1073/pnas.241507698

Boyden, E. S., Katoh, A., and Raymond, J. L. (2004). Cerebellum-dependent learning: the role of multiple plasticity mechanisms. *Annu. Rev. Neurosci.* 27, 581–609. doi: 10.1146/annurev.neuro.27.070203.144238

Boyden, E. S., and Raymond, J. L. (2003). Active reversal of motor memories reveals rules governing memory encoding. *Neuron* 39, 1031–1042. doi: 10.1016/s0896-6273(03)00562-2

Broussard, D. M., Titley, H. K., Antflick, J., and Hampson, D. R. (2011). Motor learning in the VOR: the cerebellar component. *Exp. Brain Res.* 210, 451–463. doi: 10.1007/s00221-011-2589-z

Calabresi, P., Picconi, B., Tozzi, A., and Di Filippo, M. (2007). Dopamine-mediated regulation of corticostriatal synaptic plasticity. *Trends Neurosci.* 30, 211–219. doi: 10.1016/j.tins.2007.03.001

Charlier, T. D., Cornil, C. A., Patte-Mensah, C., Meyer, L., Mensah-Nyagan, A. G., and Balthazart, J. (2015). Local modulation of steroid action: rapid control of enzymatic activity. *Front. Neurosci.* 9:83. doi: 10.3389/fnins.2015.00083

Clements, L., and Harvey, J. (2020). Activation of oestrogen receptor alpha induces a novel form of LTP at hippocampal temporoammonic-CA1 synapses. *Br. J. Pharmacol.* 177, 642–655. doi: 10.1111/bph.14880

Coesmans, M., Weber, J. T., De Zeeuw, C. I., and Hansel, C. (2004). Bidirectional parallel fiber plasticity in the cerebellum under climbing fiber control. *Neuron* 44, 691–700. doi: 10.1016/j.neuron.2004.10.031

Colciago, A., Casati, L., Negri-Cesi, P., and Celotti, F. (2015). Learning and memory: steroids and epigenetics. *J. Steroid Biochem. Mol. Biol.* 150, 64–85. doi: 10.1016/j.jsbmb.2015.02.008

Cordoba Montoya, D. A., and Carrer, H. F. (1997). Estrogen facilitates induction of long term potentiation in the hippocampus of awake rats. *Brain Res.* 778, 430–438. doi: 10.1016/s0006-8993(97)01206-7

Creutz, L. M., and Kritzer, M. F. (2004). Mesostriatal and mesolimbic projections of midbrain neurons immunoreactive for estrogen receptor beta or androgen receptors in rats. *J. Comp. Neurol.* 476, 348–362. doi: 10.1002/cne.20229

Cyr, M., Ghribi, O., and Di Paolo, T. (2000). Regional and selective effects of oestradiol and progesterone on NMDA and AMPA receptors in the rat brain. *J. Neuroendocrinol.* 12, 445–452. doi: 10.1046/j.1365-2826.2000.00471.x

Cyr, M., Ghribi, O., Thibault, C., Morissette, M., Landry, M., and Di Paolo, T. (2001). Ovarian steroids and selective estrogen receptor modulators activity on rat brain NMDA and AMPA receptors. *Brain Res. Brain Res. Rev.* 37, 153–161. doi: 10.1016/s0165-0173(01)00115-1

Di Mauro, M., Tozzi, A., Calabresi, P., Pettorossi, V. E., and Grassi, S. (2015). Neo-synthesis of estrogenic or androgenic neurosteroids determine whether long-term potentiation or depression is induced in hippocampus of male rat. *Front. Cell. Neurosci.* 9:376. doi: 10.3389/fncel.2015.00376

Di Mauro, M., Tozzi, A., Calabresi, P., Pettorossi, V. E., and Grassi, S. (2017). Different synaptic stimulation patterns influence the local androgenic and estrogenic neurosteroid availability triggering hippocampal synaptic plasticity in the male rat. *Eur. J. Neurosci.* 45, 499–509. doi: 10.1111/ejn.13455

Dickens, M. J., de Bournonville, C., Balthazart, J., and Cornil, C. A. (2014). Relationships between rapid changes in local aromatase activity and estradiol concentrations in male and female quail brain. *Horm. Behav.* 65, 154–164. doi: 10.1016/j.yhbeh.2013.12.011

Dieni, C. V., Contemori, S., Biscarini, A., and Panichi, R. (2020). *De novo* synthesized estradiol: a role in modulating the cerebellar function. *Int. J. Mol. Sci.* 21:3316. doi: 10.3390/ijms21093316

Dieni, C. V., Ferraresi, A., Sullivan, J. A., Grassi, S., Pettorossi, V. E., and Panichi, R. (2018a). Acute inhibition of estradiol synthesis impacts vestibulo-ocular reflex adaptation and cerebellar long-term potentiation in male rats. *Brain Struct. Funct.* 223, 837–850. doi: 10.1007/s00429-017-1514-z

Dieni, C. V., Sullivan, J. A., Faralli, M., Contemori, S., Biscarini, A., Pettorossi, V. E., et al. (2018b). 17 beta-estradiol synthesis modulates cerebellar dependent motor memory formation in adult male rats. *Neurobiol. Learn. Mem.* 155, 276–286. doi: 10.1016/j.nlm.2018.08.011

Diotel, N., Charlier, T. D., Lefebvre, d'Hellencourt, C., Couret, D., Trudeau, V. L., et al. (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

Dutia, M. B. (2010). Mechanisms of vestibular compensation: recent advances. *Curr. Opin. Otolaryngol. Head Neck Surg.* 18, 420–424. doi: 10.1097/MOO.0b013e32833de71f

Evinger, A. J. III, and Levin, E. R. (2005). Requirements for estrogen receptor alpha membrane localization and function. *Steroids* 70, 361–363. doi: 10.1016/j.steroids.2005.02.015

Fester, L., Brandt, N., Windhorst, S., Prols, F., Blaute, C., and Rune, G. M. (2016). Control of aromatase in hippocampal neurons. *J. Steroid Biochem. Mol. Biol.* 160, 9–14. doi: 10.1016/j.jsbmb.2015.10.009

Fester, L., and Rune, G. M. (2015). Sexual neurosteroids and synaptic plasticity in the hippocampus. *Brain Res.* 1621, 162–169. doi: 10.1016/j.brainres.2014.10.033

Fester, L., Zhou, L., Ossig, C., Labitzke, J., Blaute, C., Bader, M., et al. (2017). Synaptopodin is regulated by aromatase activity. *J. Neurochem.* 140, 126–139. doi: 10.1111/jnc.13889

Foradori, C. D., Weiser, M. J., and Handa, R. J. (2008). Non-genomic actions of androgens. *Front. Neuroendocrinol.* 29:169–181. doi: 10.1016/j.yfrne.2007.10.005

Foy, M. R. (2001). 17beta-estradiol: effect on CA1 hippocampal synaptic plasticity. *Neurobiol. Learn. Mem.* 76, 239–252. doi: 10.1006/nlme.2001.4018

Foy, M. R., Xu, J., Xie, X., Brinton, R. D., Thompson, R. F., and Berger, T. W. (1999). 17beta-estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation. *J. Neurophysiol.* 81, 925–929. doi: 10.1152/jn.1999.81.2.925

Gallicchio, L., MacDonald, R., Wood, B., Rushovich, E., and Helzlsouer, K. J. (2012). Menopausal-type symptoms among breast cancer patients on aromatase inhibitor therapy. *Climacteric* 15, 339–349. doi: 10.3109/13697137.2011.620658

Gazzaley, A. H., Weiland, N. G., McEwen, B. S., and Morrison, J. H. (1996). Differential regulation of NMDAR1 mRNA and protein by estradiol in the rat hippocampus. *J. Neurosci.* 16, 6830–6838.

Giatti, S., Garcia-Segura, L. M., Barreto, G. E., and Melcangi, R. C. (2019). Neuroactive steroids, neurosteroidogenesis and sex. *Prog. Neurobiol.* 176, 1–17. doi: 10.1016/j.pneurobio.2018.06.007

Good, M., Day, M., and Muir, J. L. (1999). Cyclical changes in endogenous levels of oestrogen modulate the induction of LTD and LTP in the hippocampal CA1 region. *Eur. J. Neurosci.* 11, 4476–4480. doi: 10.1046/j.1460-9568.1999.00920.x

Gould, E., Woolley, C. S., Frankfurt, M., and McEwen, B. S. (1990). Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. *J. Neurosci.* 10, 1286–1291.

Grassi, S., Della Torre, G., Capocchi, G., Zampolini, M., and Pettorossi, V. E. (1995). The role of GABA in NMDA-dependent long term depression (LTD) of rat medial vestibular nuclei. *Brain Res.* 699, 183–191. doi: 10.1016/0006-8993(95)00895-w

Grassi, S., Frondaroli, A., Dieni, C., Dutia, M. B., and Pettorossi, V. E. (2007). Neurosteroid modulation of neuronal excitability and synaptic transmission in the rat medial vestibular nuclei. *Eur J. Neurosci.* 26, 23–32. doi: 10.1111/j.1460-9568.2007.05645.x

Grassi, S., Frondaroli, A., Dieni, C., Scarduzio, M., and Pettorossi, V. E. (2009). Long-term potentiation in the rat medial vestibular nuclei depends on locally synthesized 17beta-estradiol. *J. Neurosci.* 29, 10779–10783. doi: 10.1523/JNEUROSCI.1697-09.2009

Grassi, S., Frondaroli, A., Scarduzio, M., Dieni, C. V., Brecchia, G., Boiti, C., et al. (2012). Influence of sex and estrous cycle on synaptic responses of the medial vestibular nuclei in rats: role of circulating 17beta-estradiol. *Brain Res. Bull.* 87, 319–327. doi: 10.1016/j.brainresbull.2011.11.008

Grassi, S., Frondaroli, A., Scarduzio, M., Dutia, M. B., Dieni, C., and Pettorossi, V. E. (2010). Effects of 17beta-estradiol on glutamate synaptic transmission and neuronal excitability in the rat medial vestibular nuclei. *Neuroscience* 165, 1100–1114. doi: 10.1016/j.neuroscience.2009.11.039

Grassi, S., Scarduzio, M., Panichi, R., Dall'Aglio, C., Boiti, C., and Pettorossi, V. E. (2013). Opposite long-term synaptic effects of 17beta-estradiol and 5alpha-dihydrotestosterone and localization of their receptors in the medial vestibular nucleus of rats. *Brain Res. Bull.* 97, 1–7. doi: 10.1016/j.brainresbull.2013.05.006

Grassi, S., Tozzi, A., Costa, C., Tantucci, M., Colcelli, E., Scarduzio, M., et al. (2011). Neural 17beta-estradiol facilitates long-term potentiation in the hippocampal CA1 region. *Neuroscience* 192, 67–73. doi: 10.1016/j.neuroscience.2011.06.078

Hansel, C., Linden, D. J., and D'Angelo, E. (2001). Beyond parallel fiber LTD: the diversity of synaptic and non-synaptic plasticity in the cerebellum. *Nat. Neurosci.* 4, 467–475. doi: 10.1038/87419

Hao, J., Janssen, W. G., Tang, Y., Roberts, J. A., McKay, H., Lasley, B., et al. (2003). Estrogen increases the number of spinophilin-immunoreactive spines in the hippocampus of young and aged female rhesus monkeys. *J. Comp. Neurol.* 465, 540–550. doi: 10.1002/cne.10837

Hasegawa, Y., Hojo, Y., Kojima, H., Ikeda, M., Hotta, K., Sato, R., et al. (2015). Estradiol rapidly modulates synaptic plasticity of hippocampal neurons: Involvement of kinase networks. *Brain Res.* 1621, 147–161. doi: 10.1016/j.brainres.2014.12.056

Hatanaka, Y., Hojo, Y., Mukai, H., Murakami, G., Komatsuzaki, Y., Kim, J., et al. (2015). Rapid increase of spines by dihydrotestosterone and testosterone in hippocampal neurons: dependence on synaptic androgen receptor and kinase networks. *Brain Res.* 1621, 121–132. doi: 10.1016/j.brainres.2014.12.011

Hatanaka, Y., Mukai, H., Mitsuhashi, K., Hojo, Y., Murakami, G., Komatsuzaki, Y., et al. (2009). Androgen rapidly increases dendritic thorns of CA3 neurons in male rat hippocampus. *Biochem. Biophys. Res. Commun.* 381, 728–732. doi: 10.1016/j.bbrc.2009.02.130

Hedges, V. L., Ebner, T. J., Meisel, R. L., and Mermelstein, P. G. (2012). The cerebellum as a target for estrogen action. *Front. Neuroendocrinol.* 33, 403–411. doi: 10.1016/j.yfrne.2012.08.005

Hogervorst, E., De Jager, C., Budge, M., and Smith, A. D. (2004). Serum levels of estradiol and testosterone and performance in different cognitive domains in healthy elderly men and women. *Psychoneuroendocrinology* 29, 405–421. doi: 10.1016/s0306-4530(03)00053-2

Hojo, Y., Hattori, T. A., Enami, T., Furukawa, A., Suzuki, K., Ishii, H. T., et al. (2004). Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons. *Proc. Natl. Acad. Sci. U.S.A.* 101, 865–870. doi: 10.1073/pnas.2630225100

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

Hojo, Y., Munetomo, A., Mukai, H., Ikeda, M., Sato, R., Hatanaka, Y., et al. (2015). Estradiol rapidly modulates spinogenesis in hippocampal dentate gyrus: involvement of kinase networks. *Horm. Behav.* 74, 149–156. doi: 10.1016/j.yhbeh.2015.06.008

Hojo, Y., Murakami, G., Mukai, H., Higo, S., Hatanaka, Y., Ogiue-Ikeda, M., et al. (2008). Estrogen synthesis in the brain–role in synaptic plasticity and memory. *Mol. Cell. Endocrinol.* 290, 31–43. doi: 10.1016/j.mce.2008.04.017

Inagaki, T., Frankfurt, M., and Luine, V. (2012). Estrogen-induced memory enhancements are blocked by acute bisphenol A in adult female rats: role of dendritic spines. *Endocrinology* 153, 3357–3367. doi: 10.1210/en.2012-1121

Inagaki, T., Gautreaux, C., and Luine, V. (2010). Acute estrogen treatment facilitates recognition memory consolidation and alters monoamine levels in memory-related brain areas. *Horm. Behav.* 58, 415–426. doi: 10.1016/j.yhbeh.2010.05.013

Jacome, L. F., Barateli, K., Buitrago, D., Lema, F., Frankfurt, M., and Luine, V. N. (2016). Gonadal hormones rapidly enhance spatial memory and increase hippocampal spine density in male rats. *Endocrinology* 157, 1357–1362. doi: 10.1210/en.2015-1959

Kang, L., Li, S., Xing, Z., Li, J., Su, Y., Fan, P., et al. (2014). Dihydrotestosterone treatment delays the conversion from mild cognitive impairment to Alzheimer's disease in SAMP8 mice. *Horm. Behav.* 65, 505–515. doi: 10.1016/j.yhbeh.2014.03.017

Kato, A., Hojo, Y., Higo, S., Komatsuzaki, Y., Murakami, G., Yoshino, H., et al. (2013). Female hippocampal estrogens have a significant correlation with cyclic fluctuation of hippocampal spines. *Front. Neural Circuits* 7:149. doi: 10.3389/fncir.2013.00149

Kawato, S. (2004). Endocrine disrupters as disrupters of brain function: a neurosteroid viewpoint. *Environ. Sci.* 11, 1–14.

Kimoto, T., Tsurugizawa, T., Ohta, Y., Makino, J., Tamura, H., Hojo, Y., et al. (2001). Neurosteroid synthesis by cytochrome p450-containing systems localized in the rat brain hippocampal neurons: N-methyl-D-aspartate and calcium-dependent synthesis. *Endocrinology* 142, 3578–3589. doi: 10.1210/endo.142.8.8327

Kramar, E. A., Chen, L. Y., Rex, C. S., Gall, C. M., and Lynch, G. (2009). Estrogen's place in the family of synaptic modulators. *Mol. Cell. Pharmacol.* 1, 258–262.

Krentzel, A. A., Barrett, L. R., and Meitzen, J. (2019). Estradiol rapidly modulates excitatory synapse properties in a sex- and region-specific manner in rat nucleus accumbens core and caudate-putamen. *J. Neurophysiol.* 122, 1213–1225. doi: 10.1152/jn.00264.2019

Leranth, C., Hajszan, T., and MacLusky, N. J. (2004). Androgens increase spine synapse density in the CA1 hippocampal subfield of ovariectomized female rats. *J. Neurosci.* 24, 495–499. doi: 10.1523/JNEUROSCI.4516-03.2004

Lisberger, S. G., and Miles, F. A. (1980). Role of primate medial vestibular nucleus in long-term adaptive plasticity of vestibuloocular reflex. *J. Neurophysiol.* 43, 1725–1745. doi: 10.1152/jn.1980.43.6.1725

Lu, Y., Sareddy, G. R., Wang, J., Wang, R., Li, Y., Dong, Y., et al. (2019). Neuron-derived estrogen regulates synaptic plasticity and memory. *J. Neurosci.* 39, 2792–2809. doi: 10.1523/JNEUROSCI.1970-18.2019

Luine, V., and Frankfurt, M. (2020). Estrogenic regulation of memory: the first 50 years. *Horm. Behav.* 121:104711. doi: 10.1016/j.yhbeh.2020.104711

Luine, V. N. (2014). Estradiol and cognitive function: past, present and future. *Horm. Behav.* 66, 602–618. doi: 10.1016/j.yhbeh.2014.08.011

Luine, V. N., and Frankfurt, M. (2012). Estrogens facilitate memory processing through membrane mediated mechanisms and alterations in spine density. *Front. Neuroendocrinol.* 33:388–402. doi: 10.1016/j.yfrne.2012.07.004

Luine, V. N., Jacome, L. F., and Maclusky, N. J. (2003). Rapid enhancement of visual and place memory by estrogens in rats. *Endocrinology* 144, 2836–2844. doi: 10.1210/en.2003-0004

MacLusky, N. J., Luine, V. N., Hajszan, T., and Leranth, C. (2005). The 17alpha and 17beta isomers of estradiol both induce rapid spine synapse formation in the CA1 hippocampal subfield of ovariectomized female rats. *Endocrinology* 146, 287–293. doi: 10.1210/en.2004-0730

Massot, C., Schneider, A. D., Chacron, M. J., and Cullen, K. E. (2012). The vestibular system implements a linear-nonlinear transformation in order to encode self-motion. *PLoS Biol.* 10:e1001365. doi: 10.1371/journal.pbio.1001365

Mensah-Nyagan, A. G., Do-Rego, J. L., Beaujean, D., Luu-The, V., Pelletier, G., and Vaudry, H. (1999). Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. *Pharmacol. Rev.* 51, 63–81.

Moffat, S. D., Zonderman, A. B., Metter, E. J., Blackman, M. R., Harman, S. M., and Resnick, S. M. (2002). Longitudinal assessment of serum free testosterone concentration predicts memory performance and cognitive status in elderly men. *J. Clin. Endocrinol. Metab.* 87, 5001–5007. doi: 10.1210/jc.2002-020419

Mohamad, N. V., Ima-Nirwana, S., and Chin, K. Y. (2018). A review on the effects of testosterone supplementation in hypogonadal men with cognitive impairment. *Curr. Drug Targets* 19, 898–906. doi: 10.2174/1389450118666170913162739

Moradpour, F., Naghdi, N., and Fathollahi, Y. (2006). Anastrozole improved testosterone-induced impairment acquisition of spatial learning and memory in the hippocampal CA1 region in adult male rats. *Behav. Brain Res.* 175, 223–232. doi: 10.1016/j.bbr.2006.08.037

Mukai, H., Takata, N., Ishii, H. T., Tanabe, N., Hojo, Y., Furukawa, A., et al. (2006). Hippocampal synthesis of estrogens and androgens which are paracrine modulators of synaptic plasticity: synaptocrinology. *Neuroscience* 138, 757–764. doi: 10.1016/j.neuroscience.2005.09.010

Mukai, H., Tsurugizawa, T., Murakami, G., Kominami, S., Ishii, H., Ogiue-Ikeda, M., et al. (2007). Rapid modulation of long-term depression and spinogenesis via synaptic estrogen receptors in hippocampal principal neurons. *J. Neurochem.* 100, 950–967. doi: 10.1111/j.1471-4159.2006.04264.x

Mulkey, R. M., Endo, S., Shenolikar, S., and Malenka, R. C. (1994). Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature* 369, 486–488. doi: 10.1038/369486a0

Murakami, G., Hojo, Y., Kato, A., Komatsuzaki, Y., Horie, S., Soma, M., et al. (2018). Rapid nongenomic modulation by neurosteroids of dendritic spines in the hippocampus: androgen, oestrogen and corticosteroid. *J. Neuroendocrinol.* 30:e12561. doi: 10.1111/jne.12561

Murakami, G., Hojo, Y., Ogiue-Ikeda, M., Mukai, H., Chambon, P., Nakajima, K., et al. (2015). Estrogen receptor KO mice study on rapid modulation of spines and long-term depression in the hippocampus. *Brain Res.* 1621, 133–146. doi: 10.1016/j.brainres.2014.12.002

Murphy, D. D., Cole, N. B., Greenberger, V., and Segal, M. (1998). Estradiol increases dendritic spine density by reducing GABA neurotransmission in hippocampal neurons. *J. Neurosci.* 18, 2550–2559.

Murphy, D. D., and Segal, M. (1996). Regulation of dendritic spine density in cultured rat hippocampal neurons by steroid hormones. *J. Neurosci.* 16, 4059–4068.

Ooishi, Y., Kawato, S., Hojo, Y., Hatanaka, Y., Higo, S., Murakami, G., et al. (2012). Modulation of synaptic plasticity in the hippocampus by hippocampus-derived estrogen and androgen. *J. Steroid Biochem. Mol. Biol.* 131, 37–51. doi: 10.1016/j.jsbmb.2011.10.004

Pan, W., Han, S., Kang, L., Li, S., Du, J., and Cui, H. (2016). Effects of dihydrotestosterone on synaptic plasticity of the hippocampus in mild cognitive impairment male SAMP8 mice. *Exp. Ther. Med.* 12, 1455–1463. doi: 10.3892/etm.2016.3470

Pape, H. C., and Pare, D. (2010). Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. *Physiol. Rev.* 90, 419–463. doi: 10.1152/physrev.00037.2009

Pettorossi, V. E., Di Mauro, M., Scarduzio, M., Panichi, R., Tozzi, A., Calabresi, P., et al. (2013a). Modulatory role of androgenic and estrogenic neurosteroids in determining the direction of synaptic plasticity in the CA1 hippocampal region of male rats. *Physiol. Rep.* 1:e00185. doi: 10.1002/phy2.185

Pettorossi, V. E., Frondaroli, A., and Grassi, S. (2011). Cyclic estrogenic fluctuation influences synaptic transmission of the medial vestibular nuclei in female rats. *Acta Otolaryngol.* 131, 434–439. doi: 10.3109/00016489.2010.536992

Pettorossi, V. E., Panichi, R., Botti, F. M., Kyriakareli, A., Ferraresi, A., Faralli, M., et al. (2013b). Prolonged asymmetric vestibular stimulation induces opposite, long-term effects on self-motion perception and ocular responses. *J. Physiol.* 591, 1907–1920. doi: 10.1113/jphysiol.2012.241182

Phan, A., Gabor, C. S., Favaro, K. J., Kaschack, S., Armstrong, J. N., MacLusky, N. J., et al. (2012). Low doses of 17β-estradiol rapidly improve learning and increase hippocampal dendritic spines. *Neuropsychopharmacology* 37, 2299–2309. doi: 10.1038/npp.2012.82

Phan, A., Suschkov, S., Molinaro, L., Reynolds, K., Lymer, J. M., Bailey, C. D., et al. (2015). Rapid increases in immature synapses parallel estrogen-induced hippocampal learning enhancements. *Proc. Natl. Acad. Sci. U.S.A.* 112, 16018–16023. doi: 10.1073/pnas.1522150112

Pike, C. J., Rosario, E. R., and Nguyen, T. V. (2006). Androgens, aging, and Alzheimer's disease. *Endocrine* 29, 233–241.

Rudick, C. N., and Woolley, C. S. (2001). Estrogen regulates functional inhibition of hippocampal CA1 pyramidal cells in the adult female rat. *J. Neurosci.* 21, 6532–6543.

Sakamoto, H., Mezaki, Y., Shikimi, H., Ukena, K., and Tsutsui, K. (2003). Dendritic growth and spine formation in response to estrogen in the developing Purkinje cell. *Endocrinology* 144, 4466–4477. doi: 10.1210/en.2003-0307

Saldanha, C. J., Remage-Healey, L., and Schlinger, B. A. (2011). Synaptocrine signaling: steroid synthesis and action at the synapse. *Endocr. Rev.* 32, 532–549. doi: 10.1210/er.2011-0004

Scarduzio, M., Panichi, R., Pettorossi, V. E., and Grassi, S. (2012). The repetition timing of high frequency afferent stimulation drives the bidirectional plasticity at central synapses in the rat medial vestibular nuclei. *Neuroscience* 223, 1–11. doi: 10.1016/j.neuroscience.2012.07.039

Scarduzio, M., Panichi, R., Pettorossi, V. E., and Grassi, S. (2013). Synaptic long-term potentiation and depression in the rat medial vestibular nuclei depend on neural activation of estrogenic and androgenic signals. *PLoS One* 8:e80792. doi: 10.1371/journal.pone.0080792

Shiroma, S., Yamaguchi, T., and Kometani, K. (2005). Effects of 17beta-estradiol on chemically induced long-term depression. *Neuropharmacology* 49, 97–102. doi: 10.1016/j.neuropharm.2005.02.002

Smith, C. C., and McMahon, L. L. (2005). Estrogen-induced increase in the magnitude of long-term potentiation occurs only when the ratio of NMDA transmission to AMPA transmission is increased. *J. Neurosci.* 25, 7780–7791. doi: 10.1523/JNEUROSCI.0762-05.2005

Smith, C. C., and McMahon, L. L. (2006). Estradiol-induced increase in the magnitude of long-term potentiation is prevented by blocking NR2B-containing receptors. *J. Neurosci.* 26, 8517–8522. doi: 10.1523/JNEUROSCI.5279-05.2006

Smith, P. F., and Curthoys, I. S. (1989). Mechanisms of recovery following unilateral labyrinthectomy: a review. *Brain Res. Brain Res. Rev.* 14, 155–180. doi: 10.1016/0165-0173(89)90013-1

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

Song, Z., Yang, H., Peckham, E. M., and Becker, J. B. (2019). Estradiol-induced potentiation of dopamine release in dorsal striatum following amphetamine administration requires estradiol receptors and mGlu5. *eNeuro* 6:ENEURO.0446-18.2019. doi: 10.1523/ENEURO.0446-18.2019

Srivastava, D. P. (2012). Two-step wiring plasticity–a mechanism for estrogen-induced rewiring of cortical circuits. *J. Steroid Biochem. Mol. Biol.* 131, 17–23. doi: 10.1016/j.jsbmb.2012.01.006

Sumner, R. L., Spriggs, M. J., McMillan, R. L., Sundram, F., and Kirk, I. J. (2018). Muthukumaraswamy SD. Neural plasticity is modified over the human menstrual cycle: combined insight from sensory evoked potential LTP and repetition suppression. *Neurobiol. Learn. Mem.* 155, 422–434. doi: 10.1016/j.nlm.2018.08.016

Tabori, N. E., Stewart, L. S., Znamensky, V., Romeo, R. D., Alves, S. E., McEwen, B. S., et al. (2005). Ultrastructural evidence that androgen receptors are located at extranuclear sites in the rat hippocampal formation. *Neuroscience* 130, 151–163. doi: 10.1016/j.neuroscience.2004.08.048

Tanaka, M., and Sokabe, M. (2012). Continuous de novo synthesis of neurosteroids is required for normal synaptic transmission and plasticity in the dentate gyrus of the rat hippocampus. *Neuropharmacology* 62, 2373–2387. doi: 10.1016/j.neuropharm.2012.02.007

Tanaka, M., and Sokabe, M. (2013). Bidirectional modulatory effect of 17beta-estradiol on NMDA receptors via ERalpha and ERbeta in the dentate gyrus of juvenile male rats. *Neuropharmacology* 75, 262–273. doi: 10.1016/j.neuropharm.2013.07.029

Toni, N., Buchs, P. A., Nikonenko, I., Bron, C. R., and Muller, D. (1999). LTP promotes formation of multiple spine synapses between a single axon terminal and a dendrite. *Nature* 402, 421–425. doi: 10.1038/46574

Tovote, P., Fadok, J. P., and Luthi, A. (2015). Neuronal circuits for fear and anxiety. *Nat. Rev. Neurosci.* 16, 317–331. doi: 10.1038/nrn3945

Tozzi, A., de Iure, A., Tantucci, M., Durante, V., Quiroga-Varela, A., Giampa, C., et al. (2015). Endogenous 17beta-estradiol is required for activity-dependent long-term potentiation in the striatum: interaction with the dopaminergic system. *Front. Cell. Neurosci.* 9:192. doi: 10.3389/fncel.2015.00192

Tozzi, A., Durante, V., Manca, P., Di Mauro, M., Blasi, J., Grassi, S., et al. (2019). Bidirectional synaptic plasticity is driven by sex neurosteroids targeting estrogen and androgen receptors in hippocampal CA1 pyramidal neurons. *Front. Cell. Neurosci.* 13:534. doi: 10.3389/fncel.2019.00534

Tsutsui, K., Ukena, K., Sakamoto, H., Okuyama, S., and Haraguchi, S. (2011). Biosynthesis, mode of action, and functional significance of neurosteroids in the purkinje cell. *Front. Endocrinol.* 2:61. doi: 10.3389/fendo.2011.00061

Tuscher, J. J., Szinte, J. S., Starrett, J. R., Krentzel, A. A., Fortress, A. M., Remage-Healey, L., et al. (2016). Inhibition of local estrogen synthesis in the hippocampus impairs hippocampal memory consolidation in ovariectomized female mice. *Horm. Behav.* 83, 60–67. doi: 10.1016/j.yhbeh.2016.05.001

Vierk, R., Glassmeier, G., Zhou, L., Brandt, N., Fester, L., Dudzinski, D., et al. (2012). Aromatase inhibition abolishes LTP generation in female but not in male mice. *J. Neurosci.* 32, 8116–8126. doi: 10.1523/JNEUROSCI.5319-11.2012

Wagner, B. A., Braddick, V. C., Batson, C. G., Cullen, B. H., Miller, L. E., and Spritzer, M. D. (2018). Effects of testosterone dose on spatial memory among castrated adult male rats. *Psychoneuroendocrinology* 89, 120–130. doi: 10.1016/j.psyneuen.2017.12.025

Warren, S. G., Humphreys, A. G., Juraska, J. M., and Greenough, W. T. (1995). LTP varies across the estrous cycle: enhanced synaptic plasticity in proestrus rats. *Brain Res.* 703, 26–30. doi: 10.1016/0006-8993(95)01059-9

Weiland, N. G. (1992). Estradiol selectively regulates agonist binding sites on the N-methyl-D-aspartate receptor complex in the CA1 region of the hippocampus. *Endocrinology* 131, 662–668. doi: 10.1210/endo.131.2.1353442

Wong, M., and Moss, R. L. (1991). Electrophysiological evidence for a rapid membrane action of the gonadal steroid, 17 beta-estradiol, on CA1 pyramidal neurons of the rat hippocampus. *Brain Res.* 543, 148–152. doi: 10.1016/0006-8993(91)91057-8

Wong, M., and Moss, R. L. (1992). Long-term and short-term electrophysiological effects of estrogen on the synaptic properties of hippocampal CA1 neurons. *J. Neurosci.* 12, 3217–3225.

Woolley, C. S. (1998). Estrogen-mediated structural and functional synaptic plasticity in the female rat hippocampus. *Horm. Behav.* 34, 140–148. doi: 10.1006/hbeh.1998.1466

Woolley, C. S., Gould, E., Frankfurt, M., and McEwen, B. S. (1990). Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons. *J. Neurosci.* 10, 4035–4039.

Woolley, C. S., and McEwen, B. S. (1993). Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. *J. Comp. Neurol.* 336, 293–306. doi: 10.1002/cne.903360210

Woolley, C. S., Weiland, N. G., McEwen, B. S., and Schwartzkroin, P. A. (1997). Estradiol increases the sensitivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input: correlation with dendritic spine density. *J. Neurosci.* 17, 1848–1859.

Woolley, C. S., Wenzel, H. J., and Schwartzkroin, P. A. (1996). Estradiol increases the frequency of multiple synapse boutons in the hippocampal CA1 region of the adult female rat. *J. Comp. Neurol.* 373, 108–117.

Yang, Y., Wang, X. B., Frerking, M., and Zhou, Q. (2008). Spine expansion and stabilization associated with long-term potentiation. *J. Neurosci.* 28, 5740–5751. doi: 10.1523/JNEUROSCI.3998-07.2008

Zhao, C., Fujinaga, R., Tanaka, M., Yanai, A., Nakahama, K., and Shinoda, K. (2007). Region-specific expression and sex-steroidal regulation on aromatase and its mRNA in the male rat brain: immunohistochemical and in situ hybridization analyses. *J. Comp. Neurol.* 500, 557–573. doi: 10.1002/cne.21193

Zhao, L., Chen, S., Ming Wang, J., and Brinton, R. D. (2005). 17beta-estradiol induces Ca2+ influx, dendritic and nuclear Ca2+ rise and subsequent cyclic AMP response element-binding protein activation in hippocampal neurons: a potential initiation mechanism for estrogen neurotrophism. *Neuroscience* 132, 299–311. doi: 10.1016/j.neuroscience.2004.11.054

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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 11 January 2021
doi: 10.3389/fendo.2020.552805

Progesterone's Effects on Cognitive Performance of Male Mice Are Independent of Progestin Receptors but Relate to Increases in $GABA_A$ Activity in the Hippocampus and Cortex

Cheryl A. Frye[1,2,3,4,5,6,7,8]*, *Vincent F. Lembo*[8] *and Alicia A. Walf*[1,5,7,9]

[1] Department of Psychology, The University at Albany-SUNY, Life Sciences, Albany, NY, United States, [2] Department of Biological Sciences, The University at Albany-SUNY, Life Sciences, Albany, NY, United States, [3] The Center for Neuroscience Research, The University at Albany-SUNY, Life Sciences, Albany, NY, United States, [4] The Center for Life Sciences Research, The University at Albany-SUNY, Life Sciences, Albany, NY, United States, [5] Institute of Arctic Biology, University of Alaska–Fairbanks, Fairbanks, AK, United States, [6] Department of Chemistry, University of Alaska–Fairbanks, Fairbanks, AK, United States, [7] IDeA Network of Biomedical Excellence (INBRE), University of Alaska–Fairbanks, Fairbanks, AK, United States, [8] Comprehensive Neuropsychological Services, Albany, NY, United States, [9] Department of Cognitive Science, Rensselaer Polytechnic Institute, Troy, NY, United States

OPEN ACCESS

Edited by:
Hubert Vaudry,
Université de Rouen, France

Reviewed by:
Xavier Xifró,
University of Girona, Spain
Valerio Magnaghi,
University of Milan, Italy

***Correspondence:**
Cheryl A. Frye
cheryl.a.frye@gmail.com

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

Received: 17 April 2020
Accepted: 02 September 2020
Published: 11 January 2021

Citation:
Frye CA, Lembo VF and Walf AA (2021) Progesterone's Effects on Cognitive Performance of Male Mice Are Independent of Progestin Receptors but Relate to Increases in $GABA_A$ Activity in the Hippocampus and Cortex.
Front. Endocrinol. 11:552805.
doi: 10.3389/fendo.2020.552805

Progestogens' (e.g., progesterone and its neuroactive metabolite, allopregnanolone), cognitive effects and mechanisms among males are not well-understood. We hypothesized if progestogen's effects on cognitive performance are through its metabolite allopregnanolone, and not actions via binding to traditional progestin receptors (PRs), then progesterone administration would enhance performance in tasks mediated by the hippocampus and cortex, coincident with increasing allopregnanolone concentrations, brain derived neurotrophic factor (BDNF) and/or muscimol binding of PR knock out (PRKO) and wild-type PR replete mice. **Experiment 1**: Progesterone (4 mg/kg, subcutaneously (SC; $n = 12$/grp), or oil vehicle control, was administered to gonadally-intact adult male mice PRKO mice and their wild-type counterparts and cognitive behaviors in object recognition, T-maze and water maze was examined. Progesterone, compared to vehicle, when administered post-training increased time investigating novel objects by the PRKO and wild-type mice in the object recognition task. In the T-maze task, progesterone administration to wild-type and PRKO mice had significantly greater number of spontaneous alternations compared to their vehicle-administered counterparts. In the water maze task, PRKO mice administered vehicle spent significantly fewer seconds in the quadrant associated with the escape platform on testing compared to all other groups. **Experiment 2**: Progesterone administered to wild-type and PRKO mice increased plasma progesterone and allopregnanolone levels ($n = 5$/group). PRKO mice had higher allopregnanolone levels in plasma and hippocampus, but not cortex, when administered progesterone and

compared to wild-type mice. **Experiment 3**: Assessment of PR binding revealed progesterone administered wild-type mice had significantly greater levels of PRs in the hippocampus and cortex, compared to all other groups ($n = 5$/group). Wild-type mice administered progesterone, but not vehicle, had increased BDNF levels in the hippocampus, but not the cortex, compared to PRKOs. Wild-type as well as PRKO mice administered progesterone experienced significant increases in maximal $GABA_A$ agonist, muscimol, binding in hippocampus and cortex, compared to their vehicle-administered counterparts. Thus, adult male mice can be responsive to progesterone for cognitive performance, and such effects may be independent of PRs trophic actions of BDNF levels in the hippocampus and/or increases in $GABA_A$ activity in the hippocampus and cortex.

Keywords: brain-derived neurotrophic factor, prefrontal cortex, allopregnanolone, hippocampus, object recognition, T-maze, memory

INTRODUCTION

Our understanding of progesterone, a gonadal hormone that is produced primarily by the ovaries in females, as well as progestin receptors (PRs) functioning, has primarily come from studies in females (1–3). Although progesterone has always been considered a "female-typical hormone," adult male rodents produce progesterone in the testes and adrenal cortex (4, 5). Male rodents have circulating levels of progesterone around 1.5–2 ng/mL (6, 7), compared to a range of 3–35 ng/mL in females that is seen throughout the estrous cycle (8). Males, compared to females, have higher levels of steroid receptor co-activators, which enhance steroid hormone action in many brain regions (9). Of note, across species, both males and females have early exposure to maternal progesterone, by which brain functioning is organized. "Male-typical" hormones, such as androgens are derived from a cholesterol-based pro-hormone, progesterone. Thus, despite conceptualization of progesterone as a female hormone, the extent to which adult males respond to progesterone is an important question.

Progesterone can exert beneficial effects for cognitive performance; however, most of the work on progesterone's cognitive effects has involved female subjects. Compared to other treatments, progesterone to rodents assessed in the Morris water maze reduced latencies to the hidden platform, increased platform crossings, and time spent swimming in the quadrant area where the platform had been during training (10). Progesterone improved reference memory acquisition and reversal learning in the Morris water maze task, compared with vehicle treatment (11). In addition, young and aged rodents administered progesterone, or its neuroactive metabolite, allopregnanolone, performed significantly better in the object recognition, object placement, T-maze, and water maze tasks compared to other groups (12, 13). Progesterone can have memory-enhancing effects among young adult mice in condition place preference, inhibitory avoidance and other tasks that may be mediated by several brain regions, including the hippocampus, prefrontal cortex (PFC), amygdala, nucleus accumbens, and cerebellum (14, 15). Thus, progesterone has beneficial effects to improve cognitive performance of female rodents across a variety of tasks. The question remains about the responsiveness of males to progesterone on cognitive performance.

To understand the role and brain targets of progestogens for cognitive performance among males, different mechanisms of actions of progesterone and its metabolic allopregnanolone should be considered. Unlike allopregnanolone, progesterone binds with high affinity to intracellular PRs (16). Progestin receptors have been localized to brain targets for learning/memory effects of progesterone in the hippocampus (17) and the frontal cortex (18). However, progesterone may be exerting its effects through its metabolite, allopregnanolone, which has greater affinity for γ-aminobutyric acid ($GABA_A$) receptors. Allopregnanolone alters functioning of many neurotransmitter targets, rather than binding to PRs, when in physiological concentrations (19–21). Female mice, administered allopregnanolone or those that were administered progesterone and could metabolize this to allopregnanolone, performed significantly better in the object recognition, object placement, T-maze and water maze tasks compared to female mice administered vehicle (12). In addition, rodents administered allopregnanolone, perform better in the water maze, a delayed nonmatching-to-sample Y-maze task, object recognition and object placement tasks, and conditioned aversion tasks and have enhanced conditioned place preference compared to controls (22–27). Moreover, PR knockout (PRKO) mice, which lack PRs throughout development (28), have been used. For example, young and/or aged PRKO and wild-type mice have increased sexual responding, decreased anxiety-like behavior, and enhanced cognition following progesterone administration, despite PRKO mice having low levels of cortical PR binding (29, 30). Cognitive enhancement among both PRKO and wild-type mice administered progesterone suggests that PRs are not necessary for progesterone's beneficial effects on cognitive performance. Thus, progesterone's beneficial effects across various cognitive tasks may be related to the capacity to produce allopregnanolone, rather than actions at PRs among females. Of interest is the effects among males.

Another non-PR target to be considered is brain-derived neurotrophic factor (BDNF). BDNF is produced both in neurons and glial cells (31, 32). BDNF is of interest as a marker of

neural plasticity, which may play a role in synaptic plasticity and learning/memory (33, 34). There is strong evidence to support the role of BDNF in synaptic plasticity and cognitive function (35) and as such, alterations in its function and/or expression have been implicated in the pathophysiology of aged-related neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, seizure disorder, major depression (34, 36–45), and a variety of stressors/events [e.g., ischemia, hypoglycemia, stressor exposure, etc. (46, 47)]. Restoring BDNF expression and/or function may be therapeutic. Furthermore, there is evidence that progesterone and other hormones have enhancing effects on BDNF expression. BDNF levels are increased and are associated with administration of progesterone and/or cognitive enhancement (12, 33, 34, 48–53). Thus, BDNF may play a role for cognitive enhancement following progesterone administration.

An important question is the responsiveness of progesterone in a mouse model that should be less sensitive to progesterone effects (adult males, low levels of progesterone, and no PRs). Notably, young males in some cases do not respond to progesterone as females do. For example, duration spent immobile in the forced swim task is not reduced with progesterone administration in young males to the same extent that it is in age-matched females; this sex difference is no longer apparent in aged mice (54). We hypothesized if progesterone's effects on learning/memory are through its metabolite allopregnanolone, and not due to traditional actions via binding to PRs, progesterone administration post-training will enhance performance in tasks mediated by the hippocampus and cortex, coincident with increasing allopregnanolone concentrations in the hippocampus and cortex, and increase BDNF levels or activity of $GABA_A$ receptors of both PRKO and wildtype mice. To test this, gonadally-intact male wild-type and PRKO mice were administered progesterone and/or oil vehicle and exp 1: cognitive behaviors (object recognition, T-maze and Water maze), exp 2: neuroendocrine factors (plasma, hippocampal and cortical progesterone and allopregnanolone levels), and trophic factors, PR binding, BDNF levels in the hippocampus and cortex, and $GABA_A$ activity in the hippocampus and cortex were assessed.

METHODS AND MATERIALS

The methods utilized for animal husbandry, determination of WT vs. PRKOs, drug administration, behavioral testing, euthanasia and tissue collection in the murine subjects in this study were approved by the Institutional Animal Care and Use Committee at the University at Albany.

Animal Housing

Subjects were adult (8–10 weeks old), male mice. Mice were group-housed (4–5 per cage) in polycarbonate cages (26 × 16 × 12 cm) in a temperature-controlled room (21 ± 1°C) in the core Laboratory Animal Care Facility at the University at Albany. The housing room was on a 12/12-h reversed light cycle (lights off 8:00 a.m.-8:00 p.m.). Mice had continuous access to Purina Mouse Chow and tap water in their home cages and were assessed during their dark phase. There were 50/12-13 mice group in one cohort and 20/5 mice group in another cohort. The first cohort of 5 per group was done to examine physiological measure around 15 generations of back crossings to bring the PRKO mice from their 129 background strain onto the c57UA strain, which were c57 mice that had been subjected to random and frequent fire alarms with changes in air pressure for 4 years.

Mouse Strain and Genotyping

PRKO mice that were back crossed onto a c57 background are not distinguishable based upon any obvious phenotypic or behavioral characteristics from c57 controls. As such, another member of the laboratory conducted genotyping, as described below, and randomly assigned them to groups, which were unknown to the individuals that were testing the animals. Subjects were wild-type (+/+) or (-/-) PRKO mice, congenic on C57BL/6 background, that were derived from heterozygous (+/–) breeder pairs from a colony that was maintained at the University at Albany. These mice were developed by Bert O'Malley's laboratory [Baylor College of Medicine, Houston, Texas; (29, 55)]. Typical polymerase chain reaction (PCR) methods, modified from Jackson Laboratory's published protocol, were utilized to determine the genotype of mice (54, 56). Briefly, genomic DNA was isolated from tail snips and analyzed by PCR. PCR was performed by denaturing the DNA at 95°C for 5 min, followed by 30 cycles of amplification: 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final primer extension step at 72°C for 10 min. The following PR specific primers were used: P1 (5′TAGACAGTGTCTTAGACTCGTTGTTG-3′), P2 (5′GATGGGCACATGGATGAAATC-3′), and a neo gene specific primer, N2 (5′GCATGCTCCAGACTGCCTTGGGAAA-3′). Primers were obtained from Integrated DNA Technologies (Coralville, IA). Bands of ∼565 and 500 base pairs were amplified for wild-type and PRKO mice, respectively. PRKO and wild-type mice were randomly assigned to receive progesterone or vehicle as described below. The individual who was testing the animals was blind to the genotype of all animals and the vehicle or progesterone administration condition.

Progesterone Administration

Crystalline progesterone was obtained from Steraloids, Newport, RI and dissolved in vegetable oil vehicle. Intact male mice were randomly assigned to receive progesterone (4 mg/kg) or vehicle (vegetable oil) by subcutaneous injection (SC) in the nape of the neck 1 h before behavioral testing in the T-maze, immediately after training in the single trial of the object recognition task, and after the last training trial in the water maze (57).

Behavioral Testing

Wild-type and PRKO mice were assigned to one hormone condition (vehicle or progesterone) and then, tested once per week in each of the behavioral tasks described below. Behavioral data were collected simultaneously by an experimenter (T-maze), the Any-Maze tracking system (Stoelting, Wood Dale, IL; object recognition), and/or both methods (water maze). On the day when mice were trained and tested, they were transported in

their home-cages on a cart to the testing area. Mice were singly-housed in a clean cage immediately before training and until the last testing trial, when they were returned to their home-cage in the vivarium.

Object Recognition

In the object recognition task, mice were trained with two identical objects, i.e., a plastic toy block or a bottle, that were placed in an open field. The objects used were those that mice showed a high and similar degree of investigating during a single, 3 min training trial (58). There were no significant differences between genotypes or treatment group for time spent investigating objects during training in the object recognition task [left object: WT: 12.1 ± 2.2 s (SEM), PRKO: 10.2 ±1.4 s (SEM); right object: WT: 11.1 ± 1.9 s (SEM), PRKO: 11.9 ±2.3 s (SEM); training data are from mice that were trained before receiving treatment]. The durations spent within 5 cm of the object, directly in contact, investigating and/or orienting toward the objects were automatically recorded using Any-Maze for the training and testing trials. Immediately after training, mice were injected with vehicle and/or progesterone. Mice were tested in this task 4 h after training. During testing, there was a novel object and a familiar object (i.e., the object that mice had been trained with) and mice freely explored in the testing chamber for 3 min. The duration of time the mice spent exploring the familiar and novel objects were recorded.

T-maze

Spontaneous alternation was assessed in the T-maze, which has a clear Plexiglas start box connected to a start arm (30.5 × 9 × 7 cm) and two goal arms (17.8 × 9 × 7 cm). Mice were placed in the start box 1 h after vehicle or progesterone treatment. The door was opened and following one forced trial (where either the left or right side was blocked in a random fashion), the number of spontaneous alternations made to each goal arm was assessed for 13 consecutive trials (max latency = 900 s). Each of the 13 trials consisted of the mouse fully returning to the start arm and then, entering the right or left goal arm (13, 59). Data were hand-collected by an experimenter and videos of trials were recorded using Any-Maze or a video-camera. The index of performance in this task is the number of successful alternating trials out of 13 possible trials.

Water Maze

The water maze was filled with 25°C tap water and was made opaque by the addition of white non-toxic tempera paint. Mice were habituated to the maze by allowing them to swim in the water maze with the hidden platform (8 × 8 cm) in it. After 1 min, mice were placed on the hidden platform for 10 s. Following habituation, mice were trained in twelve 1-min trials which were organized into 3 blocks of 4 trials with a randomized starting position in the maze represented during each of these 4 trials in the block. There were 3 different starting positions in the maze. In each trial, mice had 60 s to find the clear platform in the opaque water (hidden platform). Latencies to find the platform and distance traveled were recorded simultaneously by the experimenter and the Any-Maze tracking program. Each block of trials had a 30 min inter-trial interval. Mice were injected with vehicle or progesterone immediately after the last training trial. Before the probe trial to assess spatial memory, the hidden platform was removed from the water maze. Thirty minutes following vehicle or progesterone administration, mice were returned to the water maze at a random position. The latency to return to the quadrant that had the platform, and the duration of time spent in that quadrant, were the indices of cognitive performance in this task. Immediately after the probe trial, mice were tested in a cued trial of the water maze to assess their ability to swim to a platform in the maze. During this trial, the latency of mice to swim to a platform that is made visible and cued is determined to rule out the ability to perform the task (13, 30). There were no differences between groups in these measures (data not shown).

Tissue Collection

Immediately after testing in the water maze, mice were euthanized by cervical subluxation and decapitation. Whole brains were collected from mice and stored frozen at −70°C until brain regions were processed for enzyme-linked immunosorbent assays (ELISA), progesterone, allopregnanolone, BDNF, PR binding and muscimol binding. The cortex and hippocampus were grossly dissected from the whole brain on ice prior to steroid and BDNF measurement.

Sample Preparation

The cortex and hippocampus were dissected out and homogenized with a pestle in 500 microliters of distilled water in a microcentrifuge tube and centrifuged for 10 min at 3,000 x g. Protein concentrations in each sample were measured using a Nanodrop Spectrometer (Thermo Scientific, Federal Way, WA).

Allopregnanolone, Progesterone and BDNF ELISAs

Analyses of allopregnanolone and progesterone were per standard methods of the ELISA kits purchased from Arbor Assays (Ann Arbor, MI). Fifty microliters of homogenized sample were added to each well.

Analyses of BDNF were per standard methods of the Emax Immunoassay system [Promega, Fisher Scientifics; (12, 60, 61)]. Brain homogenates were homogenized in 10 microliters of cell lysis buffer (Qiagen) with a pestle in a microcentifuge tube. Fifty microliters of these prepared homogenates were diluted in 4 volumes of Dulbecco's Phosphate-Buffered Saline (Fisher Scientific). Diluted samples were acid-treated by adding 1 microliter of 1 N HCl, incubating for 15 min at room temperature, and then neutralizing the samples by adding 1 microliter of 1 N NaOH.

For allopregnanolone, progesterone and BDNF ELISA, 50 microliters of prepared Detection Reagent A was immediately pipetted into wells. Plates were shaken, mixed and incubated for 1 h at 37°C. Samples were then aspirated and washed 3 times with 350 microliters of 1x wash buffer. Any remaining liquid from all wells was removed completely by snapping the plate onto absorbent paper. Next, 100 microliters of prepared

Detection Reagent B was pipetted into each well and incubated for 30 min at 37°C. Plate was then aspirated and washed 5 times with 350 microliters of 1x wash buffer and any remaining liquid was removed from wells by snapping the plate onto absorbent paper. Then, 90 microliters of substrate solution was pipetted into each well and incubated for 15–25 min at 37°C and placed in the dark where the liquid turned blue. Lastly, 50 microliters of stop solution was pipetted into each well and gently tapped. The addition of the stop solution turned the liquid yellow. Immediately after, the plate was read at 450 nm on a microplate reader (Bio-tek, Thermo Scientific, Federal Way, WA).

Progestin Receptor Binding

Progestin receptors in hippocampus and cortex were investigated in $n = 5$ mice per group to confirm that backcrossing PRKOs to make them congenic on our c57UA strain did not alter their brain levels of progestin receptors. We used the tritiated synthetic competitive binding assay as previously described (54, 55) with RU5020 (promegestrone; 17α, 21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione), which has a Kd of 0.4 nM for progestin receptors (62). Progestin receptors are found in pituitary, reproductive tract and most estrogen receptor-containing brain regions (62). There are also progestin receptor sites in brain areas lacking estrogen receptors, such as the cerebral cortex, which are similar to those induced by estradiol.

Muscimol Binding

As per our previous methods (63), tissues were thawed and resuspended in 0.05 M Tris-HC1 buffer to a protein concentration of 0.8 mg protein per tube in a final volume of 0.5 ml. [^{3}H] muscimol (NET 574, spec. act. 14.72 Ci/mmol, New England Nuclear, Boston, MA, USA) at 10–100 nM concentrations was added and incubation was continued at 4°C for 30 min. Non-specific binding was determined by addition of 1 mM cold GABA as a displacer of bound [3H] muscimol. The bound and free fractions were separated by vacuum filtration through GF/C glass filters and washed twice very quickly with 0.05 M Tris buffer [89]. Filters were placed in scintillation vials to dry overnight. The next day, scintillation cocktail was added and the vials were counted for radioactivity and degradations per minute were calculated. The E_{max} of specific muscimol binding was used as the variable of interest.

Statistical Analyses

For all measures except the T-maze task, two-way ANOVAs were used to determine effects of hormone condition (vehicle or progesterone) and genotype (wild-type or PRKO). Two subjects were removed from the main cohort, as they varied more than 2 standard deviations from the mean in their group. One subject was from the wild-type progesterone group and the other was from the wild-type prko group. This resulted in an equal number of observations across groups ($n = 12$/group) and no question as to the adherence of the assumptions of the premises of the ANOVAs. A X^2-square test of independence with Yates correction was performed to examine the relationship between progesterone and genotype and the ability to alternate in the T maze. The α level for statistical significance was $p \leq 0.05$. Fisher's *post-hoc* tests were used to examine group differences.

RESULTS

Progesterone to PRKO and Wild-Type Male Mice Enhances Object Recognition

Progesterone administered mice spent significantly more time with the novel object during testing in the object recognition task. Wild-type and PRKO mice administered progesterone spent more time spent with the novel object compared to their vehicle administered counterparts [($F_{(1,44)} = 22.88$, $P < 0.001$) see **Figure 1**, top]. There were neither effects of genotype, nor significant interactions of genotype and progesterone, for time spent with the novel object during testing.

Progesterone to PRKO and Wild-Type Male Mice Results in More Spontaneous Alternation in the T-maze Task

A chi-square test of independence was performed to examine the relationship between progesterone and genotype and the ability to alternate in the T maze. The relationship between these variables was significant, according to X^2 with Yates correction (4, $N = 48$) = 8.9, $p = 0.02$. Mice, irrespective of wild type or PRKO, administered progesterone made significantly greater number of alternations in the T-maze than did vehicle-administered mice. Indeed, progesterone administered mice made approximately twice the number of spontaneous alternations than did their vehicle-administered counterparts. See **Figure 1**, middle.

Progesterone to PRKO and Wild-Type Male Mice in the Water Maze Task

Hormone condition and genotype interacted, such that PRKO, vehicle-administered mice had longer latencies to find the quadrant where the platform had been, compared to all other groups [$F_{(3,44)} = 22.87$, $P < 0.0001$]. Progesterone and genotype also interacted in that PRKO, vehicle-administered mice, spent less time in the quandrant where the platform had been located compared to all other groups [$F_{(3,44)} = 13.56$, $P < 0.0001$] See **Figure 1**, bottom. There were no significant differences for the time spent in the quadrant of the hidden platform during the probe trial and the latency to find the platform in the cued trial indicating initial performance variables were not a factor (data not shown).

Progesterone Increased Plasma Progestogens in PRKO >Wild-Type Male Mice

Progesterone condition and genotype significantly interacted to influence circulating plasma progesterone [$F_{(1,16)} = 69.77$, $P < 0.0001$] and allopregnanolone [$F_{(1,16)} = 28.59$, $P < 0.0001$]. PRKO mice administered progesterone had significantly higher circulating progesterone (see **Figure 2**, top left) and allopregnanolone (see **Figure 2**, top right) levels compared to all other groups.

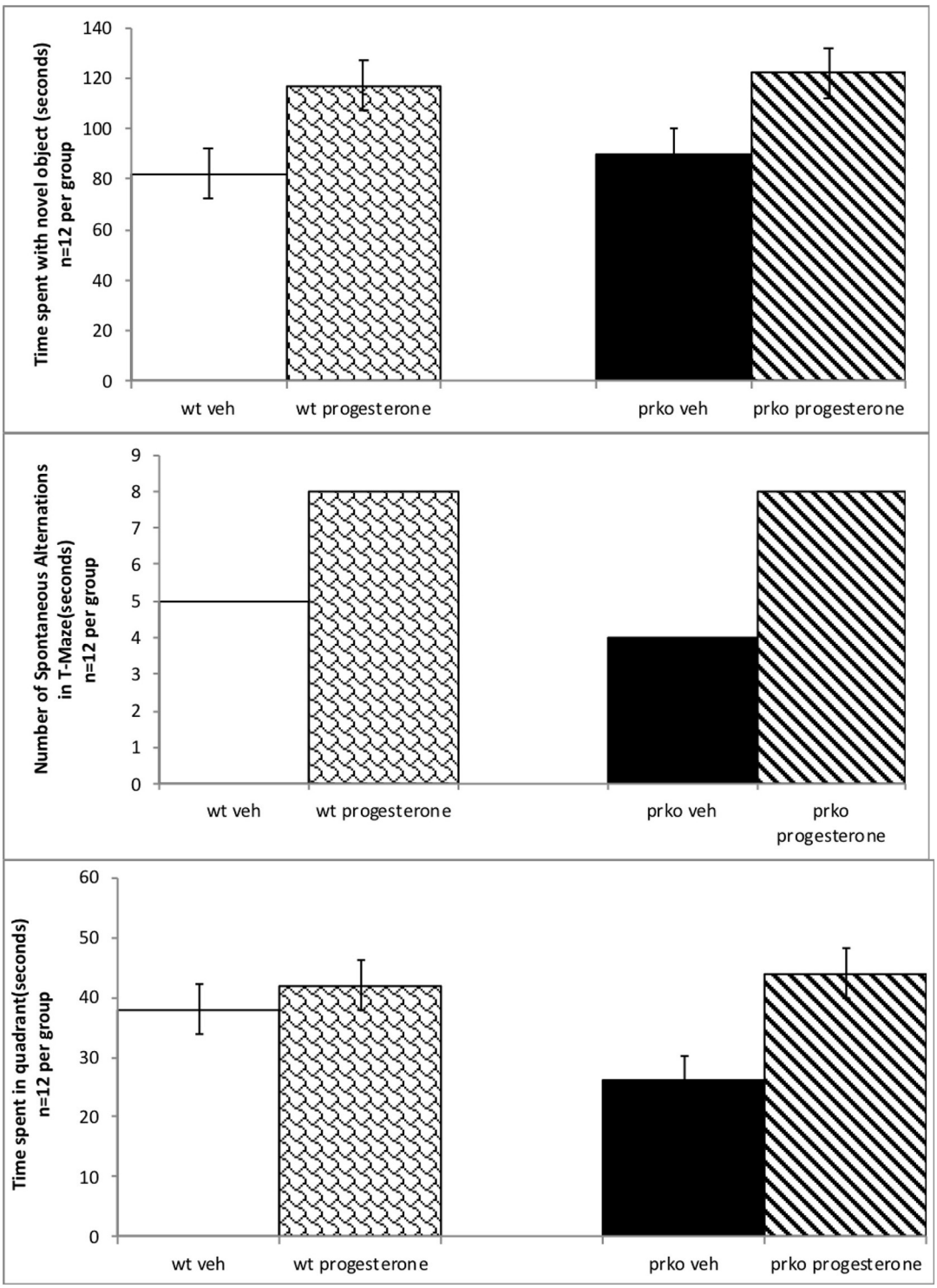

FIGURE 1 | Results from the vehicle wildtype mice are represented by white bars, P_4 wildtype by stippled bars, vehicle PRKO by black bars, and P_4 PRKO mice are represented by diagonally striped bars. There are 12 animals per experimental group. The **top** panel represents mean time (in secs) spent with novel object (±S.E.M.). Wildtype or PRKO male mice administered P_4 had enhanced cognitive performance in the object recognition task compared to vehicle administration among wild-type and PRKO mice. The **middle** panel indicates the mean number of spontaneous alternations (±S.E.M.) in the T-maze out of 13 trials. Wildtype or PRKO male mice administered P_4 had ac greater number of spontaneous alterations in the T-maze compared to vehicle administered groups. The **bottom** panel represents the mean number of seconds spent in the quadrant (±S.E.M.) where the hidden platform had been in previous trials. PRKO vehicle mice spent significantly less time in the quadrant than did all other groups.

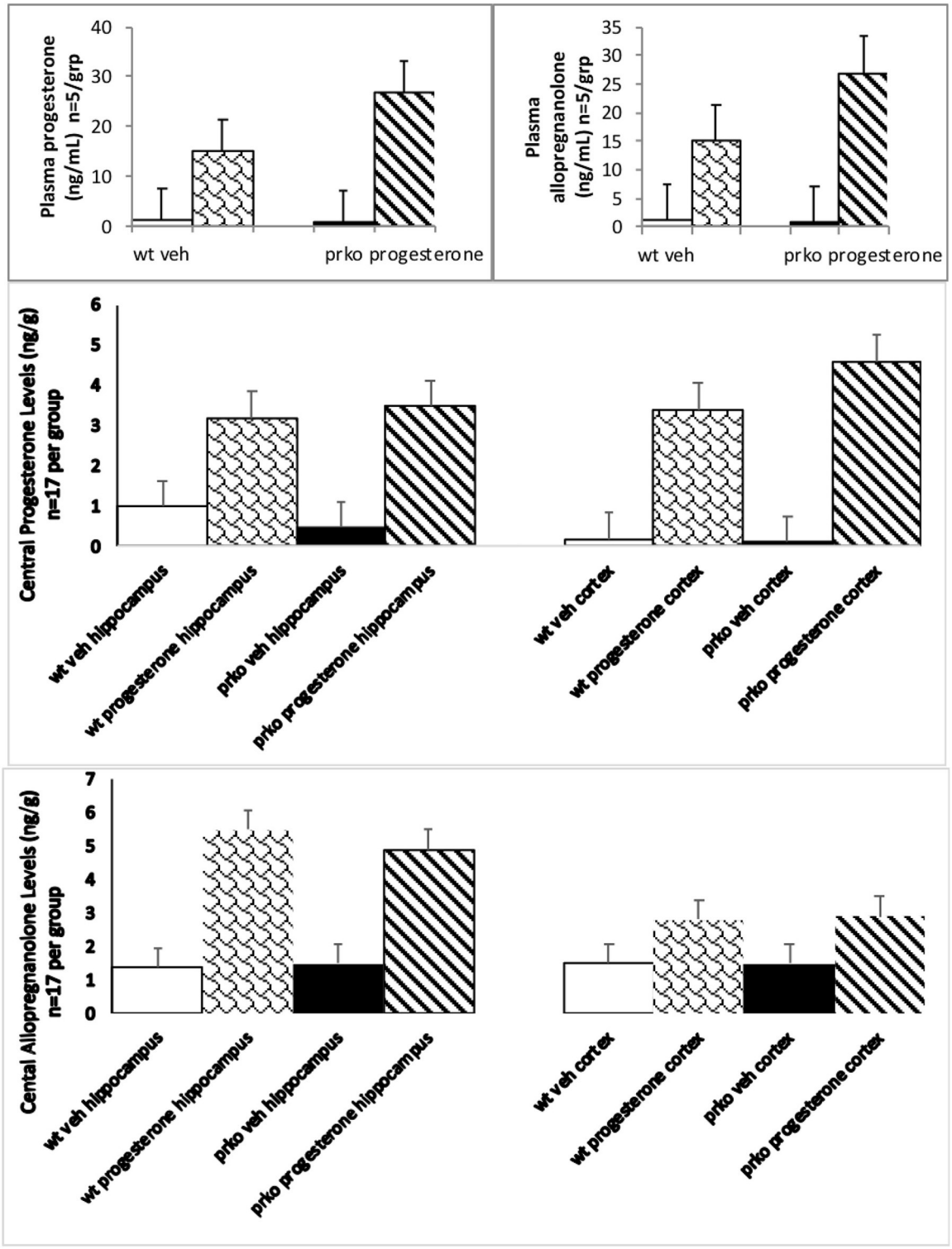

FIGURE 2 | The results from the vehicle wildtype mice are represented by white bars, P_4 wildtype by stippled bars, vehicle PRKO by black bars, and P_4 PRKO mice are represented by diagonally striped bars. The **top left** panel represents plasma progesterone levels ($n = 5$/group) whereas the **top right** panel represents plasma allopregnanolone levels ($n = 5$/group). Progesterone administration significantly increased progesterone and allopregnanolone levels for PRKO > wild-type male mice. The **middle** panel represents central levels of progesterone in the hippocampus left side and cortex right side ($n = 17$/ this and all other brain groups). The **bottom** panel represents central levels of allopregnanolone in the hippocampus left side and cortex right side ($n = 17$/group). Progesterone administration significantly increased hippocampal and cortical progesterone and allopregnanolone levels for both wild-type and PRKO male mice.

Progesterone and Genotype Interacted With Progestogens in Cortex Not Hippocampus

Progesterone [$F_{(1,64)}$ =405.86, $P < 0.01$] and genotype [$F_{(1,64)}$ =5.27, $P < 0.05$] conditions significantly influenced hippocampal levels of progesterone. Progesterone administration produced hippocampal levels of progesterone around 3.25 ng/g compared to vehicle 0.58 ng/g. PRKOs had mean levels of progesterone around 2.1 ng/g compared to wild-types 1.8 ng/g (see **Figure 2**, middle right).

Progesterone condition and genotype significantly interacted to influence cortical progesterone levels [$F_{(1,64)} = 4.80$, $P < 0.03$]. PRKO mice had significantly higher levels of progesterone in the cortex (see **Figure 2**, middle left) compared to all other groups.

Progesterone Has Effects Irrespective of Genotype to Alter Central Allopregnanolone

Progesterone [$F_{(1,64)} = 202.10$, $P < 0.0001$] condition significantly influenced hippocampal levels of allopregnanolone. Progesterone administration produced hippocampal levels of allopregnanolone around 5.2 ng/g compared to vehicle 1.4 ng/g (see **Figure 2**, bottom left).

Progesterone [$F_{(1,64)}$ =80.67, $P < 0.0001$] condition significantly influenced cortical levels of allopregnanolone. Progesterone administration produced cortical levels of allopregnanolone around 2.6 ng/g compared to vehicle 1.3 ng/g (see **Figure 2**, bottom right).

Progesterone Increased Progestin Receptor Binding Sites in Hippocampus and Cortex Cortex of Wild-Type but Not PRKO Male Mice

Progesterone condition significantly interacted with genotype to influence [^{3}H] RU5020 Emax moles/g in the hippocampus [$F_{(1,16)}$ =12.14, $P < 0.001$]. Progesterone administration produced increased PR binding to ∼3.5 moles/g compared to all other groups, which averaged 1.7 (see **Figure 3**, top left).

Progesterone condition significantly interacted with genotype to influence [^{3}H] RU5020 Emax N/g in the cortex [$F_{(1,16)}$ =252.11, $P < 0.0001$]. Progesterone administration increased PR binding to ∼4.3 moles/g among wild-types compared to all other groups, which averaged 2.2 (see **Figure 3**, top right).

Progesterone Increased BDNF Levels in Hippocampus but Not Cortex of PRKO and Wild-Type Male Mice

There was a significant main effect of progesterone to increase BDNF levels in the hippocampus [$F_{(1,46)}$ =4.70, $P < 0.008$] irrespective of genotype (see **Figure 3**, middle left).

Progesterone, compared to vehicle administration, increased BDNF levels in the hippocampus. There was neither an effect of genotype, nor an interaction between genotype and hormone condition. These effects were not observed in the cortex (see **Figure 3**, middle right).

Progesterone Increased E max Muscimol Binding in Cortex of PRKO and Wild-Type Male Mice

Progesterone administration significantly enhanced maximal ^{3}H muscimol binding in the hippocampus irrespective of genotype [$F_{(1,16)}$ =2,030.41, $P = 0.0001$]. See **Figure 3**, left. There was no difference between muscimol binding of wild-type and PRKO mice.

Progesterone administration significantly enhanced maximal ^{3}H muscimol binding in the cortex irrespective of genotype [$F_{(1,16)}$ =2,202.02, $P = 0.0001$]. See **Figure 3**, right. There was no difference between muscimol binding of wild-type and PRKO mice.

DISCUSSION

This study generally supports our a priori hypothesis that progesterone, compared to vehicle, to male PRKO and wild-type mice could improve cognitive performance. In the water maze task, wild-type mice tended to outperform PRKO mice, where wild-type mice had shorter latencies to find the hidden platform compared to PRKO mice. However, in the object recognition and T-maze tasks, progesterone improved performance in both the PRKO and wild-type mice. Progesterone administered to wild-type and PRKO mice higher progesterone levels in the hippocampus and cortex. On the contrary, PRKO mice had higher allopregnanolone in the hippocampus and cortex compared to wild-type mice. Moreover, wild-type, but not PRKO, mice had higher BDNF levels in the hippocampus with progesterone administration compared to vehicle administration. No differences were seen in the cortex for progesterone to increase BDNF levels. Yet, muscimol binding in cortex was similarly increased in wt and PRKO mice administered progesterone. Thus, progesterone's actions in wild-type and PRKO male mice to enhance cognitive performance may be associated with allopregnanolone and/or BDNF expression, in the hippocampus, or cortical increases in allopregnanolone and $GABA_A$ activity.

In the present study, progesterone improved cognitive performance in the object recognition and T-maze task among wild-type and PRKO mice. This finding extends previous published data. Progesterone treatment immediately post-training enhances object recognition in young (14), middle-aged, and aged (64) mice. Also, progesterone administration to young and/or aged mice improved cognitive performance in these tasks (13, 60). However, in the water maze task, wild-type mice tended to outperform PRKO mice. There are beneficial effects of post-training progesterone on spatial memory consolidation in the Morris water maze in mice (64). In addition, it has been previously observed that aged wild-type mice outperform PRKO mice (57). Although, PRKO mice have reduced levels of PR binding (65, 66), there are significant increases in cognitive behavior of progesterone administered PRKO and wild-type mice

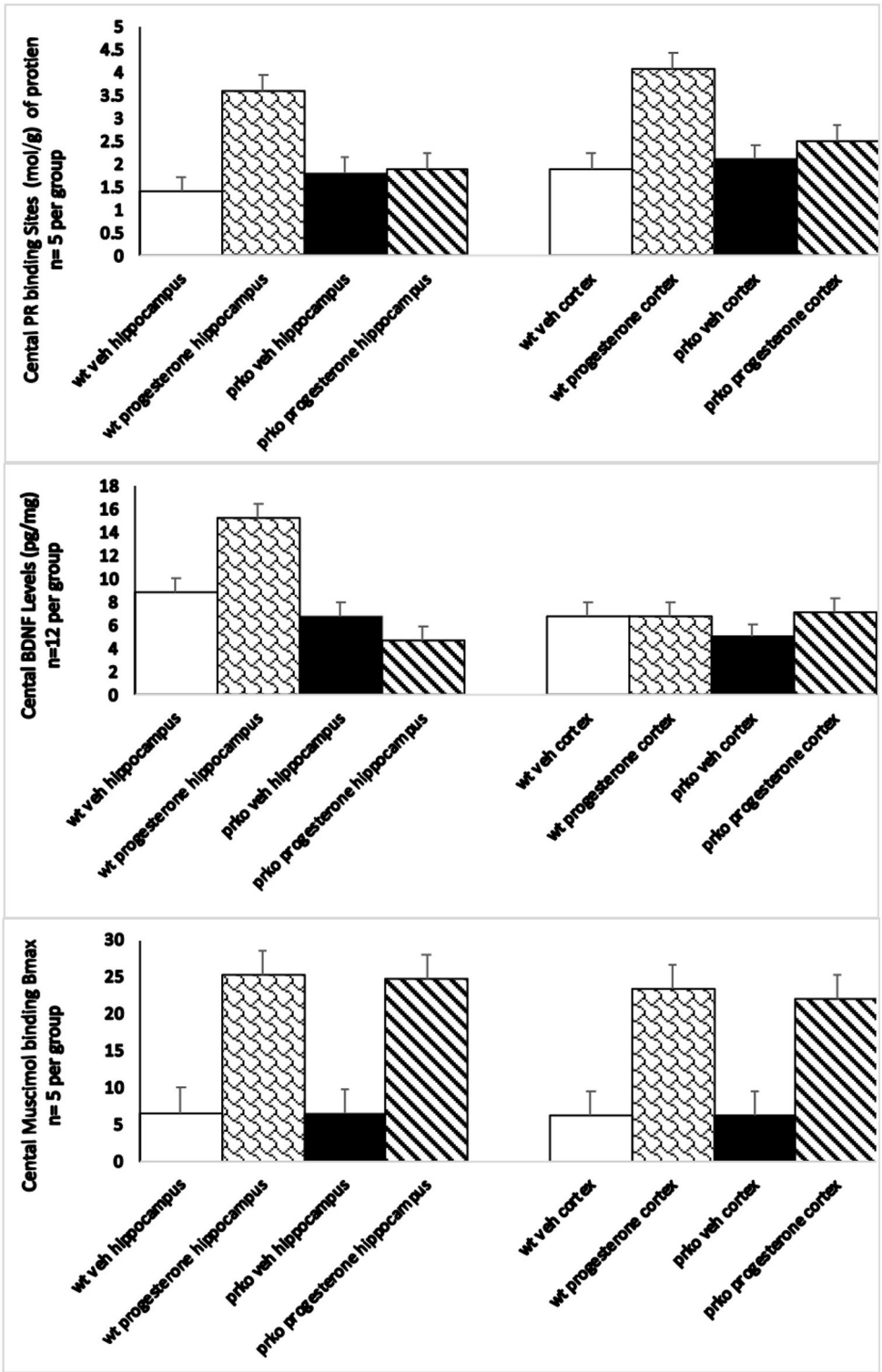

FIGURE 3 | The vehicle wildtype mice results are represented by white bars, P_4 wildtype by stippled bars, vehicle PRKO by black bars, and P_4 PRKO mice are represented by diagonally striped bars. The **top** panel represents E_{max} progestin receptor binding (n = 5/group) in the hippocampus in the **left** panel, and cortex in the **right** panel. Only wild-type mice administered progesterone showed significant increases in progestin receptor binding. The **middle** panel represents BDNF levels (n = 12/group) in the hippocampus in the **left** panel, and cortex in the **right** panel. BDNF levels were only increased in progesterone administered wild-type mice in the hippocampus. The **bottom** panel represents maximum muscimol binding (n = 5/group) in the hippocampus in the **left** panel, and cortex in the **right** panel. Both wild-type and PRKO male mice administered progesterone showed significant increases in the maximal binding of the $GABA_a$ agonist mucimol.

concomitant with increased levels of allopregnanolone in the hippocampus (29). Progesterone can also have beneficial effects in other behaviors. For example, progesterone has beneficial effects for sexual behavior, motivation, anxiety, response to drugs of abuse (24) and also, depressive-like behaviors (54). Thus, progesterone exerts beneficial effects on cognition as well as other behaviors.

Progesterone can also have neuroprotective effects in the brain. The neuroprotective effects of progesterone have been demonstrated in rodent models of neurodegeneration (67), brain ischemia/stroke (68–71), and traumatic brain injury (TBI) (72–78). Moreover, progesterone limits the extent of tissue damage and the impairment of motor functions in an animal model of stroke (77). Furthermore, progesterone has neuroprotective and cognitive enhancing effects however progesterone also can have beneficial effects in other disorders (i.e., Alzheimer's, Parkinson etc.). Thus, progesterone can have neuroprotective and cognition enhancing, which support the purpose of such investigations.

BDNF levels were increased in the hippocampus with progesterone administration to wild-type, but not PRKO, mice compared to vehicle administration. As such, these findings add and extend the literature on interactions between progesterone and growth factors. In support, progesterone failed to elicit an increase in BDNF in PRKO mice but induced an increase in BDNF levels of wild-type mice (79). This evidence has suggested that classical intracellular/nuclear PR would be the principle mediator of the effects of progesterone on BDNF expression because this effect was inhibited by the pharmacological inhibitor of PRs, Mifepristone, and was lost in PRKO mice (79). In addition, levels of BDNF in the cortex and hippocampus were lowest among mice administered a synthetic progestin, medroxyprogesterone acetate, that does not act like progesterone to induce allopregnanolone synthesis, suggesting a downstream role of this growth factor (12, 79). Indeed, progesterone increased BDNF levels; however, this may only be possible by way of its metabolite, allopregnanolone. Furthermore, among young cycling rodents, manipulation of allopregnanolone synthesis in the midbrain alters expression of BDNF in the hippocampus (61). Others have noted that allopregnanolone alters BDNF expression in other limbic structures, such as the amygdala and hypothalamus, in addition to the hippocampus (80). Together, production of allopregnanolone in the hippocampus may be required for progesterone's mnemonic effects to increase BDNF levels.

In the present study, allopregnanolone levels were increased in the hippocampus and cortex of PRKO and wild-type mice. This may be due to increased activity of the stress axis, which can interact to alter circulating and brain levels of steroids (e.g., neurosteroids), particularly among mice. Though PRKO mice had increased levels of allopregnanolone, this was not associated with increased BDNF levels, which support the notion that PRs may not be involved for cognitive improvements with progesterone administration. It must be noted that levels of allopregnanolone and progesterone are very low in male mice, but they are not completely absent. This may be because there can be *de novo* synthesis of allopregnanolone in the brain itself, in addition to metabolism of circulating progesterone. Indeed, it may be that these levels of allopregnanolone were produced via actions in the hippocampus, cortex and/or other regions in this circuit involved in the behaviors observed. Another consideration is that the behavioral effects of PRKO mice may be less related to effects on production of allopregnanolone, or even other steroids that can be derived in the periphery or brain (progesterone), but may relate more to different rates of clearance of neuroactive steroids. Moreover, these effects may be related to increased rates of progesterone conversion to allopregnanolone, which is known to increase following environmental stressors (e.g., cold water swim, restraint) as well as social challenges (e.g., mating) and mitigates stress responding [reviewed in (24, 81, 82)]. Furthermore, this may explain the difference that was shown in the water maze task (latency to find the platform). The water maze requires a high degree of physical activity (swimming), remembering where a hidden escape platform is located in the pool, and a probe trial. Although this was not directly tested in the present study, the water maze task may be associated with an increased stress response in PRKO mice, which could lead to higher levels of allopregnanolone in circulation among both progesterone and vehicle administered mice. Thus, clearance of neurosteriods and/or stress responsiveness may play an important role in allopregnanolone production. Further investigation of this is substantiated.

CONCLUSION

In conclusion, progesterone can have beneficial effects for cognitive performance among males. An important piece to this story may be progesterone's metabolite, allopregnanolone, and effects on BDNF levels in the hippocampus or $GABA_A$ receptors in the cortex.

IMPLICATIONS

Progesterone and its neuroactive metabolite, allopregnanolone, cognitive effects' among males is not well-understood and is addressed in this study. Progesterone (4 mg/kg, or oil vehicle SC) administered post-training in hippocampus and/or cortex tasks to wild type or mice lacking functional nuclear PRs. In the water maze task, wild-type mice tended to outperform PRKO mice. Progesterone enhanced performance of irrespective of genotype in the water maze, object learning and T-maze. Progesterone, but not vehicle, increased progesterone and allopregnanolone levels of wild-type and PRKO mice; albeit, PRKO mice had higher allopregnanolone levels than did wild-type in the hippocampus and cortex. Wild-type mice administered progesterone, but not vehicle, had increased BDNF levels in the hippocampus compared to PRKOs. Thus, male mice can be responsive to progesterone for learning/memory, and such effects do not require PRs, but may be associated with allopregnanolone and BDNF levels in the hippocampus or $GABA_A$ activity in the cortex.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AW: data production. VL: formal analysis. CF: funding acquisition, supervision, concept, and original draft. All authors contributed to the article and approved the submitted version.

FUNDING

Research herein was supported in part by grants National Institute of Mental Health (MH 06769801, RMH067698B) and the National Institute of General Medical Sciences of the National Institutes of Health (P20GM103395). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Assistance on the studies described herein provided by members of the laboratory, past and present, is greatly appreciated.

ACKNOWLEDGMENTS

Whitney Johnson, Carolyn Koonce, and other lab members also assisted in this project.

REFERENCES

1. Blaustein JD. Neuroendocrine regulation of feminine sexual behavior: lessons from rodent models and thoughts about humans. *Annu Rev Psychol*. (2008) 59:93–118. doi: 10.1146/annurev.psych.59.103006.093556
2. Priest CA, Pfaff DW. Actions of sex steroids on behaviours beyond reproductive reflexes. *CIBA Found Symp*. (1995) 191:74–84. doi: 10.1002/9780470514757.ch5
3. Frye CA. Neurosteroids' effects and mechanisms for social, cognitive, emotional, and physical functions. *Psychoneuroendocrinology*. (2009) 34 (Suppl. 1):S143–61. doi: 10.1016/j.psyneuen.2009.07.005
4. Fadda P, Fratta W. Stress-induced sleep deprivation modifies corticotropin releasing factor (CRF) levels and CRF binding in rat brain and pituitary. *Pharmacol Res*. (1997) 35:443–6. doi: 10.1006/phrs.1997.0155
5. Kalra PS, Kalra SP. Circadian periodicities of serum androgens, progesterone, gonadotropins and luteinizing hormone-releasing hormone in male rats: the effects of hypothalamic differentiation, castration and adrenalectomy. *Endocrinology*. (1997) 101:1821–7. doi: 10.1210/endo-101-6-1821
6. Andersen ML, Bignotto M, Machado RB, Tufik S. Different stress modalities result in distinct steroid hormone responses by male rats. *Braz J Med Biol Res*. (2004) 37:791–7. doi: 10.1590/S0100-879X2004000600003
7. Auger CJ, Vanzo RJ. Progesterone treatment of adult male rats suppresses arginine vasopressin expression in the bed nucleus of the stria terminalis and the centromedial amygdala. *J Neuroendocrinol*. (2006) 18:187–94. doi: 10.1111/j.1365-2826.2005.01400.x
8. Weisz J, Ward IL. Plasma testosterone and progesterone titers of pregnant rats, their male and female fetuses, and neonatal offspring. *Endocrinology*. (1980) 106:306–16. doi: 10.1210/endo-106-1-306
9. Bian C, Zhang D, Guo Q, Cai W, Zhang J. Localization and sex-difference of steroid receptor coactivator-1 immunoreactivities in the brain of adult female and male mice. *Steroids*. (2011) 76:269–79. doi: 10.1016/j.steroids.2010.11.009
10. Si D, Wang H, Wang Q, Zhang C, Sun J, Wang Z, et al. Progesterone treatment improves cognitive outcome following experimental traumatic brain injury in rats. *Neurosci Lett*. (2013) 11:18–23. doi: 10.1016/j.neulet.2013.07.052
11. Peterson TC, Anderson GD, Kantor ED, Hoane MR. A comparison of the effects of nicotinamide and progesterone on functional recovery of cognitive behavior following cortical contusion injury in the rat. *J Neurotrauma*. (2012) 29:2823–30. doi: 10.1089/neu.2012.2471
12. Frye CA, Koonce CJ, Walf AA. Progesterone, compared to medroxyprogesterone acetate, to C57BL/6, but not 5α-reductase mutant, mice enhances object recognition and placement memory and is associated with higher BDNF levels in the hippocampus and cortex. *Neurosci Lett*. (2013) 551:53–7. doi: 10.1016/j.neulet.2013.07.002
13. Frye CA, Walf AA. Progesterone enhances performance of aged mice in cortical or hippocampal tasks. *Neurosci Lett*. (2008) 437:116–20. doi: 10.1016/j.neulet.2008.04.004
14. Frye CA, Walf AA. Progesterone to ovariectomized mice enhances cognitive performance in the spontaneous alternation, object recognition, but not placement, water maze, and contextual and cued conditioned fear tasks. *Neurobiol Learn Mem*. (2008) 90:171–7. doi: 10.1016/j.nlm.2008.03.005
15. McEchron MD, Cheng AY, Gilmartin MR. Trace fear conditioning is reduced in the aging rat. *Neurobiol Learn Mem*. (2004) 82:71–6. doi: 10.1016/j.nlm.2004.06.002
16. Li X, Lonard DM, O'Malley BW. A contemporary understanding of progesterone receptor function. *Mech Ageing Dev*. (2004) 125:669–78. doi: 10.1016/j.mad.2004.04.007
17. Hagihara K, Hirata S, Osada T, Hirai M, Kato J. Expression of progesterone receptor in the neonatal rat brain cortex: detection of its mRNA using reverse transcription-polymerase chain reaction. *J Steroid Biochem Mol Biol*. (1992) 41:637–40. doi: 10.1016/0960-0760(92)90396-Z
18. Blaustein JD, Wade GN. Progestin binding by brain and pituitary cell nuclei and female rat sexual behavior. *Brain Res*. (1978) 140:360–7. doi: 10.1016/0006-8993(78)90469-9
19. Frye CA. Neurosteroids—from basic research to clinical perspectives. In: Rubin RT, Pfaff DW, editors. *Hormones/Behavior Relations of Clinical Importance*. San Diego: Academic Press (2009). p. 395–416.
20. Iswari S, Colas AE, Karavolas HJ. Binding of 5 alpha-dihydroprogesterone and other progestins to female rat anterior pituitary nuclear extracts. *Steroids*. (1986) 47:189–203. doi: 10.1016/0039-128X(86)90088-7
21. Majewska MD, Harrison NL, Schwartz RD, Barker JL, Paul SM. Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science*. (1986) 232:1004–7. doi: 10.1126/science.2422758
22. Farr SA, Flood JF, Scherrer JF, Kaiser FE, Taylor GT, Morley JE. Effect of ovarian steroids on footshock avoidance learning and retention in female mice. *Physiol Behav*. (1995) 58:715–23. doi: 10.1016/0031-9384(95)00124-2
23. Finn DA, Phillips TJ, Okorn DM, Chester JA, Cunningham CL. Rewarding effect of the neuroactive steroid 3 alpha-hydroxy-5 alpha-pregnan-20-one in mice. *Pharmacol Biochem Behav*. (1997) 56:261–4. doi: 10.1016/S0091-3057(96)00218-3
24. Frye CA. Progestins influence motivation, reward, conditioning, stress, and/or response to drugs of abuse. *Pharmacol Biochem Behav*. (2007) 86:209–19. doi: 10.1016/j.pbb.2006.07.033
25. Frye CA, Sturgis JD. Neurosteroids affect spatial/reference, working, and long-term memory of female rats. *Neurobiol Learn Mem*. (1995) 64:83–96. doi: 10.1006/nlme.1995.1046
26. Manshio DT, Gershbein LL. Avoidance and poke behavior in rats after gonadectomy and hormanal treatment. *Res Commun Chem Pathol Pharmacol*. (1975) 12:473–80.
27. Walf AA, Rhodes ME, Frye CA. Ovarian steroids enhance object recognition in naturally cycling and ovariectomized, hormone-primed rats. *Neurobiol Learn Mem*. (2006) 86:35–46. doi: 10.1016/j.nlm.2006.01.004

28. Lydon JP, DeMayo FJ, Conneely OM, O'Malley BW. Reproductive phenotpes of the progesterone receptor null mutant mouse. *J Steroid Biochem Mol Biol*. (1996) 56:67–77. doi: 10.1016/0960-0760(95)00254-5
29. Frye CA, Sumida K, Dudek BC, Harney JP, Lydon JP, O'Malley BW, et al. Progesterone's effects to reduce anxiety behavior of aged mice do not require actions via intracellular progestin receptors. *Psychopharmacology*. (2006) 186:312–22. doi: 10.1007/s00213-006-0309-3
30. Frye CA, Walf AA. Progesterone enhances performance of aged mice in cortical or hippocampal tasks. *Neurosci Lett*. (2010) 472:38–42. doi: 10.1016/j.neulet.2010.01.051
31. Causing CG, Gloster A, Aloyz R, Bamji SX, Chang E, Fawcett J, et al. Synaptic innervation density is regulated by neuron-derived BDNF. *Neuron*. (1997) 18:257–67. doi: 10.1016/S0896-6273(00)80266-4
32. Pruginin-Bluger M, Shelton DL, Kalcheim C. A paracrine effect for neuron-derived BDNF in development of dorsal root ganglia: stimulation of Schwann cell myelin protein expression by glial cells. *Mech Dev*. (1997) 61:99–111. doi: 10.1016/S0925-4773(96)00623-5
33. Pluchino N, Russo M, Santoro AN, Litta P, Cela V, Genazzani AR. Steroids hormones and BDNF. *Neuroscience*. (2013) 3:271–9. doi: 10.1016/j.neuroscience.2013.01.025
34. Singh M, Su C. Progesterone, brain-derived neurotrophic factor and neuroprotection n. *Neuroscience*. (2013) 239:84–91. doi: 10.1016/j.neuroscience.2012.09.056
35. Murer MG, Yan Q, Raisman-Vozari R. Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. *Prog Neurobiol*. (2001) 63:71–124. doi: 10.1016/S0301-0082(00)00014-9
36. Ferrer I, Marín C, Rey MJ, Ribalta T, Goutan E, Blanco R, et al. BDNF and full-length and truncated TrkB expression in Alzheimer disease. Implications in therapeutic strategies. *J Neuropathol Exp Neurol*. (1999) 58:729–39. doi: 10.1097/00005072-199907000-00007
37. Tapia-Arancibia L, Aliaga E, Silhol M, Arancibia S. New insights into brain BDNF function in normal aging and Alzheimer disease. *Brain Res Rev*. (2008) 59:201–20. doi: 10.1016/j.brainresrev.2008.07.007
38. Zuccato C, Cattaneo E. Brain-derived neurotrophic factor in neurodegenerative diseases. *Nat Rev Neurol*. (2009) 5:311–22. doi: 10.1038/nrneurol.2009.54
39. Connor B, Young D, Yan Q, Faull RL, Synek B, Dragunow M. Brain-derived neurotrophic factor is reduced in Alzheimer's disease. *Brain Res Mol Brain Res*. (1997) 49:71–81. doi: 10.1016/S0169-328X(97)00125-3
40. Hock C, Heese K, Hulette C, Rosenberg C, Otten U. Region-specific neurotrophin imbalances in Alzheimer disease: decreased levels of brain-derived neurotrophic factor and increased levels of nerve growth factor in hippocampus and cortical areas. *Arch Neurol*. (2000) 57:846–51. doi: 10.1001/archneur.57.6.846
41. Karege F, Perret G, Bondolfi G, Schwald M, Bertschy G, Aubry JM. Decreased serum brain-derived neurotrophic factor levels in major depressed patients. *Psychiatry Res*. (2002) 109:143–8. doi: 10.1016/S0165-1781(02)00005-7
42. Mogi M, Togari A, Kondo T, Mizuno Y, Komure O, Kuno S, et al. Brain-derived growth factor and nerve growth factor concentrations are decreased in the substantia nigra in Parkinson's disease. *Neurosci Lett*. (1999) 270:45–8. doi: 10.1016/S0304-3940(99)00463-2
43. Parain K, Murer MG, Yan Q, Faucheux B, Agid Y, Hirsch E, et al. Reduced expression of brain-derived neurotrophic factor protein in Parkinson's disease substantia nigra. *Neuroreport*. (1999) 10:557–61. doi: 10.1097/00001756-199902250-00021
44. Soontornniyomkij V, Wang G, Pittman CA, Hamilton RL, Wiley CA, Achim CL. Absence of brain-derived neurotrophic factor and trkB receptor immunoreactivity in glia of Alzheimer's disease. *Acta Neuropathol*. (1999) 98:345–8. doi: 10.1007/s004010051092
45. von Bohlen und Halbach O, Minichiello L, Unsicker K. Haploinsufficiency for trkB and trkC receptors induces cell loss and accumulation of α-synuclein in the substantia nigra. *FASEB J*. (2005) 19:1740–2. doi: 10.1096/fj.05-3845fje
46. Komulainen P, Pedersen M, Hänninen T, Bruunsgaard H, Lakka TA, Kivipelto M, et al. BDNF is a novel marker of cognitive function in ageing women: the DR's EXTRA Study. *Neurobiol Learn Mem*. (2008) 90:596–603. doi: 10.1016/j.nlm.2008.07.014
47. Lindvall O, Kokaia Z, Bengzon J, Elmér E, Kokaia M. Neurotrophins and brain insults. *Trends Neurosci*. (1994) 17:490–6. doi: 10.1016/0166-2236(94)90139-2
48. Aguirre CC, Baudry M. Progesterone reverses 17beta-estradiol-mediated neuroprotection and BDNF induction in cultured hippocampal slices. *Eur J Neurosci*. (2009) 29:447–54. doi: 10.1111/j.1460-9568.2008.06591.x
49. Gibbs RB. Levels of trkA and BDNF mRNA, but not NGF mRNA, fluctuate across the estrous cycle and increase in response to acute hormone replacement. *Brain Res*. (1998) 787:259–68. doi: 10.1016/S0006-8993(97)01511-4
50. González SL, Labombarda F, González Deniselle MC, Guennoun R, Schumacher M, De Nicola AF. Progesterone up-regulates neuronal brain-derived neurotrophic factor expression in the injured spinal cord. *Neuroscience*. (2004) 125:605–14. doi: 10.1016/j.neuroscience.2004.02.024
51. Gonzalez Deniselle MC, Garay L, Gonzalez S, Saravia F, Labombarda F, Guennoun R, et al. Progesterone modulates brain-derived neurotrophic factor and choline acetyltransferase in degenerating Wobbler motoneurons. *Exp Neurol*. (2007) 203:406–14. doi: 10.1016/j.expneurol.2006.08.019
52. Kaur P, Jodhka PK, Underwood WA, Bowles CA, de Fiebre NC, de Fiebre CM, et al. Progesterone increases brain derived neuroptrophic factor expression and protects against glutamate toxicity in a mitogen-activated protein kinase- and phosphoinositide-3 kinase-dependent manner in cerebral cortical explants. *J Neurosci Res*. (2007) 85:2441–9. doi: 10.1002/jnr.21370
53. Singh M, Meyer EM, Simpkins JW. The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female Sprague-Dawley rats. *Endocrinology*. (1995) 136:2320–4. doi: 10.1210/endo.136.5.7720680
54. Frye CA. Progesterone attenuates depressive behavior of younger and older adult C57/BL6, wildtype, and progesterone receptor knockout mice. *Pharmacol Biochem Behav*. (2011) 99:525–31. doi: 10.1016/j.pbb.2011.05.024
55. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR Jr, Montgomery CA et al. Mice lacking progesterone receptors exhibit pleiotropic reproductive abnormalities. *Genes Dev*. (1995) 9:2266–78. doi: 10.1101/gad.9.18.2266
56. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. New York, NY: Cold Spring Harbor Laboratory Press (1989).
57. Frye CA, Koonce CJ, Walf AA. Mnemonic effects of progesterone to mice require formation of 3alpha,5alpha-THP. *Neuroreport*. (2010) 21:590–5. doi: 10.1097/WNR.0b013e32833a7e14
58. Walf AA, Frye CA. Object Recognition - The role of hormones throughout the lifespan. In: Cao TP, editor. *Object Recognition*. InTech (2011).
59. Spowart-Manning L, van der Staay FJ. The T-maze continuous alternation task for assessing the effects of putative cognition enhancers in the mouse. *Behav Brain Res*. (2004) 151:37–46. doi: 10.1016/j.bbr.2003.08.004
60. Frye CA, Rhodes ME, Dudek B. Estradiol to aged female or male mice improves learning in inhibitory avoidance and water maze tasks. *Brain Res*. (2005) 1036:101–8. doi: 10.1016/j.brainres.2004.12.014
61. Frye CA, Koonce CJ, Walf AA. Involvement of pregnane xenobiotic receptor in mating-induced allopregnanolone formation in the midbrain and hippocampus and brain-derived neurotrophic factor in the hippocampus among female rats. *Psychopharmacology*. (2014) 231:3375–90. doi: 10.1007/s00213-014-3569-3
62. McEwen BS. Endocrine effects on the brain and their relationship to behavior. *Basic Neurochem*. (2012) 8:945–62. doi: 10.1016/B978-0-12-374947-5.00055-9
63. Frye CA, Duncan JE, Basham M, Erskine MS. Behavioral effects of 3a-androstanediol II: Hypothalamic and preoptic area actions via a GABAergic mechanism. *Behav Brain Res*. (1996) 79:119–30. doi: 10.1016/0166-4328(96)00005-8
64. Lewis MC, Orr PT, Frick KM. Acute progesterone enhances spatial and object memory in aged female C57BL/6 mice. *Hormones Behav*. (2008) 54:455–62.
65. Mani SK, Allen JM, Clark JH, Blaustein JD, O'Malley BW. Steroid hormone–and neurotransmitter-induced rat sexual behavior: addendum. *Science*. (1995) 268:1833. doi: 10.1126/science.7604251
66. Mani SK, Allen JM, Lydon JP, Mulac-Jericevic B, Blaustein JD, DeMayo FJ, et al. Dopamine requires the unoccupied progesterone receptor to induce sexual behavior in mice. *Mol Endocrinol*. (1996) 10:1728–37. doi: 10.1210/mend.10.12.8961281
67. Vongher JM, Frye CA. Progesterone in conjunction with estradiol has neuroprotective effects in an animal model of neurodegeneration. *Pharmacol Biochem Behav*. (1999) 64:777–85. doi: 10.1016/S0091-3057(99)00140-9

68. Cervantes M, González-Vidal MD, Ruelas R, Escobar A, Moralí G. Neuroprotective effects of progesterone on damage elicited by acute global cerebral ischemia in neurons of the caudate nucleus. *Arch Med Res*. (2002) 33:6–14. doi: 10.1016/S0188-4409(01)00347-2
69. Sayeed I, Wali B, Stein DG. Progesterone inhibits ischemic brain injury in a rat model of permanent middle cerebral artery occlusion. *Restor Neurol Neurosci*. (2007) 25:151–9.
70. Gibson CL, Coomber B, Rathbone J. Is progesterone a candidate neuroprotective factor for treatment following ischemic stroke? *Neuroscientist*. (2009) 15:324–32. doi: 10.1177/1073858409333069
71. Liu A, Margaill I, Zhang S, Labombarda F, Coqueran B, Delespierre B, et al. Progesterone receptors: a key for neuroprotection in experimental stroke. *Endocrinology*. (2012) 153:3747–57. doi: 10.1210/en.2012-1138
72. Stein DG. Brain damage, sex hormones and recovery: a new role for progesterone and estrogen? *Trends Neurosci*. (2001) 24:386–91. doi: 10.1016/S0166-2236(00)01821-X
73. Stein DG, Sayeed I. Is progesterone worth consideration as a treatment for brain injury? *AJR Am J Roentgenol*. (2010) 194:20–22. doi: 10.2214/AJR.09.3407
74. Schumacher M, Guennoun R, Stein DG, De Nicola AF. Progesterone: therapeutic opportunities for neuroprotection and myelin repair. *Pharmacol Ther*. (2007) 116:77–106. doi: 10.1016/j.pharmthera.2007.06.001
75. Brinton RD, Thompson RF, Foy MR, Baudry M, Wang J, Finch CE, et al. Progesterone receptors: form and function in brain. *Front Neuroendocrinol*. (2008) 29:313–39. doi: 10.1016/j.yfrne.2008.02.001
76. Gibson CL, Gray LJ, Bath PM, Murphy SP. Progesterone for the treatment of experimental brain injury; a systematic review. *Brain*. (2008) 131(Pt 2):318–28. doi: 10.1093/brain/awm183
77. Stein DG. Progesterone exerts neuroprotective effects after brain injury. *Brain Res Rev*. (2008) 57:386–97. doi: 10.1016/j.brainresrev.2007.06.012
78. Stein DG, Wright DW. Progesterone in the clinical treatment of acute traumatic brain injury. *Expert Opin Investig Drugs*. (2010) 19:847–57. doi: 10.1517/13543784.2010.489549
79. Jodhka PK, Kaur P, Underwood W, Lydon JP, Singh M. The differences in neuroprotective efficacy of progesterone and medroxyprogesterone acetate correlate with their effects on brain-derived neurotrophic factor expression. *Endocrinology*. (2009) 150:3162–8. doi: 10.1210/en.2008-1247
80. Naert G, Maurice T, Tapia-Arancibia L, Givalois L. Neuroactive steroids modulate HPA axis activity and cerebral brain-derived neurotrophic factor (BDNF) protein levels in adult male rats. *Psychoneuroendocrinology*. (2007) 32:1062–78. doi: 10.1016/j.psyneuen.2007.09.002
81. Maguire J, Mody I. Neurosteroid synthesis-mediated regulation of GABA(A) receptors: relevance to the ovarian cycle and stress. *J Neurosci*. (2007) 27:2155–62. doi: 10.1523/JNEUROSCI.4945-06.2007
82. Sarkar J, Wakefield S, MacKenzie G, Moss SJ, Maguire J. McEwen Neurosteroidogenesis is required for the physiological response to stress: role of neurosteroid-sensitive GABAA receptors. *J Neurosci*. (2011) 31:18198–210. doi: 10.1523/JNEUROSCI.2560-11.2011

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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 11 May 2021
doi: 10.3389/fendo.2021.652512

Altered Spontaneous Brain Activity in Women During Menopause Transition and Its Association With Cognitive Function and Serum Estradiol Level

Lemin He[1,2,3], *Wei Guo*[4], *Jianfeng Qiu*[2,5], *Xingwei An*[3*] *and Weizhao Lu*[2,5*]

[1] Department of Biomedical Engineering, College of Precision Instruments and Optoelectronics Engineering, Tianjin University, Tianjin, China, [2] Department of Radiology, Shandong First Medical University & Shandong Academy of Medical Sciences, Taian, China, [3] Academy of Medical Engineering and Translational Medicine, Tianjin University, Tianjin, China, [4] Department of Radiology, The Second Affiliated Hospital of Shandong First Medical University, Taian, China, [5] Science and Technology Innovation Center, Shandong First Medical University & Shandong Academy of Medical Sciences, Jinan, China

OPEN ACCESS

Edited by:
Neil James MacLusky, University of Guelph, Canada

Reviewed by:
Gregoy Y. Bedecarrats, University of Guelph, Canada
Maria Claudia Gonzalez Deniselle, CONICET Instituto de Biología y Medicina Experimental (IBYME), Argentina

***Correspondence:**
Xingwei An
anxingwei@tju.edu.cn
Weizhao Lu
mingming9053@163.com

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

Received: 12 January 2021
Accepted: 28 April 2021
Published: 11 May 2021

Citation:
He L, Guo W, Qiu J, An X and Lu W (2021) Altered Spontaneous Brain Activity in Women During Menopause Transition and Its Association With Cognitive Function and Serum Estradiol Level. Front. Endocrinol. 12:652512. doi: 10.3389/fendo.2021.652512

Objective: Serum hormone deficiencies during menopause transition may affect spontaneous brain activity and global cognition. The purpose of this study was to explore the differences in spontaneous brain activity between premenopausal and perimenopausal women, and to investigate the associations between spontaneous brain activity, serum hormone levels and global cognition.

Methods: Thirty-two premenopausal women (47.75 ± 1.55 years) and twenty-five perimenopausal women (51.60 ± 1.63 years) underwent resting-state functional MRI (fMRI) scan. Clinical information including Mini-Mental State Examination (MMSE), levels of estradiol (E2), free testosterone, progesterone, prolactin, follicle-stimulating hormone and luteinizing hormone were measured. Regional homogeneity (ReHo) was used to evaluate spontaneous brain activity alterations between perimenopausal and premenopausal women. Correlation analysis was used to investigate the associations between brain functional alterations and clinical measures in perimenopausal group.

Results: The results demonstrated increased ReHo value in the right lingual gyrus (LG) and decreased ReHo value in the right superior frontal gyrus (SFG) in perimenopausal women compared with premenopausal women. In perimenopausal group, ReHo of the right LG showed a negative correlation with level of E2 ($r = -0.586$, $p = 0.002$), ReHo of the right SFG showed a positive correlation with level of E2 ($r = 0.470$, $p = 0.018$) and MMSE ($r = 0.614$, $p = 0.001$).

Conclusions: The results demonstrated that women approaching menopause suffered from altered functions in brain regions related to cognitive function, working memory, the results also revealed a direct association between levels of E2 and brain functions in perimenopausal women.

Keywords: functional magnetic resonance imaging, spontaneous brain activity, menopause, estradiol, ReHo

INTRODUCTION

Perimenopause, also known as menopause transition, is the period between declined ovarian function and permanent cessation of menstruation in women (1). Erratic fluctuations in hormone levels lead to various physical manifestations which include hot flashes, night sweats, vasomotor dysfunction and vaginal dryness (2, 3). Indeed, perimenopause is regarded as "a window of vulnerability" for women (3). In addition, as the transition into menopause, many women experience cognition decline and memory loss (4). The most critical change during menopause transition is the fluctuation of hormone levels, especially the drop of estrogen levels (1, 5). There is growing evidence that serum hormone levels have effect on cognition and memory during menopause transition (6). Previous studies have investigated the effects of hormone therapy on cognition in women approaching menopause, and have found that women had better cognitive and memory performance in relevant tasks after hormone therapy (7). Hormones act throughout several cellular and molecular processes which can alter structure and function of the central nervous system *via* hormone receptors (1, 8). In the brain, hormone receptor expressions have been found in the cerebral cortex and limbic systems (8, 9). Hormones and neurosteroids also play critical roles in neural plasticity in the brain as well (10, 11).

In the recent decade, neuroimaging techniques have been applied to investigate alterations of the brain in women during menopause transition (1, 10, 12–14). Functional magnetic resonance imaging (fMRI), as a popular tool in neuroscience, has been used to study brain functional changes in women during menopause transition (1, 12). Specifically, fMRI has been applied to evaluate therapeutic effect of hormone therapy on cognitive function of women during menopause transition and have revealed that women during menopause transition may benefit from certain hormone therapy in terms of cognitive control, verbal and working memory (14–16). In addition, fMRI studies have also revealed functional activations in premenopausal and postmenopausal women related to sexual arousal (17–19). A previous study by our group revealed that perimenopausal women experienced altered intrinsic functional connectivity in regions related to sexual function (1).

For most fMRI studies related to women in menopause transition, the studies focused on brain functional changes under stimuli or after hormone therapy (14–19), few studies have focused on the hormonal fluctuations during menopause transition and its effect on spontaneous brain activity. Regional homogeneity (ReHo), one of the measures in resting-state fMRI, has been used to quantify the synchronization of a given voxel with its neighboring voxels (20). Unlike functional connectivity which involves in distant temporal correlations of fMRI signals, ReHo focuses on functional coherence of regional neural activity (21). Using ReHo analysis, researchers have identified brain functional alterations associated with Parkinson's disease, depressive disorder, schizophrenia, etc. (22, 23). In this study, we aimed to find the differences in spontaneous brain activity between premenopausal and perimenopausal groups, and to explore the association between serum hormone levels and spontaneous brain activity in perimenopausal women. Regional homogeneity (ReHo) was used to quantify spontaneous brain activity, correlation analysis was used to evaluate the association between ReHo values, serum hormone levels and cognitive function.

MATERIALS AND METHODS

Participants

This cross-sectional study received full approval from the Medical Ethics Committee of the Shandong First Medical University in accordance with the Declaration of Helsinki. All participants gave their written, informed consent before participating in this study. From June 2017 to January 2018, premenopausal and perimanopausal women were recruited by the Second Affiliated Hospital of Shandong First Medical University. Enrollment criteria for perimenopausal women included: (1) 45 - 55 years old, (2) more than 12 years of formal education, (3) right-handedness, (4) heterosexuality, (5) perimenopause was diagnosed according to the Stages of Reproductive Aging Workshop (STRAW) +10 staging system: persistent difference in consecutive menstrual cycle variable length was larger than 7 days or interval of amenorrhea was larger than 2 months (24). Enrollment criteria for premenopausal women included: (1) 45 - 55 years old, (2) more than 12 years of formal education, (3) right-handedness, (4) heterosexuality, (5) premenopause was diagnosed based on the diagnosis criterion of STRAW +10 staging system: having a regular ovulation day according to the rhythm method. Exclusion criteria for the enrolled women were: (1) history of psychiatry or neurological disorders, (2) hormone or steroid treatment in a month prior to the study, or oral contraceptive use in a month prior to the study, (3) use of antihistamines, ranitidine, black cohosh, or other drugs that modulate ovarian steroid secretion in a month prior to the study, (4) dysfunction of organs including heart, liver or kidney, (5) endocrine diseases, (6) premenstrual syndrome or premenstrual dysphoric disorder, (7) MRI contradictions. At last, 32 premenopausal women (47.75 ± 1.55 years) and 25 perimenopausal women (51.60 ± 1.63 years) were finally recruited. Upon enrollment, all subjects received a routine body examination including routine blood test, urine test, chest and abdominal CT, routine gynecological examination to rule out any possible effect on the bioavailability of steroids and brain function.

Measurement of Cognitive Function and Serum Hormone Concentrations

Cognitive function was evaluated by the Mini-Mental State Examination (MMSE). In terms of serum hormones, the concentrations of six hormones included estradiol (E2), prolactin (PRL), luteinizing hormone (LH), follicle-stimulating hormone (FSH), free testosterone (free-T) and progesterone (P) were measured during the early follicular phase (3-5 days after menstrual onset). Participants were instructed to have a good night sleep before the test. The test was carried out between 8:30

and 9:30 a.m. Venous blood samples were obtained from all subjects *via* vein puncture. The levels of E2, PRL, LH, FSH, free-T and P were measured using chemiluminescent immunoassay method by E170 Immunology Analyzer (Roche, Brussels, Belgium).

fMRI Acquisition and Processing

A 3.0T MR scanner (Discovery MR 750, GE, Milwaukee, US) with 8-channel head array coil was used to acquire fMRI data. Participants were scanned in a supine, head-first position with cushions on both sides and at top of the head to control head motion. T1-weighted structural images were obtained *via* 3D-BRAVO sequence with the following parameters: repetition time (TR) = 6.656 ms, echo time (TE) = 2.928 ms, inversion time = 450 ms, field of view (FOV) = 240 mm × 240 mm^2, slice thickness = 1 mm, slice gap = 1 mm, matrix = 256 × 256, number of signal averages = 1, flip angle (FA) = 12°, and 176 sagittal slices. Before resting-state fMRI scan, participants were instructed to open their eyes, calm breathing, keep a clear consciousness and not to engage in any specific thinking activity. To acquire resting-state fMRI data, echo-planar imaging sequence was used with the following parameters: TR = 2000 ms, TE = 30 ms, FOV = 240 mm × 240 mm^2, matrix = 64 × 64, slice thickness = 3.5 mm, slice gap = 1.2 mm, FA = 90°, scan duration = 480 s (240 volumes) and 33 axial slices.

Data Processing & Analysis for Brain Imaging (DPABI, http://rfmri.org/dpabi) was used for fMRI data preprocessing, regional homogeneity (ReHo) calculation and statistical analysis. Data preprocessing included the following steps: (1) The first 10 volumes of the fMRI data were removed to preserve steady-state data only. (2) The remaining fMRI volumes were corrected for timing differences and for head motion. Subjects with head motion more than 1 mm, head rotational motion larger than 1° or framewise displacement more than 0.5 mm were excluded from further analysis. 9 participants were excluded and they were contacted for fMRI rescans. Finally, all the enrolled women passed the thresholds. (4) The individual fMRI images were spatially registered to the Montreal Neurological Institute (MNI) standard space using Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra algorithm. (5) Nuisance covariates including head motion parameters, white matter and cerebro-spinal fluid signal were regressed out from each subject's fMRI time series. (6) A band-pass filter (0.01-0.08 Hz) was used to reduce low-frequency drifts and high-frequency noise.

After data preprocessing, ReHo map for each woman was calculated. In the present study, ReHo value of each voxel was calculated as the Kendall's coefficient concordance (KCC) of this voxel with its adjacent 26 voxels (20). ReHo value close to 1 means a given voxel and its adjacent voxels are more consistent, vice versa (20). Then, ReHo maps were spatially smoothed with a Gaussian kernel (full width half maximum = 6 mm).

Statistical Analysis

SPSS 20.0 was used for statistical analysis. Independent t-test was used for comparisons of age, education and MMSE between premenopausal and perimenopausal women. Mann-Whitney U test was used to compare serum hormone levels between the two groups. The threshold for significance was set at $p < 0.05$.

Comparison of ReHo maps between premenopausal and perimenopausal women was carried out using the statistical module of DPABI. Specifically, general linear model was used to detect whether there were differences in ReHo maps between the two groups. Age was treated as a nuisance covariate and was adjusted using linear regression. Gaussian random field (GRF) correction with voxel level $p < 0.001$ and cluster level $p < 0.05$ (two-tailed) was used to control false positives. In addition, effect size (ES) calculated as Cohen's d was used to evaluate statistical effect of the group analysis.

Associations between ReHo values and clinical measures (including MMSE, serum hormone concentrations) in perimenopausal group were evaluated by Pearson's correlation analysis. Group analysis revealed several regions with significant differences in ReHo values between the two groups. Mean ReHo values were extracted from these regions in perimenopausal group. Pearson's correlation analysis was conducted to investigate the association between mean ReHo values and serum hormone concentrations. In addition, relationship between ReHo values and MMSE was also assessed using Pearson's correlation analysis. The threshold for statistical significance was set at $p < 0.05$.

RESULTS

Demographic and Clinical Information

Demographic information and serum hormone concentrations of premenopausal and perimenopausal women are listed in **Table 1**. MMSE scores and six hormone levels were all in the normal range for premenopausal and perimenopausal women. In addition, increased levels of FSH and LH in perimenopausal women were observed, while decreased levels of PRL, E2, free-T and P in perimenopausal women were found compared with premenopausal women. Significant differences in age, MMSE, levels of PRL, FSH, LH, E2 and P were found between premenopausal and perimenopausal women. There were no significant statistical differences in education duration and level of free-T between the two groups.

ReHo Changes

Compared with premenopausal women, perimenopausal women demonstrated increased ReHo in the right lingual gyrus (LG) (GRF corrected at voxel level $p < 0.001$ and cluster level $p < 0.05$, two-tailed, ES = 0.69). Perimenopausal women also showed decreased ReHo values in the right superior frontal gyrus (SFG) compared with premenopausal women (GRF corrected at voxel level $p < 0.001$ and cluster level $p < 0.05$, two-tailed, ES = 0.78). The differences in ReHo map between the two groups are shown in **Figure 1**. The brain regions with significant differences in ReHo are identified in **Table 2**.

TABLE 1 | Demographic information and sex hormone levels between premenopausal (n = 32) and perimenopausal women (n = 25).

	Premenopausal women (n = 32)	Perimenopausal women (n = 25)	Normal range	P value
Age (years)[a]	47.75 ± 1.55	51.60 ± 1.63	–	<0.001[b]
Education (years)[a]	16.18 ± 1.31	15.84 ± 0.80	–	0.248[b]
MMSE[a]	29.28 ± 0.85	28.68 ± 1.28	27 - 30	0.038[b]
PRL (ng/ml)[a]	21.67 ± 17.71	12.93 ± 10.68	1.9-25	0.011[c]
FSH (mIU/ml)[a]	5.74 ± 4.21	52.63 ± 29.75	1.2-153	<0.0001[c]
LH (mIU/ml)[a]	7.31 ± 13.13	22.12 ± 11.32	1.1-77	0.0013[c]
E2 (pg/ml)[a]	109.70 ± 64.55	35.70 ± 22.70	20-400	<0.0001[c]
free-T (ng/dL)[a]	28.47 ± 16.35	19.13 ± 1.27	0-73	0.403[c]
P (ng/ml)[a]	2.37 ± 4.23	0.22 ± 0.06	0.1-24	0.001[c]

[a]*Variables are presented as mean ± standard deviation.*
[b]*P value was calculated via independent t-test.*
[c]*P value was calculated via Mann-Whitney U test.*
PRL, prolactin; FSH, follicle-stimulating hormone; LH, luteotropic hormone; E2, estradiol; free-T, free testosterone; P, progesterone.

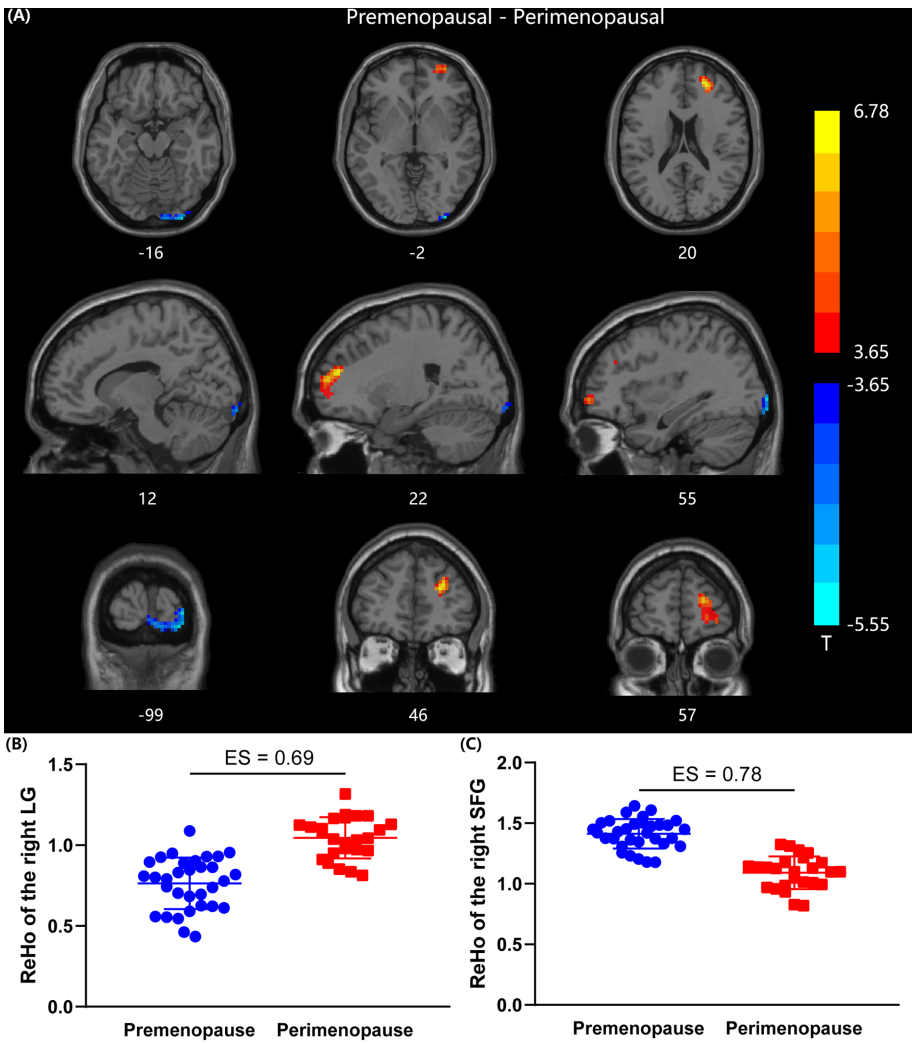

FIGURE 1 | Comparison of ReHo values between premenopausal group (n =32) and perimenopausal group (n = 25). **(A)** Two sample t-map between premenopausal women and perimenopausal women. Gaussian random field correction (two tailed, voxel-level $p < 0.001$, cluster level $p < 0.05$) was used for multiple comparisons, colorbar indicates T-score. **(B)** Scatter plot of mean ReHo value in the right LG for the two groups. **(C)** Scatter plot of mean ReHo value in the right SFG for the two groups. LG, lingual gyrus; SFG, superior frontal gyrus; ES, effect size.

TABLE 2 | Brain regions with significant differences in ReHo values between premenopausal (n = 32) and perimenopausal women (n = 25) (Gaussian random field corrected at voxel level p < 0.001, cluster level p < 0.05, two-tailed).

Brain region	BA[a]	Cluster size[b]	MNI coordinates[c]			T value[d]	Types of ReHo change[e]
			X	Y	Z		
Right LG	17,18	79	33	-99	-3	-5.430	Premenopausal < Perimenopausal
Right SFG	9,10,46	197	21	48	21	6.776	Premenopausal > Perimenopausal

[a]*BA represents Brodmann area, which is an atlas of the human cerebral cortex with 52 subregions.*
[b]*Cluster size represents number of voxels in the relevant clusters.*
[c]*MNI coordinates are the coordinates of voxels in the standard brain atlas provided by Montreal Neurological Institute.*
[d]*T value is the statistical value from independent t-test. In this column, T value represents T value of the peak voxel in the cluster.*
[e]*ReHo changes have two types, ReHo value of the premenopausal group is larger than ReHo value of the perimenopausal group, or ReHo value of the premenopausal group is smaller than ReHo value of the perimenopausal group.*
ReHo, regional homogeneity; BA, Brodmann area; MNI, Montreal Neurological Institute; LG, lingual gyrus; SFG, superior frontal gyrus.

Correlation Analyses

Correlation analysis results are shown in **Figure 2**. In perimenopausal group, ReHo of the right SFG showed a positive correlation with MMSE (r = 0.614, p = 0.001). ReHo of the right LG showed a negative correlation with the level of E2 (r = -0.586, p = 0.002), ReHo of the right SFG showed a positive correlation with the level of E2 (r = 0.470, p = 0.018). However, ReHo values did not show significant correlations with other hormone levels. In addition, ReHo value of the right LG and ReHo of the right SFG did not have a significant correlation.

DISCUSSION

Although previously viewed as a reproductive and psychological transition, a recent state-of-the-art study has pointed out that perimenopause is largely a neurological transition in nature (25). Menopausal symptoms that emerge during perimenopause indicate disruptions in multiple hormone-regulated systems (25). The fluctuations of serum hormones including sex hormones, estrogens could affect the structure and function of the central nervous system through a network of hormone receptors (8, 9, 25). Therefore, it is of clinical importance to

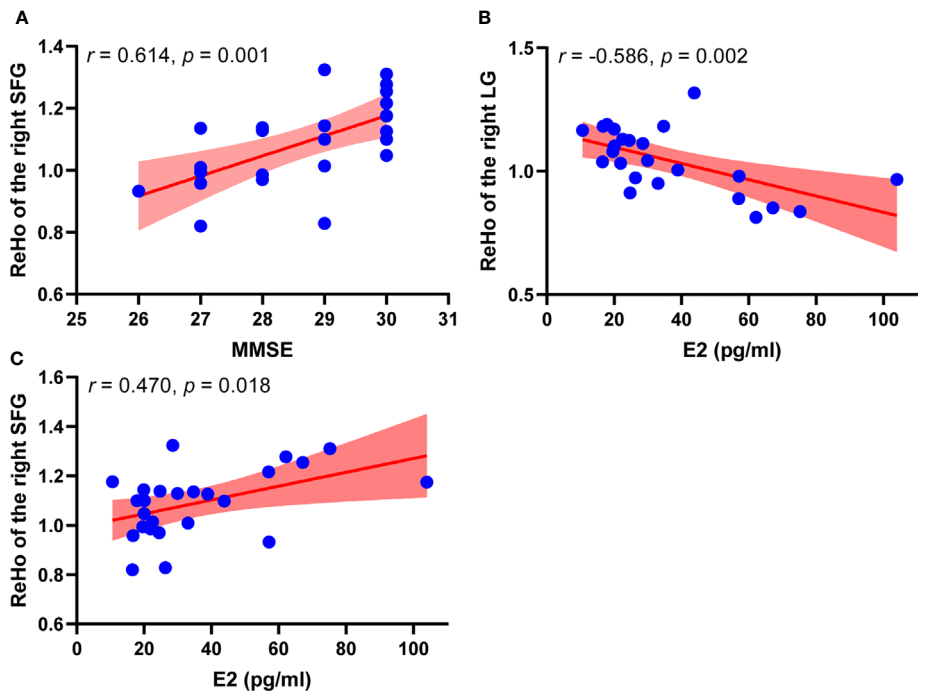

FIGURE 2 | Correlation analysis results between ReHo values and clinical information in perimenopausal group (n = 25). **(A)** Scatter plots between ReHo value of the right SFG and MMSE in perimenopausal women. **(B)** Scatter plots between ReHo value of the right LG and level of E2 in perimenopausal women. **(C)** Scatter plots between ReHo value of the right SFG and level of E2 in perimenopausal women. MMSE, Mini-Mental State Examination, LG; lingual gyrus, E2, estradiol; ReHo, regional homogeneity; SFG, superior frontal gyrus.

study brain function alterations, and to explore the association between serum hormone levels and brain functions in perimenopausal women.

For the enrolled participants in the current study, the concentrations of six hormones included E2, PRL, LH, FSH, free-T and P were measured to provide a general evaluation of endocrine status. Results indicated that concentrations of six hormones were all in the normal range for both premenopausal and perimenopausal group. However, perimenopausal women experienced increased levels of FSH and LH, and decreased levels of E2, PRL and P compared with premenopausal women. As is known, E2 and P are synthesized in the ovary, while PRL, FSH and LH are released by the pituitary gland (10, 26). In perimenopause, declined ovarian function results in the decreased release of E2 and P (1). As a result, the pituitary gland releases more FSH and LH to stimulate ovary to produce steroids (27). Different from FSH and LH, PRL secretion is not affected by the declined ovarian function (28). The decreased PRL level for perimenopausal women in this study may be a direct consequence of age-related processes in the lactotrophs (28). In addition, other factors involved in the regulation of PRL secretion, such as estrogens should also be considered (29).

In the field of neuroimaging, ReHo, an fMRI measure, has been widely used to evaluate spontaneous brain activity and brain functions at resting-state (20). In the present study, ReHo from resting-state fMRI was used to evaluate brain functional alterations in perimenopausal women, and correlation analysis was used to evaluate the relationship between serum hormone levels, cognitive function and spontaneous brain activity. The results demonstrated that perimenopausal women showed both decreased spontaneous brain activity and increased spontaneous brain activity in several brain regions compared with premenopausal women. E2 level had significant associations with ReHo of several brain regions related to cognitive function.

In this study, perimenopausal women showed decreased ReHo in the right SFG compared with premenopausal women. Furthermore, MMSE was positively correlated with ReHo of the right SFG. The SFG makes up about one third of the frontal lobe in the human brain. The SFG which includes the supplementary motor area, is involved in cognitive function and working memory (30). The decreased spontaneous brain activity in the right SFG in perimenopausal women might indicate that perimenopausal women had a greater chance of experiencing decreased cognitive function and decreased working memory than premenopausal women. SFG is also implicated in depression as a neuroimaging study has revealed that dysfunction in the SFG causes depression (31). The present finding might give potential explanation for depression in women during menopause transition. In addition, a previous voxel-based morphometry study by our group has found that perimenopausal women showed decreased gray matter volume in the right SFG compared with premenopausal women (10). There is a close relationship between structure and function of the human brain (32). Functional changes in the right SFG might have associations with structural changes in the right SFG in perimenopausal women.

The current study also revealed increased ReHo in the right LG for perimenopausal women compared with premenopausal women. The LG is part of the visual cortex which are mainly involved in visual information processing (33). However, in addition to visual function, the LG is also responsible for visual working memory processing (34, 35). Several fMRI studies have reported the role of the visual cortex in retaining visual working memory information and working memory consolidation (36, 37). Although the occipital and frontal lobe are anatomically distant, yet these two regions are highly integrated in function (38). The present findings might suggest that the LG in the occipital lobe and the SFG in the frontal lobe both experienced altered spontaneous brain activity in relation to E2 deficiency in women during menopause transition.

E2 is a form of estrogens which is implicated in numerous physiological processes (39). Studies have reported that E2 is implicated in cognitive function, mood regulation, learning, memory, etc. (39, 40). In addition, E2 plays a critical role in neurodegenerative diseases including Alzheimer's disease, dementia and stroke (41). In human, the brain expresses high levels of E2 receptors in several brain regions (42).

Present findings revealed that serum E2 level had a negative correlation with brain activity in the right LG, and serum E2 level had a positive correlation with brain activity in the right SFG in perimenopausal women. There is evidence that E2 receptors are located in the frontal cortex (13). Previous studies have reported that E2 level had an association with activity of the frontal cortex during emotion regulation and sexual stimuli (12, 43). In line with previous findings, the positive correlation between E2 level and brain activity in the right SFG suggested that E2 might positively contribute to the function of the frontal cortical system in related tasks. Davis et al. have investigated the effects of sex hormones on visuospatial function and verbal fluency in women during menopausal transition and have found that hormone therapy was associated with decreased brain activity in the lingual gyrus and occipital gyrus (44, 45). Similar phenomenon has also been reported by Neele et al. (46). It was hypothesized that with increased serum E2 level, less neural recruitment was required for task completion with the same speed and accuracy. On the contrary, when the level of E2 decreased, more neurons were needed in the occipital lobe for task completion, therefore, perimenopausal women with declined E2 level showed increased brain activity in the right LG.

Several limitations need to be addressed for this study. Firstly, clinical information and fMRI data were collected at one single time point due to limited conditions. Secondly, relatively small sample size may reduce statistical power and conclusions drawn from the results, however, effect size by Cohen's d revealed strong statistical differences, and multiple comparison correction methods enhanced reliability of the current study. Future studies will focus on a larger study sample size with more clinical information.

In conclusion, resting-state fMRI was used to assess the differences in spontaneous brain activity between premenopausal and perimenopausal women. Correlation analysis was used to evaluate the association between serum

hormone concentrations, cognitive function and spontaneous brain activity. The results demonstrated alterations of spontaneous brain activity and functional compensation in perimenopausal women compared with premenopausal women. In addition, the results also suggested that estradiol level had correlations with several regions related to cognition and visual working memory. The findings highlighted the association between estradiol and brain functions in women during menopause transition and might be helpful in understanding functional changes in the brain of women during menopause transition.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of the Shandong First Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LH and WG conceived the study, XA and WL designed the protocol. JQ conducted the statistical analyses. LH, JQ, and XA interpreted study findings and contributed to developing the manuscript. LH and WL wrote the first draft of the manuscript that was revised and approved by all authors. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Traditional Chinese Medicine Science and Technology Development Plan of Shandong Province (grant number 2019-0359), the Medicine and Health Science Development Plan of Shandong Province (202009040008), Key Research and Development Program of Shandong Province (grant number 2017GGX201010), Academic Promotion Program of Shandong First Medical University (grant number 2019QL009) and Taishan Scholars Program of Shandong Province (grant number TS201712065).

REFERENCES

1. Lu W, Guo W, Cui D, Dong K, Qiu J. Effect of Sex Hormones on Brain Connectivity Related to Sexual Function in Perimenopausal Women: A Resting-State Fmri Functional Connectivity Study. *J Sex Med* (2019) 16:711–20. doi: 10.1016/j.jsxm.2019.03.004
2. WoodS NF, Mitchell ES. Symptoms During the Perimenopause: Prevalence, Severity, Trajectory, and Significance in Women's Lives. *Am J Med* (2005) 118:14–24. doi: 10.1016/j.amjmed.2005.09.031
3. Freeman EW. Associations of Depression With the Transition to Menopause. *Menopause* (2010) 17:823–7. doi: 10.1097/gme.0b013e3181db9f8b
4. Devi G. Menopause-Related Cognitive Impairment. *Obstet Gynecol* (2018) 132:1325–7. doi: 10.1097/AOG.0000000000002963
5. Geiger PJ, Eisenlohr-Moul T, Gordon JL, Rubinow DR, Girdler SS. Effects of Perimenopausal Transdermal Estradiol on Self-Reported Sleep, Independent of its Effect on Vasomotor Symptom Bother and Depressive Symptoms. *Menopause* (2019) 26:1318–23. doi: 10.1097/GME.0000000000001398
6. Lebrun CE, van der Schouw YT, de Jong FH, Pols HA, Grobbee DE, Lamberts SW. Endogenous Oestrogens are Related to Cognition in Healthy Elderly Women. *Clin Endocrinol* (2005) 63:50–5. doi: 10.1111/j.1365-2265.2005.02297.x
7. Frizell B, Dumas JA. Examining the Relationship Between Neurosteroids, Cognition, and Menopause With Neuroimaging Methods. *Curr Psychiatry Rep* (2018) 20:96. doi: 10.1007/s11920-018-0963-2
8. McEwen BS, Milner TA. Understanding the Broad Influence of Sex Hormones and Sex Differences in the Brain. *J Neurosci Res* (2017) 95:24–39. doi: 10.1002/jnr.23809
9. Uddin MS, Rahman MM, Jakaria M, Rahman MS, Hossain MS, Islam A, et al. Estrogen Signaling in Alzheimer's Disease: Molecular Insights and Therapeutic Targets for Alzheimer's Dementia. *Mol Neurobiol* (2020) 57:2654–70. doi: 10.1007/s12035-020-01911-8
10. Lu W, Guo W, Hou K, Zhao H, Shi L, Dong K, et al. Gray Matter Differences Associated With Age and Sex Hormone Levels Between Premenopausal and Perimenopausal Women, a Voxel-Based Morphometry Study. *J Neuroendocrinol* (2018) 30:e12655. doi: 10.1111/jne.12655
11. Lentini E, Kasahara M, Arver S, Savic I. Sex Differences in the Human Brain and the Impact of Sex Chromosomes and Sex Hormones. *Cereb Cortex* (2013) 23:2322–36. doi: 10.1093/cercor/bhs222
12. Baek HS, Kim GW, Sundaram T, Park K, Jeong GW. Brain Morphological Changes With Functional Deficit Associated With Sexual Arousal in Postmenopausal Women. *Sex Med* (2019) 7:480–8. doi: 10.1016/j.esxm.2019.06.013
13. Kim GW, Park K, Jeong GW. Effects of Sex Hormones and Age on Brain Volume in Post-Menopausal Women. *J Sex Med* (2018) 15:662–70. doi: 10.1016/j.jsxm.2018.03.006
14. Berent-Spillson A, Kelley AS, Persad CC, Love T, Frey KA, Reame NE, et al. Postmenopausal Hormone Treatment Alters Neural Pathways But Does Not Improve Verbal Cognitive Function. *Menopause* (2018) 25:1424–31. doi: 10.1097/GME.0000000000001157
15. Berentspillson A, Briceno E, Pinsky A, Simmen A, Persad CC, Zubieta JK, et al. Distinct Cognitive Effects of Estrogen and Progesterone in Menopausal Women. *Psychoneuroendocrinol* (2015) 59:25–36. doi: 10.1016/j.psyneuen.2015.04.020
16. Girard R, Météreau E, Thomas J, Pugeat M, Qu C, Dreher JC. Hormone Therapy At Early Post-Menopause Increases Cognitive Control-Related Prefrontal Activity. *Sci Rep* (2017) 7:44917. doi: 10.1038/srep44917
17. Park K, Kang HK, Seo JJ, Kim HJ, Ryu SB, Jeong GW. Blood-Oxygenation-Level-Dependent Functional Magnetic Resonance Imaging for Evaluating Cerebral Regions of Female Sexual Arousal Response. *Urology* (2001) 57:1189–94. doi: 10.1016/S0090-4295(01)00992-X
18. Jeong GW, Park K, Youn G, Kang HK, Kim HJ, Seo JJ, et al. Assessment of Cerebrocortical Regions Associated With Sexual Arousal in Premenopausal and Menopausal Women by Using BOLD-based Functional MRI. *J Sex Med* (2005) 2:645–51. doi: 10.1111/j.1743-6109.2005.00134.x
19. Kim GW, Jeong GW. Menopause-Related Brain Activation Patterns During Visual Sexual Arousal in Menopausal Women: An fMRI Pilot Study Using Time-Course Analysis. *Neuroscience* (2017) 343:449–58. doi: 10.1016/j.neuroscience.2016.12.010
20. Zang Y, Jiang T, Lu Y, He Y, Tian L. Regional Homogeneity Approach to fMRI Data Analysis. *Neuroimage* (2004) 22:394–400. doi: 10.1016/j.neuroimage.2003.12.030

21. Li Z, Li C, Liang Y, Wang K, Zhang W, Chen R, et al. Altered Functional Connectivity and Brain Network Property in Pregnant Women With Cleft Fetuses. *Front Psychol* (2019) 10:2235. doi: 10.3389/fpsyg.2019.02235
22. Shen YT, Yuan YS, Wang M, Zhi Y, Wang JW, Wang LN, et al. Dysfunction in Superior Frontal Gyrus Associated With Diphasic Dyskinesia in Parkinson's Disease. *NPJ Parkinsons Dis* (2020) 6:30. doi: 10.1038/s41531-020-00133-y
23. Wolf RC, Rashidi M, Schmitgen MM, Fritze S, Sambataro F, Kubera KM, et al. Neurological Soft Signs Predict Auditory Verbal Hallucinations in Patients With Schizophrenia. *Schizophr Bull* (2020) 24:sbaa146. doi: 10.1093/schbul/sbaa146
24. Harlow SD, Gass M, Hall JE, Lobo R, Maki P, Rebar RW, et al. Executive Summary of the Stages of Reproductive Aging Workshop +10: Addressing the Unfinished Agenda of Staging Reproductive Aging. *Fertil Steril* (2012) 97:843–51. doi: 10.1016/j.fertnstert.2012.01.128
25. Brinton RD, Yao J, Yin F, Mack WJ, Cadenas E. Perimenopause as a Neurological Transition State. *Nat Rev Endocrinol* (2015) 11:393–405. doi: 10.1038/nrendo.2015.82
26. Smith MJ, Adams LF, Schmidt PJ, Rubinow DR, Wassermann EM. Effects of Ovarian Hormones on Human Cortical Excitability. *Ann Neurol* (2002) 51:599–603. doi: 10.1002/ana.10180
27. Sherman BM, West JH, Korenman SG. The Menopausal Transition: Analysis of LH, FSH, Estradiol, and Progesterone Concentrations During Menstrual Cycles of Older Women. *J Clin Endocrinol Metab* (1976) 42:629–36. doi: 10.1210/jcem-42-4-629
28. Tanner MJ, Hadlow NC, Wardrop R. Variation of Female Prolactin Levels With Menopausal Status and Phase of Menstrual Cycle. *Aust NZ J Obstet Gynaecol* (2011) 51:321–4. doi: 10.1111/j.1479-828X.2011.01321.x
29. Balint-Perić LA, Prelević GM. Changes in Prolactin Levels With the Menopause: The Effects of Estrogen/Androgen and Calcitonin Treatment. *Gynecol Endocrinol* (1997) 11:275–80. doi: 10.3109/09513599709152546
30. Zhang S, Ide JS, Li CR. Resting-State Functional Connectivity of the Medial Superior Frontal Cortex. *Cereb Cortex* (2012) 22:99–111. doi: 10.1093/cercor/bhr088
31. Sun H, Jiang R, Qi S, Narr KL, Wade BS, Upston J, et al. Preliminary Prediction of Individual Response to Electroconvulsive Therapy Using Whole-Brain Functional Magnetic Resonance Imaging Data. *Neuroimage Clin* (2020) 26:102080. doi: 10.1016/j.nicl.2019.102080
32. Damoiseaux JS, Greicius MD. Greater Than the Sum of its Parts: A Review of Studies Combining Structural Connectivity and Resting-State Functional Connectivity. *Brain Struct Funct* (2009) 213:525–33. doi: 10.1007/s00429-009-0208-6
33. Qu H, Wang Y, Yan T, Zhou J, Lu W, Qiu J. Data-Driven Parcellation Approaches Based on Functional Connectivity of Visual Cortices in Primary Open-Angle Glaucoma. *Invest Ophthalmol Vis Sci* (2020) 61:33. doi: 10.1167/iovs.61.8.33
34. Chen T, Yu W, Xie X, Ge H, Fu Y, Yang D, et al. Influence of Gonadotropin Hormone Releasing Hormone Agonists on Interhemispheric Functional Connectivity in Girls With Idiopathic Central Precocious Puberty. *Front Neurol* (2020) 11:17. doi: 10.3389/fneur.2020.00017
35. Bak Y, Nah Y, Han S, Lee SK, Shin NY. Altered Neural Substrates Within Cognitive Networks of Postpartum Women During Working Memory Process and Resting-State. *Sci Rep* (2020) 10:9110. doi: 10.1038/s41598-020-66058-x
36. Emrich SM, Riggall AC, Larocque JJ, Postle BR. Distributed Patterns of Activity in Sensory Cortex Reflect the Precision of Multiple Items Maintained in Visual Short-Term Memory. *J Neurosci* (2013) 33:6516–23. doi: 10.1523/JNEUROSCI.5732-12.2013
37. Makovski T, Lavidor M. Stimulating Occipital Cortex Enhances Visual Working Memory Consolidation. *Behav Brain Res* (2014) 275:84–7. doi: 10.1016/j.bbr.2014.09.004
38. Forkel SJ, Thiebaut de Schotten M, Kawadler JM, Dell'Acqua F, Danek A, Catani M. The Anatomy of Fronto-Occipital Connections From Early Blunt Dissections to Contemporary Tractography. *Cortex* (2014) 56:73–84. doi: 10.1016/j.cortex.2012.09.005
39. Brown AMC, Gervais NJ. Role of Ovarian Hormones in the Modulation of Sleep in Females Across the Adult Lifespan. *Endocrinology* (2020) 161:1–15. bqaa128. doi: 10.1210/endocr/bqaa128
40. Martinez-Bengochea A, Doretto L, Rosa IF, Oliveira MA, Silva C, Silva DMZA, et al. Effects of 17β-Estradiol on Early Gonadal Development and Expression of Genes Implicated in Sexual Differentiation of a South American Teleost, Astyanax Altiparanae. *Comp Biochem Physiol B Biochem Mol Biol* (2020) 248-249:110467. doi: 10.1016/j.cbpb.2020.110467
41. Vinklarova L, Schmidt M, Benek O, Kuca K, Gunn-Moore F, Musilek K. Friend or Enemy? Review of 17β-HSD10 and its Role in Human Health or Disease. *J Neurochem* (2020) 155:231–49. doi: 10.1111/jnc.15027
42. Grković I, Mitrović N. Estradiol Induces Synaptic Rearrangements. *Vitam Horm* (2020) 114:233–56. doi: 10.1016/bs.vh.2020.04.006
43. Chung YS, Poppe A, Novotny S, Epperson CN, Kober H, Granger DA, et al. A Preliminary Study of Association Between Adolescent Estradiol Level and Dorsolateral Prefrontal Cortex Activity During Emotion Regulation. *Psychoneuroendocrinology* (2019) 109:104398. doi: 10.1016/j.psyneuen.2019.104398
44. Davis SR, Davison SL, Gavrilescu M, Searle K, Gogos A, Rossell SL, et al. Effects of Testosterone on Visuospatial Function and Verbal Fluency in Postmenopausal Women: Results From a Functional Magnetic Resonance Imaging Pilot Study. *Menopause* (2014) 21:410–4. doi: 10.1097/GME.0b013e3182a065ed
45. Davison SL, Bell RJ, Robinson PJ, Jane F, Leech J, Maruff P, et al. Continuous-Combined Oral Estradiol/Drospirenone has No Detrimental Effect on Cognitive Performance and Improves Estrogen Deficiency Symptoms in Early Postmenopausal Women: A Randomized Placebo-Controlled Trial. *Menopause* (2013) 20:1020–6. doi: 10.1097/GME.0b013e318287474f
46. Neele SJ, Rombouts SA, Bierlaagh MA, Barkhof F, Scheltens P, Netelenbos JC. Raloxifene Affects Brain Activation Patterns in Postmenopausal Women During Visual Encoding. *J Clin Endocrinol Metab* (2001) 86:1422–4. doi: 10.1210/jcem.86.3.7454

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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership