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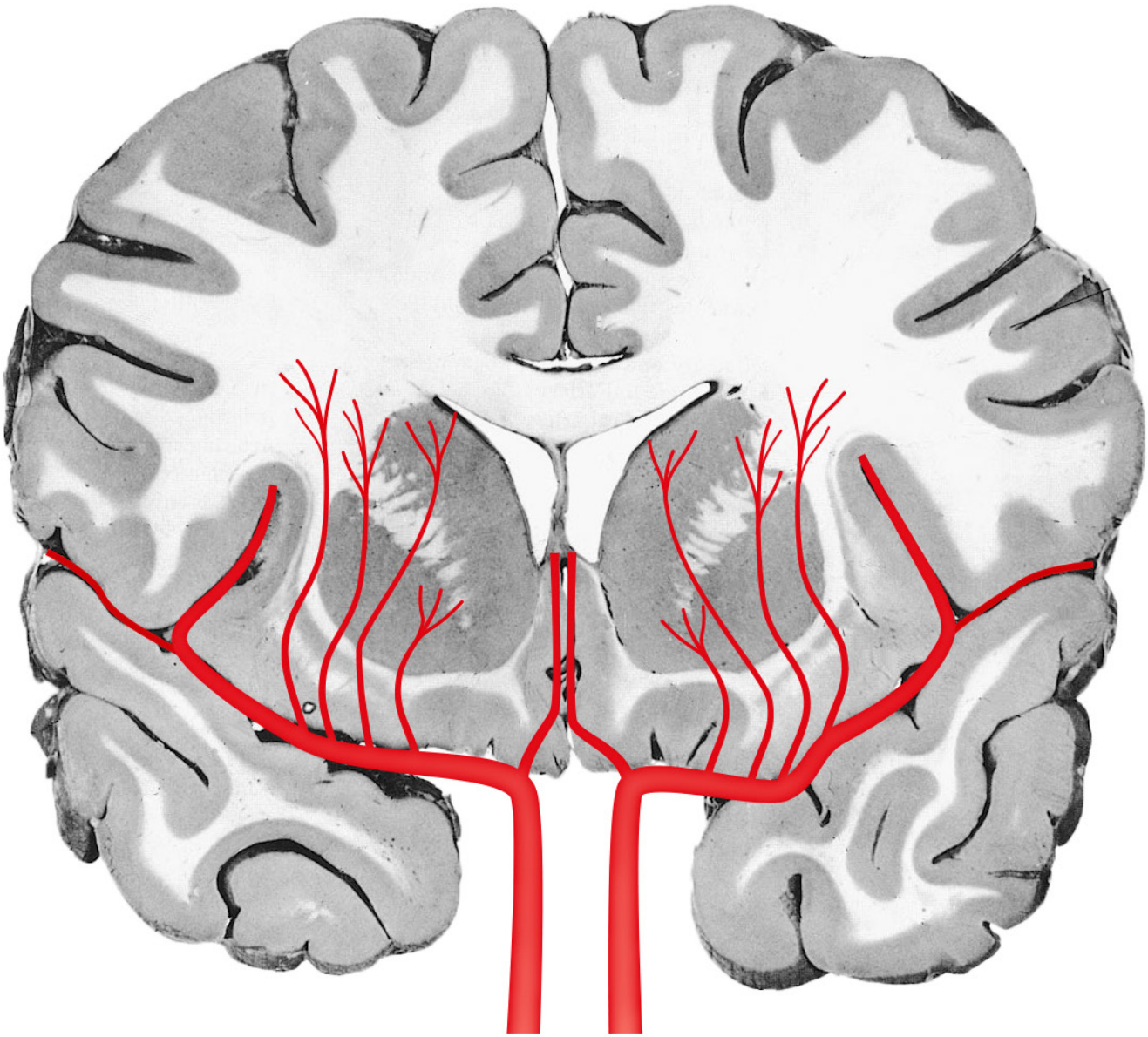

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TRENDS IN NEUROENDOCRINOLOGY

Topic Editor:
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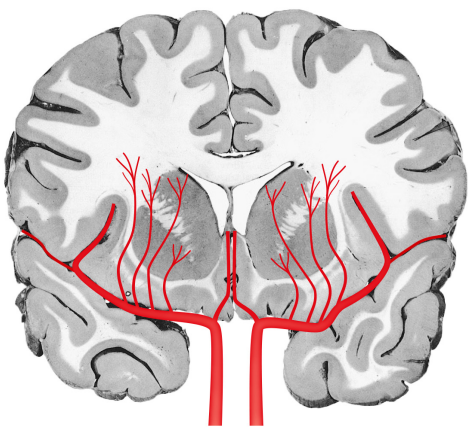

Neuroendocrinology studies interactions between the brain and the endocrine systems. Neurohormones produced by specialized brain neurons are released into the blood stream, and peripheral hormones transported to the brain influence neuronal activity.

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Neuroendocrinology is the discipline that investigates the interplay between the nervous and endocrine systems i.e. the control of endocrine glands by the central and peripheral nervous systems, the action of hormones on nerve cells and the production of hormones by the nervous system. The present Research Topic is a compilation of contributions stemming from the 8th International Congress of Neuroendocrinology (ICN2014) held in Sydney, Australia, that illustrates various facets of current neuroendocrine research.

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frontiers in Endocrinology

EDITORIAL
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Editorial: Trends in Neuroendocrinology

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The Editorial on the Research Topic

Trends in Neuroendocrinology

Neuroendocrinology is the field of research that explores the interplay between the central nervous system and the endocrine glands. The neuroendocrine system controls a number of essential physiological processes, including biological rhythms, stress, social behaviors, appetite, growth, and reproduction. The present Research Topic is a compilation of contributions stemming from the 8th International Congress of Neuroendocrinology (ICN2014) held in Sydney, NSW, Australia, that illustrates various facets of current neuroendocrinological investigations.

Most studies on circadian rhythms have been conducted on male animals only, based on the assumption that females display higher variability caused by the interaction of sex hormones with biological rhythms. The review on sex differences in circadian behavioral rhythms by Krizo and Mintz points out the need to include both female and male animals in such studies to elucidate the influence and mechanism of action of gonadal steroids on behavioral rhythmicity. This review also raises the question of the impact of sex hormone changes across the lifespan, notably during the pubertal period, on the circadian system.

The hypothalamo–pituitary–adrenal axis (also called the stress axis) is another fruitful "playground" for neuroendocrinologists. Chen et al. summarize the literature pertaining to the effects of glucocorticoid stress hormones on the nitrergic system notably in the brain. This review clarifies the complex cross-talk between the neuroendocrine stress axis and the nitrergic system that are both implicated in various pathological conditions, including anxiety and depressive disorders. In a sister review, Spiers et al. raise the important question of the effect of glucocorticoids and neuronal oxidative stress. They present the different mechanisms through which cortisol or corticosterone induces oxidative stress, particularly in the hippocampus.

There is now strong evidence that oxytocin influences social behavior in various animal models and even in human. Miller and Caldwell examine the organizational role of oxytocin in the postnatal and peripubertal periods on the brain and behaviors. This review highlights the developmental effects of oxytocin on sexual, affiliative, parental, and aggressive behaviors, as well as on non-social behaviors, such as nociception and addiction. It also investigates the neurochemical systems that mediate the effects of oxytocin on these behaviors.

Given the effects of oxytocin on prosocial behaviors, a role of oxytocin in the etiology and symptom severity of schizophrenia has been hypothesized. In this context, Rich and Caldwell analyze the possible implication of the oxytocin system in the negative symptoms and deficit in social cognition associated with schizophrenia, and discuss its potential for the treatment of schizophrenia.

Reciprocally, early-life adversity and social environment can affect the oxytocinergic system. Alves et al. review the influence of prenatal and postnatal stressors as well as maternal mental health on the development of the oxytocin system in animal models. For studies in human infants, the major

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challenge is clearly to collect plasma or CSF samples suitable for measurement of oxytocin levels.

Vasopressin, like oxytocin, acts both as a neurohormone released by the neural lobe of the pituitary and as a neurotransmitter/neuromodulator within the brain. Bester-Meredith et al. review the evidence that links vasopressin with the processing of olfactory, auditory, taste, and visual information, and explore how alteration of sensory processing can shape behavioral responses to these stimuli.

The role of neuroendocrine factors in the control of feeding behavior and energy homeostasis has been extensively studied. Thus, the implication of neurotransmitters and neuropeptides in the regulation of the hypothalamic centers that govern appetite and energy expenditure is now relatively well understood (1–7). Méquinion et al. introduce the different animal models that can be used to decipher the physiological, metabolic, and neurobiological alterations associated with anorexia nervosa.

Steroid hormones, including glucocorticoids, mineralocorticoids, androgens, and estrogens, exert their genomic actions through transcription factors known as nuclear receptors. They can also act via membrane receptors that mediate rapid, non-genomic signaling. Rainville et al. describe the various candidates for membrane estrogen and glucocorticoid receptors and focus on the contribution of non-genomic signaling in the control of hypothalamic-driven behaviors by steroid hormones.

Insulin does not only act on liver, muscle, and adipose tissue to regulate glucose homeostasis, but also exerts a central effect on neurophysiological processes. Akintola and van Heemst review the current knowledge on the role of insulin in the central nervous system and the potential implication of insulin signaling in the brain for healthy longevity. The neurotrophin-induced gene VGF encodes a precursor protein that is exclusively expressed in neuronal and neuroendocrine cells. VGF is processed by prohormone convertases to generate a series of biologically active neuropeptides. Lewis et al. describe the various effects of VGF-derived peptides on energy homeostasis, water balance, reproduction, nociception, memory, and learning.

In fish, as in mammals, reproduction is finely regulated by complex neuroendocrine mechanisms. Prasad et al. review the role of serotonin in the control of the reproductive system in teleost fish. Their report provides evidence for coordinated actions of the serotonergic system at different levels of the hypothalamo–pituitary–gonadal axis, supporting the functional significance of serotonin in the control of fish reproduction.

I wish that this Research Topic becomes a major set of references for neuroendocrinologists and raises the interest of other scientists who are not yet working in this fertile domain.

AUTHOR CONTRIBUTIONS

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MINI REVIEW ARTICLE
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Sex differences in behavioral circadian rhythms in laboratory rodents

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There is a strong bias in basic research on circadian rhythms toward the use of only male animals in studies. Furthermore, of the studies that use female subjects, many use only females and do not compare results between males and females. This review focuses on behavioral aspects of circadian rhythms that differ between the sexes. Differences exist in the timing of daily onset of activity, responses to both photic and non-photic stimuli, and in changes across the lifespan. These differences may reflect biologically important traits that are ecologically relevant and impact on a variety of responses to behavioral and physiological challenges. Overall, more work needs to be done to investigate differences between males and females as well as differences that are the result of hormonal changes across the lifespan.

Keywords: estrogens, testosterone, locomotor activity, ovariectomy, castration

INTRODUCTION

There has been a longstanding bias against the use of females in basic research involving common animal models, arising out of the belief that females show higher variability in results due to the influence of hormonal cycles (1). However, a recent meta-analysis of publications involving the use of mice across a variety of biomedical research areas concluded that this assumption was without merit (2). The failure to include both females and males, therefore, can result in researchers missing important information on sex differences in biology without any resulting gains from limiting themselves to the use of a single sex. The study of biological rhythms is no exception to this critique, as a large majority of recent work in this area has failed to include females (3). In this review, we look at sex differences in basic parameters of circadian rhythms and hypothesize about their underlying mechanisms and biological relevance.

CIRCADIAN PERIOD

The period of the circadian clock represents the time it takes to complete one cycle under constant environmental conditions, and is usually close to but not exactly 24 h. Sex differences in period are highly species-specific, but even when present the differences are generally modest. Free-running period in rats and golden hamsters is longer in males than females (4, 5); however, the differences in period are very small, whereas in *Octodon degus* period is longer in females by approximately half an hour (6, 7). In mice with a C57BL/6J background, there does not appear to be a sex difference in free-running period (3).

Despite the limited nature of the sex differences, gonadal hormones have a significant impact on circadian period. Ovariectomy lengthens circadian period in rats and hamsters, and period is then shortened by replacement of estradiol (8, 9). However, no change in period is apparent in mice after ovariectomy (3), though estradiol, an estrogen receptor α (ERα) agonist, or an estrogen receptor β (ERβ) agonist shorten period in ovariectomized mice (10). In contrast, reports on the effect of castration on period are more varied. While one study showed no effect in mice (11), others indicate that it lengthens period in this species (12–14). It appears that this effect may be dependent on the presence of constant dim red light (as opposed to true constant darkness) (14). Castration does not result in a change in period in hamsters (15) or adult degus (16).

Given the modest, if any, sex differences in circadian period, it is appropriate to ask whether the differences that are seen are biologically relevant. There is natural variation in circadian period across species and individuals, but circadian period must be under stabilizing selection to keep it close to 24 h. Period length does influence the amplitude of responses of the circadian clock to stimuli (17), so average period for a species may reflect an optimization for responses to external stimuli rather than an optimization for period length. There does not seem to be an overarching theory that explains interspecies variation in period. It is likely that sex differences in period reflect the influence of gonadal steroids, either through direct action on the central circadian clock in the suprachiasmatic nucleus (SCN) or via actions that modify behavioral feedback on the SCN. Organizational effects of gonadal hormones may also play an important role, but in this review we are focusing on potential activational effects. That said, it is remarkable that sex differences are so small, given the considerable influence of gonadal steroids on circadian period.

ONSET OF ACTIVITY

The timing of activity onset represents the most obvious difference in circadian rhythms between the sexes. Variability in activity onset is considerably greater in females than in males, and this variability is closely tied to the phase of the estrous cycle in mice (3), hamsters (8, 18, 19), and rats (20, 21). Variability in the onset of activity in females appears to be largely mediated by ERβ, at

least in mice (10, 22). Activity onset is most advanced before ovulation, corresponding to elevated estradiol levels, and then delayed afterward. The functional significance, if any, of this variation is unknown. These effects could be caused by direct effects of estrogens on the phase of the underlying circadian clock and/or by changes in effector systems that cause the threshold for onset of locomotor activity to occur slightly earlier or later depending on the hormonal environment. The idea that the underlying clock in the SCN shifts a little on each day of the estrous cycle under the direct influence of estradiol is supported by the fact that period is shortened by replacement of estradiol in ovariectomized animals (8, 9, 23). However, it is also possible that clock output is unchanged, but downstream brain regions responsible for generating the motivation for locomotor activity are slightly more or less sensitive depending on the level of estrogens present. For example, estrogens upregulate dopamine receptor 1 in the striatum (24, 25), which could result in increased motivation for wheel-running activity, resulting in a slightly earlier onset of activity.

PHOTIC RESPONSES

There are a number of potential mechanisms by which biological sex, via gonadal steroids, can influence the photic sensitivity of the circadian clock. However, it is not known if the effects that have been found thus far are biologically important, and these effects may vary dramatically by species. In *Octodon degus*, females adjust to a 6-h advance of the light–dark cycle significantly faster than males (26). In mice, females have larger phase shifts to light (3), while gonadectomized male mice have larger phase shifts than gonadally intact male mice (27). The lengthening of period that occurs when animals are housed in increasing intensities of constant light is also potentiated in gonadectomized animals (14). Female mice lacking estrogen receptor alpha show increased phase shifting responses to light (28). These data are consistent with the idea that both estradiol and testosterone act to reduce the phase shifting effects of light. The functional significance of this is unknown.

NON-PHOTIC RESPONSES

There has been little work done investigating sex differences in non-photic influences on entrainment. A couple of studies have been done on the influence of the estrous cycle on circadian responses in Syrian hamsters, but not with direct comparisons to male animals. Females show an estrous-cycle dependent modulation of activity level in response to a non-photic stimulus such as a cage change or novel wheel exposure, but this difference in behavioral activation results in only modest variability in the size of non-photic phase shifts (29). However, they did note that large shifts during proestrus caused a 1-day delay in the estrous cycle. A similar delay was observed in response to phenobarbital treatment on proestrus, suggesting that large phase shifts caused the circadian clock to "miss" generating the daily signal needed for the GnRH surge (30). However, in order to demonstrate a true sex difference in non-photic responses, it will be necessary to conduct experiments with direct comparisons between males and females, and it is important that this be done in additional species to see if there are common responses. In degus, there are sex differences in the effect of odor on circadian reentrainment rates to shifts in the light/dark cycle, and these effects are influenced by estrogen, progesterone, and testosterone (31, 32).

FOOD ENTRAINMENT

There has been very little research on sex differences in food entrainment. When rodents are placed on a restricted feeding schedule, such that food is only available for a limited period of time each day during an animal's normal sleep period, they show a behavioral response known as food anticipatory activity (FAA). This FAA generally takes the form of increased behavioral activation for a period of about 3 h prior to food availability. FAA is particularly notable when animals are provided with a running wheel, as wheel-running during FAA can be more intense than normal nocturnal running. This activity is thought to be stimulated by the action of a circadian clock, as food availability that is timed in non-circadian intervals (e.g., 18 h) does not result in FAA (33). In addition, FAA persists for several cycles under conditions of total food deprivation after entrainment to timed restricted feeding, and also does so in the absence of the SCN (34, 35). Little work has been published concerning female responses to timed restricted feeding. Rats will entrain their activity rhythms to restricted feeding if in constant dark, but there is no evidence for or against a sex difference in the ability to entrain to restricted feeding. A few studies have investigated the role of the reward system on entrainment to feeding using palatable foods. To date sex differences in FAA have only been identified in mice. When receiving a high fat food as a snack, male mice exhibit anticipatory activity and females do not (36). However, females show activity at the time of previous food delivery on subsequent days, suggesting that the females are still timing the arrival of the food but are not showing the anticipatory activity. There is some evidence to suggest that female motivation for sugary/fat foods is modulated by the estrous cycle (37). This could impact the response of female mice to a palatable food cue during *ad libitum* conditions. The fact that circadian clock-driven anticipatory activity can occur under both normocaloric and hypocaloric conditions suggests that there are multiple drivers of FAA, a motivational circuit and a homeostatic circuit (38).

PUBERTAL EFFECTS ON RHYTHMS

Puberty represents a period of substantial changes in physiology and endocrine profiles. Given that gonadal steroids have an impact on adult circadian behavior it is reasonable to hypothesize that the circadian system would be responsive to this dynamic endocrine environment. Pubertal changes in circadian phase have been noted in mice (39, 40) and rats (41, 42). However, these studies were limited to male or female subjects and therefore do not address the issues of sex differences. The role of gonadal steroids during the pubertal period on circadian development has been investigated in the rat and the degus (43–47). During the pubertal period, rats and degus (both male and female) have a bimodal distribution of locomotor activity during their active phase. By adulthood, activity in intact male rats and degus changes to a unimodal activity pattern. Pre-pubertal GDX in rats leads to a less extreme bimodal distribution that is maintained into adulthood, suggesting that gonadal steroids are responsible for the consolidation of activity to the beginning of the

active phase. In males, GDX results in a loss or reduction in pubertal-related changes in circadian parameters, whereas GDX in females results in a more variable response (45). In degus, pre-pubertal GDX of both males and females stabilizes circadian phase and the bimodal distribution of activity persists into adulthood as seen in intact female degus (46). These studies taken together provide evidence for the developmental role of gonadal steroids during puberty in setting circadian behavioral rhythm parameters.

SITE OF ACTION

A direct action of gonadal steroids on the SCN would be most likely be mediated by one or more of the steroid hormone receptors: ERα, ERβ, androgen receptor (AR), progesterone receptor (PR), or G protein-coupled estrogen receptor 1 (GPER1). ERα, ERβ, and AR are all expressed in the SCN (13, 48–51), with sexual dimorphisms present in ERβ, and AR (51). For a full review of the neuroanatomical aspects of sexual dimorphism in the circadian system, see (52). In addition, the SCN receives input from other estrogen receptor-positive regions of the brain (53), providing another potential mechanism for steroid-modulation of SCN function. Finally, it is possible that signals from some peripheral organs may be sexually dimorphic, and when activated they may alter rhythmic function in a sex-specific manner.

CONCLUSION

There is a clear need for further research to understand how biological sex and gonadal hormones can regulate behavioral rhythmicity. Sex differences in basic behavioral activity rhythms are modest in scope; however, this may not be the case if the system is challenged. For example, there are substantial sex differences in the brain's reward system (54) that could interact with circadian clocks in such a way that result in differential responses of the circadian clock to addictive drugs.

In general, the data reviewed in this article suggests that most initial research studies on the circadian system should be carried out using both male and female animals. If no sex differences in the results are observed, researchers can then decide whether their approach will work best using a single or mixed sex approach. For example, in experiments where precision of the onset of activity is critical, it may be appropriate to conduct studies in males, though modern mathematical techniques for ascertaining rhythm phase make this less of an issue than when activity rhythms were assessed by visual inspection of actograms. Failure to make use of both males and females in studies may result in important physiological and behavioral phenomenon remaining undiscovered.

Finally, most studies that look at female steroid hormone effects on circadian rhythms make use of experimental methods involving gonadectomy and hormone replacement. While such studies yield valuable information about the mechanisms of hormone influences on rhythms, they do not represent the normal physiological variation that occurs across a normal estrous cycle. It is understandable that this has occurred, given the increase in animal numbers needed and workload involved in measuring estrous cycle phase, however, such studies will become increasingly important as we learn more about the potential influence of gonadal hormones on behavioral circadian outputs.

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Response of the nitrergic system to activation of the neuroendocrine stress axis

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Exposure to stressful stimuli causes activation of the hypothalamic-pituitary-adrenal axis which rapidly releases high concentrations of glucocorticoid stress hormones, resulting in increased cellular metabolism and spontaneous oxygen and nitrogen radical formation. High concentrations of nitrogen radicals, including nitric oxide, cause damage to cellular proteins in addition to inhibiting components of the mitochondrial transport chain, leading to cellular energy deficiency. During stress exposure, pharmacological inhibition of nitric oxide production reduces indicators of anxiety- and depressive-like behavior in animal models. Therefore, the purpose of this review is to present an overview of the current literature on stress-evoked changes in the nitrergic system, particularly within neural tissue.

Keywords: anxiety, depression, hypothalamic-pituitary-adrenal axis, glucocorticoids, nitrergic system, nitric oxide, peroxynitrite, reactive nitrogen species

INTRODUCTION

An acute stress response is mediated by the tripartite activation of the sympatho-adrenal-medullary (SAM), hypothalamic-spinal-adrenal (HSA), and hypothalamic-pituitary-adrenal (HPA) axes. The first of these axes to respond is the autonomic SAM system, consisting of several hypothalamic and brainstem nuclei, notably including the locus ceruleus (Jansen et al., 1995). The locus ceruleus is the primary source of central noradrenergic signaling, functioning via the ascending noradrenergic bundle and descending through preganglionic neurons in the intermediolateral cell column (IML) of the spinal cord to innervate the adrenal medulla (Sara, 2009; Ulrich-Lai and Herman, 2009). Through the combined action of catecholamines, this system promotes increased arousal and vigilance and is responsible for the rapid generation of the "fight-or-flight" response (Jansen et al., 1995). The paraventricular nucleus (PVN) of the hypothalamus is considered the apex of the HPA stress response as release of corticotropin-releasing hormone from the parvocellular neurosecretory neurons triggers anterior pituitary corticotrophs to release the pro-opiomelanocortin fragment, adrenocorticotropic hormone (ACTH), into the circulation. However, the PVN also facilitates corticosterone release directly through the HSA stress axis via adrenocortical innervation from the IML, and indirectly via an alternative stress pathway involving prolactin release (Buijs et al., 1999; Lowry, 2002; Ulrich-Lai et al., 2006; Jaroenporn et al., 2009). This ultimately sensitizes the adrenal gland to ACTH, resulting in corticosterone release from the *zona fasciculata* of the adrenal cortex thereby exerting the characteristics downstream cellular and metabolic effects of stress (Buijs et al., 1999; Lowry, 2002; Weiser et al., 2011). Adrenal glucocorticoids accelerate cellular metabolism to increase available energy which consequently increases free radical formation in specific regions of the central nervous system (Spiers et al., 2013). This stress-induced increase in radical production, including nitric oxide (NO) formation, leads to oxidative and nitrosative stress (Chen et al., 2014). Furthermore, the toxic metabolite of NO, peroxynitrite, is capable of inhibiting components of the mitochondrial respiratory chain, leading to cellular energy deficiency (Sarti et al., 2012). Since dysfunction of the nitrergic system has been implicated in the neuropathogenesis of several stress-related disease states, the present review summarizes our current understanding and advances relating to the impact of stress on the nitrergic system.

NITRIC OXIDE BIOSYNTHESIS AND FUNCTIONS

Nitric oxide, a gaseous free radical belonging to the family of reactive nitrogen species (RNS), is synthesized through the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) in the presence of oxygen, NADPH, and cofactors such as tetrahydrobiopterin (Andrew and Mayer, 1999). There are three main isoforms, each with a specific distribution profile; neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III) (Stuehr, 1999). Though nNOS is predominantly active in the cytosol of central and peripheral neurons for signaling and regulation, it has also been found in the sarcolemma and cytoplasm of all muscle fibers (Frandsen et al., 1996). Interestingly, nNOS is present in the hippocampus, hypothalamus, pituitary, and adrenal gland, suggesting co-localization with the HPA axis (Lai et al., 2005; Gadek-Michalska et al., 2012). Furthermore, several studies have demonstrated transcriptional regulation of nNOS by glucocorticoids in the hippocampus, implicating its importance in the stress response, although the upstream promoter of NOS1 does not carry a glucocorticoid responsive element (López-Figueroa et al., 1998; Reagan et al., 1999; Zhou et al., 2011). There are four nNOS splice variants, α, β, γ, and μ, with nNOSα being the most dominant and

therefore being physically and functionally coupled to the glutamate receptors of the N-methyl-D-aspartate (NMDA) subtype through their mutual post-synaptic density-95/discs-large/zona occludens-1 (PDZ) binding motif (Eliasson et al., 1997). Within the hippocampus, local calcium influx through NMDA receptors can trigger the production of NO, which subsequently activates its receptor, soluble guanylyl cyclase, leading to release of second messenger cyclic guanosine monophosphate (cGMP) (**Figure 1**). This NO-cGMP signaling has been implicated in the induction of hippocampal long-term potentiation which is known to be one of the principal mechanisms in learning and memory (Schuman and Madison, 1991; Arancio et al., 1996; Kelley et al., 2010). The nNOSμ mainly localizes in the skeletal muscles, with nNOSμ-deficient muscles being myopathic (Percival et al., 2008). The β variant lacks the PDZ domain while nNOSγ has very little to no enzymatic activity (Eliasson et al., 1997). Endothelial NOS contains a putative shear stress responsive element in the promoter region of the NOS3 gene while the protein is membrane-bound to the golgi apparatus and caveolae, producing NO mainly in the endothelium of blood vessels responsible for vasodilation and smooth muscle relaxation (Smith et al., 2006). The inducible form of NOS responds at the transcriptional level to inflammatory factors (Zamora et al., 2000; Aktan, 2004). Within the central nervous system, the iNOS-mediated release of NO by astrocytes and microglia has a major role in antimicrobial and tumoricidal activity in response to various inflammatory signals (Hua et al., 2002; Brantley et al., 2010). Moreover, upon transcriptional activation, this soluble subtype can produce micromolar levels of NO and is known to be associated with diseases such as artherosclerosis, rheumatoid arthritis, diabetes, septic shock,

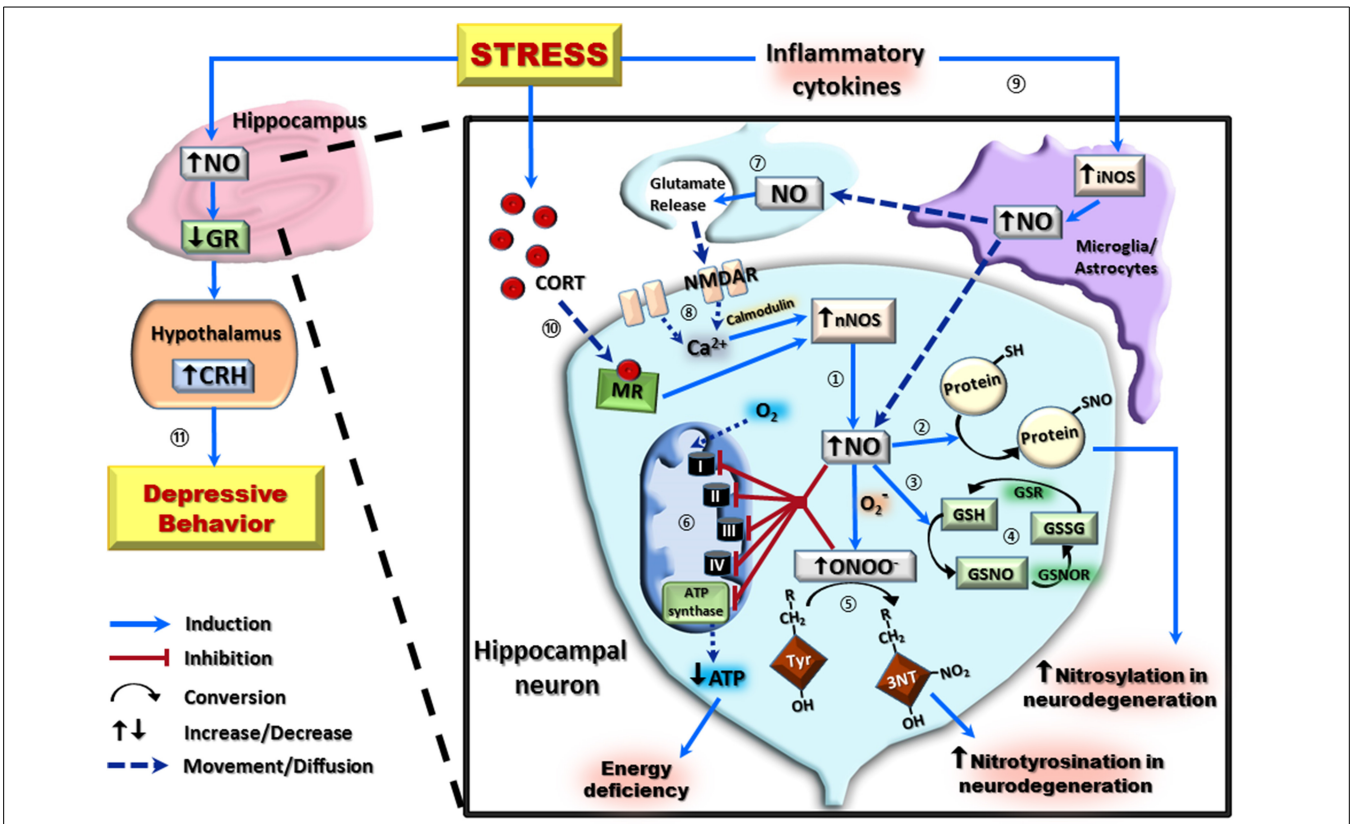

FIGURE 1 | A schematic representation of the nitrergic system and its downstream effects in hippocampal neurons following stress exposure. In hippocampal neurons, the majority of nitric oxide (NO) production occurs via the conversion of L-arginine to L-citrulline by the neuronal isoform of nitric oxide synthase (nNOS) ①. High concentrations of NO can then covalently bond with protein thiol groups (protein-SH) to form S-nitroso-proteins (protein-SNO) ② or interact with the reduced form of glutathione (GSH) forming S-nitrosoglutathione (GSNO) ③. This can be regenerated back to GSH via an initial conversion to oxidized glutathione (GSSG) by S-nitrosoglutathione reductase (GSNOR), and subsequent reduction of GSSG by glutathione reductase (GSR) ④. Interaction of NO with the superoxide radical (O_2^-) results in the formation of the neurotoxic radical, peroxynitrite ($ONOO^-$) which irreversibly reacts with protein tyrosine (Tyr) residues to form 3-nitrotyrosine (3-NT) ⑤. Increased NO and $ONOO^-$ are capable of causing cellular energy deficiency by inhibition of all components of the electron transport chain (Complex I–IV), including ATP synthase, resulting in decreased ATP production ⑥. Both post-synaptically produced NO, and NO produced by the inducible isoform of nitric oxide synthase (iNOS), can act as a neurotransmitter on pre-synaptic neurons ⑦. This pre-synaptic NO causes glutamate release, which activates post-synaptic NMDA receptors (NMDAR) to increase calcium (Ca^{2+}) concentration and, in the presence of calmodulin, further potentiate nNOS-derived NO ⑧. Stress exposure increases NO by activating inflammatory cytokines to potentiate glial/astrocyte iNOS activity ⑨, and by increasing circulating corticosterone (CORT) which induces nNOS activity via a mineralocorticoid receptor (MR)-mediated pathway ⑩. This increase in NO results in downregulation of hippocampal glucocorticoid receptors (GR) and subsequently increases hypothalamic corticotropin-releasing hormone (CRH) to induce depressive-like behaviors ⑪.

and multiple sclerosis (Kuhlencordt et al., 2001; Hill et al., 2004; Maki-Petaja et al., 2008; Heemskerk et al., 2009; Soskic et al., 2011). Both nNOS and eNOS are constitutively active isoforms producing low concentrations of NO (in the nanomolar range) over long periods and are activated by calcium ions though transient binding to the calcium-binding protein, calmodulin (Knott and Bossy-Wetzel, 2009). Comparatively, the inducible form of NOS can produce high concentrations of NO in relatively short periods and is calcium independent due to a high binding affinity to calmodulin (Aktan, 2004). The inorganic ions, nitrate and nitrite (NO*x*), were previously thought to be the end products of NO metabolism. However, recent studies have demonstrated a NOS-independent pathway in which NO can be produced by reducing NO*x*, a reaction catalyzed by xanthine reductase under low oxygen tension and low pH environment. The NO produced by this nitrate-nitrite-NO pathway may have similar roles to NO generated from the L-arginine-NOS pathway representing an important secondary pool (see review by Lundberg et al., 2008).

NITROSATIVE STRESS

High levels of NO and its derivatives are destructive to cellular components such as proteins, lipids and DNA. Nitric oxide can react directly with molecular oxygen to produce two relatively strong oxidants, nitrogen dioxide and dinitrogen trioxide. However, at physiological levels of NO these reactions are relatively slow. A primary reaction in the production of RNS is the combination of NO and superoxide anions to form the highly reactive metabolite, peroxynitrite, a potent neurotoxin (Lipton et al., 1993). It has been suggested that NO and peroxynitrite can disrupt adenosine 5′-triphosphate (ATP) synthase and almost all components of the mitochondrial respiratory chain (Almeida and Bolanos, 2001; Sarti et al., 2012). These RNS reversibly or irreversibly inhibit mitochondrial oxygen consumption, particularly at complex IV (also known as cytochrome *c* oxidase), and may lead to cellular energy deficiency and ultimately cell death in pathological conditions (Sarti et al., 2012). Inhibition of cytochrome *c* oxidase by NO and peroxynitrite causes neuronal dysfunction and, in addition to high iNOS expression, has been observed in the cortex of Alzheimer's patients (Mutisya et al., 1994; Haas et al., 2002).

S-nitrosylation is the covalent attachment of NO to the thiol side chain of the amino acid cysteine, forming other NO derivatives termed *S*-nitroso-proteins. Under physiological conditions, it has been demonstrated that NO is converted to the nitrosonium ion which subsequently S-nitrosylates the NMDA receptor, thereby preventing glutamate excitotoxicity by blocking calcium influx, promoting cell survival (Lipton and Stamler, 1994). Excessive production of NO can be counteracted by conjugation with reduced glutathione, forming the stable adduct *S*-nitrosoglutathione which has important role in signal transduction and regulation of a variety of protein functions (Klatt and Lamas, 2000; Anand and Stamler, 2012). Abnormal *S*-nitrosylation to proteins such as apolipoprotein E, cyclin-dependent kinase 5, dynamin-related protein 1, parkin, peroxiredoxin 2, protein disulfide isomerase, heat-shock protein 90, and X-linked inhibitor of apoptosis have all being linked to neurodegenerative conditions such as Alzheimer's and Parkinson's diseases (Anand and Stamler, 2012). Lastly, peroxynitrite provokes protein nitrotyrosination, an irreversible chemical addition of a nitro group to the tyrosine residue in target proteins generating 3-nitrotyrosine. This post-translational modification usually impairs the normal physiological function of the proteins and therefore nitrotyrosination has been used as a marker in several neurodegenerative conditions such as amyotrophic lateral sclerosis (Peluffo et al., 2004). These aspects of the nitrergic system have been summarized in **Figure 1**.

STRESS-EVOKED MODULATION OF THE NITRERGIC SYSTEM

It has been generally accepted that psychophysiological stress is associated with upregulation of NOS mRNA expression and enzymatic activity. For example, a single 6 h acute immobilization stress induces upregulation of iNOS expression and activity in the cerebral cortex which is mediated by the NMDA receptor and subsequent activation of the transcriptional factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Madrigal et al., 2001). The acute stress-induced activation of the NMDA receptor also increases tumor necrosis factor-alpha (TNFα) via upregulation of TNFα-convertase. Antagonism of TNFα-convertase prevents the stress-induced translocation of NF-κB and subsequent iNOS expression, thus confirming the involvement of TNFα (Madrigal et al., 2002). This is also supported by Shirakawa et al. (2004) who demonstrated glutamatergic activation and not catecholaminergic drive of the hypothalamic paraventricular nucleus to be responsible for the acute stress-induced increase in NO metabolites. Interestingly, biting activity is capable of suppressing the stress-induced increase in hypothalamic nNOS mRNA expression in rats (Hori et al., 2005). A single 2 h acute restraint stress significantly increases the density of neurons expressing nNOS visualized by nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry in the amygdaloid nucleus, an effect delayed by 5 days in the hippocampus and entorhinal cortex (Echeverry et al., 2004). Predator-induced post-traumatic stress significantly increases nNOS positive neurons and total NO*x* in the medial prefrontal cortex 7 days after the 10 min predator stress treatment (Campos et al., 2013). Conversely, Chakraborti et al. (2014) demonstrated that acute restraint stress causes a reduction in total NO*x* and an increase in the major endogenous NOS inhibitor, asymmetric dimethylarginine, in whole brain homogenates. This suggests that the stress-induced NO*x* increases in regions such as the hippocampus and hypothalamus may hold a high degree of functional significance. These biochemical changes in NO*x* and asymmetric dimethylarginine were observed alongside anxiety-like behavior and were more pronounced in male compared to female rats. The pharmacological blockade of estrogen biosynthesis exacerbated these biochemical and behavioral changes in females, suggesting that the observed sex differences are due to a protective role of estrogen. Interestingly, bilateral injection of an NMDA receptor antagonist, NOS inhibitor, or NO scavenger into the dorsal hippocampus attenuated autonomic responses such as hypertension and tachycardia following a 60 min acute restraint stress, suggesting that NMDA/NOS activation within the hippocampus plays a role in

autonomic modulation during stress (Moraes-Neto et al., 2014). Another study from the same group proposed a glutamatergic NMDA receptor-NO-cGMP signaling pathway in modulating contextual fear conditioning within the dorsal hippocampus, where intra-hippocampal injection of NMDA receptor antagonist DL-AP7, NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO), and cGMP inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), attenuated the fear-conditioned response (Fabri et al., 2014).

Chronic immobilization stress has been shown to increase NO*x*, iNOS activity, and peroxynitrite-induced 3-nitrotyrosine accumulation in cortical neurons (Olivenza et al., 2000). Notably, de Pablos et al. (2014) recently found a degree of regional specificity associated with this chronic stress-induced iNOS expression, with little to no constitutive expression in the substantia nigra following 9 days of unpredictable stress exposure. However, this same unpredictable stress model potentiates iNOS expression following exposure to exogenous immunostimulatory stressors such as lipopolysaccharides. Recent studies in several animal paradigms have demonstrated that inhibitors of NOS significantly modulate stress-related behaviors. In support of these findings, the commercially available antidepressant paroxetine, a selective serotonin reuptake inhibitor, also possesses NOS inhibition capability (Finkel et al., 1996). Wegener and Volke (2010) have reviewed and summarized these studies including data on each of the NOS inhibitor's specificity and potency, and their anxiolytic- and antidepressant-like properties. Chronic unpredictable mild stress increases plasma nitrite levels and iNOS mRNA expression in the cortex, in addition to damaging cortical neurons and inducing depressive-like behavior (Wang et al., 2008; Peng et al., 2012). These effects can be attenuated or prevented using NOS inhibitors, which was demonstrated by intra-hippocampal injection of the selective iNOS inhibitor, aminoguanidine, resulting in suppression of the chronic unpredictable mild stress-induced depressive-like behavior in rats (Wang et al., 2008). Regional infusion of a selective nNOS inhibitor 7-nitroindazole (7-NI) into the hippocampus showed antidepressant-like effects similar to those with the iNOS inhibitor, aminoguanidine (Joca and Guimaraes, 2006). Likewise, the anxiogenic-like behavior observed in rats during ethanol withdrawal is inhibited by administration of the selective iNOS inhibitor, 1400W, into the dorsolateral periaqueductal gray (Bonassoli et al., 2013). The data with intra-cerebral NOS inhibition is further supported by studies using systemic treatment. Intraperitoneal injection of 1400W increases survival of cortical neurons and decreases the depressive-like behavior in mice (Peng et al., 2012). The nNOS inhibitor 1-(-2-trifluoromethylphenyl)-imidazole (TRIM) given systemically 30 min prior to testing induces anxiolytic-like behavior shown by increased time spent in the light compartment of a light-dark compartment test (Volke et al., 2003). Furthermore, TRIM administration decreased the immobility time in the forced swimming test, demonstrating an antidepressant-like effect comparable to the tricyclic antidepressant imipramine. In agreement with these observations, Ulak et al. (2008) injected TRIM intraperitoneally 50 min before a forced swim test and showed the involvement of the serotonergic system in the antidepressant-like actions of TRIM. This was further clarified in a later study in which the serotonin type II receptors were found to be responsible for this effect (Ulak et al., 2010). Furthermore, Joung et al. (2012) demonstrated that following a 2 h immobilization stress, the selective inhibitor 7-NI produced its anxiolytic-like effects shown by an increase in the time spent on the open arms of the elevated plus-maze through the direct reduction of NO metabolites in the PVN and locus ceruleus. A less specific NOS inhibitor, L-N^G-Nitroarginine methyl ester (L-NAME), injected systemically 30 min prior to testing shows protective effects against chronic swim stress-induced impairment of passive avoidance learning and hyperalgesia in rats (Nazeri et al., 2014). In a similar vein, Ferreira et al. (2012) performed behavioral, genomic, and proteomic analyses in rats and suggested that the antidepressant-like effects of NOS inhibition may involve the expression of additional factors including members of the glutathione redox system.

Genetic animal models have also contributed to the current understanding of nitrergic changes in stress. Thus, inhibition of NO production by nNOS gene deletion in mice suppressed hippocampal neurogenesis and exhibited antidepressant-like properties while nNOS over-expression in the hippocampus was essential for chronic stress-induced depression (Zhou et al., 2007). Recently, a number of studies have proposed a regulatory role of NO on the limbic HPA stress axis. Zhang et al. (2010) used mice lacking the nNOS gene to demonstrate an anxiolytic-like phenotype when tested using an elevated plus-maze, similar to normal mice treated with intra-hippocampal microinjection of the selective nNOS inhibitor 7-NI. The authors proposed a signaling pathway involving the activation of serotonin type IA receptors which mediate, via an unknown mechanism, the downregulation of hippocampal nNOS, leading to a decrease in NO and subsequent inhibition of cAMP response element-binding (CREB) protein phosphorylation. A follow up study elucidated further the link between NO and the HPA axis by showing that chronic mild stress and glucocorticoid exposure lead to hippocampal nNOS overexpression via activating hippocampal mineralocorticoid receptor (MR) (Zhou et al., 2011). The excessive nNOS-derived NO significantly downregulated local glucocorticoid receptor (GR) expression through either the soluble guanylyl cyclase/cGMP or peroxynitrite/extracellular signal-regulated kinase (ERK) signaling pathways. The significant downregulation of GR in the hippocampus leads to an elevation in hypothalamic corticotropin-releasing hormone and the depressive-like behaviors in mice as illustrated in **Figure 1**. It is important to note that nNOS deletion, infusion of intrahippocampal nNOS inhibitor, and NO-cGMP signaling blockade prevented the chronic mild stress-evoked behavioral modification. Interestingly, this chronic glucocorticoid-induced MR-nNOS-NO pathway is exclusive to the MR-rich hippocampus and drives HPA axis hyperactivity through impaired negative feedback (Zhu et al., 2014).

The considerable body of evidence from animal models is progressively expanding and supported by modest but significant clinical studies. Several reports have shown that increased levels of NO metabolites are present in depressed and autistic patients (Suzuki et al., 2001; Sogut et al., 2003; Lee et al., 2006). Patients with recurrent depressive behavior displayed higher plasma NO*x* concentrations which were associated with cognitive impairment

(Talarowska et al., 2012). Galecki et al. (2010, 2011) discovered single nucleotide polymorphisms in exon 22 of the NOS2A gene (iNOS) and exon 29 of the NOSI gene (nNOS) in depressed Caucasian individuals. Furthermore, three single nucleotide polymorphisms located at the regulatory region of NOSI gene are responsible for the susceptibility of an individual to depressive disorders (Sarginson et al., 2014).

SUMMARY

A growing body of evidence suggests that the etiology of anxiety and depression-related conditions can be derived from the sensitization of particular stress-related circuits that are "primed" following exposure to a short-term stressor. The duration for stress-related circuitry priming far exceeds responses to adrenergic and glucocorticoid-mediated stress responses. Understanding the mechanisms underlying the induction of this long latency will provide a significant link between stress and the pathogenesis of anxiety and depressive disorders. The nitrergic system has been implicated in regulating both short and long-term activation of the stress response, with a variety of NOS inhibitors demonstrating potent anxiolytic and antidepressant activity. The intrinsic cross talk between neuroendocrine stress and nitrergic system activation is now an important physiological consideration. Further understanding the role of this system is important in identifying early players in stress-induced pathological conditions.

AUTHOR CONTRIBUTIONS

Author Hsiao-Jou Cortina Chen managed the literature searches, wrote the first draft of the manuscript, and produced the graphic. Author Jereme G. Spiers, Conrad Sernia and Nickolas A. Lavidis critically revised the manuscript. All authors have approved the final version of the manuscript for journal submission.

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Activation of the hypothalamic-pituitary-adrenal stress axis induces cellular oxidative stress

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Glucocorticoids released from the adrenal gland in response to stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis induce activity in the cellular reduction-oxidation (redox) system. The redox system is a ubiquitous chemical mechanism allowing the transfer of electrons between donor/acceptors and target molecules during oxidative phosphorylation while simultaneously maintaining the overall cellular environment in a reduced state. The objective of this review is to present an overview of the current literature discussing the link between HPA axis-derived glucocorticoids and increased oxidative stress, particularly focussing on the redox changes observed in the hippocampus following glucocorticoid exposure.

Keywords: corticosterone, hypothalamic-pituitary-adrenal axis, oxidative stress, reactive oxygen species, redox status, stress

INTRODUCTION

The acute neuroendocrine response to adverse stress stimuli is characterized by the tripartite activation of the three stress axes including the autonomic sympathetic nervous system, the direct neural innervation of the adrenal cortex, and a cascade of hypothalamic hormonal messengers. Both the sympathetic system and the hypothalamic spinal adrenal axis utilize direct neural innervations of the adrenal medulla and cortex respectively to release adrenal catecholamines and prime the adrenal cortex for subsequent hormonal activation (Jansen et al., 1995; Buijs et al., 1999). This is initiated by neurosecretory neurons in the paraventricular nucleus of the hypothalamus, which release both corticotropin-releasing hormone and arginine vasopressin into the portal circulation of the pituitary gland. These two factors synergistically act on pituitary corticotroph cells to stimulate the release of the pro-opiomelanocortin peptide fragment, adrenocorticotropic hormone, into the circulation. Adrenocorticotropic hormone activates the melanocortin 2 receptor in the *zona fasciculata* of the adrenal cortex to initiate *de novo* synthesis and release of glucocorticoids, primarily cortisol in humans and corticosterone in rodents (Spiga et al., 2011). Together, this hormone cascade constitutes the hypothalamic-pituitary-adrenal (HPA) axis and is the primary system underlying stress physiology.

The physiological effects of corticosterone in the brain are canonically mediated through a near-ubiquitously expressed (with the exception of the suprachiasmatic nucleus of the hypothalamus) low affinity (KD $\approx$ 5.0 nM) glucocorticoid receptor (GR), and a regionally specific high affinity (KD $\approx$ 0.5 nM) mineralocorticoid receptor (MR) (Reul and de Kloet, 1985; Rose et al., 2012). Typically, these receptors reside in the cytoplasm heterocomplexed with heat shock proteins and immunophilins, which maintain the affinity of the hormone-binding domain (Pratt and Toft, 1997). The lipophilic steroid hormones are cell membrane permeable and bind these receptors, causing the dissociation of the chaperone proteins and translocation into the nucleus where the activated receptor complex forms GR and MR homo- or hetero-dimers that interact with specific glucocorticoid responsive elements in the promoter regions of genomic DNA. Both GR and MR elicit equivalent activity at glucocorticoid responsive elements and these interactions can result in transcriptional activation or repression of target genes depending on the cellular context (De Kloet et al., 1998). Transcriptional repression can also be mediated through protein-protein interactions specifically with activated GR and transcription factors such as NFκB, offering a possible mechanism through which delineation of receptor function occurs between the GR and MR (van der Burg and van der Saag, 1996; De Kloet et al., 1998). Termination of the HPA response to stress is mediated through multiple negative feedback loops and utilizes both genomic and non-genomic actions of the GR (Calogero et al., 1988; Groeneweg et al., 2011). In circulation, adrenal glucocorticoids reach peak total plasma concentrations approximately 30 min after activation of the HPA axis (Qian et al., 2011). At the cellular level, these hormones act in conjunction with catecholamines to facilitate glucose availability and increase metabolic rate, which in turn increases spontaneous production of free radicals (Teague et al., 2007; Du et al., 2009).

FREE RADICAL PRODUCTION

The process of aerobic metabolism utilizes oxygen to generate ATP in the mitochondrial electron transport chain (Halliwell and Gutteridge, 1989). During this process, 1–3% of all electrons "leak" from the electron transport chain to react with oxygen, generating superoxide radicals instead of being reduced to water (Liu et al., 2002; Muller et al., 2004; Cash et al., 2007). Although this occurs at both complex I and complex III of the electron transfer chain, the majority occurs at complex I where it is facilitated by succinate (Liu et al., 2002) (**Figure 1**). Most of the cellular superoxide is produced inside the inner mitochondrial membrane where the mitochondrial concentration of superoxide can be between 5–10 times that of the cytosol or nucleus

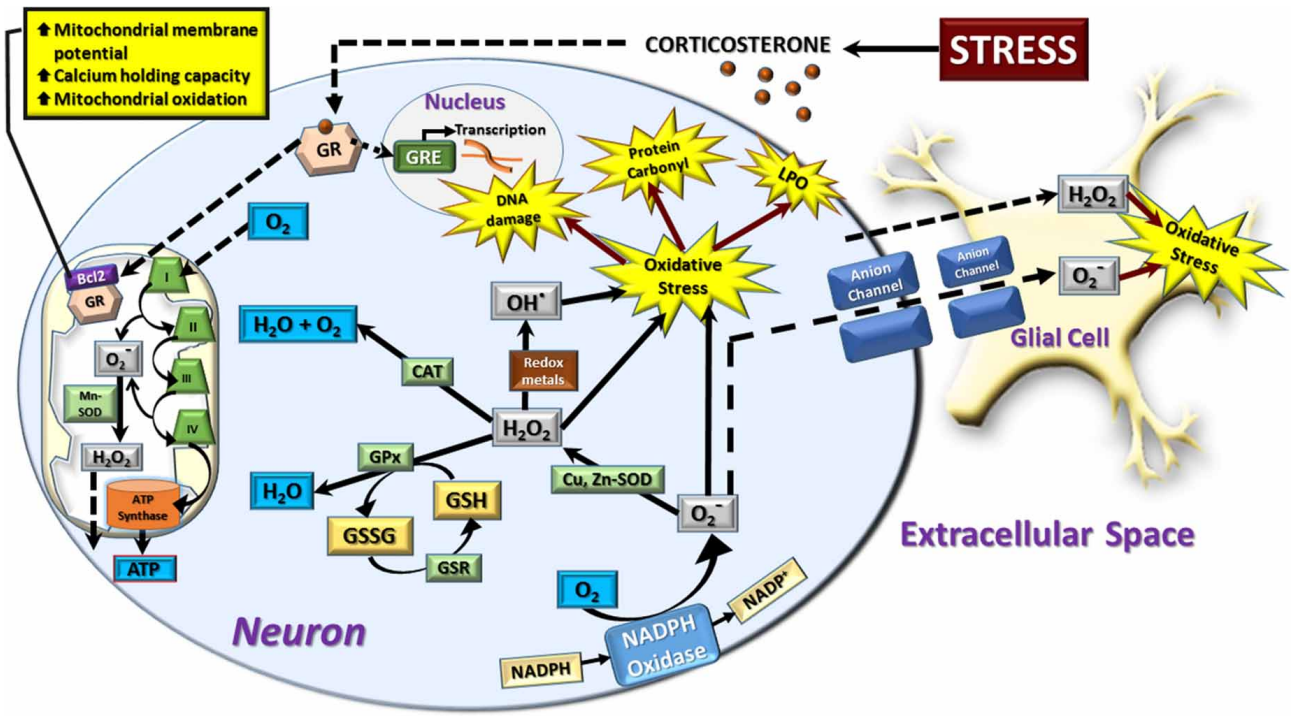

FIGURE 1 | Schematic representation of neural redox reactions. Stress causes an increase in corticosterone which activates cytosolic glucocorticoid receptors (GR). These translocate into the nucleus to modulate gene transcription through glucocorticoid responsive elements (GRE), or co-localize with the anti-apoptotic Bcl-2 protein and translocate into the mitochondria. This increases mitochondrial membrane potential, calcium holding capacity, and mitochondrial oxidation. The increase in cellular metabolic rate promotes ATP synthesis in addition to spontaneous superoxide (O_2^-) production via complex I and III of the electron transport chain. This is dismutated to hydrogen peroxide (H_2O_2) by manganese superoxide dismutase (Mn-SOD) and can be further converted to hydroxyl radical (OH^-) or reduced to water by the mitochondrial antioxidant pathway. In the cytosol, a major source of superoxide production is via the oxidation of NADPH via NADPH oxidase. Cytosolic superoxide is dismutated to hydrogen peroxide by copper, zinc-superoxide dismutase (Cu, Zn-SOD). Hydrogen peroxide is neutralized by catalase (CAT) or glutathione peroxidase (GPx) which oxidizes the reduced form of glutathione (GSH) to oxidized glutathione (GSSG). GSH is then regenerated from GSSG via the glutathione reductase (GSR) enzymatic system. Hydrogen peroxide can also interact with superoxide radicals and/or transition metals such as Fe^{2+} or Cu^{2+} to produce the highly toxic hydroxyl radical by Haber-Weiss and Fenton chemistry. An increase in the production of superoxide, hydrogen peroxide, and hydroxyl radicals leads to a state of cellular oxidative stress which causes oxidative damage to DNA, protein carbonyl formation, and membrane lipid peroxidation (LPO). Hydrogen peroxide is membrane permeable and moves freely from mitochondrial to cytosolic compartments, in addition to traversing the extracellular space to affect neighboring neurons and glial cells. Superoxide radicals can also induce oxidative stress in neighboring cells by diffusing through membrane-bound anion channels. The majority of neuronal and astrocytic reactive oxygen species are produced by mitochondrial oxidation, while other cell types such as microglia rely heavily on the cytosolic NADPH-oxidase system to produce a respiratory burst in response to invading pathogens. However, in comparison to glial cells, neurons display a relatively poor expression of endogenous antioxidants, making them more vulnerable to oxidative stress.

(Cadenas and Davies, 2000). The remainder of mitochondrial superoxide is primarily formed by complex III on both sides of the mitochondrial membrane and by extra-mitochondrial flavoenzymes (Zimmerman and Granger, 1994; Cadenas and Sies, 1998; Brand et al., 2004). Superoxide then undergoes spontaneous or enzymatic dismutation via superoxide dismutase (SOD) to generate hydrogen peroxide. Although hydrogen peroxide is relatively stable, subsequent interactions with superoxide radicals and/or transition metals such as Fe^{2+} or Cu^{2+} induce production of the highly toxic hydroxyl radical by Haber-Weiss and Fenton chemistry. This radical has been suggested to cause more damage to biological systems than any other reactive oxygen species (ROS) due to the extreme reactivity and very short *in vivo* half-life of ≈9–10 ms (Pastor et al., 2000).

Outside the mitochondrion, there are three major processes responsible for the production of free radicals, principally in the form of reactive oxygen and nitrogen species. The first process involves the production of hydrogen peroxide as a by-product of fatty acid catabolism by peroxisomes (Ames et al., 1993; Wanders and Waterham, 2006). Although technically not a free radical, hydrogen peroxide is still classed as a ROS for its role in Fenton and Haber-Weiss chemistry (Cimen, 2008). Within the peroxisome, the majority of hydrogen peroxide is neutralized via canonical catalase activity or peroxidation to another catalase substrate (Wanders and Waterham, 2006; Valko et al., 2007). However, under some conditions hydrogen peroxide can avoid degradation and escape the peroxisome, ultimately leading to cellular and nucleic acid damage (Kasai et al., 1989). The second process involves the reliance of the innate immune system on the ability of phagocytic cells such as neutrophils to engulf and digest foreign pathogens. Following the encapsulation of the foreign body into a phagosome, neutrophils increase their

oxygen consumption specifically to supply the dormant NADPH-oxidase with molecular oxygen (Dahlgren and Karlsson, 1999). This enzyme catalyzes the oxidation of NADPH to form two superoxide radicals which, together with reactive metabolites of superoxide including hydrogen peroxide and hypochlorite, constitutes the respiratory burst responsible for killing the pathogen (Ames et al., 1993; Dahlgren and Karlsson, 1999; Stadtman et al., 2007; Valko et al., 2007). Hayashi et al. (2008) have also demonstrated that NADPH-oxidase derived ROS can also be produced via a non-genomic mechanism following aldosterone administration in rat cardiac myocytes. The third process involves redox metals such as $Fe^{2/3+}$, Cu^{2+}, and Mn^{2+} which are essential for electron transfer in many enzymatic reactions, including the antioxidant enzymes of the oxidative cascade. However, these transitional metal ions can also undergo reactions resulting in the production of hydroxyl radicals (Rovira et al., 2007).

THE ENDOGENOUS ANTIOXIDANT SYSTEM

In order to neutralize ROS, cells use a suite of enzymatic and non-enzymatic antioxidants, ultimately attempting to neutralize the radical by reduction to water. In the typical ROS reduction cascade, SOD is the top-tier antioxidant, catalyzing the dismutation of this radical to hydrogen peroxide. This is achieved through the transfer of electrons across the catalytic metal core of the enzymes to reduce the superoxide radicals. In mammals, the two main isoforms of SOD are the copper, zinc-SOD which are found throughout most cell compartments, and the manganese-SOD that is specific for mitochondria. Catalase enzymes are centered around an iron-containing ferriheme group that acts as the transition metal during the reduction of hydrogen peroxide. Access to this active site is fairly specific as the channel opening is narrow and does not allow the passage of large molecules. High concentrations of superoxide anions are able to inactivate catalase by oxidizing the heme group in the active site. To prevent this, catalase binds NADPH to maintain this group in the reduced state (Nordberg and Arner, 2001; Fridovich et al., 2007). Hydrogen peroxide can also be reduced directly by both peroxiredoxins, which allow the oxidation of an active cysteine thiol group to degrade one molecule of hydrogen peroxide into two molecules of water, and the glutathione-glutathione peroxidase system. Reduced glutathione (GSH) is the most abundant intracellular thiol-based antioxidant which protects cells against oxidative stress by acting as a substrate for the selenium-containing glutathione peroxidase, subsequently forming oxidized glutathione disulphide (GSSG). In turn, GSSG is regenerated to GSH by glutathione reductase in a NADPH-dependent mechanism (Barycki et al., 2007) (**Figure 1**). The cellular concentrations of this soluble tripeptide range from 1 to 11 mM in the cytosol, 3–15 mM in the nucleus, and 5–11 mM in the mitochondria, although mitochondrial GSH requires membrane transport even against a concentration gradient (Shen et al., 2005; Valko et al., 2007). Glutathione also acts as a substrate for the glutaredoxins, which reduce proteins that have been glutathionylated by reducing GSSG to a mixed disulphide protein and GSH. As the occurrence of mixed disulphides increases with increasing concentrations of GSSG, the ratio of the reduced to oxidized fractions (GSH/GSSG) of GSH within cells is often used as a reliable indicator of redox imbalance and has been shown to strongly influence cell cycle progression in proliferating cells (Menon et al., 2003; Öztürk and Gümüslü, 2004; Rose et al., 2012). The transcription factor nuclear factor-erythroid-2-related factor 2 (Nrf2) is essential for the coordinated induction of cytoprotective enzymes and related proteins in response to oxidative and electrophilic stresses (Itoh et al., 1999; Uruno and Motohashi, 2011). This transcription factor regulates a battery of redox genes such as the glutathione synthesis enzyme gamma-glutamylcysteine synthetase, glutathione peroxidase, glutathione disulphide reductase, glutathione *S*-transferase, thioredoxin-1, and heme oxygenase-1 through their antioxidant response element (Rushmore et al., 1990; Inamdar et al., 1996; Moinova and Mulcahy, 1999; Kim et al., 2003; Kwak et al., 2003). Under basal conditions, Nrf2 activity is sequestered in part by the actin-associated Keap1 protein within the cytoplasm. Activation of Nrf2 in response to oxidative and electrophilic agents is thought to be initiated by disruption of this Nrf2-Keap1 complex, releasing Nrf2, which translocates into the nucleus to regulate the expression of downstream targets.

PHYSIOLOGICAL ROLE OF REACTIVE OXYGEN SPECIES

Although there is a general negative connotation associated with ROS production, they have important cellular functions under normal physiological conditions. Even low levels of the extremely reactive hydroxyl radical have been shown to activate guanylate cyclase, stimulating the production of a cGMP second messenger cascade (Mittal and Murad, 1977). In fact, the physiological roles of ROS vary significantly, ranging from specific oxidations of cysteine groups affecting enzyme activity and function, to cellular redox sensing in the determination of cell differentiation fate (Nicotera et al., 1985; Dalton et al., 1999; Wang et al., 2011). Progression of the cell cycle itself has demonstrated dependence on radicals produced by NADPH-oxidase modulating mitogenic pathways (Burhans and Heintz, 2009). Several transcription factors are also regulated directly by ROS-induced modifications, thereby modulating the downstream expression of several gene families (Dalton et al., 1999). Notably, the dimerized protein products of immediate-early response genes FOS and JUN, AP-1, is activated by ROS through redox reactions and post-translational modification of the individual FOS and JUN proteins (Buscher et al., 1988; Abate et al., 1991; Devary et al., 1991). Under normal conditions, any excessive ROS not participating in these physiological functions are reduced by the antioxidant system. However, an imbalance between the production of ROS and the ability of the antioxidant defense system to readily detoxify the reactive intermediates, termed oxidative stress, leads to damage of biological macromolecules and dysregulation of normal metabolism (Sies, 1997; Nordberg and Arner, 2001).

ADRENAL GLUCOCORTICOIDS AND OXIDATIVE STRESS

Increased secretion of adrenal glucocorticoids following physical and/or psychological stress exposure subsequently liberates glucose through gluconeogenesis, glycogenolysis, and lipolysis (Teague et al., 2007). Although increased metabolism alone generates ROS, glucocorticoids have demonstrated both direct and indirect modulatory roles in the onset of oxidative stress.

Furthermore, both chronic oxidative stress and glucocorticoid exposure promote gliogenesis over neurogenesis in hippocampal neural stem cell progenitors and may be the direct result of accumulated mitochondrial oxidative stress (Wang et al., 2011; Chetty et al., 2014). Glial cells play an important but poorly understood role in the modulation of neuronal redox state. It has recently been shown that astrocyte-derived L-lactate potentiates NMDA receptor activity by modulating neuronal redox status (Yang et al., 2014). This neuron-glia interaction can also increase noradrenaline release from the locus ceruleus and hypothalamic ATP production (Cortes-Campos et al., 2011; Tang et al., 2014). Furthermore, Reyes et al. (2012) have demonstrated that neuronal NADPH oxidase-derived superoxide can traverse the extracellular space to modulate the redox state in neighboring neurons and astrocytes (**Figure 1**). However, in comparison to astrocytes, neurons are known to have relatively poor expression of endogenous antioxidants, making them highly susceptible to oxidative stress.

Glucocorticoids induce neuronal oxidative stress directly through enhanced mitochondrial respiration and oxidative phosphorylation. This was demonstrated clearly in a study by Du et al. (2009), showing that acute incubation of cortical neurons with corticosterone increased mitochondrial oxidation, membrane potential, and calcium-holding capacity in a dose and time-dependent manner (**Figure 1**). This was further clarified by You et al. (2009) using the oxidation product dichlorofluorescien as a ROS indicator in organotypic hippocampal slice cultures exposed to the synthetic glucocorticoid, dexamethasone, and the glucocorticoid receptor antagonist, RU486. The single and combination use of these compounds demonstrated that hippocampal neuronal death, marked by propidium iodide, was selectively induced by glucocorticoid exposure, while other steroid hormones had no effect. Acute incubation with dexamethasone increased the hippocampal oxidative status by approximately 200% in a dose dependent manner, an effect that was ameliorated with pre-treatment of RU486 or the ROS-scavenger, N-acetyl-L-cysteine. Furthermore, 4 h of dexamethasone incubation, induced the highest increase in oxidative status, with concurrent gene expression up-regulation of the ROS-producing enzyme NADPH-oxidase, while the antioxidant enzyme glutathione peroxidase was significantly down-regulated (You et al., 2009). This indicates that the increase in oxidative status is produced by a glucocorticoid-dependent and transcriptional increase in pro-oxidative drive, with concurrent inhibition of the antioxidant defense system, ultimately leading to increased neuronal cell death. Cortical and hippocampal neural cultures have also established that 24 h of glucocorticoid exposure increases basal ROS production and exacerbates the concomitant ROS produced by adriamycin redox cycling, which negatively affects survival in hippocampal neurons (McIntosh and Sapolsky, 1996). This is supported by *in vivo* evidence that administration of exogenous corticosterone over a 14 day period increases hippocampal oxidative indicators including lipid peroxidation and protein carbonyls, while the enzymatic antioxidants SOD, catalase, and glutathione peroxidase activities are all decreased (Sato et al., 2010). This study, performed by Sato et al. (2010), also demonstrated increased apoptosis and decreased glucocorticoid receptor expression, a hallmark of chronically high glucocorticoids, in the hippocampus. Interestingly, this study also utilized a serum measurement of iron-induced superoxide formation in blood serum, establishing that this peripheral marker increased with chronic corticosterone administration. Further *in vivo* studies have shown that both corticosterone administration and restraint stress for 21 days induce an overall decrease in GSH in addition to SOD, catalase, glutathione transferase, and glutathione reductase activities in whole brain, liver, and heart tissues (Zafir and Banu, 2009). This study, by Zafir and Banu (2009), also demonstrated that these treatments increased lipid peroxidation and oxidized protein carbonyl groups in the same tissues. However, in the brain, this overall oxidative increase is likely due to increases in specific subregions. For example, Mendez-Cuesta et al. (2011) used an acute immobilization stress to induce increased lipid peroxidation and decreased SOD activity in a highly oxidative-vulnerable region, the striatum. Interestingly, they found that the decrease in SOD activity was exclusively due to the mitochondrial isoform, manganese-SOD, while the copper, zinc-SOD showed little change. Furthermore, Lucca et al. (2009) used a chronic mild stress regime to show that SOD decreased and oxidized protein carbonyl groups increased in several cerebral regions including the striatum and hippocampus, while the cerebellum remained largely unaffected. We have also observed the induction of regional specific oxidative stress in the hippocampus but not the amygdala following an acute restraint stress, demonstrating that this process is not exclusive to chronic stress exposure (Spiers et al., 2013). Recently, exposure to glucocorticoids or dexamethasone demonstrated inhibitory action over the Nrf2-dependent antioxidant pathway, causing an increase in hepatic and osteoblastic cell ROS, an effect attenuated by exogenous sulforaphane (Kratschmar et al., 2012; Lin et al., 2014).

Interestingly, ROS attenuate the glucocorticoid-induced down-regulation of pro-opiomelanocortin in pituitary corticotrophs, thereby promoting an increase in HPA axis activity via reduced negative feedback (Asaba et al., 2004). The expression and nuclear internalization of GR have also demonstrated susceptibility to highly pro-oxidative environments (Okamoto et al., 1999; Zhou et al., 2011). Using a cultured fluorescently labeled chimeric GR, Okamoto et al. (1999) demonstrated that nuclear translocation of GR following acute dexamethasone treatment is impaired in the presence of hydrogen peroxide. This effect was reduced in the presence of exogenous antioxidants or following substitution of serine for a redox-sensitive cysteine residue. The dissociation of heat shock proteins from the cytosolic GR was also impaired in a pro-oxidative environment, indicating that there may be multiple redox regulatory roles involved in the cellular response to glucocorticoids (Okamoto et al., 1999 reviewed in Tanaka et al., 1999). These observations highlight that maintenance of a balanced redox state is critical for normal cellular homeostatic function within the neuroendocrine system.

CONCLUSIONS

Although oxidative stress and elevated glucocorticoids are both observed in a number of chronic pathologies, the delineation between physiological function and pathological insult is complex and remains unclear. In particular, the role of ROS in neuron-neuron and neuron-glia interactions is an area that requires

attention. Based on the observations of increased gliogenesis in the presence of high levels of glucocorticoids, the mobility of ROS in the extracellular space, and the relative paucity of neuronal endogenous antioxidants, we suggest that glia may be playing an active role in response to neuronal oxidative stress. This may have important implications for neurodegenerative conditions involving redox-sensitive regions such as the hippocampus. These observations highlight that maintenance of a balanced redox state is critical for normal cellular homeostatic function and relies heavily on hormonal cues from the neuroendocrine stress system.

AUTHOR CONTRIBUTIONS

Author Jereme G. Spiers managed the literature searches and wrote the first draft of the manuscript. Author Hsiao-Jou Cortina Chen produced the graphic and revised the manuscript. Author Conrad Sernia and Nickolas A. Lavidis critically revised the manuscript. All authors have approved the final version of the manuscript for journal submission.

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Oxytocin during development: possible organizational effects on behavior

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Oxytocin (Oxt) is a neurohormone known for its physiological roles associated with lactation and parturition in mammals. Oxt can also profoundly influence mammalian social behaviors such as affiliative, parental, and aggressive behaviors. While the acute effects of Oxt signaling on adult behavior have been heavily researched in many species, including humans, the developmental effects of Oxt on the brain and behavior are just beginning to be explored. There is evidence that Oxt in early postnatal and peripubertal development, and perhaps during prenatal life, affects adult behavior by altering neural structure and function. However, the specific mechanisms by which this occurs remain unknown. Thus, this review will detail what is known about how developmental Oxt impacts behavior as well as explore the specific neurochemicals and neural substrates that are important to these behaviors.

Keywords: affiliative behavior, aggressive behavior, estrogen receptor alpha, maternal behavior, oxytocin receptor, parental behavior, sexual behavior, vasopressin

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Introduction

In the landmark paper by Phoenix et al. (1), the organizational effects of gonadal steroids on sexual behavior in guinea pigs (*Cavia porcellus*) were established and resulted in the formulation of the "organizational/activational hypothesis of sexual differentiation." This hypothesis, which states that perinatal exposure to gonadal steroids is important for the sexual differentiation of the brain and behavior, is part of the foundation on which the field of behavioral neuroendocrinology has been built. Given the importance of this concept, it is perhaps not surprising that in the last 55 years this hypothesis has been extended to include another critical time period – puberty – as well as other hormones and behaviors, with one of these hormones being oxytocin (Oxt) (2–4).

Oxytocin is a mammalian neurohormone composed of nine amino acids, known for its peripheral effects on parturition and lactation (5–7), as well as its central neuromodulatory effects on social behaviors such as affiliative, aggressive, and parental behaviors (8, 9). While much of the work on Oxt has focused on its involvement in the acute modulation of behavior, there is also evidence that exposure to Oxt during early life is important for the proper development of neural pathways and subsequent sex-specific behaviors. These latter observations have led researchers to hypothesize that Oxt has organizational effects on the brain (3, 10). This is an exciting possibility as developmental exposure to Oxt appears to impact many of the species-specific and sex-specific social behaviors it is known to modulate in adulthood – thus, a reevaluation and broadening of our understanding of Oxt's effects seems warranted. To encourage this shift in paradigm, in this review we will highlight recent research on how developmental exposure to Oxt affects behavior as well as the specific neurochemicals and neural substrates that underlie these behaviors.

Oxytocin's Postnatal and Peripubertal Effects on Behavior

Noonan and colleagues (10) were the first to hypothesize that Oxt might have organizational effects on the brain and behavior. Their work demonstrated that a single intracisternal injection of Oxt in postnatal day (PND) 3 rats (*Rattus norvegicus*) significantly increases novelty-induced grooming at 4 months of age (10) [grooming is known to be directly enhanced by Oxt administration in adults (11)]. However, when a study by Boer and colleagues failed to replicate the aforementioned findings in an open-field test (12), investigations into the organizational effects of Oxt largely fell by the wayside. Yet, in the last 12 years, there has been a resurgence of research in this area, with studies in numerous species providing converging evidence that Oxt during development can have permanent effects on the brain and behavior (3, 13–29) (see **Table 1**). The implications are profound, as they are changing the way that we think about how Oxt works – no longer as just a neuromodulator, but rather as a neurohormone that contributes to the development of behaviors that are essential for survival.

Sexual, Affiliative, and Aggressive Behaviors

Work in prairie voles (*Microtus ochrogaster*) has shown that there are clear behavioral consequences when Oxt is manipulated during early postnatal development, and that these effects are sex specific; this latter observation is perhaps not surprising since it is well known that Oxt expression is sexually dimorphic in numerous species, including prairie voles (30–34). In female prairie voles, a single injection of Oxt on PND1 results in increases in intrasexual aggression in adults, suggesting strengthened pair bond formation (18), whereas an injection of both Oxt and an Oxt receptor antagonist (OTA), also on PND1, decreases the frequency of mating bouts in both adult males and females (20). A single postnatal injection of Oxt also increases intrasexual aggression in adult female mandarin voles (*Lasiopodomys mandarinus*) after exposure to a male (35). In male prairie voles, an injection of Oxt at both low (1 mg/kg) and high (4 mg/kg) doses on PND0 increases partner preference and social contact in adults compared to controls (19, 36); however, a dose of 2 mg/kg does not (36). Male mandarin voles that receive a single postnatal injection of Oxt increase their mounting behavior at PND60 (37). So, at least in voles, developmental Oxt appears to increase pair bond formation in females, and increase affiliative behaviors in males, whereas the effects on sexual behavior appear to be stimulatory in both males and females; though they may be dose dependent in a manner that is not linear.

In mice (*Mus musculus*), there are sex differences in the effects of neonatal Oxt manipulation on affiliative behaviors, though the findings differ from observations in prairie voles. Female mice administered an OTA (3 μg/20 μL) on the day of birth have decreases in social approach when tested at 8–15 weeks (29) as measured in a three chambered apparatus based on that developed by Crawley (38). On the other hand, male mice administered an OTA on PND0 display social approach behaviors similar to what is observed in the control conditions (29). So, in mice it appears that Oxt exposure in neonate females may promote affiliative behaviors, while having no effect in males. Exploration of why there may be no effect in male mice can be found in the section titled "Potential Effects of Oxytocin During Fetal Development."

The aforementioned impact of developmental Oxt on behavior is not limited to rodents. Postnatal intranasal Oxt administration in 2.5- to 8-week-old pigs (*Sus scrofa*) increases intrasexual aggressive behavior and decreases social contact (39), which is similar to what is observed in female prairie voles (18). [Pigs were selected as an experimental model because their neuroanatomy, as well as their physiology and development, is more similar to humans than rodent models (40).] This particular study is unusual in that it is the only one to use an ungulate animal model, as well as one of only a few studies to use intranasal Oxt administration. Because of the uniqueness of this species, it is not known if these findings are broadly applicable to other species.

There is also evidence that Oxt's developmental effects on sociability may not be limited to the perinatal period, but may also extend into peripubertal development (2). Bowen and colleagues administered daily Oxt injections to male rats from PND33 to PND42 and then tested them in a social interaction test on PND55. Oxt-treated males spent more time in close proximity to conspecifics and made more active social contacts than controls (41). Researchers from this same research group later conducted an experiment in which male rats were given Oxt injections every 3 days from PND28 to PND55. When tested in a social interaction test at PND70, their behavior appeared similar to what was described above, with their spending more time in close proximity to conspecifics than control animals (42). There have also been studies investigating the effects of peripubertal intranasal Oxt on behavior in rodents. Male prairie voles administered low, medium, and high doses of intranasal Oxt daily during the approximate time between weaning and sexual maturity, from PND21 until 42, display increases in social contact during the treatment window, and those given low and medium doses increase their preference for strangers when tested from PND43–60 (43). In female BTBR mice (a model of autism spectrum disorders), daily intranasal Oxt treatment from PND21 to 50, followed by testing on PND55, increases time spent sniffing a novel mouse over a novel object, essentially rescuing "sociability" to levels observed in wild-type mice (44). Another study found that chronic intranasal Oxt from 12 to 23 weeks reduced social behavior in adult male mice when they were tested 1 h after Oxt administration (45). While the studies described above may differ somewhat in terms of the details of their findings, it does appear that intranasal Oxt treatment during peripubertal development facilitates social interactions in both males and females. However, it is not clear how long lasting these effects are. Given that the peripubertal period is another critical developmental window for the organizational effects of hormones (2), more work focused on these types of questions is needed, especially as intranasal Oxt is being considered as a treatment for various neurodevelopmental conditions (46–48), in particular children and adolescents diagnosed with autism spectrum disorders (49–52).

Parental Behavior

In male prairie voles, treatment with an OTA in neonates decreases alloparental behaviors. Specifically, males injected with an OTA

TABLE 1 | A summary of the design and findings of studies investigating how developmental manipulation of oxytocin activity affects long-term behavioral expression.

Species	Treatment	Age at treatment	Age at assay	Behavioral outcomes in females	Behavioral outcomes in males	Reference
Prairie vole	3.0 µg Oxt i.p.	PND0	PND60–90	ND	Rescued social behavior diminished by saline, ↑ partner preference	(19)
Prairie vole	0.3 µg OTA i.p.	PND0	PND60–90	ND	Rescued social behavior diminished by saline	(19)
Prairie vole	3.0 µg Oxt i.p.	PND0	Adult	↑ Aggression, ↓ social behavior after exposure to male	↔	(18)
Prairie vole	0.3 µg OTA i.p.	PND0	Adult	↔	↔	(18)
Prairie vole	3.0 µg Oxt i.p.	PND0	PND8	↔	↔	(23)
Prairie vole	0.3 µg OTA i.p.	PND0	PND8	↓ Ultrasonic vocalizations after isolation	↔	(23)
Prairie vole	3.0 µg Oxt i.p.	PND0–7	PND8	↔	↔	(23)
Prairie vole	0.3 µg OTA i.p.	PND0–7	PND8	↑ Ultrasonic vocalizations after isolation	↔	(23)
Prairie vole	3.0 µg Oxt i.p.	PND0	PND21	↔	↔	(14)
Prairie vole	0.3 µg OTA i.p.	PND0	PND21	↔	↓ Parental behavior, ↑ pup-directed aggression	(14)
Prairie vole	3.0 µg Oxt i.p.	PND0	PND60	↔	↔	(14)
Prairie vole	0.3 µg OTA i.p.	PND0	PND60	↔	↔	(14)
Prairie vole	3.0 µg Oxt i.p.	PND0	PND75	↓ Mating bout frequency	ND	(20)
Prairie vole	0.3 µg OTA i.p.	PND0	PND75	↓ Mating bout frequency, ↑ litter production success	ND	(20)
Prairie vole	1.0 mg/kg Oxt i.p.	PND0	PND55–69	↔	ND	(16)
Prairie vole	2.0 mg/kg Oxt i.p.	PND0	PND55–69	↔	ND	(16)
Prairie vole	4.0 mg/kg Oxt i.p.	PND0	PND55–69	↑ Pup retrievals	ND	(16)
Prairie vole	8.0 mg/kg Oxt i.p.	PND0	PND55–69	↑ Preference for stranger	ND	(16)
Prairie vole	750 nL CMV-Oxtr NAcc-specific	PND21	PND60–88	↑ Parental behavior, ↑ preference for partner	ND	(57)
Prairie vole	0.08 IU/kg Oxt intranasally	PND21–42	PND43–60	↔	↑ Preference for stranger	(43)
Prairie vole	0.80 IU/kg Oxt intranasally	PND21–42	PND43–60	↔	↑ Preference for stranger	(43)
Prairie vole	8.00 IU/kg Oxt intranasally	PND21–42	PND43–60	↔	↔	(43)
Mandarin vole	3.0 µg Oxt s.c.	PND0	PND60–90	↑ Aggression after exposure to male	↑ Social contact	(35)
Mandarin vole	3.0 µg Oxt s.c.	PND0	PND60–90	↑ Preference for partner, suppressed maintenance of preference, ↓ aggression toward stranger	↑ Mounting of partner, ↓ aggression toward stranger	(37)
Rat	1.0 µg/2.0 µL Oxt intracisternally	PND3–4	PND120	↑ Novelty-induced grooming	↑ Novelty-induced grooming	(10)
Rat	1.0 mg/kg Oxt s.c.	PND10–14	PND60–94	↑ Weight gain, ↑ tail-flick withdrawal latency	↑ Weight gain, ↑ tail-flick withdrawal latency	(63)
Rat	1.0 mg/kg Oxt i.p., 0.5 µg EB	PND0–7	PND75	↓ Sexual receptivity	ND	(28)
Rat	1.0 mg/kg Oxt i.p., 5.0 µg EB	PND0–7	PND75	↓ Sexual receptivity	ND	(28)
Rat	1.0 mg/kg Oxt i.p., 10.0 µg EB	PND0–7	PND75	↔	ND	(28)
Rat	0.1 mg/kg OTA i.p., 0.5 µg EB	PND0–7	PND75	↓ Sexual receptivity	ND	(28)
Rat	0.1 mg/kg OTA i.p., 5.0 µg EB	PND0–7	PND75	↔	ND	(28)

(Continued)

TABLE 1 | Continued

Species	Treatment	Age at treatment	Age at assay	Behavioral outcomes in females	Behavioral outcomes in males	Reference
Rat	0.1 mg/kg OTA i.p., 10.0 µg EB	PND0–7	PND75	↔	ND	(28)
Rat	1.0 mg/kg Oxt i.p.	PND33–42	PND50–72	ND	↑ Open-field exploration, ↑ social interaction, ↓ ethanol consumption	(41)
Rat	0.5 mg/kg Oxt i.p.	PND28–55	PND70–72	ND	↑ Social proximity	(42)
Rat	1.0 mg/kg Oxt i.p.	PND28–55	PND70–72	ND	↑ Social proximity	(42)
Rat	0.5 mg/kg TGOT i.p.	PND28–55	PND70–72	ND	↔	(42)
Rat	1.0 mg/kg TGOT i.p.	PND28–55	PND70–72	ND	↔	(42)
Mouse	2.0 µg Oxt s.c.	PND0	PND1–3	Rescued feeding behavior in Magel2$^{-/-}$ mice	Rescued feeding behavior in Magel2$^{-/-}$ mice	(64)
Mouse	3.0 µg OTA s.c.	PND0	PND1–3	Lethal feeding deficiency	Lethal feeding deficiency	(64)
Mouse	3.0 µg Oxt i.p.	PND0	8–15 weeks	↔	↔	(29)
Mouse	0.3 µg Oxt i.p.	PND0	8–15 weeks	↔	↔	(29)
Mouse	3.0 µg OTA i.p.	PND0	8–15 weeks	↓ Parental care	↓ Parental care	(29)
Mouse	0.3 µg OTA i.p.	PND0	8–15 weeks	↔	↔	(29)
Mouse	0.15 IU Oxt intranasally	12–23 weeks	1 h post	ND	↓ Social behavior	(45)
Mouse	0.30 IU Oxt intranasally	12–23 weeks	1 h post	ND	↓ Social behavior	(45)
Mouse	0.80 IU Oxt intranasally	PND21–50	PND55	Rescued diminished social sniffing in BTBR mouse	↔	(44)
Pig	50.0 µg Oxt intranasally	PND1–3	2–8 weeks	↑ Aggression	↑ Aggression	(39)

ND, no data; EB, estradiol benzoate.

within 24 h of birth and later tested on PND21 decrease their parental care, as measured by fewer retrievals, less time spent huddling over pups, and increases in pup-directed attacks. These effects also appear to be transient, as they are not observed when the same animals are tested again on PND60 (14). In laboratory mice, treatment with an OTA also reduces alloparental care, but in both sexes. Specifically, treatment with an OTA on PND0 decreases the total number of pups retrieved by females and increases pup retrieval latencies in males (29). Oxt treatment also increases the responsiveness of females to pups, as measured by approach times, although this effect is dose dependent, with the lowest dose of Oxt resulting in longer approach times compared to saline controls (16). Thus, it appears that Oxt signaling is important for normal displays of alloparental care, which is consistent with its role in lowering the threshold for maternal care in rodents (53–56).

Work by Keebaugh and Young has utilized viral vectors to overexpress the Oxtr and study the organizational effects of Oxt on behavior during puberty. By injecting an adeno-associated viral vector into the nucleus accumbens (NAcc) shell of female prairie voles at PND21 they were able to facilitate alloparental care, as measured by reductions in approach times and increases in time spent licking and grooming pups compared to controls (57). This gene delivery approach has helped to identify a specific neural substrate – the NAcc – on which Oxt may act. The NAcc, which is a part of the brain's reward circuit, is known to be important in numerous motivated behaviors – it not only expresses the Oxtr but is also one of the regions in which the Oxtr is more highly expressed in biparental prairie voles compared to non-monogamous species that do not exhibit biparental care (58–62).

Other Behaviors

While only a few studies have investigated Oxt's organizational effects on other behaviors, these studies confirm that Oxt's developmental effects are not limited to its impact on social behavior. In rats, repeated administration of Oxt between PND10 and PND14 results in weight gain in both males and females, increases in the gut hormone cholecystokinin, and longer withdrawal latencies in the tail-flick test at PND60, which suggests an increase in pain threshold (63). Male and female mice with a genetic disruption that models Prader–Willi syndrome usually exhibit a lethal feeding deficiency. This feeding deficiency can be rescued with an injection of Oxt on PND0, or induced in wild-type mice by injecting an OTA 1–1.5 h after birth (64). These observations are consistent with what is observed in Oxt and Oxtr knockout (−/−) mice. These mice develop late-onset obesity in the absence of hyperphagia (65, 66). However, these developmental effects of Oxt differ from what is observed in adults, where Oxt is hypothesized to have anorexigenic effects (67). Since developmental Oxt appears to impact aspects of energy homeostasis, which in turn can affect behavior, additional work in this area is warranted.

Finally, in the study by Bowen and colleagues (41) mentioned in the previous section, peripubertal Oxt administration also affects anxiety-like behavior and ethanol consumption later in life. Male rats given daily Oxt injections from PND33 to PND42 and tested in an emergence test on PND50 traveled further and spent more time in the open-field compared to controls, which would suggest

that Oxt had an anxiolytic effect. When tested on PND72 and later, Oxt-treated males consumed significantly less alcohol (i.e., beer) than controls, while water consumption remained unaffected (41).

Summary

It is apparent that Oxt in early postnatal development and during puberty can result in long-lasting changes in behavior, including behaviors that have traditionally been associated with Oxt's acute neuromodulatory effects, such as affiliative and sexual behaviors, as well as non-social behaviors, such as nociception and ethanol consumption. While there is a lack of consensus in the data, this is in part a reflection of the difficulty of these types of studies, as there are many experimental possibilities that could result in very different outcomes. Particularly crucial is the timing window for Oxt administration, the dose of Oxt, and the behavioral endpoints measured. It is also important to note that Oxt's organizational effects appear to be sex- and species-specific, which is consistent with the complexity of its neuromodulatory role in adulthood. Thus, future behavioral work will need to continue to take a broad approach to identify Oxt's potential organizational effects, as some of these behavioral changes, such as energy metabolism and anxiety, could affect a variety of other behaviors. In the meantime, rigorous investigation of Oxt's organizational effects on neural structure and function may help clarify mechanisms by which Oxt affects brain development and ultimately behavior.

Oxytocin's Developmental Effects on Neurochemicals and Neural Substrates

The behavioral effects of postnatal and peripubertal Oxt must be rooted in structural and functional modifications to neurons, such as changes in gene expression, axonal guidance, or cell morphology. Therefore, in this section, we review what is known about how Oxt may be impacting these systems.

Effects on Estrogen Receptor Alpha

It is well established that gonadal steroids play a significant role in regulating Oxt activity (68–71). While androgens and progesterone modulate Oxt and Oxtr expression (72–75), it is the estrogens that seem to have the greatest impact on the Oxt system (68, 70, 74, 76–78). Further, these effects are not unidirectional, with the Oxt system altering gonadal steroid systems, specifically the expression of estrogen receptor α (ERα). Neonatal injections of Oxt increase ERα expression in the ventromedial hypothalamus (VMH) of adult females and Oxt treatment on PND0 increases the number of ERα-immunoreactive (ERα-ir) cells in the VMH of 3-week-old prairie voles (26). These effects appear to be rapid since neonatal prairie voles treated with Oxt also have increases in ERα-ir (24, 79) and ERα mRNA expressions (79). Similar to prairie voles, rats that are repeatedly administered Oxt from PND0 until PND7 have increases in ERα-ir at PND75 (28) and neonatal Oxt manipulation increases the expression of ERα in the hippocampus (79), ventral lateral septum (LS), and central nucleus of the amygdala (24). These results differ from reports in females where a single injection of an OTA on PND1 decreases expression of ERα in the medial preoptic area (MPOA) of adult female prairie voles, as well as increases ERα expression in the BNST, and possibly decreases expression in the medial amygdala (MeA) (26) and repeated administration of an OTA from PND0 to PND7 decreases the expression of ERα in the MPOA of adult female rats (28).

Effects on the Oxytocin and Vasopressin Systems

Early exposure to relevant stimuli is known to permanently alter the responsiveness of a hormone receptor; this phenomenon is known as *hormonal imprinting* (80). Based on this observation, it is reasonable to suspect that early Oxt exposure could affect the development of the Oxt system itself. Data from several species clearly support this idea; however, the findings are not consistent between sexes and species – in keeping with Oxt's known intersexual and interspecific variation. In female prairie voles, neonates treated with Oxt and an OTA have increases in Oxt immunoreactivity (Oxt-ir) within the PVN by 3 weeks of age. Yet, in males, Oxt has no effect on Oxt-ir, but treatment with an OTA does decrease arginine vasopressin (Avp) immunoreactivity in the PVN (27).

In addition to changes in peptide expression, neonatal manipulation of Oxt impacts Avp 1a receptor (Avpr1a) binding in a sexually dimorphic manner. Specifically, in female prairie voles, Oxt treatment on PND0 decreases Avpr1a binding in the MPOA, BNST, LS, cingulate cortex (CgCtx), and medial thalamus on PND60, but in males it increases Avpr1a binding in the CgCtx. OTA treatment in females decreases Avpr1a binding only in the BNST and CgCtx, while in males it decreases Avpr1a binding in the MPOA, BNST, and LS (15). Taken together, the delivery of Oxt or an OTA during early postnatal life appears to have nearly opposite effects on adult Avpr1a expression in females and males. Similar to what was discussed in terms of behavioral effects, there does appear to be a "critical period" of organization that extends into peripubertal development, with peripubertal Oxt administration increasing Oxtr mRNA expression in the hypothalamus (41) and plasma Oxt levels (42) in adult male rats.

Other Effects

While studies on the organizational effects of Oxt on other neurochemical systems are few and far between, there is evidence that perinatal exposure to Oxt can influence the functioning of the stress axis. Female prairie voles administered Oxt on PND1 have reductions in baseline plasma corticosterone by PND8 compared to animals treated with saline or an OTA (23). In neonatal pigs, repeated Oxt treatment increases adrenocorticotropic hormone (ACTH) at 8 weeks of age and decreases responsiveness in the dexamethasone suppression test at 11 weeks; indicative of dysregulation of the glucocorticoid response. However, pigs that receive Oxt have less blunting of the cortisol response than controls (39). So, not only is Oxt able to acutely regulate stress responses (81, 82) but appears to also be involved in the development of long-term responsiveness to stress.

There are also reported effects of neonatal Oxt on adrenergic and serotonergic receptors, which may contribute to the aforementioned effects of Oxt on feeding and social behaviors (see Other Behaviors). In rats, chronic neonatal Oxt treatment alters α_2 adrenergic receptor (α_2r) kinetics in PND130 male rats; these changes are dependent upon the nutrition of the dam to which the pups were born. Specifically, Oxt treatment rescues

the affinity (described by the dissociation constant K_d), which is decreased in placebo treated neonates of food-restricted dams compared to offspring of dams fed *ad libitum,* of the α_2r for its ligand within the nucleus of the solitary tract (NTS) and increases the number of α_2r binding sites (described by B_{max}) in the hypothalamus and amygdala of offspring born to food-restricted dams compared to controls. In pups born to dams fed *ad libitum* Oxt treatment decreases the affinity of the α_2r for its ligand in the hypothalamus, and increases the number of binding sites in the hypothalamus and NTS (83). In male prairie voles, neonatal Oxt administration also results in increases in serotonergic axon density in the anterior hypothalamus, cortical amygdala, and VMH at PND21 (21), as well as decreases dopamine turnover in the hypothalamus as well as serotonin turnover in the hypothalamus, medulla oblongata, and striatum of 4-month-old female rats (84). Thus, not only does developmental Oxt exposure affect many different neural circuits, these effects can also be highly dependent on the state of the dam. This is in keeping with a large body of research showing that maternal physiology, particularly stress (85–87) and nutrition (88–90), can greatly impact the neural and behavioral development of offspring. The previously described studies have opened the door to exploring what role developmental Oxt might play in these effects.

Summary

The organizational effects of Oxt extend to many different neurochemical systems including the gonadal steroids, the Oxt and Avp systems, and the stress axis, which suggests that Oxt's effects are widespread and complex (**Figure 1**). The brain regions most commonly affected by developmental Oxt exposure are the VMH, MPOA, BNST, LS, PVN, and several nuclei of the amygdala. Oxt or the Oxtr is expressed in some of these regions (68, 91), though their expression in these brain areas often varies depending on species, age, and sex. What is particularly interesting about the aforementioned brain nuclei is that many of them are a part of the "social behavioral network," which is comprised of neuroanatomical areas or "nodes" that are interconnected, express gonadal hormone receptors, appear to be influenced by Oxt, and are important in the regulation of many types of social behaviors (92, 93). Work from Bruce Cushing's laboratory suggests that the BNST and MeA are particularly important for the developmental effects of Oxt and ERα on social behavior (24, 26, 94) but further investigation into the entire social behavioral network is needed.

Potential Effects of Oxytocin During Fetal Development

While research into the neonatal and peripubertal developmental effects of Oxt has been ever increasing, an area that remains largely unexplored is the potential for Oxt to have organizational effects during embryonic development. These potential effects are relevant, in part, because of the increased use of Oxtr agonists, such as Pitocin, and antagonists, such as Atosiban, during human pregnancy in order to manage labor timing (95, 96). While the developmental consequences of these interventions have been reviewed elsewhere (95), the possible behavioral implications of exposing human fetuses to exogenous Oxt or Oxtr antagonists should not be ignored. Because of the complexity and ethical considerations in humans our best hope for elucidating the role of fetal Oxt is the use of animal models.

In mice, there is evidence that *in utero* exposure to Oxt is important for normal intermale aggressive behavior in adulthood. Specifically, male Oxt$^{-/-}$ mice that are born to null mutant dams show heightened aggressive behavior in adulthood (97, 98). While this phenotype cannot be rescued if the pups are cross-fostered to wild-type dams (99), it is not observed when male mice are born to heterozygous dams. One of the key differences between pups born to null mutant dams versus those born to heterozygous dams is the absence or presence of maternal Oxt. The hypothesis that the maternal Oxt may be signaling in the fetal brain is supported by studies using male Oxtr knockout (Oxtr$^{-/-}$) mice, which lack Oxtr signaling throughout development. These mice also have heightened aggressive behavior in adulthood (99, 100). However, male forebrain Oxtr knockout (Oxtr Fb/Fb) mice (101, 102), in which the *Oxtr* gene is excised 21–28 days after birth, have normal aggressive behavior in adulthood (100). These data suggest that Oxt signaling via the Oxtr during fetal development might be important for displays of aggressive behavior, and perhaps other behaviors in adulthood.

Unfortunately, very little is known about the developmental time course of the Oxt system in rodents. In rats, Oxt mRNA is observed as early as embryonic day (E) 15.5 in the PVN and E18.5 in the SON (103). The mRNA for the Oxt carrier protein neurophysin-I is available as early as E16 in the PVN and SON,

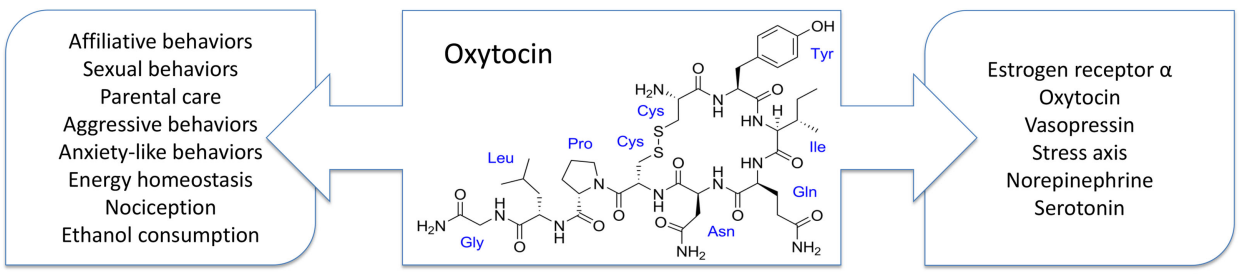

FIGURE 1 | Developmental exposure to oxytocin is known to affect many behaviors (left) and neurotransmitter and neurohormone systems (right). These behavioral effects are often species and sex specific, which is consistent with oxytocin's neuromodulatory role in adults. However, how these behavioral changes are rooted in the observed alterations in neurochemistry remains unknown.

and the Oxt peptide is seen by PND7 in the SON and PVN and E21 in the pituitary (104). Prairie voles also have a postnatal increase in Oxt peptide expression, with the number of Oxt expressing neurons significantly increasing from PND1 to PND21 in both males and females (27). While less is known about the development of the Oxtr, in rats, Oxtr binding has been identified as early as E14 in undifferentiated neurons (104). In mice, a study by Hammock and Levitt (105), which focused primarily on Oxtr binding during postnatal development, found Oxtr binding in the brains of E18.5 C57BL/6J mice. However, this was the only embryonic time point that was examined. It is plausible that the "critical window" for the manipulation of the Oxt system is not the same in males as in females, since there could be sex differences in its development. Therefore, future research in this area should consider the potential for sex differences in the development of the Oxt system, as it may help to inform the conclusions that are drawn from the data.

Conclusion

Compared to what we understand about Oxt in adulthood, research into its role in development is still in its early stages and there is much to do before we really have a handle on its effects on the brain and behavior. For instance, what other behaviors are affected? How conserved are Oxt's effects across species, or within a particular sex? How are these organizational changes grounded in alterations of neuronal structure and function? How broad is the "critical window" for Oxt's effects? What are the implications for human offspring? Therefore, examination of the mechanisms that may underlie behavioral changes, including the identification of specific neural substrates, is of the utmost importance.

This is an exciting time in behavioral neuroendocrine research due to increased interest in Oxt and the social brain as well as great advancements in the foundational work, which has methodically examined the neuromodulatory effects of Oxt in animal models. However, there is still much we do not understand about the Oxt system, and filling this "knowledge gap" becomes more vital as the interest in using Oxt in clinical settings continues to increase. For the last several years, intranasal Oxt has been marketed as the "cuddle" or "love" hormone, and is being used in a variety of contexts as a therapeutic agent to promote prosocial behaviors in humans. Much of this work has been performed in the absence of dose response studies, without serious consideration of the potential developmental or long-term effects, and with little attention paid to where in the brain these effects might be mediated. Thus, it is perhaps not surprising that more recent studies in humans suggest that Oxt's effects are nuanced (as the data from animal models would suggest) and that intranasal Oxt treatment can have undesirable effects (106–108). In light of this, basic research on the Oxt system becomes ever more critical, particularly since our understanding of the developmental role of Oxt is expanding. An important first step is to home in on specific circuits, focusing on studies that will shed light on the interactions between numerous brain regions and behaviors. This approach will allow scientists to elucidate the specific mechanisms of Oxt's organizational effects on behavior – be they genetic, epigenetic, or neuroanatomical – which can then be used not only to inform human studies but also identify any conserved mechanisms between sexes and species.

Author Contributions

Both TM and HC conceived of and drafted the work.

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REVIEW
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A role for oxytocin in the etiology and treatment of schizophrenia

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Schizophrenia is a chronic debilitating neuropsychiatric disorder estimated to affect 51 million people worldwide. Several symptom domains characterize schizophrenia, including negative symptoms, such as social withdrawal and anhedonia, cognitive impairments, such as disorganized thinking and impaired memory, and positive symptoms, such as hallucinations and delusions. While schizophrenia is a complex neuropsychiatric disorder with no single "cause," there is evidence that the oxytocin (Oxt) system may be dysregulated in some individuals. Further, treatment with intranasal Oxt reduces some of the heterogeneous symptoms associated with schizophrenia. Since Oxt is known for its modulatory effects on a variety of social and non-social behaviors, it is perhaps not surprising that it may contribute to some aspects of schizophrenia and could also be a useful therapeutic agent. In this review, we highlight what is known about Oxt's contributions to schizophrenia and schizophrenia-related behaviors and discuss its potential as a therapeutic agent.

Keywords: dopamine, early life stress, glutamate, social cognition, sensorimotor gating

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Introduction

Schizophrenia, a chronic and debilitating neuropsychiatric disorder, affects 1% of the population worldwide (1). According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders, schizophrenia is characterized by a combination of negative symptoms, cognitive dysfunction, and positive symptoms (2). Negative symptoms of schizophrenia include deficits in social behaviors such as social withdrawal, anhedonia, and flattened affect. Cognitive impairments include disorganized thinking and impaired executive function, working memory, and attention (3, 4). Lastly, the positive symptoms of schizophrenia include hallucinations, paranoid delusions, and disorganized speech. Unfortunately, while current antipsychotic medications are effective at ameliorating the positive symptoms, they are not very effective at treating the negative symptoms and cognitive dysfunction associated with schizophrenia, which tend to be more pervasive and persistent (5–7).

Current antipsychotic therapies are based on the dopamine hypothesis of schizophrenia, which proposes that increases in dopamine transmission in the mesolimbic dopamine pathway, and decreases in its activity in the prefrontal cortex contribute to many of the observed symptoms (8–10). As such, typical antipsychotics are dopamine 2 (D2) receptor antagonists, which only reduce positive symptom severity. Atypical antipsychotics on the other hand are reported to alleviate the positive symptoms as well as some of the negative symptoms associated with schizophrenia. These medications inhibit the serotonin 2A receptor (5-HT_{2A}), and to a lesser extent D_2 receptors and other neurotransmitter systems associated with schizophrenia, such as the adrenergic and cholinergic systems (11). However, two large clinical studies, the Clinical Antipsychotic Trials of Intervention and Effectiveness (CATIE) and the Cost Utility of the Latest Antipsychotic Drugs in Schizophrenia Study (CUtLASS) found no

significant difference between the ability of typical and atypical antipsychotics to reduce the negative symptoms and cognitive dysfunction of schizophrenic patients (12–15). Thus, it is important to better understand the neurochemistry of the negative symptoms and cognitive dysfunction as they often precede the onset of the positive symptoms and act as better predictors of therapeutic outcome (16, 17). Due to the various combinations of symptoms and the wide range of symptom severity, diagnosis and treatment of schizophrenia are difficult; making it extremely important to elucidate which neurological factors may contribute to schizophrenia as well as identify treatments that can effectively lessen symptom severity.

Schizophrenia

Schizophrenia is a heterogeneous group of disorders, and as such no single gene can explain its pathophysiology. Hence, it is not surprising that several neurotransmitter and neuropeptide systems, beyond dopamine, have been implicated in its symptomology (**Figure 1**) [for review, see Ref. (8, 18–20)]. In addition to the dopamine hypothesis, there is the glutamate hypothesis, which supposes that it is the hypofunctioning of *N*-methyl-D-aspartate (NMDA) receptors that contribute to the negative symptoms and cognitive impairments associated with schizophrenia (19). Researchers studying the cholinergic and gamma aminobutyric acid (GABA) systems have found that these neurotransmitter systems may also play a role in both the psychotic and cognitive deficits found in schizophrenia patients (20, 21); while serotonin (5-HT) is mainly implicated in only the cognitive dysfunction associated with schizophrenia (22–24). Cannabinoids and monoamine oxidase, which modulate some of these neurotransmitter systems, also appear to also play a role in the negative symptoms and cognitive deficits (25, 26). Since many neuropeptides are often co-released with these neurotransmitters, they likely have a role to play as well. Some of these neuropeptides are neurotensin, cholecystokinin, corticotropin-releasing factor, neuropeptide y, and orexin (18). One neuropeptide that interacts with several of the aforementioned neurotransmitter and neuropeptide systems is the nonapeptide oxytocin (Oxt). Further, there is evidence that Oxt may be important to the etiology, symptom severity, and potential treatment of schizophrenia. First, in schizophrenic patients, there are reports of disruptions in the Oxt system that are affected by treatment with antipsychotics (27, 28). Second, treatment with Oxt as an adjunctive therapy is known to lessen symptom severity in some (29, 30). Third, animal models of schizophrenia suggest that Oxt may be involved in all three symptom domains (31–35).

Oxytocin

Oxt is a nine amino acid peptide hormone, synthesized primarily in neurons of the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei. To date, a single seven-transmembrane G-protein coupled receptor, known as the Oxt receptor (Oxtr), is thought

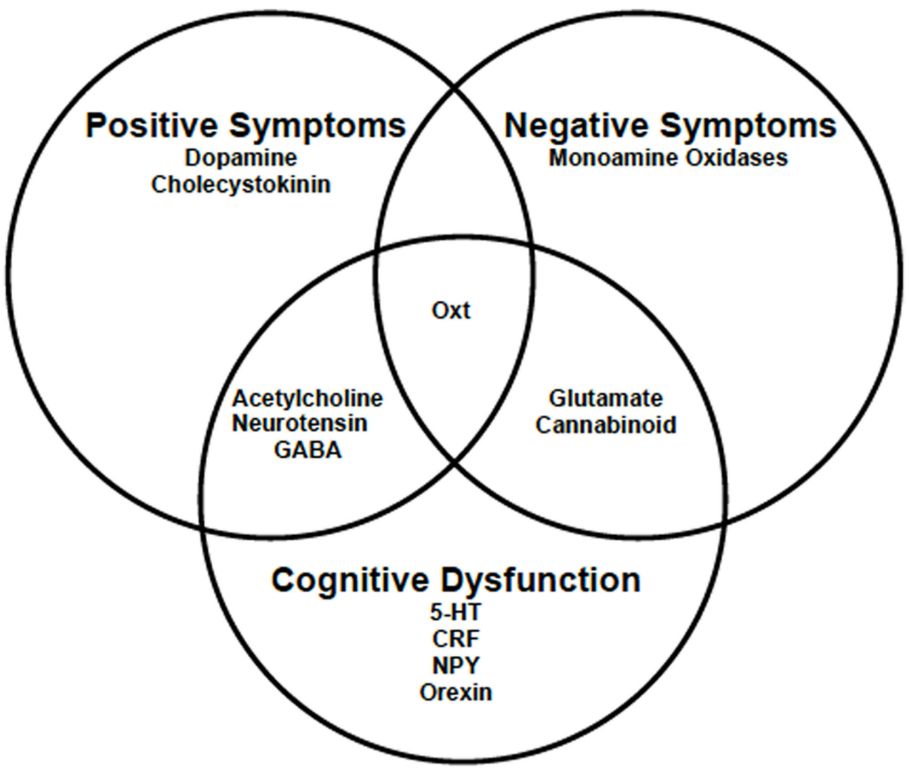

FIGURE 1 | The symptom domains of schizophrenia and the neurotransmitter and neuropeptide systems known to play a role. Research suggests that Oxt may play a role in all three symptom domains associated with schizophrenia. 5-HT, serotonin; CRF, corticotropin-releasing factor; GABA, gamma aminobutyric acid; NPY, neuropeptide y; Oxt, oxytocin.

to mediate the actions of Oxt; although Oxt can also bind to the vasopressin (Avp) 1a and 1b receptors [for review, see Ref. (36)]. The Oxt system is involved in regulating a variety of behaviors [for review, see Ref. (37)] and implicated in aspects of learning and memory, such as spatial and non-spatial memory (38–41). However, more commonly, Oxt is known for its importance to the neuromodulation of social behaviors such as social memory and social recognition, affiliative behaviors, and aggression [for review, see Ref. (37, 42)]. Social behaviors are evolutionarily important because they reduce stress and anxiety (43, 44) and in humans, Oxt facilitates prosocial behaviors and increases feelings of trust and empathy (45–47). Given the effects of Oxt on social behaviors, it is perhaps not surprising that research has focused on the role of Oxt in neuropsychiatric disorders that are characterized by disruptions in social functioning.

Abnormalities in the Oxytocin System

Due to the negative symptoms associated with schizophrenia, and the effects of Oxt on prosocial behaviors, researchers hypothesize that Oxt dysregulation may contribute to the etiology and symptom severity of schizophrenia (29, 30). This hypothesis is supported by studies indicating that disruptions in the Oxt system are linked to the pathophysiology of schizophrenia (28, 48, 49). Altered levels of Oxt are reported in patients with schizophrenia (50, 51). However, the data are conflicting with some studies reporting an increase in Oxt and the Oxt carrier protein neurophysin I (50, 51) and another reporting no change in Oxt levels in cerebral spinal fluid (CSF) (52). However, patients with higher plasma levels of Oxt have less severe positive symptoms and exhibit fewer social deficits (53, 54).

Recently it has been reported that single nucleotide polymorphisms (SNPs) of the *OXT* and *OXTR* genes may contribute to symptom severity and treatment efficacy in schizophrenic patients (55–57). SNPs of the *OXTR* gene are associated with the severity of symptoms and the improvement of the positive symptoms of schizophrenia following treatment with antipsychotics (27, 28). Additionally, post-mortem analysis of brain tissue from unmedicated schizophrenia patients found altered neurophysin immunoreactivity (ir) in the PVN, internal palladium, and substantia nigra (58). Most recently, in patients with schizophrenia and polydipsia, decreases in plasma Oxt were found to correlate with the ability to correctly identify facial emotions (48) as well as malformations in brain areas that mediate neuroendocrine responses such as the anterior lateral hippocampus and amygdala (Amg) (59). Together, these data suggest that alterations in the function of the Oxt system may underlie all three symptom domains associated with schizophrenia. Given the dysregulation of the Oxt system in patients with schizophrenia, Oxt has been studied as a candidate for use as a therapeutic.

Human studies suggest that Oxt may have antipsychotic properties [for review, see Ref. (60, 61)]. Previous work found that injections of Oxt reduce the symptoms of psychosis and anhedonia in patients with schizophrenia (62, 63). Due to the ease of delivery, researchers are now utilizing intranasal administration of Oxt. It should be noted that there is an ongoing debate in the field on whether or not intranasal administration of Oxt is able to cross the blood–brain barrier, but there is evidence that intranasal administration increases Oxt concentrations in CSF in humans and animal models (64–66). In healthy patients, intranasal Oxt increases holistic processing, divergent thinking, and creative cognition (67), and studies in patients diagnosed with schizophrenia report that intranasal Oxt can be beneficial. Specifically, intranasal Oxt can facilitate social cognition (30, 68–70) and alleviate some of the cognitive deficits and positive symptoms in patients with schizophrenia (69). Yet, intranasal Oxt may be most effective as an adjunctive therapy to already prescribed antipsychotics, where chronic treatment is able to ameliorate some of the negative symptoms and the cognitive deficits, as well as the positive symptoms (30, 71, 72). While this research suggests that Oxt treatment has the potential to improve symptoms in all three domains, where in the brain and how these effects are mediated remains unknown. Animal models for schizophrenia are being used to determine where and how Oxt treatment may improve symptoms associated with schizophrenia.

Oxytocin in Humans and Animal Models

There are inherent challenges when studying a multifaceted disorder such as schizophrenia. Therefore, reliable animal models are necessary to understand and develop viable treatments. A good animal model must have phenotypic overlaps with either a behavior or a molecular characteristic of the disease. In humans, schizophrenia is characterized by several endophenotypes, including impairments in social behaviors such as emotion processing, social perception, attributional bias, and theory of mind [for review, see Ref. (73)]. Schizophrenic patients also have deficits in sensorimotor gating, as measured by prepulse inhibition (PPI) of the acoustic startle reflex [for review, see Ref. (74)], and cognitive deficits in verbal and visual memory, and impaired cognitive flexibility [for review, see Ref. (75)]. There are also neuromotor abnormalities such as dysmetria, eye tracking dysfunctions, and saccadic eye movements, which are typically associated with the positive symptoms of schizophrenia [for review, see Ref. (76–78)], as well as structural abnormalities in total brain volume and the volume of specific brain regions including, but not limited to, the hippocampus, the lateral ventricles, and the prefrontal cortex [for review, see Ref. (77, 79)] Co-morbid anxiety disorders are found in 38% of schizophrenia patients, and studies have reported increases in violent behaviors in schizophrenic patients (80–82). While changes in anxiety-like behavior and aggression have not been proposed as animal models for schizophrenia, several existing animal models of schizophrenia result in altered anxiety-like and aggressive behavior (83–91). Further, atypical antipsychotics have been found to reduce anxiety and reverse psychosis-induced aggression in patients with schizophrenia (92–96). Therefore, the examination of anxiety-like and aggressive behaviors seems warranted.

Currently, over 20 animal models are being used to assess the heterogeneous symptoms associated with schizophrenia (97). To study the specific contributions of the Oxt system, several models have been developed. The first utilizes perinatal stress, since research in humans suggests that exposure to adverse environmental conditions during perinatal development increases the risk for schizophrenia (98). Stress during the perinatal period is known to induce the behavioral and molecular characteristics

of schizophrenia and is commonly used to model the negative symptoms of schizophrenia (85, 86, 99). The second employs the pharmacological disruption of the dopaminergic and glutamatergic systems, since the pathophysiology of schizophrenia suggests that there is dysfunction in both of these systems. Treatment with amphetamine (AMP), an indirect dopamine agonist, or phencyclidine (PCP), an NMDA receptor antagonist, induces hyperlocomotor activity, which corresponds with the positive symptoms of schizophrenia (100–103). Further, PCP treatment induces both negative symptoms and cognitive dysfunction, such as social withdrawal (104–106), impaired PPI (107), and cognitive deficits (108). The third uses gene knockout, since schizophrenia is a genetic disorder with high levels of heritability. Recently, it has been reported that genetic mutations in Oxt genes are associated with schizophrenia (55). It is for this reason that mice with genetic disruptions of their Oxt systems, such as Oxt and Oxtr knockout mice (Oxt−/− and Oxtr−/−, respectively) have been used to determine their potential contributions to the symptoms associated with schizophrenia. While no single model is sufficient to encompass all of the heterogeneous symptoms of schizophrenia, together these models can help us to better understand the role that Oxt may play in schizophrenia. It should be noted that several of these models are not specific to schizophrenia, and the data are relevent for other neuropsychiatric disorders (109). Currently, all of the aforementioned models are being used to study the relationship of Oxt to the negative symptoms of schizophrenia, and while some have been used to study the cognitive deficits and positive symptoms, more research is needed.

Deficits in Social Behaviors

Oxt has a well-characterized role in the neural regulation of social behaviors [for reviews, see Ref. (37, 42, 63, 110, 111)]. It is therefore not surprising that Oxt is studied for its potential contributions to the modulation of the negative symptoms of schizophrenia (**Table 1**). This section is broken up according to the approaches described in the previous section, as there is far more data on the contributions of Oxt to deficits in social behaviors than there are for the other symptoms associated with schizophrenia.

Perinatal Stress

Research in humans has demonstrated that there is a positive correlation between perinatal exposure to a stressful environment and increased risk of schizophrenia (98). In rodents, maternal separation modifies aggressive behavior, and decreases social recognition, anxiety-like, and depression-like behaviors (85, 86, 99); with the effects of early life stress on aggression and Oxtr distribution being sex specific in both mice and rats. Following maternal separation, male mice exhibit decreases in aggression (83, 84) and increases in Oxt-ir in the PVN (84). However, in female mice, maternal separation results in increases in maternal aggression and decreases in Oxt-ir cells in the PVN (83). Similar to mice, in male Long Evans rats, early life stress results in decreases in intermale aggression, and in male Wistar rats, prolonged maternal separation results in increases in Oxt-ir in the Amg (122), increases in Oxtr binding in the medial pre-optic area (MPOA) and ventromedial hypothalamus (VMH), and decreases in Oxtr binding in the lateral septum (LS), agranular cortex, and caudate putamen (CP) in adulthood (123). Early life stress in female Wistar rats results in increases in aggression (134, 135). Data from another rodent species, mandarin voles, have shown that neonatal social isolation results in increases in Oxt-ir in the PVN until post natal day (PND) 8 and the SON until PND4 in both sexes (124). Further, in vole pups that have been isolated from their fathers there is a downregulation of Oxt-ir neurons until PND14, but these decreases do not persist (124).

In addition to maternal separation, prenatal stress can also cause behavioral effects in rodents that are reflective of symptoms of schizophrenia. Adult male rats subjected to prenatal stress and reared by stressed mothers display lower levels of aggression and social behaviors, and increases in anxiety-like behaviors (125, 126, 136). However, when non-stressed mothers rear pups that are exposed to stress during the prenatal period, the deficits in aggressive behaviors and increases in anxiety do not persist (125). Further, these effects appear to be due to Oxt, as an injection of Oxt into the central amygdala (CeA) is able to restore the social deficits exhibited by male rats subjected to prenatal stress (126). Male offspring raised by their prenatally stressed mothers also have reductions in Oxtr mRNA, fewer Oxt positive magnocellular neurons in the PVN, and increases in Oxtr binding in the CeA (125, 126). These morphological changes in Oxt system are not found when non-stressed dams raise the pups.

The behavioral differences observed between species, strain, and sex that result from stress during the perinatal period appear to be a result of alterations in the Oxt system. Many of the changes in the Oxt system are found within the neuronal network that mediates aggression: the MPOA, LS, anterior hypothalamus, VMH, medial amygdala (MeA), and bed nucleus of the stria terminalis (BNST) (137). There are also changes found in the Oxt system in the PVN, and it is known that stress can modulate aggression via the PVN (137). In males, perinatal stress results in decreases in aggression and increases in Oxt-ir and Oxtr binding (83, 84, 122, 134). However, in females, increases in aggression coincided with decreases in Oxt signaling (83, 134, 135). These sex differences in aggression and Oxt may be a result of estrogen-mediated sex differences in Oxtr regulation (138, 139).

Low levels of licking/grooming (LG) maternal behavior are associated with decreases in estrogen receptor-alpha (ERα) and Oxtr levels in the MPOA in female offspring (138, 139). Further, the interactions of estrogens and the Oxt system may result in changes to the dopamine system, as females reared by low LG dams have fewer dopamine neurons in the VTA (140). Research using dopamine agonists to model schizophrenia suggest that there are important interactions between the Oxt and dopaminergic systems to social cognition. Taken together, the data from perinatal stress models suggest that there can be long-lasting disruptions of Oxt neurochemistry, which may lead to impairments in behaviors that are similar to the negative symptoms of schizophrenia.

Pharmacological Disruption

Pathophysiological studies utilizing dopamine agonists and NMDA receptor antagonists have reaffirmed the importance of Oxt to social cognition in patients with schizophrenia. A study

TABLE 1 | Oxt and social deficits associated with schizophrenia.

Animal model	Species	Main findings	Author	Relevant findings in humans	Author
Early life stress	Mouse	↓ Intermale aggression ↑ Maternal aggression ↑ Oxt-ir in PVN in males ↓ Oxt-ir in PVN in females	Tsuda et al. (84) Veenema et al. (83)	↓ Oxt in CSF in adult females with history of childhood abuse	Heim et al. (112)
	Rat	↑ Oxt-ir with prolonged separation in males	Oreland et al. (122)	↓ Plasma Oxt in adult males exposed to early life stress	Opacka-Juffry and Mohiyeddini (113)
		↑ Oxtr binding in MPOA and VMH ↓ Oxtr binding LS, AG, and CP	Lukas et al. (123)		
	Mandarin Vole	↑ Oxt-ir until PND8 in PVN and PND4 in SON after social isolation ↓ Oxt-ir until PND 14 in PVN after paternal deprivation	Wang et al. (124)	↑ Plasma Oxt in adult females exposed to trauma in childhood following psychosocial challenge	Pierrehumbert et al. (114)
Prenatal stress	Rat	↑ Aggression and Anxiety ↓ Social recognition and social interaction ↓ Oxt-ir in PVN	de Souza et al. (125)	↓ Plasma Oxt in children exposed to early neglect following interactions with their mother compared to controls	Fries et al. (115)
		↓ Social recognition and social interaction ↑ Oxtr binding CeA ↓ Oxt mRNA in PVN Oxt administered to CeA reversed social deficits	Lee et al. (126)		
Dopamine agonist	Prairie Vole	Subchronic AMP treatment ↓ Pair bond formation ↓ Oxtr-ir in mPFC/PLC Oxt administered to PLC restores pair bond formation	Young et al. (127)	APO treatment ↓ Plasma neurophysin in patients with schizophrenia compared to controls	Legros et al. (116)
NMDA antagonist	Rat	Chronic PCP treatment ↓ Social interaction ↓ Oxt mRNA in PVN ↑ Oxtr binding CeA Oxt administered to CeA restores social deficits	Lee et al. (105)	Higher plasma Oxt levels in patients with schizophrenia results increased social cognition and fewer negative symptoms	Goldman et al. (48) Rubin et al. (53) Rubin et al. (54) Strauss et al. (117) Strauss et al. (118)
Dysregulation of the Oxt system – Oxt and Oxtr knockout mice	Mouse	↓ Social memory and Social recognition in Oxt and Oxtr−/− mice	Ferguson et al. (128) Nishimori et al. (31) Takayanagi et al. (32)		
		Oxt administration to Amg restores deficits in social recognition in Oxt−/− mice	Winslow and Insel (33)	↓ Plasma Oxt in male patients with schizophrenia and increased negative symptoms	Jobst et al. (119)
		↑ Social withdrawal in Oxtr−/− mice in visible burrow paradigm ↑ Social withdrawal in Oxtr−/− mice in three-chamber test	Pobble et al. (129, 130)	Lower CSF Oxt in male schizophrenic patients corresponds with increased negative symptoms	Sasayama (120)
		↑ Intermale Aggression Oxt and Oxtr−/− mice	Winslow et al. (131) Dhakar et al. (90)		
		↓ Maternal aggression Oxt−/− mice	Young et al. (91)		
		↓ Initiation Maternal Behavior Oxtr−/− mice and Oxtr FB/FB	Macbeth et al. (132) Rich et al. (133)	↓ Plasma Oxt in patients with schizophrenia after trust exercise compared to controls	Keri et al. (121)
		↓ Ultrasonic vocalization in Oxt−/− mice pups	Winslow et al. (131)		

on drug addiction and social behaviors provides insight into the role of Oxt, dopamine, and social behaviors (127). Specifically, in prairie voles, repeated subchronic AMP exposure inhibits pair bond formation (127), decreases Oxtr-ir in the mPFC, and reduces Oxtr activation in the PLC; which is important for partner preference formation (127, 141). Additionally, Oxt direct infusion into the PLC is able to restore AMP-induced impairment in partner preference and alter dopamine levels in the nucleus accumbens (NAcc) (127). Administration of PCP induces social dysfunctions in animals that mimics the negative symptoms associated with schizophrenia [for review, Ref. see (142, 143)]. Oxt mRNA expression is reduced in the PVN of rats and Oxtr binding is increased in the CeA following chronic PCP treatment (105). Further, PCP-induced deficits in social interactions are increased by bilateral infusions of Oxt to the CeA (105). While these data suggest that the interaction of Oxt with both dopamine and glutamate is important for social behavior, the specific mechanisms that mediate these effects remain unclear.

Research on sex behavior in rats suggests that the dopaminergic and Oxt systems can modulate each other (144–146), and the Oxtr is located throughout the mesolimbic dopamine pathway (147, 148). Thus, researchers have hypothesized that Oxt and dopamine may work together to affect on how an individual perceives the salience of social cues [for review, see Ref. (149)]. However, the connection between these two systems and their role in schizophrenia remains murky. Likewise, the link between the Oxt and glutamate systems is also poorly understood. In rat SON preparations, application of both Oxt and Avp inhibits glutamate release (150). However, in cultured rat olfactory bulb neurons glutamate transmission is facilitated (151). More recently, it has been found that in the CeA, Oxt and glutamate are co-released from Oxt neurons (152). More research is still needed to determine how and where Oxt may interact with these neurotransmitter systems to affect social cognition in patients with schizophrenia.

Genetic Disruptions

The use of genetic tools, including Oxt−/− and Oxtr−/− mice have significantly contributed to our understanding of the role of Oxt in the social deficits observed in patients with schizophrenia. Male Oxt−/− and Oxtr−/− mice fail to develop social recognition memory, in essence having social amnesia (31, 32, 128, 153) (**Figure 2**). Further, an injection of Oxt into the MeA of Oxt−/− mice is able to restore social recognition (33, 154). These deficits in social memory are not specific to males, as female Oxt−/− mice do not show a normal Bruce effect (155, 156). Oxtr−/− mice also display behaviors similar to the negative symptoms of schizophrenia across multiple testing scenarios. In a visible burrow system, which provides a more natural habitat for rodents, Oxtr−/− mice have reductions in social interaction behaviors, spending more time alone and self-grooming than controls (129, 130). In a three-chamber test for sociability

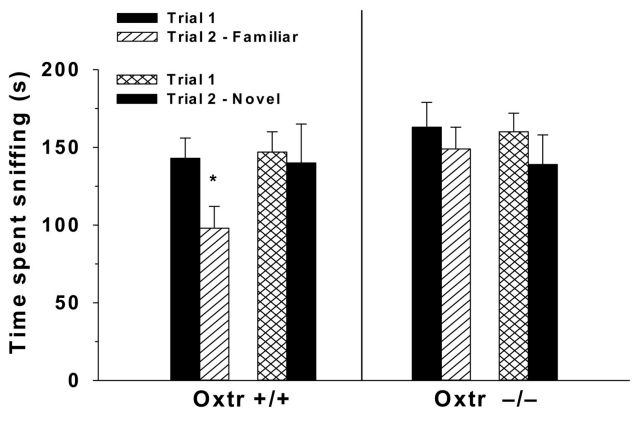

FIGURE 2 | **Oxtr−/− mice have impaired social recognition**. In a two-trial discrimination task performed over 2 weeks, Oxtr+/+ (n = 8) and Oxtr−/− males (n = 8) were exposed to overiectomized BALB/C female mice during trial 1, and then 30 min later during trial 2 they were exposed to a familiar female on week 1. During week 2 of testing after trial 1, mice were exposed to a novel female during trial 2. Oxtr−/− mice fail to discriminate between the familiar and novel female spending approximately equal amounts of time sniffing both; compared to Oxtr+/+ mice that spend more time sniffing the novel female. Reprinted with permission from Endocrinology, Lee et al. (153).

Oxtr−/− mice display increases in social withdrawal (129, 130) and in a social proximity test they display reductions in the frequency of nose-to-nose and nose-to-anogenital behaviors (129, 130). These data suggest that a functional Oxt system is necessary for normal social interactions, and that dysregulation of Oxt in schizophrenia could contribute to some of the negative symptoms.

Research also suggests that Oxt is important for other social behaviors, such as aggression and maternal behavior. Some studies have reported increases in violent behaviors in schizophrenic patients; however, it remains unclear whether this is a symptom of schizophrenia or rather co-morbid disorders (80, 81). Oxt−/− and Oxtr−/− mice have increases in aggressive behavior, and given the dysregulation of the Oxt system in schizophrenia, a functional Oxt system could be important for normal aggressive behavior (32, 90, 131, 153, 157, 158). Specifically, male Oxt−/− mice have heightened aggression when born to null mutant dams, but not when they are born to heterozygous dams (131, 157). Oxtr−/− mice also have heightened intermale aggression, but Oxtr FB/FB do not (32, 90, 153, 158). These data suggest that Oxt exposure during development may have persistent effects on aggressive behavior. Therefore, it could be that developmental Oxt contributes to the etiology of schizophrenia; however, more research is needed before such a claim can be made.

While there are no reported deficits in maternal behavior in patients with schizophrenia, the cognitive impairments associated with schizophrenia may lead to reductions in the ability to acquire necessary parenting skills (159–161). In animal models of schizophrenia, evidence suggests that decreases in maternal behaviors result in the development of behaviors similar to those found in other animal models of schizophrenia (83, 125, 126, 136, 162). Oxtr−/− and Oxtr FB/FB display deficits in the initiation of maternal behavior (32, 132, 133) and Oxt−/− mice pups emit fewer ultrasonic vocalizations when separated from nest; all of which suggest that Oxt contributes to social behavior in rodents (33, 131).

Impaired Cognition

The Oxt system may also be important to the cognitive dysfunctions associated with schizophrenia. One endophenotype of schizophrenia is impaired sensorimotor gating, i.e., the inability to "filter or gate" information (163, 164). Across species, sensorimotor gating can be measured using PPI of the startle reflex. The startle reflex is a defensive response to an abrupt, relatively intense stimuli (165). The neural circuitry that underlies PPI is known as the cortico-striato-pallido-pontine (CSPP) circuit (166). In humans, PPI is measured using electromyographic recordings from eye blink responses (167). In rodents, it is measured using the whole body flinch reflex of an animal to the startle stimulus (168). Patients with schizophrenia not only have reduced PPI but also show less habituation of the startle reflex compared to controls (169). In Brown Norway rats, which have a naturally low PPI, Oxt but not its structural analog carbetocin, is able to significantly increase PPI (170).

Stress during the perinatal period may contribute to deficits in PPI, though there have been contradicting reports, with one group reporting deficits in PPI and another group finding no changes in PPI, some data suggests that early life stress reduces

PPI levels in adulthood (162, 171). Changes in the Oxt system have been reported following early life stress, and may have an impact on perinatal stress-induced reductions in PPI, though more research is necessary (83, 122, 123). Further, in models that pharmacologically disrupt PPI, exogenous Oxt is known to reverse these deficits (170). Specifically, in rats, subcutaneous Oxt injections are able to restore deficits in PPI induced by AMP, an indirect dopamine agonist, and dizocipline (MK-801), a specific NMDA receptor antagonist, but not apomorphine (APO), a direct dopamine agonist (172). Finally, genetic disruptions in the Oxt system suggest that a lack of endogenous Oxt appears to be important in the regulation of PPI, as Oxt−/− mice have increased PCP-induced deficits in PPI (173) (**Figure 3**). This further suggests that the effects of endogenous Oxt on PPI may be specific to the glutamatergic system.

Oxt is likely to also contribute to the cognitive deficits associated with schizophrenia, such as impaired spatial memory and cognitive flexibility (3, 174). Similar to the cognitive deficits found in schizophrenia, Oxtr−/− mice display reduced cognitive flexibility, as measured by an inability to alter their behavior during the reversal phase of a *t*-maze task (175). Since the Oxtr is abundant in the hippocampus of mice, it may be important for memory (176). However, there are divergent reports of Oxt's effects on spatial memory, suggesting that Oxt may have brain region-specific effects (38, 177). *In vitro*, hippocampal slices treated with Oxt are able to maintain long-term potentiation longer than untreated slices (38). In mouse dams, a central injection of Oxt is able to improve reference memory on a radial arm maze, but does not affect their short-term memory during acquisition, suggesting that Oxt only improves long-term spatial memory (38). As Oxt can improve anxiety in virgin mice when administered to the Amg or VMH, the effects of Oxt on reference memory may be due to its actions in these brain regions. However, there was no effect on their open-field activity, which suggests direct action on hippocampal neurons (38). Further, dams that receive an intracerebroventricular (i.c.v.) injection of an Oxt antagonist have reductions in reference memory compared to controls (38). But, in rats, Oxt injections into the nucleus basalis of Meynert (NBM) impair spatial memory, as measured by a Morris water maze, while an Oxtr antagonist injected into the NBM facilitates spatial memory (177). Given that disruptions in Oxt signaling appear to contribute to multiple aspects of cognition, and that Oxt may affect memory formation, it is plausible that Oxt may play a role in the cognitive deficits associated with schizophrenia.

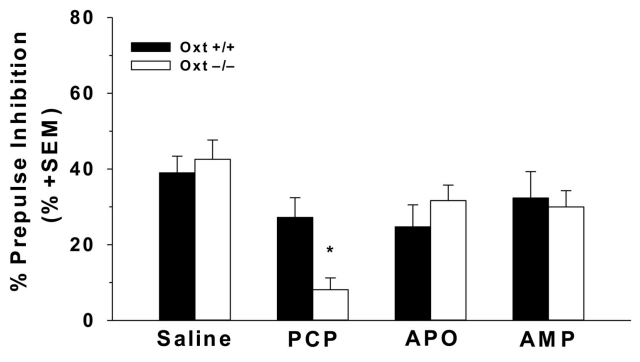

FIGURE 3 | Oxt−/− mice have greater PCP-induced deficits in sensorimotor gating. The acoustic startle of male Oxt+/+ (n = 12) and Oxt−/− mice (n = 8) was measured using the whole body reflex flinch in reaction to a startle tone using startle chambers (SR-LAB; San Diego Instruments, San Diego, CA, USA). Mice were administered either an i.p. injection of 10 mg/kg AMP and APO, a subcutaneous injection 6 mg/kg PCP, or an equivalent volume of 0.9% saline as a control 15 min prior to testing. Testing session consisted of 60 trials, including no stimulus trials, pulse-alone trials, and prepulse + pulse trials. The testing sessions began and ended with the presentation of five 120 db pulse-alone tones. The middle 50 trials consisted of: 10 no pulse tones trials, 30 prepulse + pulse trials at 3, 6, and 12 db above background, and 10 pulse-alone tones at 120 db. A repeated measures design was used with each animal receiving 0.9% saline, AMP, APO, and PCP, with a minimum of 3 days between each trial. Oxt−/− mice display greater reductions in the average percent PPI across three prepulse levels (3, 6, and 12 db above background) following an injection of PCP compared to Oxt+/+ mice. There were no genotypic differences in PPI following injection of AMP or APO. Adapted and reprinted with permission from Macmillan Publishers Ltd: Molecular Psychiatry, Caldwell et al. (173).

The effects of Oxt dysregulation and Oxt treatment on the cognitive dysfunction found in patients with schizophrenia are poorly understood. Studies in both humans and animal models suggest that a functional Oxt system is required for normal sensorimotor gating and cognitive flexibility. The effects Oxt on sensorimotor gating may be specific to the glutamatergic system (172, 173), with mice lacking the obligatory NMDA receptor 1 subunit having impaired PPI (178). Unfortunately, as previously discussed, how these two systems interact remains unclear. The Oxt system is coupled to phospholipase c-β1 (PLC-β1) and glutamate is known to regulate PLC-β1 (36, 179–182). Abnormal expression patterns of PLC-β1 are found in patients with schizophrenia (183, 184). Further, studies using PLC-β1 knockout (PLCβ1−/−) mice find impaired PPI and deficits in working memory (185, 186). Therefore, the PLC-β1 may reflect a point of convergence for the Oxt and glutamate systems in the regulation of sensorimotor gating.

The effects of Oxt treatment on spatial learning are also ambiguous. While research suggests that Oxt in the hippocampus facilitates learning, it impairs memory when injected to the NBM (38, 177). However, while neuronal deficits in the hippocampus have been found, no reductions in neuronal density have been observed in the NBM in patients with schizophrenia (187). So, it is not clear whether or not this brain region is important to the pathophysiology of schizophrenia. In addition to Oxt's effects on the cognitive deficits, it may also play a role in the positive symptoms associated with schizophrenia.

Neuromotor Abnormalities

In animal models, psychotic symptoms similar to the positive symptoms of schizophrenia can be manifested in rodents by treatment with dopamine agonists and NMDA receptor antagonists, which cause hyperlocomotor activity. While hyperlocomotor activity does not have direct face validity for the positive symptoms of schizophrenia, it does have construct validity as psychotomimetics cause similar neurotransmitter activity in animal models as is found in human schizophrenic patients. However, the behavioral effects are not necessarily similar; though some suggest that hyperlocomotor activity is comparable to some positive symptoms such as grossly disorganized behavior and psychomotor agitation (188, 189). Further, established

antipsychotics, which reduce positive symptoms of schizophrenia, consistently reduce the hyperactivity associated with pharmacological agents such as AMP, cocaine, ketamine, and PCP. The antipsychotic efficacy of Oxt is supported by pharmacological manipulations that induce aspects of schizophrenia. During studies on addiction, Oxt decreases drug-induced hyperlocomotor activity (34, 35) while pretreatment with Oxt is able to attenuate the hyperlocomotor activity caused by cocaine, an indirect dopamine agonist (35) (**Figure 4**). In another study, which examined the effects of Oxt on addiction, i.c.v. injections of Oxt reduce methamphetamine-induced increases in locomotor activity (34). Other research also suggests that Oxt and the glutamatergic system may interact to affect the positive symptoms associated with schizophrenia. In addition to its behavioral effects, PCP induces the excessive release of glutamate within the medial prefrontal cortex (mPFC), which when blocked, suppresses hyperlocomotion (190, 191). Oxt has been found to reduce the PCP-induced symptoms associated with psychosis (173), as well as suppress glutamate release within the mPFC (192). Therefore, Oxt could suppress the hypofunction of glutamate specifically within the mPFC to protect against PCP-induced symptoms of psychosis. Genetic disruptions of the Oxt system also provide evidence that endogenous Oxt may affect locomotor activity, as there is hyperlocomotor activity in infant Oxtr−/− mice; however, this effect is not persistent (32).

Oxytocin and the Pharmacology of Schizophrenia

Oxt is known to interact with several other neurotransmitter systems that are important in the etiology and treatment of schizophrenia, such as GABA and 5-HT (193). During parturition, Oxt has been found to modulate GABAergic inhibition in rodent models for autism spectrum disorder (ASD) (194). Given that ASDs and schizophrenia share similar endophenotypes, Oxt may also modulate GABA signaling in schizophrenic patients as well. However, further research is necessary to elucidate the role of the interactions of the Oxt system and GABAergic system to the symptomology of schizophrenia. Oxt and 5-HT are known to modulate one another, and both are important for numerous social behaviors and mood (195–197). Specifically, Oxt may exert anxiolytic effects via Oxtr activation in 5-HT neurons (195). Current atypical antipsychotics may provide further evidence for the interactions between the Oxt and 5-HT systems and schizophrenia.

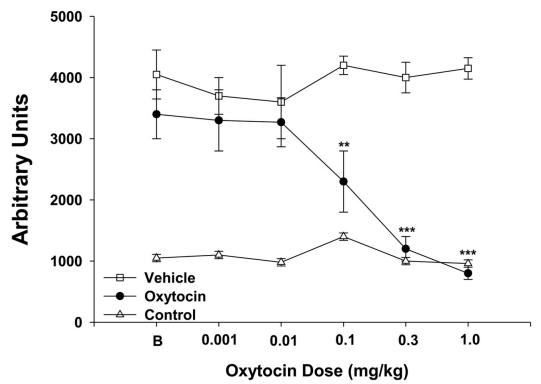

FIGURE 4 | Oxt dose-dependently decreases locomotor activity in self-administering methamphetamine rats. Oxt ($n = 5$) was administered IP in ascending doses (0.001, 0.01, 0.1, 03, 1 mg/kg) over five consecutive days and equivalent amounts of vehicle ($n = 5$) were administered. Only the animals treated with Oxt or vehicle self-administered methamphetamine, and the control ($n = 8$) was used to determine baseline levels of locomotor activity. B = baseline day before oxytocin testing began. $^{**}p < 0.01$ and $^{***}p < 0.001$. There was no difference between rats treated with Oxt compared to the control group at 0.3 and 1 mg/kg Oxt dose. All other comparisons between Oxt treatment and the control group and the vehicle treatment and control groups were significant. Data are shown as mean ± SEM. Adapted and reprinted with permission from Elsevier: Carson et al. (34).

Some of the currently used atypical antipsychotics are known to interact with the Oxt system. The atypical antipsychotics, amperozide and clozapine, increase plasma levels of Oxt, but the typical antipsychotic haloperidol does not (198). Amperozide and clozapine are both a 5-HT_{2A} antagonists, and to lesser extent D2 antagonists, that are reported to decrease both the negative and positive symptoms associated with schizophrenia (199–202). Whereas, the D2 specific antagonist, haloperidol, only appears to alleviate positive symptoms of schizophrenia (203, 204). Further, some atypical antipsychotics cause activation of Oxt cells as measured by cFos ir. Clozapine increases cFos activation in Oxt cells in the PVN, but again, haloperidol treatment does not (205). Similar to the effects of Oxt, in rodents, clozapine attenuates the reduction of cognitive flexibility caused by the sub-chronic PCP treatment (206), and is able to restore normal levels of PPI to brown Norway rats (207). This evidence further supports a role of the Oxt in the cognitive deficits found in schizophrenic patients. In humans, clozapine attenuates both the negative symptoms and cognitive dysfunctions found in patients with schizophrenia (208–212). Therefore, the ability to reduce the social and cognitive deficits may be associated with the ability of clozapine to increase Oxt levels. Further, the specific interactions between the Oxt and serotonergic systems may be important to the social and cognitive deficits found in patients with schizophrenia. However, additional research is necessary to assess how Oxt may affect the symptom domains associated with schizophrenia through its interactions with other neurotransmitters systems.

Conclusion

Given the importance of the Oxt system to the modulation of social behaviors, it is not surprising that across animal models of schizophrenia, Oxt has been implicated in the negative symptoms and deficits in social cognition. Data suggests that developmental, drug induced, and genetic disruptions in the Oxt system lead to the symptoms associated with the negative symptoms observed in schizophrenic patients. However, further research is needed to elucidate the specific mechanisms whereby Oxt exerts these effects. Human and animal models also suggest that research is needed to determine if Oxt can work as a therapeutic agent to improve the social behavior deficits observed in patients with schizophrenia. Oxt also appears to be a contributor to the cognitive and positive symptom domains of schizophrenia; though much more work in this area is needed. While Oxt

does not "cause" schizophrenia, its putative impact to all three symptom domains suggests that it may be an important player to the etiology, and perhaps even an effective treatment, of schizophrenia. Using animal models, future research will need to focus on elucidating of the mechanisms of Oxt dysregulation and the interactions between Oxt and other neurotransmitter systems that may contribute to the symptoms associated with schizophrenia.

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Early social environment affects the endogenous oxytocin system: a review and future directions

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Endogenous oxytocin plays an important role in a wide range of human functions including birth, milk ejection during lactation, and facilitation of social interaction. There is increasing evidence that both variations in the oxytocin receptor (OXTR) and concentrations of oxytocin are associated with differences in these functions. The causes for the differences that have been observed in tonic and stimulated oxytocin release remain unclear. Previous reviews have suggested that across the life course, these differences may be due to individual factors, e.g., genetic variation (of the OXTR), age or sex, or be the result of early environmental influences, such as social experiences, stress, or trauma partly by inducing epigenetic changes. This review has three aims. First, we briefly discuss the endogenous oxytocin system, including physiology, development, individual differences, and function. Second, current models describing the relationship between the early life environment and the development of the oxytocin system in humans and animals are discussed. Finally, we describe research designs that can be used to investigate the effects of the early environment on the oxytocin system, identifying specific areas of research that need further attention.

Keywords: oxytocin, early-life environment, research design, individual differences, mother–infant bonding

THE ENDOGENOUS OXYTOCIN SYSTEM

Oxytocin, a mammalian hormone, is a naturally produced neuropeptide with nine amino acids. Oxytocin is mainly produced in magnocellular neurons in the hypothamalic paraventricular and supraoptic nuclei (1). The magnocellular neurons release oxytocin into circulating blood via the pituitary gland, while the parvocellular oxytocin neurons release oxytocin in other areas of the central nervous system (CNS). Recent evidence has demonstrated that oxytocinergic axons ending in forebrain regions including the central amygdala and nucleus accumbens may originate exclusively in the magnocellular neurons of the paraventricular nucleus (2). Evidence also indicates that central projections of hypothalamic oxytocin neurons may be widespread, and that oxytocin release from local axonal endings may be able to control region-associated behaviors (2). Oxytocin is also produced in a number of peripheral tissues and organs, such as the uterus, ovaries, testis, vascular endothelium, and the heart (3). For a review of the anatomy and functional aspects of the oxytocin system, see Kiss and Mikkelson (4).

Oxytocin is released in response to a range of internal and external stimuli. Historically, it is known that oxytocin is released in response to vaginocervical and nipple stimulation and plays important roles in mammalian uterine contraction and lactation. Oxytocin also plays a crucial role in the milk ejection reflex. In lactating women, oxytocin peaks during the morning and then declines until the beginning of the afternoon, and it is released in a cyclic manner, with salivary oxytocin concentrations at their highest within 30 min before feeding begins (5). This indicates that the brain may be able to release oxytocin in anticipation of future behaviors and interactions.

More recent data demonstrate the pro-social role of oxytocin, including its role in social and emotional regulation (6), orgasm (7), regulating stress, and anxiety and facilitation of pair, maternal and infant bonding (8–12). Interestingly, the release of oxytocin is dependent on the social context. For example, calves more readily release oxytocin when suckling milk directly from their mother's udder than when drinking the same milk from a bucket (13). Another example shows that oxytocin administration combined with psychological support of a friend lowered salivary cortisol concentrations and was correlated with decreased anxiety and increased calmness in human males (8).

Large individual differences exist in basal concentrations of oxytocin and in response to stimulation [for a review, see Ref. (12)]. Basal individual oxytocin concentrations in humans have been found to vary by thousands of pg/ml within the same study (14). Individual differences, such as genetic variation of the oxytocin receptor (OXTR) may explain some of the variation. Expression of the OXTR also varies in a sex-specific way. It is therefore possible that an interaction with the estrogen system plays a role that warrants further attention and investigation (15).

OXYTOCIN AND THE EARLY SOCIAL ENVIRONMENT

The early-life environment is critical for development in humans and other mammals [for a review on the long-term impact of

early-life events, see Ref. (16)]. A significant component of the early-life environment is mother–infant bonding. Social experiences in the early-life period and bonding form the basis for healthy social and emotional development, possibly of the mechanism that manages stress and resilience (11). The well-documented protective associations between secure attachments and later social functioning and behavior underscore the need to understand the origins of attachments and identify the specific individual differences that influence attachment and development (17). Current research highlights the link between oxytocin concentrations and a specific set of maternal bonding behaviors and attitudes in humans (18–24). There is evidence to suggest that oxytocin plays an important role in facilitation of mother–infant bonding [for reviews, see Ref. (10, 25)] and that the early social environment can shape the developing oxytocin system. There is substantial literature documenting the relationship between oxytocin and the early-life environment in animals (26–28); however, minimal research has focused on this area in humans. Importantly, there is only limited data about the normal development of the oxytocin system in humans over time (11).

Oxytocin plays an important role in priming mammals to form social bonds, but in turn, the early social environment may also be able to shape the development of the oxytocin system [for a review, see Ref. (11)]. Interestingly, studies have demonstrated correlations between infant and parental oxytocin concentrations and parenting behaviors in child–parent interactions (14, 29). The following section will review the influence of prenatal and postnatal environment on the oxytocin system. Additionally, maternal mental health problems will be addressed as they can negatively affect the mother–infant bond.

PRENATAL ENVIRONMENT

In humans, prenatal stressors (including substance use, maternal depression, and chronic stress) can lead to a range of abnormal neurodevelopmental outcomes for infants [for a review, see Ref. (30)]. A recent human study found that male and female fetuses respond differentially to chronic maternal stress, indicating that sex may be an important factor influencing fetal development (31).

Lee and colleagues (32) demonstrated that a regime of prenatal stressors in pregnant rats caused numerous changes in the oxytocin systems of adult male offspring (less oxytocin mRNA in the paraventricular nucleus, but increased OXTR binding in the central amygdala). Prenatally stressed rats showed disturbed social behavior, which could be normalized with local oxytocin administration in the central amygdala. Cross fostering the pups did not normalize the behavior.

Drug abuse, including smoking, heavy consumption of alcohol, and illegal substances, can adversely affect fetal development and can also impair infant health and development. Alcohol can cause numerous growth impairments, and is especially well known for its harmful effects on the developing nervous system. Tobacco and cannabis smoking are also notorious for fetal growth-restricting and gestation-shortening effects (33). There is ample evidence on the long-term effects of prenatal drug use (ethanol, stimulants, and nicotine) on rodent offspring, focusing on social behavior and drug use and changes to the oxytocin system (33). Exposure to cocaine in the prenatal period in rats affects both the oxytocin system and social behavior and increases susceptibility to addiction later in life (34, 35).

POSTNATAL ENVIRONMENT

The oxytocin system continues to develop after birth, and this development may be critical for providing humans with the skills for healthy social functioning (25). There is extensive research focusing on the long-term effects of early-life adversity and social environment on the oxytocin system; however, most of these studies use animal models. Differences in rearing conditions and bonding behavior can influence adult social and parental behavior in prairie voles (9). Additionally, quality of maternal behavior has been linked to differences in oxytocin concentrations and OXTR expression observed in animal models (9, 36). For example, high levels of maternal licking resulted in increased plasma oxytocin concentrations in neonatal rats (37). Furthermore, Kojima and colleagues (38) found that maternal skin-to-skin contact stimulates rat pups' central oxytocin concentrations. Early-life adversity and differences in the early social environment may also adversely affect the expression and concentration of the OXTR. Veenema (26) provides a thorough review on the effects of early-life manipulations in rodents on the distribution and expression of oxytocin and vasopressin receptors. Bales and Perkeybile (39) also provide a good review of the effect of early experience on the OXTR system.

Human studies with infants and their parents have demonstrated how oxytocin levels rise in response to social interaction and how infant and parental oxytocin concentrations correlate (39). These findings have been supported in previous research measuring cerebrospinal fluid (CSF) oxytocin concentrations, which have shown that higher infant CSF oxytocin concentrations were positively correlated with active initiation and interest in parental social interaction (40). The human oxytocin system seems to be receptive to both positive and negative early social experiences, such as separation (41). Infants who experienced high affect parent–infant synchrony (i.e., monitoring and responding) showed increase oxytocin saliva measures compared to infants reared in the presence of low affect parent–infant synchrony (42). This suggests that the early environment may directly affect peripheral oxytocin concentrations in humans. Importantly, a study by Wismer Fries and colleagues (43) reported significant social deficiencies and low oxytocin concentrations in children reared in extremely aberrant social environments. These results indicate that the early environment may influence cross-generation transmission of human social attachments and behavior. They also support the notion that peripheral oxytocin measurements may be correlated to social behavior in both infants and adults.

MATERNAL ANXIETY, STRESS, AND DEPRESSION

Maternal mental health problems can greatly influence the mother–child bond as they affect the way they perceive their child's needs and cues, their stress resilience, and their general emotional availability.

Research has shown that mothers with postpartum anxiety report significantly lower bonding with their infants than healthy mothers (44). Depressed mothers are more likely to perceive their infant's behavior negatively than healthy mothers (45). Depression

can also play an important role in influencing the early-life environment through its effect on maternal behavior and mother–infant bonding. Research shows that the most significant predictor of lower postnatal maternal attachment was depressive symptoms experienced during the final stages of pregnancy and in the postnatal period (18, 46). Despite this emerging evidence, a recent review of the literature (47) identifies that mechanisms underlying maternal stress, depression and anxiety, and their effects on infant outcomes are poorly understood. Due to its important role in mother–infant bonding, oxytocin could be involved in these mechanisms; however, further study is needed.

Interestingly, a recent pilot study found that exogenous administration of oxytocin stimulated protective behavior in mothers with postnatal depression (48). Eapen and colleagues (49) also found an association between lower plasma oxytocin levels in the post partum period and separation anxiety and depression during pregnancy. Further study is needed to determine the relationship between maternal depression and oxytocin, how this relationship may affect infants, and the potential role of exogenous oxytocin in treating depression.

CONSIDERATIONS FOR RESEARCH

The previous sections have introduced the endogenous oxytocin system and its relationship to early environmental and social factors. The present section will identify limitations in the current literature and provide suggestions for future research.

This review has established that there is evidence showing that early social environment in animals is correlated with altered oxytocin concentrations. Particular attention to maternal behaviors during the postnatal period is warranted given the established link between mother–infant dyadic interactions and later physical, emotional, and social health. Studies by Feldman and colleagues (14, 18, 41, 42) have provided insight into the range of saliva and plasma oxytocin concentrations that should be expected in both mothers and babies. However, very little is still known about the normal development of the oxytocin system into childhood and adolescence.

Additionally, a proposed direction of research would be to determine how an early adverse social environment in humans affects the oxytocin system. Of interest are changes in basal concentrations, differences in reactivity of the oxytocin system under stress or social interaction and changes in OXTR characteristics (e.g., investigation of epigenetics, binding affinity, up- or downregulation of receptors, and methylation).

Up- or downregulation of the number of OXTRs, localization and sensitivity of OXTRs are currently difficult to research in humans, as there is no radio-active ligand that can be used in this process. Therefore, a number of different research techniques are needed to investigate these changes through other means, including the collection of biological samples during observational studies and psychological testing undertaken in large cohort studies, and further refinements of assay methodologies.

Epigenetic changes will be of interest to investigate when determining positive or negative effects of early social environment on the developing oxytocin system. Epigenetics refers to the regulation of DNA transcription without alteration of the original sequence. DNA methylation is an important epigenetic modification in response to, e.g., oxygen deprivation, trauma, or drug use. A recent study found that traumatic experiences and stressful life events in early life were associated with higher methylation of the NR3C1 gene (50). Although the topic of epigenetics has not been thoroughly explored in the present review, there is promising research indicating its importance (28, 51–53).

SAMPLE COLLECTION AND MEASUREMENT

Extensive discussion in the field addresses the most suitable and reliable method of collecting and analyzing oxytocin samples, both from the periphery and centrally. Different methods of sample collection and measurement contribute to the large range in oxytocin concentrations reported in the literature (54).

Human research into oxytocin is significantly more difficult than animals due to the inability to non-invasively assess brain oxytocin pathways and concentration. However, peripheral oxytocin concentrations can be tested through plasma, urine or saliva. These measures possibly may not directly correspond with brain concentrations; however, research has shown that changes in behavior are linked to changes in peripheral oxytocin concentrations (14, 54). There is also increasing evidence suggesting that peripheral oxytocin is reflective of changes in central concentrations. For example, a recent study of human children found that plasma oxytocin concentrations significantly and positively predicted CSF oxytocin concentrations (55).

Collecting samples of oxytocin from infants provide an additional challenge to researchers. CSF, urine, and plasma samples are generally unsuitable in these situations as the collection method is invasive and painful; furthermore, it is unlikely that parents will provide consent for their infant's participation in a study with these extraction methods. Finding correlations between oxytocin concentrations in urine and social behavior has shown some success in both animal and human studies (56, 57). However, this collection method may still not be appropriate when attempting to determine an acute infant increase in oxytocin in response to a parent-infant interaction, as urine concentrations represent accumulated oxytocin concentrations and are not always readily available for collection, especially in infants. Saliva, therefore, would appear to be the most ideal method of measuring oxytocin concentrations in infants, with adoption of this method resulting in significant associations between infant and parent oxytocin concentrations and social behavior (42). Recent research from our group (unpublished) has supported previous studies, finding that oxytocin concentrations can be determined from both adult and infant saliva samples. Adults are able to provide this sample by simply spitting into a tube. Saliva from infants can be collected by collecting passive drool from the baby's mouth with a syringe and then dispensing into a tube. Parents have also willingly provided infant consent for this form of collection as it is a minimally invasive and completely painless extraction method. A limitation of this process, however, is that some infants are more accepting of syringing than others, influencing the collection time. This process of collection could also potentially be difficult if the infant is distressed.

Further refinement of oxytocin assay methodologies is also needed. A comparison of enzyme immunoassays (EIA) and radio immunoassays (RIA) of human plasma samples with and without

extraction found that without extraction, plasma measured by EIA was more than 100 times higher than in extracted plasma, and the correlation between them was minimal ($p = 0.54$) (58). The same study found that when using an RIA, the majority of samples (90%) were below the level of detection. This could provide some insight into the general inconsistency of results across different studies, indicating the need to refine oxytocin assay methodologies. Currently, comparing group differences within experiments is the most reliable approach.

Specificity of the role of oxytocin provides a challenge in determining the link with early adversity. Oxytocin and vasopressin are very similar in structure, and the two hormones have a high affinity for each other's receptors (59). It is suggested that early social experiences influence sensitivity to both the oxytocin and vasopressin systems (28). This emphasizes the need for further understanding of the systems interacting with oxytocin and the relationship they may have with early adversity and development in humans.

While focusing on the effect of early life social experiences on infants and their developing oxytocin system, it is important to acknowledge that the oxytocin system has numerous bilateral interactions (e.g., dopamine and HPA-axis) that are likely to affect behavior [for a review, see Ref. (12)]. Changes to the oxytocin system will also influence these systems and vice versa. Additionally, systems like the HPA-axis will be affected by environmental influences as well. Researchers focusing on long-term effects of early social environments need to keep these considerations in mind and try to address all the dimensions.

CHARACTERIZATION AND MEASUREMENT OF EARLY ADVERSITY

Another research challenge is measuring early adversity itself. First, there is little consensus on the term, and second, it is conceptualized differently across different disciplines, i.e., neglect, abuse, toxic stress, disorganized attachment, reduced bonding, etc. Detecting and quantifying early adversity is challenging when relying on questionnaires. Observing single mother–child interactions in a laboratory setting or home environment can provide great insight into the early social environment. Assessing attachment status is often not an option, as extensive training is needed. Mothers of infants who may be experiencing early adversity may be reluctant to participate in behavioral observations, although large cohort studies may be able to incorporate this in sub-groups. The next challenge is selecting a tool that can be used to score behavior in a dyad and quality of the interaction (e.g., reciprocity), which is objective, intuitive, and has large inter-rater reliability. There are few scales that have been validated for this purpose meeting all these criteria.

CONCLUDING REMARKS

Review of the literature indicates that the early-life is a vital period for healthy development in humans, and that oxytocin is an important regulator of emotional development. Specifically, investigating the effect of early adversity on the endogenous oxytocin system is important in understanding normal behavior and social disorders. Understanding this effect can increase knowledge of how the early environment appears to change the way humans are hardwired to respond in social and stressful situations. A number of animal studies have been conducted; however, more human research specifically focusing on this relationship is necessary incorporating a large range of study designs. Future studies must consider the obstacles that prevent conducting this research, such as collecting appropriate peripheral oxytocin measures and selecting a suitable tool to score both infant and maternal behavior.

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Vasopressin proves es-sense-tial: vasopressin and the modulation of sensory processing in mammals

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As mammals develop, they encounter increasing social complexity in the surrounding world. In order to survive, mammals must show appropriate behaviors toward their mates, offspring, and same-sex conspecifics. Although the behavioral effects of the neuropeptide arginine vasopressin (AVP) have been studied in a variety of social contexts, the effects of this neuropeptide on multimodal sensory processing have received less attention. AVP is widely distributed through sensory regions of the brain and has been demonstrated to modulate olfactory, auditory, gustatory, and visual processing. Here, we review the evidence linking AVP to the processing of social stimuli in sensory regions of the brain and explore how sensory processing can shape behavioral responses to these stimuli. In addition, we address the interplay between hormonal and neural AVP in regulating sensory processing of social cues. Because AVP pathways show plasticity during development, early life experiences may shape life-long processing of sensory information. Furthermore, disorders of social behavior such as autism and schizophrenia that have been linked with AVP also have been linked with dysfunctions in sensory processing. Together, these studies suggest that AVP's diversity of effects on social behavior across a variety of mammalian species may result from the effects of this neuropeptide on sensory processing.

Keywords: vasopressin, sensory, olfaction, social behavior, hearing, gustation, social learning

INTRODUCTION

In social species, survival depends on navigating complex social cues. An individual must be able to use sensory cues to distinguish between familiar and unfamiliar individuals. These sensory cues must then be filtered in a way that directs the animal to make an appropriate behavioral response. Although interpretation of sensory cues is influenced by many neural pathways, the neuropeptide arginine vasopressin (AVP) appears to be critical for making these key social distinctions in mammals. This neuropeptide has been associated with pair bonding, aggression, parental care, social memory formation, and stress responses in multiple species of rodents [reviewed in Ref. (1)], possibly due to its broader role in integrating sensory input. In addition, the distribution of AVP and its receptors changes throughout the lifespan. During development, AVP immunoreactivity and receptors show remarkable plasticity in response to environmental influences, such as the quality and quantity of interactions with peers and parents (2–10). Therefore, early developmental experiences may shape how an animal interprets sensory cues related to social behavior throughout its lifespan.

The focus of this article is to review evidence that links AVP with the processing of sensory information and to explore whether this alteration of sensory processing leads to diverse behavioral effects across different mammalian species. This paper describes the distribution of AVP and its receptors within sensory organs and within brain areas that receive direct sensory input. We also describe anatomical pathways containing AVP and its receptors that connect the primary sensory cortices with brain areas that process social cues and that direct complex forms of social behavior. We are proposing that the sex-specificity and species-specificity of AVP effects on behavior in mammals result from the variation in the pattern of distribution of AVP and its receptors in sensory pathways. The convergence of sensory input with vasopressin pathways that travel both within the brain and outside of the brain suggests that the effects of AVP on behavior may be mediated by both central pathways that alter the valence of social stimuli and peripheral pathways that alter physiological responses to social stimuli.

BACKGROUND

SEXUAL DIMORPHISM IN AVP: DIFFERENCES BETWEEN PARVOCELLULAR AND MAGNOCELLULAR NEURONS

Arginine vasopressin has been localized in parvocellular and magnocellular neurons that are widely distributed in the central nervous system (11). AVP within a pathway that originates in the medial amygdala (MA) and bed nucleus of the stria terminalis (BNST) and projects to the lateral septum (LS) has been associated with complex social behavior in mammals [reviewed in Ref. (1)]. Early exploration into the association between this pathway and sex-specific patterns of social behavior originated with the identification of sexual dimorphism in AVP immunohistochemistry. In rats (*Rattus rattus*), prairie voles (*Microtus ochrogaster*), meadow voles (*Microtus pennsylvanicus*), and CD1 mice (*Mus musculus*), males show more AVP-immunoreactive (AVP-ir) staining in the BNST and its projections to the LS than do females (12–14). The sexual dimorphism in AVP appears to be testosterone-dependent because castration reduces AVP immunoreactivity in male rats and testosterone implants increase AVP immunoreactivity in female

rats (15–17). Although AVP is found only in mammals, similar sexual dimorphism has been observed in the homologous arginine vasotocin (AVT) pathways of birds, reptiles, and amphibians (18). Although the role of AVT in regulating sensorimotor processing in the amphibian *Taricha granulosa* has been explored elsewhere (19), the role of AVP in regulating sensory processing in mammals has received less attention.

Because AVP immunoreactivity is more pronounced in the male brain, it has been suggested that AVP may regulate male social behavior, and that oxytocin may serve a similar role in the female brain. Early evidence supported the contention that AVP was a key regulator of male social behavior during pair-bonding and aggressive encounters, but more recent evidence also implicates AVP in regulating female social behavior (9, 20–30). The role of AVP in regulating species-specific social behavior in both males and females in different ways in a variety of mammalian species suggests that this neuropeptide serves a broader function in behavioral regulation.

Although behavioral studies usually focus on AVP in the parvocellular pathway described above, AVP produced within magnocellular neurons of the hypothalamus also regulates physiological functions that may indirectly influence an animal's behavioral responses to social stimuli. After its release from the posterior pituitary gland, AVP also enters into the bloodstream where it produces hormonal effects on a variety of peripheral tissues. The magnocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus and of the supraoptic nucleus (SON) appear to be the main sites of AVP production in this pathway and can be distinguished from the parvocellular neurons by measuring their voltage-gated currents (31). Within the bloodstream, AVP is more commonly known as anti-diuretic hormone (ADH), a hormone that increases blood pressure and decreases ion concentrations within the blood by lowering the amount of water that is excreted in urine. AVP also modulates cardiac function by increasing activity of the sympathetic neurons that innervate the heart and decreasing activity of parasympathetic neurons [reviewed in Ref. (32)]. Although the changes in blood pressure and heart rate produced by AVP in response to activation of magnocellular neurons are often overlooked in studies of social behavior, the coupling of these effects with central effects can be critical for normal emotional responses (33). Appropriate responses to social stimuli may be more likely to occur when alterations in heart rate and blood pressure create an optimal level of arousal that allows the animal to focus on subtle social cues.

DISTRIBUTION OF THREE TYPES OF AVP RECEPTORS

Arginine vasopressin produces different effects within the central and peripheral nervous systems because it binds to three main categories of receptors: V1a, V1b, and V2 receptors. Although V2 receptors and both subtypes of V1 receptors are found within the peripheral nervous system, only V1 receptors are found within the central nervous system. Despite the early suggestion that binding of AVP to V1a receptors was responsible for all of the behavioral effects of this neuropeptide, more recent evidence using receptor knockouts indicates that central V1b receptors also are critical for social behavior [(1); for review of V1b receptors, see Ref. (34, 35); for review of V1a receptors, see Ref. (36–38)]. Adding an additional layer of complexity to our understanding of the central effects of AVP is the observation that AVP can bind to oxytocin receptors and that oxytocin may act as an agonist at AVP receptors because of the similarities between the structures of these peptides and their G-protein linked receptors [(39); reviewed in Ref. (40)]. However, the impact of this potential peptide cross-reactivity on social behavior is unclear as it has not been well studied.

Although V1a receptor distribution often varies between closely related species with different mating systems [e.g., Ref. (41–44)], consistent patterns of differences between monogamous and non-monogamous species have not yet been identified. Monogamous California mice (*Peromyscus californicus*) show elevated V1a receptor binding in the LS in comparison to non-monogamous white-footed mice (*Peromyscus leucopus*), but studies in voles have found an opposite pattern with higher V1a receptor binding in the non-monogamous species (42, 45, 46). Despite the lack of consistency in patterns of variability across species, social behaviors related to monogamy can be altered using manipulations of AVP within a single species. In prairie voles, a monogamous species, reduction of V1a gene activity using RNA interference reduces partner preference and other behaviors related to monogamy (47). However, the lack of consistency in V1a receptor distribution patterns between closely related species has led to multiple hypotheses about the reasons for these differences. Although it has been suggested that interspecies variation in V1a receptor distribution may result from differences in aggression, different ecological pressures that alter spatial distributions of animals within their habitats, and/or other social pressures, none of these explanations seem to fit all of the existing data (38, 42, 44, 48, 49). Therefore, it seems reasonable to hypothesize that AVP pathways may serve a broader function related to social behavior because of the large amount of sex and species variation in this system.

VASOPRESSIN AND SENSORY SIGNALING

VASOPRESSIN AND OLFACTION

Olfactory brain circuitry: overview

Two separate olfactory systems exist within the rodent brain that process socially relevant olfactory information. Both of these pathways contain AVP and its receptors (**Figure 1**). In the main olfactory system, input travels from the main olfactory bulb (MOB) to the anterior olfactory nucleus, piriform cortex (PC), and amygdala for additional processing (50). This multi-step pathway brings olfactory information to be processed in multiple areas of the cerebral cortex, including the anterior olfactory nucleus and PC. The anterior olfactory nucleus, a cortical area adjacent to the olfactory bulb that is part of the main olfactory pathway, contains AVP neurons that are activated by the exposure to social odors in rats but not by exposure to other odor cues (51). A parallel system, the accessory olfactory system, brings information from the vomeronasal organ (VNO) into the accessory olfactory bulb (AOB), which innervates the MA, BNST, and cortical amygdala (52). Although it was originally assumed that only the accessory olfactory system processed pheromones and other socially relevant odors, more recent evidence suggests that social information is processed by both pathways (52).

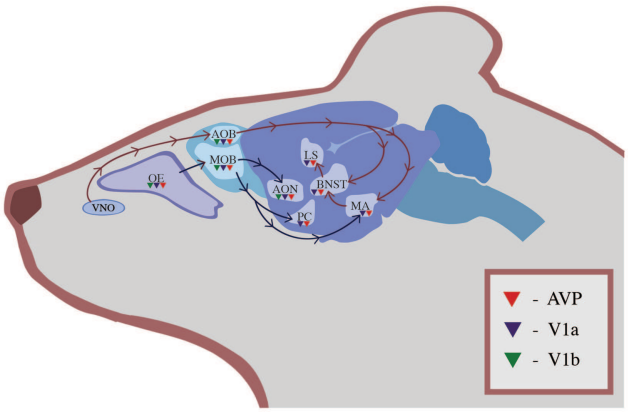

FIGURE 1 | The distribution of AVP (red triangles), V1a receptors (blue triangles), and V1b receptors (green triangles) in olfactory regions of the rodent brain. Information in the main olfactory system travels from the OE to the MOB and then is processed in the accessory olfactory nucleus, PC, and MA. Information in the accessory olfactory system travels from the VNO to the AOB and then to the MA, BNST, and LS.

This redundancy allows olfactory input to be processed simultaneously by non-dimorphic brain areas and by sexually dimorphic, AVP-rich areas such as the BNST, amygdala, and LS. For example, in male rats, exposure to the odors of a receptive female held behind a perforated plastic partition leads to Fos expression in the AVP neurons of the MA (53). The presence of this neuronal marker of gene activation in the MA indicates that the odor of the estrous female is driving activation of the MA and the interconnected hypothalamic brain areas that are associated with reproductive behavior. A different pattern of neural activation occurs in the brain of a female exposed to the odor of an estrous female. Similarly, female rough skin newts (*T. granulosa*) that have been treated with testosterone and AVT show male-typical responses to female odors, suggesting that manipulations of AVP and its homologs can alter olfactory processing of social cues. The ability of AVP to lead to differentiated responses to complex social odors would be favored by natural selection because these differentiated responses allow mammals to target their behavioral responses toward other individuals in a way that maximizes individual fitness. However, the specific role of AVP in driving complex behavioral responses to social odors has yet to be elucidated.

Arginine vasopressin alters olfactory processing in rats from the moment that olfactory information enters the brain via the MOB or the AOB, but this processing occurs in a variety of complex ways. In rats, the olfactory bulb contains a population of AVP neurons with V1b receptors that does not project outside of the olfactory bulb and that may regulate their own activity (54). This modulation within the bulb could decrease or increase sensitivity to social odors. Social odors also activate OR37 receptors in the main olfactory system, which provide direct, monosynaptic input into both the magnocellular and parvocellular AVP neurons of the PVN and the SON (55). It is not clear, however, whether activation of these receptors leads to release of AVP into the bloodstream, activation of brain areas associated with social behavior, or both. V1a and V1b receptors within the olfactory bulb are also associated with neurons that transmit information outside of the olfactory bulb, and additional V1a and V1b receptors have been localized in the olfactory epithelium (OE) and targets of the olfactory pathways such as the PC (56–58). The presence of AVP and its receptors in so many olfactory processing areas indicates that AVP is able to modulate olfactory information, as the information is evaluated by areas of the brain that direct behavioral responses.

Olfactory brain circuitry: interactions between olfactory pathways

Arginine vasopressin's influence on olfactory processing extends beyond the main and accessory olfactory systems. Although some processing of social odors appears to occur in a sexually dimorphic way to allow males and females to respond differentially to sexual cues, other processing of social odors follows similar neural pathways in both sexes. In mice, anatomical evidence suggests that olfactory cues may stimulate the non-dimorphic cells of the MA and activate the magnocellular cells of the SON (14). SON or PVN activation has the potential to lead to release of AVP in the bloodstream where it may alter the behavior of the animal by affecting the arousal level of the animal due to changes in blood pressure or autonomic activation. However, even though this pathway is not sexually dimorphic, modulation of the activity of this pathway might occur in a sexually dimorphic way through interactions with other neurotransmitter systems or with AVP in other neural pathways that are sexually dimorphic.

Our understanding of how AVP alters olfactory processing has been extended by the use of functional magnetic resonance imaging (fMRI) in addition to traditional neuroanatomical techniques. In this paradigm, fMRI BOLD responses are used to monitor changes in neural activation. Central administration of a V1a receptor antagonist in conjunction with the presentation of a male intruder increases BOLD responses in the anterior olfactory nucleus and in the infralimbic prefrontal cortex and decreases BOLD responses in the cortical amygdala in dams (27). The finding indicates that blocking AVP receptor binding during a threatening situation alters how olfactory cues are processed within the brain. In addition, the alteration in infralimbic prefrontal cortex activation is intriguing because this brain area has been implicated in the regulation of fear and autonomic responses to olfactory stimuli and projects to the BNST and LS (59). Because the BNST and LS have been associated with maternal aggression in rodents (22, 23), this pattern of neuronal activation may indicate readiness to attack an intruder. Although we are only beginning to understand how complex behavioral responses are integrated across the entire brain during social situations, these findings suggest that AVP's modulation of olfactory systems may have important implications for an animal's fitness. When AVP receptors are blocked, the typical fear response to an olfactory threat is diminished.

AVP and olfactory processing in Syrian hamsters

Arginine vasopressin also has been demonstrated to play a critical behavioral role in the interpretation of chemical signals during social interactions in Syrian hamsters (*Mesocricetus auratus*). In both male and female Syrian hamsters, infusions of AVP

into the BNST, LS, or anterior hypothalamus increase the frequency of flank marking (60–62). V1a receptor binding in targets of this pathway, such as the ventromedial hypothalamus, is downregulated in response to social cues. Male Syrian hamsters who win repeated aggressive encounters show less submissive behavior and greater V1a receptor binding in the ventromedial hypothalamus than socially subjugated males (6). Therefore, social experience during development in Syrian hamsters leads to plasticity in AVP receptor distribution that shapes the production of olfactory signals. Although it is not known whether AVP also alters the interpretation of olfactory signals in this species, social odor preferences in both females and males are eliminated with lesions in brain areas that contain AVP and its receptors (63, 64). This plasticity indicates that social experiences that alter the distribution of AVP or its receptors within the brain may also shape a mammal's ability to produce and respond to olfactory signals.

Despite parallels between the role of AVP in regulating scent-marking behavior in male and female Syrian hamsters, the social context of AVP release may constrain scent mark production differently in males and females. For example, infusions of AVP or a V1a receptor antagonist into the anterior hypothalamus produce opposite effects in males and females. In female, but not male Syrian hamsters, AVP infusions decrease aggression whereas the V1a receptor antagonist infusions increase aggression (65). Together these results indicate that although AVP may shape the production of olfactory signals like flank marking, the social context of these signals determines how this peptide will influence social behavior. Selection pressures, therefore, can lead to sex differences in how AVP influences the processing of olfactory signals.

AVP and social recognition

The role of AVP in regulating the interpretation of olfactory signals has also been investigated in other rodents using various social recognition paradigms [reviewed in Ref. (50, 52, 66–69)]. A typical protocol involves the exposure of a rodent to an unfamiliar juvenile conspecific. After a delay of 30-120 min and infusion of AVP or one of its antagonists, the animal then is returned to a testing arena that contains either the same conspecific, a novel animal, or both [e.g., Ref. (70, 71)]. Exposure to the odor of a familiar animal typically leads to less olfactory exploration than exposure to the odor of a novel animal.

Arginine vasopressin influences social recognition via two separate pathways: one pathway that is contained entirely within the olfactory bulb and a second pathway that is sexually dimorphic and leads to the LS. Infusion of AVP into the olfactory bulb enhances social recognition in male rats (72). Although infusions of a commonly used V1a receptor antagonist in the olfactory bulb does not eliminate social recognition, performance in a social recognition test is impaired in male rats after infusions of OPC-21268, a non-peptide V1 receptor antagonist that diffuses more widely (54, 72). In both sexes, infusion of a V1a receptor antagonist into the LS decreases olfactory exploration of a novel same-sex juvenile rat (73). Although this finding is somewhat surprising because AVP immunoreactivity is lower in the LS in female rats, V1a receptors are more abundant in females (73). Therefore, the lower AVP content of the LS in females may be counteracted by the presence of additional receptors in this brain area. These findings also indicate that activation of AVP neural pathways may be important for social recognition in both sexes despite anatomical differences between the sexes.

Although initial processing of the olfactory cues used in social recognition occurs within the olfactory bulb, more complex processing occurs in sexually dimorphic AVP pathways. Innate variation in the production of AVP and its receptors within these areas leads to individual variation in social recognition. Female mice who were categorized as "high recognizers" because of strong performance in a social recognition test expressed less mRNA for AVP, V1a receptors, and V1b receptors in the lateral amygdala than "low recognizers," although no differences were identified in the BNST or MA (74). The ability to perform well during a social recognition task appears to result from downregulation of AVP in the lateral amygdala even if AVP in other brain areas like the LS is essential for processing the olfactory cues related to the social recognition task. In addition, in some rodent species, AVP pathways used in social recognition are responsive to changes in the environment that alter gene expression through epigenetic mechanisms that change the packaging of DNA and histones within the nucleus of a cell. For example, environmental influences such as maternal separation have been shown to alter methylation of DNA and to alter AVP gene expression (75). Similarly, administration of a histone deacetylase inhibitor like valproic acid creates epigenetic modifications that can alter olfactory processing. In female mice, valproic acid masculinizes AVP fiber density in the LS and increases the attraction of a female toward same-sex odors (76). This finding suggests that epigenetic mechanisms that are shaped by the prenatal and postnatal environment can alter AVP distribution within the brain and modify an animal's social behavior by modulating responses to olfactory stimuli.

The effects of central release of AVP on social recognition may be amplified by the simultaneous release of AVP into the bloodstream. During a social recognition test in mice using an intact male mouse as a stimulus animal, AVP is released in the SON as measured by microdialysis (77). Because AVP release from the SON leads to elevation of plasma AVP, this indicates that central activation and peripheral activation of AVP pathways may be linked. Lesions of the MA also disrupt the release of AVP from the SON, suggesting that olfactory processing by the pathway projecting from the MA to the LS may activate release of AVP from the SON (77). This connection between the MA and the magnocellular cells of the SON may modulate aggressive responses while elevating levels of AVP in the bloodstream (14).

Despite compelling data indicating that AVP regulates social recognition in both sexes, elimination of one type of AVP receptor does not block social recognition consistently. In male mice, a null mutation in the V1a or the V1b receptor leads to impairments in social recognition [V1a: (78) and V1b: (79)]. Deficits caused by a null mutation in the V1a receptor can be reversed by re-introducing the V1a receptor into the LS using a viral vector (80). However, other studies have failed to identify any impairment in social recognition in male V1a receptor knockout mice despite mild olfactory impairments (81). Female mice with a null mutation of the V1a receptor also display a normal Bruce effect, which is the loss of a pregnancy after the odor of urine of an unfamiliar male

activates the vomeronasal system (82). In contrast, female mice with a null mutation in the V1b receptor do not show a normal Bruce effect, indicating that the V1b receptor may be more critical for this response in females (82). Inconsistencies between studies may result from the involvement of multiple receptor types and neurotransmitter systems in processing socially relevant olfactory signals.

Evolutionary significance of olfactory processing

The ability to discriminate between the odors of a mate, offspring, and an unfamiliar individual is important for individuals from social species. The evolutionary significance of olfactory investigation, however, may be compounded by the ability to extract additional complex information from odors. Male rats not only can discriminate between individuals but also avoid the odors of ill conspecifics. This avoidance response requires intact AVP neural pathways because exposure to these odors upregulates mRNA for V1a and V1b receptors in the MA, but does not occur if a rat receives a microinfusion of a V1a or V1b receptor antagonist into the MA (83). Although an increase in c-fos mRNA in the olfactory bulb and BNST indicated that those brain areas were activated by exposure to illness-related odors, V1a or V1b receptor binding was not elevated in these brain areas. Therefore, processing of illness-related odors in the AVP system occurs specifically in the MA, a brain area associated with fear. This example illustrates the idea that AVP pathways may assist with creating a complex emotional and behavioral response to the odor of a conspecific that varies depending on the conspecific's familiarity, sex, health, age, and other individual characteristics.

AVP AND AUDITORY PROCESSING

Neural processing of auditory signals in birds, fish, and frogs

Although AVP has most commonly been associated with processing of olfactory signals, AVP also assists with processing of auditory signals. A clear link between auditory processing and a non-mammalian AVP homolog, AVT, has been established in birds, frogs, and fish. For example, pairing male and female zebra finches (*Taeniopygia guttata*) increases AVT mRNA in the PVN and BNST of both sexes, and the magnitude of the increase in mRNA production in males is associated with the quantity of singing behaviors (84). Male zebra fiches that choose to sing to a female behind a wire barrier also have more AVT-immunoreactive neurons in the BNST than non-singers, again indicating a linkage between auditory signal production and social behavior in this species (85). In addition to these correlational linkages between AVT and singing behavior, direct manipulations of AVT using intraventricular infusions also increase singing behavior in female sparrows (*Zonotrichia leucophrys gambelii*; 86). Even though these studies do not indicate whether AVT influences auditory processing, an additional study in Lincoln's sparrows (*Melospiza lincolnii*) demonstrates that sparrows alter the effort used in song production and show changes in AVT-immunoreactivity depending on the quality of songs to which they are exposed (87).

Evidence linking AVT to auditory processing also has been found in fish and frogs. AVT is present in brain areas responsible for auditory integration in the plainfin midshipman fish (*Porichthys notatus*), a species where males vocalize to attract mates and during nest defense (88, 89). Similarly, AVT is more abundant in auditory processing areas of male bullfrogs (*Rana catesbeiana*) in comparison to female bullfrogs (90). Because calls are only produced by males in this species, this sexual dimorphism may indicate that AVT plays a key role in assessing the salience of auditory signals that are being produced by conspecifics. Manipulations of the AVT system support this idea because AVT infusions change call properties of túngara frogs (*Physalaemus pustulosus*), possibly interfering with communication between the sender and the recipient (91, 92). Similarly, in the gray tree frog (*Hyla versicolor*), auditory cues shape the effects of AVT on calling behavior because AVT only alters call quality when a male is in close proximity to another male (93). Together, these results indicate that AVT modulates calling behavior in response to auditory cues and allows animals to produce calls that are appropriate for a particular social context.

Neural processing of auditory signals in mammals

The distribution of AVP and its receptors within brain areas associated with auditory processing has been studied in less detail in mammals, possibly because much of the AVP research has focused on rodents that use olfaction as the primary sense for social assessments. Although it is known that female, but not male, guinea pigs (*Cavia porcellus*) display AVP-ir staining in the auditory brainstem, the function of this sexual dimorphism is unknown (94). In the few rodent species where the functional importance of AVP on vocalization has been studied, a role for both V1a and V1b receptors has been identified. Female mice with a null mutation in the V1b receptor show decreased ultrasonic vocalizations during resident-intruder aggressive encounters (95). Although vocal production is affected, it is not known whether auditory processing also differs between control mice and mice with a null mutation in the V1b receptor. Anatomical studies using singing mice (*Scotinomys teguina* and *Scotinomys xerampelinus*) that vocalize in social contexts have identified the presence of V1a receptors in brain areas used for vocal production and auditory responses (49). In both species of singing mice, V1a receptors are expressed in the medial geniculate nucleus, which is the region of the thalamus that processes auditory information (49). In addition, both species display V1a receptor binding in two brain areas associated with vocalization, the periaqueductal gray and anterior hypothalamus, with more extensive binding in the more vocal species, *S. teguina* (49). These findings are intriguing because they implicate AVP in the give-and-take of information that occurs during social communication. In mammalian species where vocalizations are used in social situations, selection may favor a role for AVP in modulating these signals.

Although the effects of central AVP on auditory processing have not been studied in humans due to methodological limitations, human genetic variation in V1a receptor haplotypes has been linked with auditory processing and communication. The number of repeats in the RS3 microsatellite marker for the V1a receptor has been positively linked with prepulse inhibition, a startle response that is suppressed in individuals with schizophrenia and other disorders of social communication (96, 97). This phenotype has been linked more closely to auditory communication in studies examining the relationship between V1a receptor

variation and an individual's musical aptitude. In families containing either a professional or active amateur musician, the ability to detect structural changes in abstract sounds is linked with RS1 and RS3 microsatellite markers for the V1a receptor (98). Interest in music, another marker of interest in listening to auditory cues, is also associated with V1a receptor distribution in humans: the RS1 haplotype is most strongly associated with listening to music regularly at the present time and the RS3 haplotype is most prominently associated with listening to music regularly throughout the lifespan (99). Although studies using viral vectors to manipulate V1a receptor activity cannot be performed in humans, these correlational results indicate that central processing of auditory signals in humans is modulated by AVP and its receptors.

AVP as a hormone and acoustic processing in the ear

In addition to affecting neural processing of auditory signals, AVP in the bloodstream also affects hearing. Peripheral injections of AVP in rats create short-term hearing impairment as measured by evoked auditory brainstem responses to sound (100). In the inner ear, AVP also alters hearing by binding to V2 receptors and reducing the number of aquaporin-2 membrane channels, channels that increase water permeability (101). AVP binding to V2 receptors thus leads to hearing impairment through the accumulation of excess water in the membrane of the endolymphatic sac (102, 103). Because insulin interacts with the signaling pathway that regulates V2 receptors, disruption in this pathway in diabetic patients has been linked to hearing loss in humans (103). Similarly, excess endolymphatic fluid due to excess plasma AVP levels or V2 receptors in the inner ear produces the symptoms of Menière's disease, a disorder in humans that results in intermittent hearing loss, vertigo, and tinnitus (104–106). Therefore, release of AVP into the bloodstream in response to social or stressful stimuli may have a secondary effect of reducing sensitivity to sound cues.

Although excess AVP in the bloodstream may hamper the ability to detect auditory cues, smaller elevations in plasma AVP may increase recall of auditory information due to increased arousal. Peripheral administration of AVP can enhance performance in learning tasks due to elevation of heart rate, blood pressure, and other sympathetic nervous system activity [reviewed in Ref. (107)]. Administration of AVP via intranasal infusions in healthy, non-depressed elderly humans increases recall of auditory information, possibly due to this increase in arousal (108). Natural variation in AVP in humans also is correlated with performance on auditory learning tasks. In humans with major depression, plasma concentrations of AVP correlate positively with auditory memory as measured by a 10-WLLA test for audio recall (109). Diabetes insipidus, a disorder characterized by a mutation in the vasopressin prohormone that leads to lower plasma levels of AVP, is associated with decreased performance on a test of verbal memory in humans (110). Although elevations of AVP in the bloodstream can lower detection of auditory signals, once those signals are detected, AVP can enhance recall of information provided in those signals.

AVP AND PROCESSING OF TASTE INFORMATION

AVP and conditioned taste avoidance

A key to understanding the evolution of taste aversion in mammals and its link to social behavior has been provided by studies of gustatory learning in *Caenorhabditis elegans*, a nematode that utilizes chemoattraction to locate low salt environments (111). Modulation of this ability occurs through the action of a vasopressin/oxytocin-like neuropeptide, nematocin. When exposed to preferred low salt environments in the absence of food, worms with a null mutation for nematocin or its receptor are not able to learn to avoid these environments (111). Individuals lacking nematocin or its receptors also exhibit deficiencies in male mating behavior and other forms of social behavior due to altered activity of mechanosensory neurons (112). These findings implicate the ancient neuropeptide, nematocin, in the integration of sensory input into complex motor output in the model organism *C. elegans*. This link between AVP, taste, and social behavior seems to be preserved in more complex organisms like birds and mammals. In the zebra finch (*Taenioypygia guttata*), intraseptal infusion of a V1 antagonist reduces gregariousness and increases the latency to feed in the presence of a novel stimulus (113). However, in mammals, it is unclear whether the effects of AVP on taste also modify social behaviors.

Arginine vasopressin within the bloodstream may alter the processing of taste in mammals through a mechanism similar to the one described above for auditory learning. By altering arousal through activation of the sympathetic nervous system, AVP improves an animal's ability to learn to avoid an aversive stimulus. In rats, taste aversion to saccharine lasts longer if administration of desglycinamide-lysine vasopressin occurs prior to pairing of the taste of saccharine with nausea induced by injections of LiCl (114). Other studies, however, have demonstrated the opposite relationship with AVP injections leading to faster extinction of learned responses. The timing of AVP administration appears to be critical because the learned association disappears more rapidly if AVP is administered after the acquisition process, regardless of whether the AVP is administered peripherally or centrally (115–117). However, dosage of AVP does play a critical role because high doses are capable of inducing taste aversion without administration of LiCl following consumption of a sucrose solution (117). Although peripheral AVP injections produce similar effects as central AVP infusions, this effect still seems to result in part from the release of AVP from the neurons of the PVN. AVP is released in the PVN during extinction of taste avoidance responses in rats that have not been deprived of water as part of the testing protocol (118). Because radioimmunoassay shows no changes in AVP content in brain areas that have been associated with conditioned taste avoidance such as the medial septum, LS, insular cortex, or MA, this increase in AVP content in the PVN is most likely related to the osmoregulatory properties of AVP in the PVN (118). However, future studies may clarify the role of neural AVP in regulating taste aversion. Taken together, these data indicate that AVP can either improve sensory learning or hasten extinction of learned responses depending on the timing of AVP release.

AVP and modulation of taste

One molecular mechanism that AVP may use to modify taste is through modulation of the activity of epithelial sodium ion channels (ENaCs) that regulate salty and sour tastes [**Figure 2**; (119)]. ENaCs are found in the renal collecting duct, urinary

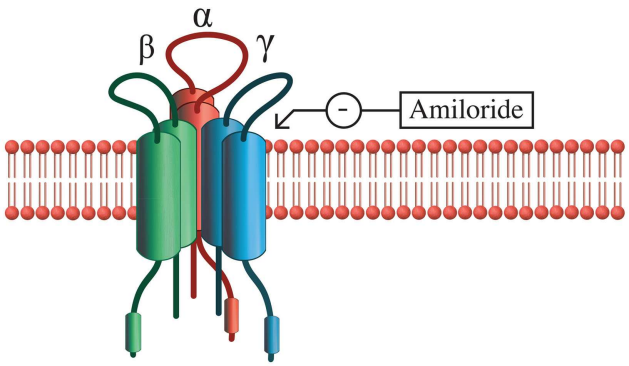

FIGURE 2 | Epithelial Na^+ channel structure. ENaCs are composed of three subunits: α, β, and γ, and transport salt in a variety of epithelial tissues in the body. The α subunit alone is capable of functioning as a sodium transporter, but the addition of β and γ subunits increase the efficiency of this process. Sodium transport activity of ENaCs can be blocked with amiloride or enhanced with AVP.

bladder, lung, and taste buds and consist of three subunits that form a pore that selectively permits sodium to cross the membrane [reviewed in Ref. (120)]. In fungiform taste cells of hamsters, AVP increases sodium ion currents in ENaCs, indicating that the threshold for stimulation of these cells is being lowered (121). Therefore, AVP in the bloodstream may increase sensitivity to salty or sour tastes through its action on the ENaCs in taste cells. Under conditions where blood volume is low, this response may be part of a homeostatic mechanism to increase water retention in the blood by enhancing the palatability and consumption of salty food (121, 122). Although high doses of AVP appear to inhibit salt intake by making animals feel ill, central administration of lower doses of AVP stimulate salt intake whereas V_1 receptor antagonists inhibit salt intake (123). At the neural level, ENaCs also appear to be involved in regulating salt intake because they are co-localized with AVP in the magnocellular neurons of the SON and PVN, cells that assist with osmoregulation (124). Interestingly, these neural sodium channels are similar to ENaCs of the tongue in that both exhibit sensitivity to amiloride, a potassium-sparing diuretic that inhibits taste responses (124). In the brain, ENaCs may act as sensors of salt concentrations on the cells of the PVN and SON and lead to alterations in neural firing rates in response to fluctuations in salt concentration in the cerebrospinal fluid (124). Although it is unknown how ENaC activation by AVP may alter complex social behavior in mammals, these data indicate that behavioral responses that lead to release of AVP from the PVN and SON may also increase sensitivity to taste.

Disruption of taste sensitivity may be an important clue in diagnosing diseases that are accompanied by AVP dysregulation, such as syndrome of inappropriate ADH secretion (SIADH) (81, 125). In patients with SIADH related to lung cancer, the excess AVP alters taste perception (81, 126, 127). In patients with low serum sodium levels due to the effects of AVP on blood osmolality, correction of these levels led to improvement of taste function (81, 126, 127).

AVP AND VISUAL PROCESSING

Although visual information feeds into many brain areas that contain AVP, such as the BNST and LS (128, 129), the effect of AVP on processing of visual information has received little attention. Comparative analysis of visual opsin sequences in a variety of vertebrate species indicates that the visual opsins and AVP receptors are found in a shared genomic region that was duplicated twice during vertebrate evolution (130). However, the behavioral significance of the linkage between these sequences has not been examined. In chickens, a visual opsin has been co-localized with AVT in the neurons of the PVN, SON, and BNST (131). Although the function of opsins outside of the eye is unknown, these pigments may allow these neurons to respond to light and assist with coordination of circadian rhythmicity in behavior (131).

Connections between AVP and visual processing have been made in studies of visual learning, although these studies have focused more on AVP enhancement of sensory learning instead of AVP alteration of visual processing [e.g., Ref. (132, 133)]. In non-mammalian species, however, it has been demonstrated that AVT, a homolog of AVP, stimulates interest in visual cues associated with reproduction in rough skin newts (134). Additional studies in mammalian species that rely on visual cues during social interactions will help to clarify the role of AVP in visual processing.

CONCLUSION AND FUTURE DIRECTIONS

A social animal is bombarded with sensory cues throughout his or her lifetime. Even at birth, animals are usually attracted to the odors of their mothers but avoid unfamiliar odors. These and similar sensory cues are channeled through the central nervous system of the animal leading to output in the form of the social behaviors that are necessary for that individual's survival. In social rodents who heavily utilize their olfactory senses to distinguish threat from other environmental stimuli, AVP has been localized within the olfactory bulb itself and within the targets of the olfactory pathways. Therefore, as AVP neurons of the main and accessory olfactory systems are activated, they may modulate this olfactory input and direct it to the appropriate sites in the brain that regulate behavioral output. In addition, olfactory pathways also send information to the magnocellular neurons of the PVN and SON, which release AVP into the bloodstream. Additional research may clarify the degree of interplay between these two systems. The relationships between AVP in the olfactory system and other neurotransmitter systems and other sensory systems also should be explored in greater detail.

Although evidence also indicates that a relationship exists between AVP and other sensory modalities, these relationships have not been studied as extensively as the relationship between AVP and olfactory processing. Because most studies examining the role of AVP in sensory processing have been performed in rodents that use olfaction as the primary sense, other sensory modalities have received less attention. At this time, it is not known whether AVP in brain areas associated with mammalian visual, taste, or auditory processing shows the same level of plasticity in response to social or hormonal cues that shape behavior. It is also unknown whether manipulations of AVP can produce broad alterations in sensory processing in a variety of modalities in mammalian species with different social systems. How an animal is able to filter these

social cues and make complex behavioral decisions needs to be explored in additional detail. However, it is clear that AVP modulates sensory processing through a variety of neural and hormonal pathways that are critical for the expression of species-typical social behavior.

Arginine vasopressin may be essential for integration of sensory input during complex forms of social behavior in mammals. As described earlier in this paper, fMRI has been used to assess neural activation in maternal rats presented with the threat of a male intruder (27). When a V1a antagonist is administered prior to exposure to the intruder, the gustatory cortex and olfactory areas of the brain show enhanced BOLD responses in females (27). This finding indicates that AVP is involved in regulating multiple sensory responses during complex social interactions, but should be explored under other social contexts and in additional species.

Studies using peripheral and central injections of AVP have demonstrated that the hormonal effects of AVP can shape gustatory, visual, and auditory learning through its effects on general levels of arousal. In addition, levels of AVP within the bloodstream can influence sensory processing in the ear and tongue through modulation of sodium channels. Within the ear, excess AVP has been associated with impaired hearing due to excess endolymph accumulation. In the tongue, AVP appears to modulate the activity of sodium channels known as ENaCs. Whether AVP shows similar ability to regulate visual signal transduction in a similar way has yet to be explored.

The apparent consequence of this fine-tuning of sensory input is that an animal is able to display appropriate social behavior in response to particular environmental stimuli. Animals with null mutations in vasopressin or its receptors demonstrate sensory deficits that appear to be potentially correlated with deficits in social behavior. In addition, the plasticity in AVP pathways during development that has been demonstrated in a variety of rodent species (2–4, 8, 135–137) may affect social behavior through alterations in the processing of sensory signals. However, the impact of developmental plasticity in AVP on sensory processing in multiple modalities has not yet been explored.

In humans, attempts have been made to link AVP with disorders that affect social behavior and involve sensory processing issues such as autism and schizophrenia [reviewed in Ref. (138)]. Because it is impossible to directly manipulate AVP or its receptors within specific brain areas in humans, the relationship between AVP and behavioral disorders has been assessed by correlating plasma AVP levels or V1a receptor promoter polymorphisms with behaviors associated with these disorders. Although the relationship between AVP and social behavior disorders has been difficult to establish in males, plasma levels of AVP have been linked to severity of psychosis in women with schizophrenia (139). Similarly, in girls with autism, plasma levels of AVP have been linked to the intensity of repetitive behaviors (140). Genetic linkages between AVP and the likelihood of developing a disorder of social behavior have also been identified in humans. A specific genetic polymorphism in the V1a receptor promoter was associated with increased susceptibility of psychopathology in children that had been exposed to war (141). The latter finding suggests that although certain V1a genotypes may predispose an individual to developing a disorder related to social behavior, exposure to environmental stressors also plays a role in the manifestation of the disorder. Therefore, it is possible that early social experiences may create life-long changes in the way that an individual perceives sensory cues in the surrounding world.

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The use of animal models to decipher physiological and neurobiological alterations of anorexia nervosa patients

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Extensive studies were performed to decipher the mechanisms regulating feeding due to the worldwide obesity pandemy and its complications. The data obtained might be adapted to another disorder related to alteration of food intake, the restrictive anorexia nervosa. This multifactorial disease with a complex and unknown etiology is considered as an awful eating disorder since the chronic refusal to eat leads to severe, and sometimes, irreversible complications for the whole organism, until death. There is an urgent need to better understand the different aspects of the disease to develop novel approaches complementary to the usual psychological therapies. For this purpose, the use of pertinent animal models becomes a necessity. We present here the various rodent models described in the literature that might be used to dissect central and peripheral mechanisms involved in the adaptation to deficient energy supplies and/or the maintenance of physiological alterations on the long term. Data obtained from the spontaneous or engineered genetic models permit to better apprehend the implication of one signaling system (hormone, neuropeptide, neurotransmitter) in the development of several symptoms observed in anorexia nervosa. As example, mutations in the ghrelin, serotonin, dopamine pathways lead to alterations that mimic the phenotype, but compensatory mechanisms often occur rendering necessary the use of more selective gene strategies. Until now, environmental animal models based on one or several inducing factors like diet restriction, stress, or physical activity mimicked more extensively central and peripheral alterations decribed in anorexia nervosa. They bring significant data on feeding behavior, energy expenditure, and central circuit alterations. Animal models are described and criticized on the basis of the criteria of validity for anorexia nervosa.

Keywords: genetic models, environmental models, anorexia nervosa, acute stress, social stress, food restriction, activity/hyperactivity

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Introduction

Eating disorders represent a large field of investigation in industrialized societies where food intake behaviors and quality of food become indisputable and incoherent. Research projects are currently focused on obesity, a dramatic consequence of overconsumption of fat and carbohydrates. However, populations of these societies also suffer of other dramatic but underinvestigated

eating disorders. These eating disorders are presently defined according to the American Manual of Psychiatry DSM-5 (1) and divided into three main subtypes of eating disorders: anorexia nervosa (AN), bulimia nervosa (BN), and binge eating disorder (BED). The main characteristics of these three subtypes are summarized in **Table 1**. As usually mentioned by psychiatrists, the subtype determination at the time of diagnosis should be considered carefully since the majority of women with AN crossed over between the other subtypes [BED or BN; (2)].

Anorexia is said to be restrictive if during the past 3 months the person has not engaged in recurrent bulimic crises or purging behavior (i.e., self-induced vomiting or abuse of laxatives, diuretics, or enemas). One might consider this restrictive AN (AN-R) subtype as an awful eating disorder as the chronic refusal to eat leads to severe and sometimes irreversible complications for the whole organism, until death. AN-R is considered as a multifactorial disease with a complex etiology. The dramatic physiological and psychological consequences on health generated by the low food intake might lead to central and/or peripheral reprograming that permits the organism to endure in a first step, this reduced energy supply. A better understanding of the different facets of this disease becomes an urgent necessity to find novel therapeutic approaches complementary to the classic psychological therapies.

The objectives of this review are first to present briefly the main pathophysiological alterations observed in AN-R patients, then to introduce the different animal models that are currently used or could be used to better apprehend the physiological, metabolic, and neurobiological dysfunctions associated with AN-R, and finally to discuss the potential contribution of these models for understanding the pathology.

Physiological Alterations in Restrictive Anorexia Nervosa: From Neurobiology to Genetic Polymorphisms

The two faces of anorexia, physiological and psychological, which were first used to describe the disease, then were neglected by the psychiatrists and psychologists for years are now more and more widely accepted by numerous clinicians and practicians to interfere.

Physiopathological Alterations

The recent DSM-5 (2013) suggests diagnosing AN-R by three major criteria. The first criterion is a severe and persistent restriction of energy intake leading to significantly low body weight in context of what is minimally expected for age, sex, developmental trajectory, and physical health. The gradual loss of weight can reach more than 50% of the initial body weight. The second criterion is the intense fear of gaining weight or of becoming fat. The third criterion is a disturbance in the way AN patients experience their body weight or shape (dysmorphophobia), associated with persistent lack of recognition of the seriousness of the current low body weight. Another important criterion, amenorrhea or the absence of at least three menstrual cycles, was removed in the DSM-5. This criterion was deleted since it cannot be applied to patients of different age and gender. Moreover, some data describe individuals who exhibit all other symptoms and signs of AN, but still report some menstrual activity (5, 6).

Anorexia nervosa has one of the highest mortality rates of all psychiatric diseases (7, 8). In a 21-year follow-up study, Löwe et al. (9) showed that 16% of AN patients deceased due to consequences of the illness. Among them, about 50% died because of somatic complications and the other 50% committed suicide. In fact, the course of AN is extremely variable, with approximately 50–60% of individuals with AN that recover, 20–30% that partially recover, and 10–20% that remain chronically ill (9, 10). Among the different clinical studies conducted on AN patients, low ionic plasma concentrations, symptomatic hypoglycemia, and anemia are often associated with lymphopenia that can generate opportunistic infections or hepatic cytolysis in some cases. However, contradictory results were published concerning essential amino acid levels in plasma of AN patients and healthy controls (11–13). Modifications in essential metabolites might be related to the generalized amyotrophy often described in AN patients. Moreover, increase in the metabolic hormone levels (like ghrelin or cortisol) is often observed, and the endocrine function of adipose tissue is modified resulting in increased circulating levels of adiponectin and decreased concentrations of leptin (14, 15). Usually, AN-R patients also showed a nutritionally acquired hepatic resistance to GH with decreased production of IGF-1 and increased GH levels. Such increase is due to (i) a reduction of IGF-1 feedback on pituitary and hypothalamus GH secretion and (ii) high levels of ghrelin, a GH secretagogue (16). Additionally, osteoporosis,

TABLE 1 | Main characteristics of the mean eating disorders: anorexia nervosa (AN), bulimia nervosa (BN), binge eating disorder (BED).

	AN	BN	BED
BMI	$<17.5\,kg/m^2$	$>17.5\,kg/m^2$; $<25\,kg/m^2$	$>17.5\,kg/m^2$
Lifetime prevalence	1.9–2.6% (3)	0.5–1.5% (4)	2–3.5% (4)
DSM-5	Distorted body image, excessive dieting	Recurrent episodes of binge eating followed by inappropriate purging behaviors (self-induced vomiting)	Recurrent episodes of eating significantly more food in a short period of time than most people would eat under similar circumstances, feelings of lack of control
Personality traits	Anxiety, fear to gain weight, avoidance, perfectionist, poor self-esteem, compulsivity dysmorphophobia	Anxiety, avoidance, poor interoceptive awareness, ineffectiveness, self-directedness, stress reactivity, perfectionism	Anxiety, poor self esteem, harm avoidance, impulsivity
Comorbidities	Anxiety, depression, TOC, addiction, phobia	Anxiety, depression, TOC, addiction, phobia (obesity)	Anxiety, depression, TOC, addiction, phobia, obesity

another main complication of AN affecting 20–50% of cases, has been observed and is often irreversible (17, 18). Behavioral changes like physical (or intellectual) hyperactivity observed in 31–80% of the cases might also be associated with AN (19). Finally, disordered fluid intake is currently associated with AN-R, 54% of patients drinking excessively, and 28% drinking restrictively (20). This leads to relatively frequent renal complications (21).

Neurobiological Alterations

Anorexia nervosa is often associated with psychiatric comorbidities like depression, anxiety, obsessive–compulsive or personality disorders, and drug abuse (22). It becomes more and more accepted that AN-R resembles an addictive behavior disorder linked to food deprivation, weight loss, or physical activity. In fact, neuroimaging studies have first pointed out morphological changes affecting gray and white matters (23). The systematic review of Phillipou et al. (24) summarizes a number of brain differences, which are reported in AN patients. The neural profile of AN corresponds to a predominant imbalance between the reward (meso-cortico-limbic system) and inhibition (prefrontal cortex) systems of the brain. Recent data of Kullmann et al. (25) suggest that AN patients showed a reduced connectivity in the brain areas involved in the cognitive control and an increased connectivity in regions important for salience processing. The demonstrated altered integrity of the inferior frontal cortex might contribute to the physical hyperactivity developed by AN patients due to its role in the general behavioral inhibition like motor response. Furthermore, dysfunction of the central monoaminergic systems has been related. The review of Bari and Robbins (26) describes the implication of these systems as pathological neural substrates of diseases. They underline that prefrontal noradrenergic neurotransmission is involved in the inhibition of an already initiated response whereas dopaminergic system appears to modulate motor readiness for both inhibition/activation and reward, respectively at the level of the dorsal and ventral striatum. Dopamine has been associated with the expression of an appetitive reward system (27), and probably works in mutual opponency with a system that signals the prediction of punishment instead of reward. Serotonin neuromodulation might contribute to the more affective part of the inhibition behavior and/or the wanting behavior. Serotonin has a critical role in the adaptation of animals to aversive events, in the inhibition of appetite, and in anxious and obsessive behaviors, as well as in depression. Furthermore, harm avoidance is a temperament trait highly observed in AN patients (28), that reflects inhibition and anxiety and involves both dopamine and serotonin (5-HT) neurotransmission (29). AN patients show decreased dopaminergic metabolite levels in the cerebro-spinal fluid as well as increased dopaminergic D2/D3 receptor density (30, 31). Similarly, levels of serotonin markers like blood serotonin contents, plasma tryptophan are lower in AN patients compared to non-eating disordered subjects (32). Brain imaging studies using serotonin-specific radioligands have consistently shown 5-HT1A receptor binding is increased in cortical and limbic structures in ill and recovered AN patients (33, 34), whereas 5-HT2A receptor binding remains normal in ill patients (33). 5-HT transporter activity is also increased in recovery AN patients (35). The basal hyperfunctioning of the serotonergic pathway described in these various studies may be related not only to alteration in the reward process of food intake but also to anxiety, behavioral inhibition, and body image distortions (29, 36, 37).

Finally, one might also consider the involvement of the endocannabinoid neurotransmission in the neurobiological changes observed in AN patients. As reviewed by Monteleone and Maj (38) in a positron emission tomography study, AN patients showed a dysregulated endocannabinoid tone with enhanced plasma anandamide (AEA) levels and an increased number of cannabinoid type 1 receptors (CB1) in the insula and inferior frontal and temporal cortex of underweight AN patients. These data underline or suggest that altered food intake in AN patients may be a consequence of aberrant reward processing combined with an exaggerated cognitive control [see review in Ref. (39)]. Consequently, the current psychopharmacologic strategy in the treatment of AN uses typical and atypical antipsychotics, tetrahydrocannabinol, anticonvulsants, antidepressants, which modulate the synaptic signals of these neuromediators, but which have been illusive for decades [see Ref. (40)]. Thus, dissecting the mechanisms of action of the different neuropeptides/neurotransmitters involved in the regulation of food intake, as well as in the motivational aspects of feeding, becomes a necessity to open new perspectives for an efficient therapy of this disease complementary to the psychological approaches.

Genetics

As clearly summarized by Scherag et al. (41), formal genetic studies suggested a substantial genetic influence in eating disorders and particularly in AN. The possible involvement of genetic components was strengthened by several twin and family studies concluding that AN presents genetic etiological components for 33–84% of the patients (42–45). Beside this, genome-wide linkage screens have been performed in order to identify unknown genes involved in AN. In the following paragraphs, presented data without reference to publication where cited in Scherag et al. (41).

Investigation on the genes directly involved in the regulation of feeding and energy expenditure was performed. The *leptinergic–melanocortinergic* system includes several key factors of the regulation of food intake and body weight. Surprisingly, despite the anorexigenic role of the leptin hormone, critically involved in the regulation of energy balance and adaptation of organism to semi-starvation, mutation analysis of the leptin gene and of the leptin receptor gene did not show any association with AN (46, 47). Agouti related peptide (AgRP), an orexigenic peptide, acts downstream of leptin through inhibition of central melanocortin receptors (MC receptors). Several studies concluded that the Ala67Thr AgRP polymorphism is significantly associated with AN. However, the involvement of this polymorphism in AN patients remains to be determined. This mutation would cause a lower inhibition the MC4R, a decrease in food intake, and would increase the risk of developing anorexia (48, 49). Brain-derived neutrophic factor (BDNF) is indirectly involved in the negative control of food intake. Low plasma levels of BDNF were determined in acute patients with AN. Several studies found that variants of BDNF and BDNF receptors (TrkB) are associated with AN. Moreover, AN patients often display high plasma levels of *adiponectin*, an adipocyte hormone known to play a role in the regulation of food intake and energy expenditure. Recently, a German study showed that several single nucleotide polymorphisms

within the adiponectin (AdipoQ) locus were associated with adiponectin serum levels or eating behavior (50), but there is no convincing published study on the linkage between adiponectin gene polymorphism and AN.

Among the neurotransmitters suspected, genes involved in the *serotonergic* and *dopaminergic* systems have been pointed out. An overexpression of serotonin was suggested in AN. An association was shown with AN for serotonin transporter, serotonin receptors, and tryptophan hydroxylase 2 expressions. Moreover, positive but non-significant associations were also observed for dopamine D2 and D4 receptors and catechol-O-methyltransferase genes. The *norepinephrine* system was also investigated as low norepinephrine serum levels were always measured in recovery AN patients. Variants of the norepinephrine transporter gene that could lead to a lower norepinephrine reuptake have been associated with AN.

The *endocannabinoid system* is particularly involved in the regulation of appetite, food intake, and energy balance. Cannabinoids stimulate food intake through activation CB1. A study on 52 families showed that an allele of CB1 gene was more often transmitted in the restricted AN group. Moreover, in the Japanese population, Ando et al. (51) showed an association of a polymorphism of fatty acid amide hydrolase, which role is to inhibit the activity of the main CB1 ligand (N-arachidonoyl-ethanolamide), with AN.

As a first general comment on these data, it is to note that for several genes there is no evidence to suggest that any of the polymorphisms identified has a functional consequence on the biological activity or expression of the resulting protein. This may lead us to ponder these data when we try to establish linkages between polymorphisms and physiology or etiology. A second conclusion is that most of the polymorphisms that were shown to be associated with AN are related to the central nervous system, and particularly factors involved in the regulation of energy balance.

Inputs of Animal Models of AN

Development of appropriate animal model of AN appears to be something difficult given the complex etiology. Although psychological factors play a pivotal role in the development of AN, a better understanding of the biological basis of this eating disorder can help to improve current treatments additional to therapies currently used by psychologists and psychiatrists. However, due to obvious ethic reasons, all the aspects of AN remain difficult to assess rendering necessary to develop relevant animal models. Thus, in rodents, different genetic and environmental models have been developed with varying degrees of success.

Genetic Models

Two categories of genetic models are commonly used: models presenting spontaneous mutations and genetically engineered models that can be constitutive or conditional.

Spontaneous Mutations

Anx/anx mice

This model has been extensively studied and described (52). The mutant mice *anx/anx* emerged spontaneously in the Jackson Laboratories (Bar Harbor, USA) in 1976. These mice are characterized by an emaciated appearance, a reduction in food intake, and early death 3–5 weeks after the birth (53). Moreover, serotonergic hyperinnervation and decrease in the striatal dopamine concentration and its metabolites may contribute to alterations in the locomotor and reward systems (54, 55). The *anx/anx* phenotype is associated with an approximative 50% downregulation of the gene *Ndufaf1* in the hypothalamus. It encodes a protein required for assembly of mitochondrial complex I (56). These mice exhibited several deviations in the hypothalamic neurotransmitter and neuropeptidergic systems involved in the regulation of food intake and energy metabolism, with a down-regulation of anorexigenic peptides POMC and CART and variations in the expression of the orexigenic NPY and AgRP peptides in the arcuate nucleus (54, 57–59). The reduction of the leptin peak, usually observed around postnatal day 8, could alter the arcuate neuronal development (60). These data are associated with mitochondrial dysfunction and neurodegeneration/neuroinflammation processes (52, 56, 61, 62). All these data suggest that this natural genetic model of anorexia represents an excellent model of anorexia–cachexia syndrome characterized by an inflammatory response that might be useful to dissect mechanisms that lead to physiological dysfunctions observed in AN. Here, the main limitations of this genetic anorexia model are: (i) the premature death of the mice before reaching puberty and (ii) effects observed on both male and female mice.

Lou/C rats

Lou/C rat is a rat substrain obtained from a Wistar rat selection at the Louvain University (Belgium). Lou/C rats are mainly characterized by a long life span until 35 months in male and 40 months in female (63, 64). These rats present the particularity to be resistant to diet-induced obesity and age-induced obesity since they exhibited a spontaneous food restriction, by eating fewer calories per day than Wistar rats in standard chow diet (63). The decreased food intake level is associated with a lower body weight (65, 66) itself associated with high energy expenditure and high sympathetic tone in the white and brown adipose tissues (67). Interestingly, Lou/C rats develop also an osteoporosis related to age associated with increased bone marrow adiposity (68). Lou/C rats mimic leptin, insulin, ghrelin, GH, and IGF-1 alterations observed in AN patients (66, 69–71). At central level, Lou/C rats present an upregulation of the hypothalamic AgRP, NPY, and orexin mRNA, and a down-regulation of leptin and ghrelin receptors in the arcuate and ventromedial hypothalamic nuclei (70).

Even if this rat strain presents various common alterations observed in AN patients, it is a more suitable model of healthy aging (64, 72).

Genetically Engineered Mice

Beside spontaneous mutation models, various genetically engineered models have been developed. In humans, genomic association studies have shown that various gene polymorphisms seem particularly linked to AN (see "Genetics"). In view of these data, we have summarized results from studies on animal models with modified genes encoding molecules involved in neuropeptidergic circuits and monoaminergic systems. For a more complete overview, animal models based on genetic alterations of peripheral factors are also presented (**Table 2**). It is

TABLE 2 | Presentation of the most pertinent model to decipher subtle peripheral and central mechanisms that might be involved in anorexia nervosa.

Gene	Main peptide functions	Gene alteration mimicking AN alteration	Main induced alterations	Reference	Comments related to AN alterations
Leptin or Leptin receptor	Regulation of energy balance, food intake	Deficiency	Hyperphagia, obesity, diabetes	(73–76)	No mimicking the main AN alterations, models of obesity and diabetes
PYY	Anorexigenic in response to food intake	Overexpression	Reduced food intake after short fasting, normal body weight, and energy expenditure	(77, 78)	No mimicking the main alterations
Ghrelin	Orexigenic, energy balance	Overexpression	Increased food intake but normal body weight	(79)	No mimicking the main alterations
Goat and ghrelin	Activation of ghrelin (acylation)	Overexpression	Decreased energy expenditure but normal food intake and body weight	(80)	No mimicking the main alterations
Pancreatic polypeptide	Regulation of gastric emptying, . . .	Overexpression	Modest decrease of food intake and body weight	(81)	Slightly mimicking food intake and body weight alterations
Cholecystokinin	Satiation peptide	Deficiency	Low lipid absorption, normal food intake, and body weight	(82, 83)	No mimicking the main alterations
Neuropeptide Y	Orexigenic, decrease in energy expenditure and anxiety	Deficiency	Normal food intake and body weight	(84)	No mimicking the main alterations
Neuropeptide Y	Orexigenic, decrease in energy expenditure and anxiety	Destruction of NPY neurons in adults	Decreased food intake and body weight	(85)	Mimicking the voluntary food restriction and body weight decrease
Y2/Y4 receptor	Orexigenic, decrease in energy expenditure and anxiety	Deficiency	Normal food intake, lower body weight, higher activity, and energy expenditure; lower anxiety- and depression-related behavior for Y4	(86, 87)	Mimicking the body weight decrease
Agouti-related peptide	Orexigenic, decrease in energy expenditure	Destruction of AgRP neurons in adults	Decreased food intake and body weight	(85)	Mimicking the voluntary food restriction and body weight decrease
Melanin-concentrating hormone (MCH)	Orexigenic, regulation of physical activity	Deficiency	Decreased food intake and body weight, increased activity	(88, 89)	Mimicking voluntary food restriction, body weight decrease, and high activity
Cannabinoid type 1 receptor (CB1)	Orexigenic, regulation of energy expenditure	Deficiency in hypothalamus of adult	Normal food intake but lower body weight gain associated with a greater energy expenditure	(90, 91)	Mimicking the low body weight
5-HT4	Serotonin receptor	Deficiency	Voluntary food restriction following restraint stress; reduction of novelty-induced exploratory activity	(92)	Mimicking the voluntary food restriction
5-HT4		Knockdown in Accumbens nuclei	Increase food intake in fed mice	(93)	No mimicking the main alterations
5-HT1B	Serotonin receptor	Deficiency	Decrease food intake	(94)	Mimicking the voluntary food restriction
5-HT1A	Serotonin receptor	Deficiency or chronic agonist treatment	Decrease food intake	(95)	Mimicking the voluntary food restriction
Tyrosine hydroxylase	Production of dopamine	Deficiency in dopaminergic neurons	Strong hypophagia and hypoactivity; need of dopamine treatment to survive	(96)	Mimicking the voluntary food restriction but not the hyperactivity tendency
BDNF	Neurotrophin factor which stimulates growth and differenciation of neurons	No model of overexpression	Inhibit food intake	(97, 98)	No mimicking the main alterations
M3 receptor	Acetylcholine receptor or muscarinic receptor	Deficiency	Decrease food intake, lower body weight; hypoactivity	(99)	Mimicking voluntary food restriction and some endocrine alterations
CRH	Stress reaction	Deficiency	Decrease food intake, lower body weight	(100)	Mimicking the voluntary food restriction and low body weight
CRH		Central overexpression	Increase food and water intake; increase body temperature and heart rate	(101)	No mimicking the main alterations

noteworthy that the interest of all these models is discussed independently of the purpose of the original studies and thus of their intrinsic interest.

Peripheral factors: hormones involved in the regulation of the energy metabolism

As a consequence of *leptin* anorexigenic function, leptin-deficient (*ob/ob*) or leptin receptor-deficient (*db/db*) mice display a phenotype of hyperphagia and obesity [see reviews in Ref. (76, 102)]. Even if the plasma levels of leptin are low in AN, these genetic models did not mimic pathology alterations. Mice overexpressing the *ghrelin* peptide in their stomach show higher plasma levels of bioactive (acyl) and total (acyl and non-acyl) ghrelin. They display a slight increase in food intake but not in body weight (79). To increase acyl-ghrelin plasma levels, it might be necessary to also increase the expression of GOAT (ghrelin O-acyltransferase), enzyme involved in the ghrelin acylation. Contradictory results were obtained for GOAT expression levels in stomach after 12–36 h of fasting, whereas chronic and severe food restrictions (21 days, 70% restriction) increase GOAT expression in rat (103). Mice overexpressing GOAT display higher concentrations of acyl-ghrelin without any changes in body weight or food intake (80). Thus, engineering genetic alterations of the ghrelin system in mice did not succeed in mimicking AN alterations despite the essential role of this hormone in the maintenance of glucose homeostasis on food restriction condition (104–106). The anorexigenic peptide *PYY* is physiologically released in response to food intake and its plasma levels increased in patients with AN. Mice overexpressing PYY display normal weight gain and food intake (77). These observations could suggest that this model should be excluded from the list of AN models, but a recent study (78) showed that when PYY overexpression begins in adult mice, it induces a reduced food intake after 24-h fasting. However, these mice display no significant difference of body weight or energy expenditure when compared to wild type mice. The *pancreatic polypeptide* (PP) produced in pancreas after food intake inhibits gastric emptying, and contributes to the important satiety effect of cholecystokinin (CCK). Baseline PP concentrations were similar between AN patients and healthy controls (107) or higher in AN patients (108), but these concentrations increased much more in AN patients than in controls after a meal test (107, 108). Transgenic mice over-expressing PP display a slightly lowered body weight associated with a modest reduction of food intake (81). *CCK* is a gut hormone stimulated by fatty meals and inducing satiety. It is also involved in the control of gastrointestinal motility and in anxiety behaviors. The response of CCK to a meal test was four-fold lower in AN patients than in healthy control group (107). Interestingly, CCK deficient mice display a normal food intake and a normal body weight when fed a basal diet (82, 83). Thus, once again, this model does not mimic the main alterations observed in patients with AN.

Neuropeptidergic systems

Modifications in the expression of neuropeptides permit to generate central alterations that might explain mechanisms giving rise to some of the symptoms described in AN patients.

In the arcuate hypothalamic nucleus, the two populations of orexigenic and anorexigenic neurons and their receptors, respectively the AgRP/NPY and αMSH/CART (α-melanocyte stimulating hormone/cocaine amphetamine related peptides) neurons, have been the focus of numerous studies in an attempt to better understand the finely tuned regulation of food intake. During fasting, NPY and AgRP gene expressions are up-regulated, and αMSH and CART gene expressions are down-regulated in hypothalamus. Moreover, various experiments suggest that NPY/AgRP inhibits directly the activity of αMSH neurons through a corelease of GABA, as well as an action on MC4R-bearing cells. Inactivation of genes encoding NPY, AgRP, or both has little effect on energy balance (109). Mice KO for *NPY* present significant changes neither in their body weight nor in their food intake, but become hyperphagic following food deprivation (110, 111). Surprsingly, mice KO for both Y2 and Y4 receptors exhibited a reduction in adiposity and an increase in lean mass, but without significant changes in food intake. Energy expenditure and physical activity were significantly increased in Y4-KO and particularly in Y2-KO/Y4-KO (87). Such models might be valuable to study the involvment of NPY and its receptors in the modulation of body composition and energy metabolism that are dramatically disturbed in AN. Contrary to the Y2 and Y4 receptors, the Y1-KO and Y5-KO mice develop the late-onset obesity with an increase in food intake and adiposity (112–114). This implies compensatory mechanism in feeding behavior in these KO mice and underlines the complexity of the NPY-food intake regulation system. Selective acute deletion of *AgRP* neurons in the adult mouse inhibits feeding and can lead to starvation not observed when the ablation is performed in neonatal mice before AgRP neurons are mature (85). Wu et al. (115) show in Ay/a mice no discernable effect on the anorexia phenotype caused by AgRP neuron ablation, suggesting that excessive activation of the melanocortin signaling is not responsible for starvation. Compensatory mechanisms may occur and hide the potential role of certain peptides (116, 117). Unfortunately, in these models, physiological data are rarely presented, their use are of interest to better understand the dialog existing between these populations of neurons by deciphering the involvement of their receptors in specific conditions. These approaches can highlight the main homeostatic pathway disturbed in AN.

The lateral hypothalamus contains *MCH* (melanin concentrating hormone) orexigenic neurons, described to be essential in the control of food intake and physical activity (88). In his review, Macneil (118) points out the various mouse models where disruption of MCH signaling results in altered energy homeostasis. Indeed, targeted inactivation of the MCH gene in mice induces reduced body weight and leanness due to hypophagia associated with an increased metabolic rate, despite reduced amount of both leptin and arcuate nucleus proopiomelanocortin mRNA (88). KO MCH mice also display an increased running-wheel activity during dark period (89). In the Promch/ataxin-3 mouse, 60–70% of MCH-expressing neurons degenerate in the first few weeks of life. Thus, at the age of 7-week, mice developed reduced body weight due to hypophagia and increased energy expenditure, body length, fat mass, lean mass, and leptin levels (119). Similarly, the $Mchr1^{-/-}$ mice were less susceptible to diet-induced obesity,

and the leanness was a consequence of hyperactivity and altered metabolism. The manipulation of the MCH system remains one of the most interesting to reproduce many of the symptoms described in AN. The progressive degeneration of an orexigenic neuronal population induces a voluntary food restriction that impacts the overall physiology of the animal.

The lateral hypothalamic area also comprises another population of orexigenic neurons: the *orexin/hypocretin (Hcrt)* neurons which are implicated in various functions altered in AN. Indeed, in a neuron-ablated strategy, the orexin/ataxin-3 transgenic mice severely reduced the formation of food anticipatory activity (FAA) under food restriction conditions (120). Furthermore, in a recent study, Ramanathan and Siegel (121) report gender differences in Hcrt KO mice. Hcrt KO females had increased body weight associated with increases in various components of the body composition, despite a decreased food and water intake not observed so drastically in the males. This promising model remains complex to interpret in the case of AN, because of the multiple roles in which orexin is involved.

Among the other neuropeptidergic systems involved in AN, the *cannabinoid* system must be pointed out. Mice invalidated for CB1 in hypothalamus showed a significant weight loss associated with greater energy expenditure despite a normocaloric food intake in standard diet (90). The mechanisms involved in such adaptations need to be more investigated since pharmacological manipulation of the endocannabinoid system is currently discussed as potential strategy for the treatment of anxiety disorders, depression, and AN (122, 123). Similarly, the *opioïd* system is known to play a role in the control of homeostatic and hedonic pathways. Thus, mice knockout for the opioid receptors like the μ-receptor display no significant difference in body weight, food intake, locomotor activity, or dark respiratory quotient when fed with regular chow diet compared to wild type mice, but they are resistant to diet-induced obesity and display more important weight loss during food deprivation (124–126). They also show a decrease in food motivation as demonstrated in an operant paradigm for chow diet or sucrose pellets, and a reduction of FAA in a daily scheduled food access compared to wild type mice (127, 128). These models might be of interest more specially to dissect the complex mechanisms that regulate the non-homeostatic aspects of the feeding in AN patients.

Neurotransmitters: dopamine and serotonin

As mentioned above, in AN patients, neuroimaging studies as well as dosages in the cerebro-spinal fluid report alterations in the serotonergic and dopaminergic systems.

Concerning the *serotonergic* system, pharmacological treatments that increase serotonin disponibility lower consumption of food in humans and rodents (129, 130). The model of mice genetically modified for 5-HT4 receptors has been extensively studied as a model of anorexia (131). Briefly, these mice were characterized by a voluntary food restriction, only following restrained stress, and by an attenuation of novelty-induced exploratory activity (92). Conversely, the knockdown of 5-HT4 receptor in nucleus accumbens increases food intake only in fed mice (93). Likewise, mice lacking 5-HT1B receptor food restricted (20%, 3 days) eat less than the wild type mice when standard food ration is given. They also show an increased locomotion (94). Mice lacking 5-HT1A receptor or wild type mice chronically treated subcutaneously with a 5-HT1A receptor agonist display a decrease of their food intake (95). The interpretation of data obtained from manipulation of the serotonergic system is rendered difficult due to the large number of receptors and the various effects they have depending of their location at the synaptic level and in the brain. Thus, to better elucidate the role of serotonin in the feeding behavior, it is preferable to use conditioned deletion or the cre-lox technology to avoid large effects that might be more the result of compensatory mechanisms than a true action of the neurotransmitter.

Concerning the *dopaminergic* system, Szczypka et al. (96) used initially a gene-targeting strategy to inactivate specifically the tyrosine hydroxylase (TH) gene in dopaminergic neurons, sparing the production of dopamine as a precursor for adrenaline and noradrenaline. These mice, called "dopamine deficient mice," became hypophagic and died from starvation at 34 days because they showed locomotor deficiencies. Routine treatment with L-DOPA restored a food intake similar to wild type mice. Using viral strategy (96, 132–134), the involvement of dorsal striatum and accumbens nucleus has been demonstrated in locomotion and motivation, respectively, underlining the importance of dopamine to execute behaviors necessary to seek and ingest properly food. In AN, dopamine deficiencies might contribute to alterations in the accomplishment of these behaviors. Moreover, motivation aspects of feeding are also under the influence of medial prefrontal cortex and amygdala as recently demonstrated and involved D1 and D2 receptors (135, 136). These recent data emphasize the complexity of the regulation of feeding motivation, complete brain imaging data obtained in humans in these brain regions, and point out the need of more targeted pharmacological treatments (137).

Other genes

Other genes are also studied in the case of AN and are potential targets involved in the maintenance and/or evolution of the disease, like BDNF (brain-derived neurotrophic factor), CRH (corticotropin-releasing hormone), the glutamate receptors, the muscarinic type receptors even if they are also involved in a variety of functions (138, 139) rendering difficult to dissect precisely their actual role in the regulation of hunger/feeding. BDNF, a neurotrophic factor, is also a central regulator of energy balance, since BDNF suppresses food intake by acting on hypothalamic neurons (97, 98). Unfortunately, to our knowledge, no studies on hypothalamic overexpression of BDNF and feeding behavior are described in the literature. Investigating the CRH system in the case of AN is rendered difficult, even if the link is obvious, since AN patients often present stress-related disorders like anxiety and depression. Among the genetically modified models, the CRH-KO mice model described by Jacobson (100), the mice fed with chow diet present a decreased food intake associated with a lowered body weight loss than mice fed with restricted protein diet. In the opposite, mice who overexpress central CRH display changes in autonomic variables, like increased body temperature and heart rate, as well as increased food and water consumption, when compared with wild type mice (101). Thus, as detailed along the review, the HPA axis plays a key role in the regulation of the homeostatic and non-homeostatic aspects of the AN altered feeding,

but the precise role remains to be determined in this case due to numerous brain areas involved. Muscarinic receptors (M1 to M5) are involved in acetylcholine signaling and in various functions at peripheral and central level (140). The M3 receptor has been associated with alterations that are observed in AN. Indeed, M3 KO mice are hypoactive and display a voluntary food restriction associated with lower body weight compared to wild type mice. These transgenic mice also present lower fat deposits associated with reduction of plasma leptin and insulin concentrations. Moreover, M3 KO mice present an up-regulation of AgRP and down-regulation of POMC and MCH in hypothalamus compared to control (99). Due to the large distribution of these receptors in the CNS, targeted strategies of gene deletion must be chosen to assess precisely their involvement in the regulation of food intake (141).

Conclusion

The main results obtained on mouse models in which one gene expression was modified to follow the alteration of the corresponding protein levels described in AN patients was summarized on the **Table 2**. These lead us to mention that most of these models are more relevant for obesity or display no specific phenotype related to AN. Interestingly, this table points out that most of the alterations related to these genes induce phenotypes very different of the pathologic ones. This could be linked to the fact that alterations of factors in AN patients appear often to be opposite to the physiological and behavioral alterations obtained in these genetic models. As examples, the plasma levels of leptin and ghrelin, respectively, low and high in AN patients, might normally lead to an increase in food intake, which is not the case in the disease, reflecting a physiological adaptation that is not well-perceived at the central and/or peripheral levels.

Thus, even if these genetic models gave comprehensive informations about some mechanisms related to the processes regulating homeostatic and non-homeostatic regulation of food intake, these models are most often used on short term protocols and do not allow to follow the physiological and neurobiological evolutions of the phenotype while restrictive AN is usually a chronic disease. Furthermore, they focus on certain aspects of the disease such as hypophagia, hyperactivity, or motivational disturbances without taking into account a general view of the whole body functioning. To circumvent these drawbacks, the use of "environmental" model allows us to reconsider some of these aspects.

Environmental Models

Various environmental animal models have been proposed to mimic various symptoms of AN. These models are usually based on qualitative or quantitative modifications in the pattern of distribution of the meal, including period of quantitative food restriction or limited time of food access as well as exposure to chronic or acute stress.

Animal Model Based on One Inducing Factor

Dietary restriction models

Various studies have focused on adaptations induced by dietary restriction to determine contribution of energy imbalance or nutriment deficiency in changes observed in AN patients. Some studies focused on life span, cancer prevalence, or metabolic syndrome have brought data useful for understanding AN-related alterations [see review in Ref. (142)]. Altogether, the different feeding paradigms lead to various but complementary results.

Food restriction (FR)

Most of the studies using chronic FR used mild restriction protocols. Restricted animals were fed usually 30–40% less than *ad libitum* control ones. However, it must be noted that in animal facilities, rodents are usually overfeed of about 30% compared to their physiological needs resulting in a significant weight gain over the time and leading to the use of overweight animals as reference (143). In FR protocols, body weight changes are age and gender dependent. Breeding weaned mice onto 30% FR lead to gain weight, even less rapidly than control ones (144). On the contrary, feeding adult mice (10 weeks of age) with 30% FR induces a loss of 20% of their body weight in 1 week (145). Thus, such FR models should be considered as valuable models of balanced feeding as shown by the induced longer lifespan (146).

In the quantitative food restriction models, the severity of the restriction generates various levels of weight loss associated with modifications of energy expenditure and respiratory quotient (145, 147–150). Indeed, long-term 30% FR in mice leads to a significant shift to carbohydrate metabolism during the meal (145). In rats, a 30% FR applied during 48 h or 14 days induced a significant body weight loss associated with decrease in plasma leptin concentrations, but only acute food deprivation leads to a decrease in glycemia and plasma insulin concentrations. At central level, both protocols induce up-regulation of hypothalamic AgRP and NPY mRNA associated with down-regulation of POMC mRNA (151). A 30% FR applied for 9 weeks in 3–week-old mice impacts bone mineral content more rapidly than when it is applied in older mice (9–14 weeks old) (144, 152, 153). Food restriction is associated with emotional impairments (154). C57Bl/6 mice subjected to a 20% caloric restriction for 8–12 days exhibit an anxiety-like behavior (155). Moreover, in 20–30% FR rats for 7–10 days, a decrease in dopamine levels in the nucleus accumbens occurs associated with an impairment of the expression of genes related to the dopamine (156). These alterations could be involved in reward sensitivity and emotional and motivation related behaviors observed in AN patients.

Alternate feeding experiments

Alternate feeding experiments with animal fed 1 day every two days appeared to induce alterations close to that observed on 40% FR models. Mice under alternate feeding from 12 to 65 weeks of age displayed a 20% increase in their body weight while this increase reached 60% for control mice (157).

Severe food restriction

Severe food restriction studies (50–70% restriction) are much less common (158, 159). Because of their severity, these studies are often shorter while numerous changes need several weeks to develop (160, 161). However, in a 50% FR on a long term protocol, mice show a decrease in energy expenditure after a meal associated with a decrease in lipid oxidation (150). Severe FR on 5-week protocol induced emotional impairments on rats.

They showed increased anxiety like behavior, decreased serotonin turnover in the hippocampus and hypothalamus, and a decreased expression of 5-HT reuptake transporter in the raphe nucleus (162). Alterations of dopamine and DOPAC levels in septum and hypothalamus are associated with conditioning fear and control in food intake (163–167). The dopaminergic signaling was also shown to be modified (168) in the mesolimbic circuitry, strongly involved in the modulation of the motivational aspects of the food intake. Altogether, these protocols mimic various AN symptoms such as body weight loss associated with alterations in reproductive function, metabolic, endocrine, and neuro-endocrine systems (**Table 3**). Moreover, these models bring very interesting informations about the potential mechanisms sustaining physiological alterations observed in AN, and due to chronic caloric restriction, but they do not take into account two other major components widely described in AN, namely stress and physical activity. Other models have been developed to determine the role and involvement of both of these factors.

Time-restricted feeding (TR)

Time-restricted feeding consists in *ad libitum* energy intake, but within few hours each day. Recently, Rothschild et al. (179) wrote a comprehensive review on the links between TR and metabolic diseases in animal models and human. Sherman et al. (219) showed that a 3-h food access each day for 16 weeks induces a food intake 15% lower and a body weight increase a half lower in adult male mice compared to their *ad libitum* control mice. But in these experiments, restricted animal are fed during the light period. Longer durations of daily food access were also studied, but they had a lower impact on food intake and body weight. Most of the time-restricted studies demonstrated slight or no changes in body weight gain when compared to control group, but an improvement of markers of metabolic disease risks. They also pointed out the link between disruption of the molecular circadian clock and metabolic disorders even under high fat diet (219, 220). These models mimic neither severe food restriction nor body weight decrease described in AN. TR feeding also leads to a reduction in the anxiety-like behavior and alteration of the serotonin system of rats (176, 221). The authors suggest that the decrease in the essential amino acid tryptophan in the hypothalamus may be the consequence of plasma tryptophan decreases, and thus contribute to the decrease in the serotonin synthesis. The related hypothalamic variations are suggested to provoke a compensatory upregulation of postsynaptic 5-HT receptors to precipitate AN.

Low fat diet

Animal models based on low fat diet (4–5% of fat/g) could take into account the fact that patients with AN not only reduce their food intake, but also select their foods. But two main difficulties limit the use of these models to study AN. First, foods with 4% of fat are commonly used as low fat diet, even if this is the fat level suggested for standard rodent food, while 10% fat diets usually lead to overweight with time and age. Second, almost all studies focused on comparisons between high-fat and low-fat diet consequences or focused on the effects of low-fat diet on obese mice.

Fat-free diet

The first studies conducted on rats submitted to fat-free diet during from 60 days to 6 weeks display a decrease of body weight (80% compared to control), a lower growth with emaciation appearance associated with increase of water intake, no difference in food intake compared to control rats (180, 181). It was also described impairment of reproductive function in male and female rats (181, 222). Respiratory quotient measured in rats under fat-free diets (1 month) but submitted to carbohydrate access following 14 h of fasting evidenced a shift to lipid metabolism (182). Variations of plasma lipid induced by low-fat diet and fat-free diet are sensed by neurons of ventromedial hypothalamus (223–225). However, to our knowledge, only the study of Staszkiewicz et al. (185) showed an upregulation of AgRP and NPY expression in low-fat diet group. In parallel, a lower dopamine signaling is described in rats submitted during two generations of α-linolenic acid deficient diet compared to normal chow diet as well as a lower 5-HT2 binding was observed till in the frontal cortex, even if no significant difference was observed concerning body weight between groups (183, 184, 226, 227). Moreover, although these neurotransmitters are known to be related to anxiety- and depression-like behaviors, no behavioral test was conducted in these studies.

Low carbohydrate diet

Patients with AN also select food with low carbohydrate content in the aim to reduce their calorie intake. But in rats, low carbohydrate diets moderately impact body weight (171), and mice on a zero-carbohydrate diet significantly gain more weight than animals consuming standard chow, despite similar caloric intake. These zero-carbohydrate fed mice also exhibited metabolic disruptions, while low carbohydrate diets in humans induce greater weight loss than isocaloric food (228). These results do not lead to consider low carbohydrate diet fed mice as relevant models for AN. Finally, studies on high/low fat or high/low carbohydrate diets revealed great differences in the use of fat and carbohydrate between mice and humans.

Indispensable amino acid deficient diet (IAA)

Indispensable or essential amino acids are neither synthesized nor stored in organisms. In AN patients, one might consider that severe food restriction may alter the concentrations of plasma essential amino acids and might have drastic nutritional consequences (229). Several studies examining plasma amino acid levels display conflicting results in AN with higher, lower, or no significant differences compared to controls (11–13). However, a decrease in plasma tryptophan and a decrease in the tryptophan/large neutral amino acid ratio in acutely underweight AN patients are usually observed (32, 230–233). Thus, animal models based on essential amino acid restriction do not appear to be suitable models for AN. However, they could mimic some induced alterations, because essential amino acid restriction induces an adaptive behavior of food deprivation or because they are related to tryptophan. Various protocols have been developed using more commonly threonine, leucine, or valine deficient diets (189). Interpretation of changes observed should be taken with caution, since some alterations are related to energy deficit and others are related to the amino acid deficiency itself. In particular, valine

TABLE 3 | Environmental models: main physiological and neurobiological changes observed in rodent models manipulated for one or several factors.

	Inducing factors	Duration	Body weight and tissues	GH/IGF-1	Reproduction	Energy metabolism and appetite regulating hormones	Stress	Central impact (neuropeptides/ neurotransmitters)	Key references
Restrictive anorexia nervosa	Not well known	Months to years	20–25% under normal weight (↘fat mass); osteoporosis	GH resistance (↗GH ↘IGF-1); ↗→SRIF in CSF; ↗↘SRIF in blood	Amenorrhea; ↘LH, FSH, E_2	↘ Energy expenditure; ↘Leptin; ↘Insulin; ↗Ghrelin (acyl- and desacyl-ghrelin); ↗ adiponectin; ↘Glycemia	Anxiety-related behaviors and mood disorders; ↗Cortisol; ↘ACTH; →CRH	Morphological alteration of white and gray matter; ↗AgRP↗NPY; →αMSH in blood; ↘Dopamine metabolites in CSF, ↘D2/D3 density; ↘Serotonin markers	(30, 31, 169, 170) (review), (14, 18, 38) (review), (32)
Animal models									
Mild food restriction	30–40% food restriction	Months to a year	0–20% of weight loss (↘→lean mass, ↘fat and bone masses)	↘ GH; ↘IGF-1; →GHRH	→ GnRH	↘Energy expenditure; ↘Leptin, insulin; ↘→Ghrelin total, ↘Desacyl-ghrelin; →Adiponectin; ↘→Glycemia	Anxiety-like behavior; →ACTH; ↗Corticosterone	↗AgRP↗NPY; ↘POMC; ↘Dopamine and DOPAC in septum; ↗DOPAC/dopamine ratio in hypothalamus	(145, 151, 152, 155, 164, 171–173)
Severe food restriction	50–70% food restriction	24 h to 60 days	Until 20% of weight loss (↘lean, fat masses, ↘bone mass)	↘GH; ↘IGF-1; ↗FGF-21	Stop estrus cycle; ↘LH, ↘FSH	↘Leptin, insulin; ↗Ghrelin (acyl- and desacyl-ghrelin); ↘Glycemia (15 days); ↗Free fatty acids; ↘Ketone bodies; →Triglycerides; ↘Energy expenditure	↗Corticosterone	↗AgRP↗NPY; ↘POMC; ↘Dopamine and DOPAC in septum; ↘DOPAC/dopamine ratio in hypothalamus	(150, 156, 164, 171, 174, 175)
Time-restricted feeding	6–1 h food access/day	Until 16 weeks	Lower body weight gain than control to 25% of weigh loss	?	?	↘Insulin; ↘Glycemia; ↘Triglycerides	↗→Corticosterone; →CRH; →ACTH	↘Anxiety-like behavior; ↘Serotonin in hypothalamus; Circadian clock disturbances	(176–179) (review)
Low fat and fat-free	Reduced fat intake	Two generations	20% of weight loss	?	Disruption of reproductive function	↘→Energy expenditure	?	↗AgRP↗NPY; ↗Dopamine signaling; ↘D2 binding, 5HT2A binding in frontal cortex	(180–185)
Low carbohydrate	Reduced carbohydrate intake	4 weeks	No modification or increase according food composition	↗GH; ↘GH receptor in liver; ↘IGF-1; ↘SRIF	?	↘Insulin fasted; →Ghrelin total, Acyl-ghrelin; ↘Glycemia fasted	?	?	(171, 186)
Low essential amino acids/protein	Reduced essential amino acid protein intake	2 days to 6 weeks	Until 30% under control weight	↘IGF-1; ↗SRIF	Stop estrus cycle	↘Insulin; ↗Ghrelin (acyl- and desacyl-ghrelin); ↘Glycemia; ↘Triglycerides	?	No anxiety and depression-like behaviors; ↘Serotonin turnover in brainstem, hippocampus, prefrontal cortex; involvement of anterior piriform cortex in aversion observed	(187–190) (review)

(Continued)

TABLE 3 | Continued

	Inducing factors	Duration	Body weight and tissues	GH/IGF-1	Reproduction	Energy metabolism and appetite regulating hormones	Stress	Central impact (neuropeptides/ neurotransmitters)	Key references
Dehydration-induced anorexia	Hyperosmolar drink (2.5% NaCl)	4 days to 2 weeks	Until 69% of the body weight of controls	?	?	↘Leptin, insulin; ↘TSH, T_3	↗Corticosterone; ↘CRH, CRH-R2	↗NPY; ↘POMC; ↗ORX; ↗TRH	(191–193)
Restraint stress and immobilization	Slight contention 30 min to 6 h/day	1–42 days	15% of weight loss (↘lean, fat masses, ↘bone mass)	↘→GH	↘ LH; ↘Testosterone	↗Energy expenditure	↗Corticosterone; ↗→CRH; ↗→CRH-R1	↗NPY, ↗AgRP; ↗POMC; ↗MCH, ↗ORX	(194–199)
Cold exposure	Exposure to 4 to −15°C	24 h to 4 weeks	Low body weight loss (↘lean, fat masses)	?	?	↘Leptin insulin; ↗Glycemia; ↗Free fatty acids	↗Corticosterone	↗MCH; ↗TRH	(105, 200–202)
Chronic mild stress	Random stress	5 days to 8 weeks	No or low body weight loss (↘ fat mass)	?	?	↘Leptin, insulin	↗CRH	↘NPY	(203–205)
Social stress	Group of rodent with an organization into a hierarchy	2 weeks and recovery phase	10–15% of body weight loss (↘fat mass)	?	?	↘Leptin, insulin	↗Corticosterone; ↗ACTH; ↗CRH	↗NPY; ↘Preproenkephalin in nucleus accumbens; ↗D2 binding in striatum	(206–208) (review), (209)
Activity-based anorexia (ABA)	Voluntary physical activity and time-restricted feeding	3–14 days	Stopped over 20–25% of weight loss (↘lean and fat masses)	?	Stop estrus cycle	↘Leptin, ↘insulin; ↗Ghrelin (acyl- and desacyl-ghrelin); ↘Glycemia; ↘Free fatty acids	↗Corticosterone; ↗Adrenal gland mass; →CRH	↗AgRP, ↗NPY; ↘POMC; ↘CART; ↗Dopamine during feeding in accumbens nuclei; ↘Serotonin in accumbens nuclei	(49, 210–215) (review), (216) (review)
Food restriction and wheel (FRW)	Voluntary activity and food restriction	15–55 days	18–22% of weight loss (↘lean, fat, and bone masses)	?	Stop estrus cycle	↘Leptin; ↗Ghrelin (acyl- and desacyl-ghrelin); ↘Glycemia (15 days); ↗Free fatty acids; ↘Ketone bodies; →Triglycerides; ↘Energy expenditure	↗Corticosterone (15 days) = Corticosterone (55 days)	?	(150)
Separation-based anorexia (SBA)	Stress related to separation and time-restricted feeding	Until 10 weeks and recovery phase	Until 28% of weight loss (↘lean and fat, ↘bone masses)	↗GH; ↘IGF-1	Stop estrus cycle	↘Leptin; →Glycemia	↘ACTH; ↗Glucocorticoïd	↗MHPG/norepinephrine in hippocampus; ↘Dopamine in hippocampus	(161, 217, 218)

↗ increase; ↘decrease; →no changes of expression or concentration according to the compartment studied; ? not well-documented.

5HT2A, serotonin receptor 2A; ACTH, adrenocorticotropic hormone; AgRP, agouti related peptide; CART, cocaine and amphetamine regulated transcript; CRH, corticotropin-releasing hormone; CRH-R, corticotropin-releasing hormone-receptor; D2 receptor, dopamine receptor 2; DOPAC, 3,4-dihydroxyphenylacetic acid; E2, estradiol; FSH, follicle stimulating hormone; GH, growth hormone; GHRH, growth hormone-releasing hormone; IGF-1, insulin-like growth factor 1; LH, luteinizing hormone; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; ORX, orexin; POMC, pro-opiomelanocortin; SRIF, somatostatin; TRH, thyrotropin-releasing hormone; αMSH, alpha-melanocyte-stimulating hormone. The gray parts of the table point animal models induced by several factors.

deficient diet induced food restriction, a greater weight loss than for other IAA diets (approximatively 20% of their initial body weight), and increased of plasma acylghrelin and des-acylghrelin concentrations after 6 days of protocol (188). However, this valine deficient diet must be taken with caution since it leads to neurotoxicity not observed with an isoleucine deficient diet for example (189, 234–236). In another series of experiments using a combination of IAA deficient diets, Narita et al. (189) showed after 15 days of protocol, a decrease of glycemia, plasma triglycerides, leptin, insulin, and IGF-1 levels as well as a blockage of the estrous cycle in diestrus stage. Chronic tryptophan deficient diets (until 6 weeks) in rodents also lead to a progressive decrease of body weight (237–239). In contrast to acute deficient diet, no anxiety- or depressive-like behavior was observed in rodent despite a decrease in tryptophan concentration and serotonin turnover in brainstem, hippocampus, and prefrontal cortex (238–241). Indeed, no sucrose preference was observed in acute deficient rats while an increase of sucrose consumption was observed in mice after 5 weeks of tryptophan deficiency (239, 241). Unfortunately, to our knowledge, no studies determine the alterations of brain circuits regulating energy homeostasis. Rodents also develop different strategies to overcome the amino acid unbalance, including stopping the ingestion of food, change in the choice of food; they also develop a foraging behavior (to find complementary food); they establish an aversion with a learning phase and memorization of taste and smell to avoid the consumption of deficiency food in future (190, 242–244).

Dehydration-induced anorexia

As highlighted in part I, AN patients present relatively frequent osmoregulation impairment and renal complications due to their drink intake behaviors (21, 245, 246). Gutman and Krausz (247) pointed out a drastic decrease of food intake after acute subcutaneous injection of a hypertonic solution in rat. A "dehydration-induced anorexia" (DIA) model was developed by Watts (248). It consisted in a scheduled consumption of a hyperosmolar solution of NaCl (2.5%). This protocol has been tested for 4–14 days (192, 248). It provokes reduced food intake with a negative energy balance that is similar to those seen in pair-fed food-restricted animals: weight up to 69% under the body weight of control rodents, increased corticosterone, lowered leptin and insulin plasma levels (191). The food restriction is due a change in the pattern of food intake, a reduction of meal duration, and an inhibitory effect on gastric motility (249, 250). At the central level, DIA and pair-fed groups share up-regulation of NPY and down-regulation of POMC mRNA in the arcuate nucleus, and up-regulation of orexin mRNA in the lateral hypothalamic area only in pair-fed groups (191, 193). Beside, a down-regulation of CRH mRNA expression in the paraventricular nucleus and higher plasma corticosterone levels are observed only in DIA group (192). This model displays some common alterations also observed in AN. However, the drastic changes in osmolarity, which are not always observed in patients, might limit the use of DIA to decipher the central and peripheral mechanisms that can lead to chronic renal failure.

Stress models

A growing body of literature associated stress and anxiety as critical factors in the development of eating disorders like AN (251). Several animal models have been developed to evaluate mechanisms linking response to stressful events and alterations of food intake. In this section, we will not discuss data related to anorexia induced by the administration of lipopolysaccharides or endotoxemia. The most extensive studies concern restraint stress, cold exposure, or chronic mild stress (CMS).

Restraint stress

In rodents, limiting movements for a determined period (30 min to 6 h) every day generates a stress and a body weight decrease depending on the duration and type of immobilization (195). Indeed, animals are immobilized in a plastic tube or by attaching the four limbs to metal mounts with adhesive tape. Body weight loss up to 15% impacts both lean and fat masses, and is associated with a voluntary food restriction after an acute stress session (like 2 h), or when the stress is repeated (195, 252–254). Moreover, this low body weight is maintained even after a recovery period (199). Repetition of the restraint stress induces long lasting increased plasma corticosterone, ACTH and ghrelin concentrations, and decreased plasma leptin and insulin concentrations (255–258). In long duration experiments, bone physiology alterations are also observed (259). Rats exhibit an increase of energy expenditure and body temperature during the stress followed by return to control values (199, 260). At central level, noticable changes in the activation and/or expression of genes involved in the control of food intake are described. Acute restraint stress increases the number of activated neurons in several brain areas compared to controls, while repeated stress effects are lowered probably because of habituation (261–263). In these studies, modifications in the activation of the HPA axis are the most documented. Restraint stress protocols increase plasma corticosterone concentrations, which are associated with an increased activation and expression of CRH in the paraventricular nucleus (198, 264). However, such increases are not anymore observed when the stress is repeated (198, 199, 265). Considering the anorexigenic effects of intracerebroventricular injections of CRH, this peptide has been suspected to be responsible for the voluntary food restriction observed in this type of protocol (265). Both acute and repeated restraint stress in rats induce decreased number of neurons immunoreactive for Fos and AgRP in arcuate nucleus, while the number of neurons immunoreactive for Fos and MC4R increases in the lateral hypothalamic area but decreases in the arcuate nucleus on the long term (263). Such reduction in MC4R cell activation may signify a desensitization of feeding regulatory pathways in the arcuate nucleus after repeated stress exposure that may be indicative of a shift toward more orexigenic behaviors, as signals promoting feeding become more prominent. In another study where a 2-week chronic restraint stress is applied on mice, inhibition of food intake occurs until the end of the first week and is associated with also an up-regulation of POMC mRNA in the arcuate nucleus (258). The data obtained with NPY are less clear since acute restraint stress increases NPY mRNA expression in the arcuate nucleus. This expression is normal in the case of chronic stress (266). Thus, the relative balance between orexigenic and anorexigenic pathway activation appears to be dependent on whether the stress is acute or repeated. Finally, stress induces a very rapid degradation of GH (267) and thus a decrease in

plasma GH concentrations (268, 269). The release of somatostatin, a major inhibitor of the GHRH release, increases in the median eminence level following acute restraint stress, and thus might be a major factor in this GH drop (270).

These results suggest that food intake may be increased or decreased as a consequence of stress, and may play a role in eating disorders from anorexia to binge-eating leading to obesity and other stress-associated metabolic disorders. Once again, this psychological stress impacts differentially the brain areas involved in the regulation of food intake rendering difficult to use such protocol to study precisely the mechanisms involved in AN.

Cold exposure

One hypothesis on the origins of hyperactivity often observed in AN is that it would be a form of thermoregulatory behavior. Studies on the effects of ambient temperature or heat treatment on AN patients displaying hyperactivity strengthen this hypothesis (271, 272). Cold exposure is a physiological stress used to determine mechanisms involved in control of body temperature. The protocol used temperature exposure from 4°C to -15°C and for a duration ranging from 24 h to 4 weeks. Usually, relatively low body weight loss is observed and is not always associated with a decrease in food intake (200–202, 273–276). This body weight loss is associated with a decrease in both lean and fat masses and an increase in brown adipose tissue mass (202, 277–279). Short term exposure (1–24 h) or long term exposure (8 days) to cold stress (at 4°C) increases blood glucose, plasma adrenaline, and corticosterone concentrations, and decreases plasma leptin and insulin concentrations (200, 276, 279, 280). Cold exposure also leads to increased glucose uptake by peripheral tissues associated with increased liver glycogen, lipolysis in white and brown adipose tissues, and concomitant to lipogenesis in these tissues (200, 279, 281). Activation of lipolysis in the different fat depots involves the sympathetic nervous system as suggested by an increased noradrenergic turnover (276, 277). Lower temperatures (under 0°C) during 2 weeks induced in mice, a more important body weight loss associated with higher food intake and lower body temperature (202). Cold exposure leads to activation of numerous brain areas involved in thermoregulation located in the hindbrain (282, 283), in the hypothalamus, and in the forebrain (280, 284). Cold exposure during 4 days (4°C) leads also to increase of MCH expression in the hypothalamus (201), suggesting the involvment of this neuropeptide directly or indirectly in such variations. However, the origin of these variations is unclear but they are probably due to the role of MCH in control of energy expenditure (201, 285).

Chronic mild stress

Depression is another sign classically observed in AN. The most valuable animal model of depression like behavior was developed by Willner et al. (203). This model called CMS consists to expose rodents to mild stress applied randomly and daily during 3 to 9 weeks. In this kind of protocol conducted on rodents, the body weight is slightly diminished but, a notable reduction of sucrose consumption, sign of anhedonia, is described (203, 204, 286, 287). The body weight loss concerns decreased subcutaneaous and visceral fat mass associated with decreased plasma leptin and insulin concentrations. However, these changes are not specific to CMS protocol because they are also observed in the "weight match" control group (205). At central level, CMS animals exhibit up-regulation of CRH in paraventricular nucleus while its expression is reduced in the "weight match" group (205). Other peptide expressions also are altered, with especially a down-regulation of NPY in the arcuate nucleus (204). The CMS is described to have anxiogenic effects through a stronger neuronal activation in various brain areas, as well as a decreased neurogenesis in the hippocampus (288). Recent reviews (289–291) pointed out a role of ghrelin in depression and anxiety, even if it is again still a subject of debate. Its receptor is present in structures known to be involved in mood disorders like hippocampus and amygdala. The model presents the advantages to mimic alterations of the stress axis and anhedonia for palatable food associated with a slight body weight loss. However, the complexity of the stress procedure and a rapid recovery limit the interest of CMS to mirror AN.

Social stress

Another kind of acute/chronic stress is related to rodent social interactions. The main models are based on social defeat stress and the visible burrow system (VBS). The social defeat stress was first used as a model of anxiety and depression (292). A rodent (intruder) is placed in the home cage of another rodent (resident). The interactions between the two animals are usually rapid and lead to aggressive behaviors, with a dominant and a subordinate. The defeat social stress leads to a markedly decrease of body weight in animal following 1 h session of stress (206). The repetition of this stress induces also a higher reactivity to an acute restraint stress with increased plasma corticosterone and ACTH concentrations, but with a normalization of values after stress (293). A decrease in locomotor activity is also observed associated with reduced social interaction in the presence of a non-aggressive rodent (206, 293). An increased nocturnal food intake is noticed and not observed in the case of VBS protocol (209, 293). The VBS protocol induces a more complex social defeat stress since it is based on the establishment of a hierarchy in a group of male rats leading to dominance hierarchies with offensive and defensive behaviors (294). At the end of the confrontation period, a dominant male rat (DOM) takes the ascendancy over other rats qualified like subordinate males (SUB). VBS protocol induces decrease of body weight associated with a decrease of food intake only in SUB male rats (208, 209, 295). The body weight reduction is associated only with a decrease in subcutaneous fat mass, whereas lean mass is unchanged and visceral fat mass is increased (208). The pattern of food intake is modified with a decrease of meal duration (209). SUB rats display also endocrine changes with a decrease of plasma leptin and insulin concentrations compared to DOM and control rats (296, 297). Studies related to alterations at the central level have mainly focused on the HPA axis, particularly affected in the SUB rats, with increased plasma corticosterone concentrations correlated with increased expression of CRH in the paraventricular nucleus and amygdala (208, 295, 296, 298). The chronically elevated corticosterone levels may create an orexigenic drive through upregulation of NPY and AgRP in the SUB rats as well as the loss of fat mass seen in both DOM and SUB, which indicates a negative energy balance, and may also create an

orexigenic drive through similar mechanisms. Such observations are validated by the behavior observed in a recovery phase where the rodents become hyperphagic and increase drastically their fat mass (299). These observations render the model inadequate for studying the recovery period after food restriction even if the model generates transiently a food restriction during the protocol. It should be underlined that a noticable decrease of palatable food is observed as in the CMS protocol in the recovery phase (207, 208, 300). These changes have been attributed to alterations in dopamine transporter binding and dopamine receptor (D2) binding which are reduced or increased respectively in the striatum and accumbens nucleus in SUB group (207). The VBS protocol also leads to changes in the SUB serotonergic system in various brain areas involved in the modulation of stress (294, 301). This model is interesting to study the impact of chronic social stress on food intake and its homeostatic and non-homeostatic regulation. But the main drawbacks are: the absence in human of such notion of subordinate and dominant; the recovery period which shows a binge-eating behavior that is rare in recovered AN patients; the short term duration (around 15 days) excluding the development of long term alteration like osteoporosis. Finally, there is currently no or few data about the regulation of energy balance. The VBS model presents an important limit that reduces its use to study AN: it is applicable only on males.

Animal Model Based on Several Inducing Factors

Separation-based anorexia

Separation-based anorexia model is another model of chronic social stress not often used until now. This model is based on stress produced by a physical separation of mice belonging to the same group and associated with a food restriction or a time-restricted feeding (TR) (217). This study was initially conducted on Sabra female mice. Only few studies were published on this strain of mouse with high body weight. Food was provided during the light phase for 1 h a day. Control mice with the same feeding schedule lost 10% of their day 0 body weight within 18 days, and daily ate 2.84 g of food. Separated and time-restricted mice lost 28% of their initial body weight, and daily ate 2.33 g of food. In this group, 21% of mice died before reaching the targeted body weight loss of 33–35%. Separated and time-restricted mice ate 65% of the daily requirements and reach the same level of body weight loss than mice fed 40% of the daily requirements without being separated. These data suggested that separation of mice increases metabolic demands. This first study was followed by two studies on the same model and conducted by the same team. Both of them dealt with the effects of tyrosine treatments on central nervous system functions. Hao et al. (218) showed that SBA mice display an increase in 3-methoxy-4-hydroxyphenyglycol/norepinephrine ratio, an up-regulation of the cholinergic signaling, and a decrease in the dopamine concentration in hippocampus. In 2002, the effects of tyrosine treatments on HPA axis were studied on this model (302). This second central study pointed out a specific pattern of central alterations in SBA mice when compared to FR and active mice despite similar body weight loss. To allow studies on long term metabolic and central adaptations on a usual mouse strain, we recently adapted this model to C57Bl/6 young adult female mice. Food access was progressively reduced from 6 to 2 h a day within 2 weeks and then maintained at 2 h a day for up to 8 weeks (161). We have shown that this protocol induces significant weight loss with a reduction from 20 to 25% of initial body weight. Interestingly, the body weight loss observed in SBA group is not attributable to the timed food access as SBA mice eat only 10% less than *ad libitum* group. Moreover, such a difference in body weight is not observed in the TR group without separation. We suspect that this difference is partly due to rising energy costs both through the separation-induced stress and higher thermogenesis needs caused by the separation. Body weight loss is related to a decrease in lean mass and visceral and subcutaneous fat masses. In parallel, SBA mice present a blocking of their reproductive function and bone mass gain. Like in AN patients, various endocrine changes are observed. Thus, SBA mice display lower plasma leptin concentrations. Furthermore, disruption of the GH/IGF-1 associated with alteration in bone physiology was observed at 2 and 10 weeks. At metabolic level, protocol induces an up-regulation of several genes (UCP1, PGC1a, Prdm16) especially in the subcutaneous adipose tissue of SBA mice, suggesting the emergence of beige/brite adipocytes in this specific fat depot. Moreover, after 10 weeks of SBA, protocol mice were submitted to a 10-week recovery period with free food access in normal cage. During this recovery period, mice correct their various alterations including body weight, food intake, reproductive function, body composition, endocrine factors, and adipose tissue metabolism. However, SBA mice maintain low plasma leptin concentrations and low leptin expression in visceral fat tissue despite a full normalization of fat mass (161).

This long term model appears interesting as it mimics numerous central and peripheral alterations described or suggested in AN, and allows a recovery study. However, the increased energy expenditure related to chronic stress and high needs of thermogenesis does not match the decrease usually described in patients.

Activity models

In 1967, Routtenberg and Kuznesof developed a protocol, where rats isolated in a cage were allowed to have a timed food access, 1 h per day, combined to a voluntary activity. This model later named activity-based anorexia (ABA)/self-starvation/semistarvation-induced hyperactivity/food restriction-induced hyperactivity/wheel-induced feeding suppression model produces a rapid weight loss, close to 25% of their initial weight within days and food intake, physical hyperactivity, hypothermia, impaired estrous cycle in females, and increases in HPA axis activity (215, 303, 304). Moreover, rats eat less than inactive rats fed with the same schedule. This procedure led rapidly to a "self-starvation" or self-deprivation behavior resembling to that observed in restrictive AN patients and leading rapidly to the death of animals due to the voluntary privation of food (around 7 days). It is currently the most well-known animal model of anorexia (216, 305) and has been adapted to mice (306, 307). Recently, Lewis and Brett (308) reduced progressively the food access duration to maintain mice longer than 7 days. Following this new protocol, Jésus et al. (309) demonstrated alterations of intestinal permeability. In many aspects, all these models mimic numerous physiological alterations observed in AN. However, as specified in the review of Klenotich and Dulawa (310), the ABA paradigm

is strongly dependent on the rodent strain, on age and gender (307, 311), on temperature [increasing the temperature to 32°C strongly reduces the ABA behavior, (312)], and on the time of the day the animals receive food. In fact, Boakes and Juraskova (313) and Boakes (210) demonstrated that the "self-starvation" observed in ABA rats might reflect both the reduced palatability of the dry chow for a dehydrated animal and satiety signals from a stomach full of water. Finally, in all these protocols, rodents were isolated in their cage to permit individual metabolic and physiological measures, but isolation creates a social stress adding on the physiological stress of food deprivation, rendering the protocol more drastic. Thus, all these studies present limitations that maintain a distance with AN. Recently, we have developed a modified ABA model on female mice, named here Food Restriction and Wheel (FRW) model that aims: (i) to prevent the social stress by using two mice per cage and (ii) to follow on the long term (up to 10 weeks) physiological alterations induced by a combination of physical activity and a food restriction of 50% (150). All of these activity models present metabolic, endocrine, and neurobiological alterations that might be the basis to study adequately some of physiological mechanisms altered in AN patients. Finally, they all exhibited a FAA, which occurs between 2 and 5 h before food intake distribution, and which is also described in AN patients (314).

The body weight loss observed both in ABA and FRW rodents is related to decrease of lean and subcutaneous/visceral fat masses after 7–14 days of protocol (150, 315). Physical activity at short term exacerbates decreased fat mass and has no protective effect on bone composition and lean mass (150, 211, 213, 316). When the protocol is maintained on the long term (55 days) like in FRW protocol, physical activity participates to body weight stabilization and to a significant slight body weight regain compared to pair-fed group (150). The long term protocol induces alterations in the bone mineral content leading in AN patients to osteoporosis. Indeed, in FRW mice, physical activity, currently described to stimulate bone formation, did not prevent on long term protocol the termination of bone mass acquisition induced by food restriction. Similar data were also described in SBA female mice subjected to a protocol of chronic stress associated with caloric restriction as previously mentioned. Such data confirmed the absence of protective effect of activity on bone mineral content in AN. In the ABA model, Pardo et al. (315) underline a differential tissue-specific expression pattern of ghrelin and leptin receptor at peripheral level reflecting tissue specific mechanisms to control energy homeostasis. The study of intestinal barrier indicates that the ABA protocol generates an increased colonic permeability associated with altered tight junction expression (309). These recent data open new windows to decipher the impact of gut microbiota in the deregulation of energy metabolism as well as the hepatic injury occurring in AN patients.

Besides alterations in various peripheral tissues, numerous endocrine changes are similar to that described in AN patients. Overall, ABA mice present lower plasma leptin and insulin concentrations and higher total plasma ghrelin and corticosterone concentrations (212, 215, 317). Moreover, energy metabolic factors are also changed in ABA/FRW mice with, in particular, an increase of free fatty acid and a decrease of glycemia (150, 213). On the long term, most of the endocrine alterations persist like lower plasma leptin concentrations, higher plasma total ghrelin concentrations still associated to lower glycemia, plasma ketone bodies, and higher free fatty acid in FRW mice (150). As highlighted previously, food restriction might induce shift in the energy metabolism regulations. Combination of food restriction and voluntary physical activity leads to a higher carbohydrate metabolism and a lower fat oxidation during the light period like the *ad libitum* control groups whereas at long term, FRW mice adopt a similar profile than the pair-fed group with a lipid metabolism more prominent. These changes point out the complexity of the peripheral regulation of nutrient and energy supplies, engaging probably hormones like leptin or ghrelin, which act on adipose tissues, muscles, or liver, might contribute to the changes/reduction in energy expenditure observed in FRW and pair-fed controls both at short and long term.

Central alterations are also observed in ABA protocols with an up-regulation of AgRP and NPY mRNA expression associated with a down-regulation of POMC and CART expression in the arcuate nucleus compared to control mice (211, 318–320). Surprisingly, no differences were observed concerning MCH and orexin expression in lateral hypothalamic area or CRH expression in paraventricular nucleus (211). However, until now, there is no study that evaluates potential changes in the expression of ghrelin and leptin receptors in ABA mice. Such information might be of importance since GHSR KO mice or intracerebroventricular injection of GHSR1a antagonist decreased the behavior of FAA and did not modify the food intake (321). Likewise, chronic subcutaneous or intracerebroventricular leptin injections lead to lower running wheel activity associated or not with reduction of food intake (322–324). Such fundamental researches are conducted to aim finding potential treatment using leptin or ghrelin to reduce hyperactivity frequently associated with AN, and leading to its excessive to emaciated phenotype. Indeed, intracerebroventricular injection of αMSH, whose release in hypothalamus is stimulated by leptin, enhances the ABA phenotype (325). Likewise, the specific sites of action of ghrelin and/or leptin in the ABA protocol should also be clarified. As an example, injections of ghrelin agonist in the lateral dorsal tegmental nucleus or its target, the ventral tegmental area, stimulate locomotor activity and food intake (326, 327). ABA mice are also shown to exhibit higher concentrations of noradrenaline, serotonine, but lower dopamine concentrations in the mediobasal hypothalamus compared to pair-fed and control groups (302, 328, 329). Moreover, Verhagen et al. (214) showed in the nucleus accumbens of ABA rats a lower circadian serotonergic activity without any changes for the circadian dopamine activity compared to control. These monoamines were suggested to play a role in voluntary food restriction in ABA rodents and in comorbidities observed in AN patients [i.e., depression or obsessive compulsive disorders; (137)]. It was suggested that reduction of physical activity is due to inhibition of serotonin release *via* 5HT1A autoreceptors in raphe nucleus (329–333). The opioid and endocannabinoid systems are also modified in the ABA model with increased plasma βendorphin concentration and pituitary βendorphin content in rats (334). This hyperendorphinism in the hypothalamo-pituitary-adrenal axis was linked to the auto-addiction hypothesis of AN. Furthermore, intraperitoneal

injections of ABA mice with Δ9-tetrahydrocannabinol, an exogene ligand of cannabinoid receptors, increase their food intake, attenuate the body weight loss, reduce the energy expenditure, but increase the mortality rate compared to ABA mice vehicle-treated (308, 335). Due to the large distribution of endocannabinoid and opioid receptors in the brain, further studies are needed to clarify more precisely the mechanisms involved and the finely tuned interactions between all these homeostatic and non-homeostatic structures.

The ABA/FRW protocols also affect two other main endocrine functions: stress and reproduction. In ABA rodents, like in FRW mice (on the short and long term), a disruption of estrus cycle, vaginal closure, and reduction of ovaries size, and also hormone disturbances including a decrease of plasma testosterone and luteinizing hormone concentrations have been noted (150, 329, 336, 337). Reproduction axis is normalized when rodents are placed in recovery conditions, which reflect that reproductive disturbance is the result of energy unbalance (337). Concerning the HPA axis, ABA protocols induce on the short-term increased plasma corticosterone and ACTH concentrations and adrenal gland hypertrophy, but no significant modification of CRH expression in paraventricular nucleus compared to controls (211, 318, 338). Intracerebroventricular injection of CRH antagonist injection during the protocol leads to blunt the ABA phenotype (318). Furthermore, ABA adrenalectomized rats do not display increased wheel running activity (212). Once again, these data suggest that HPA axis is essential to apparition of ABA phenotype and point out the role of the glucocorticoids in the pathophysiology of AN. Somatotrope axis is another axis disrupted in AN patients, but in our knowledge there is no study using ABA protocol or associated protocols showing such alterations.

As mentioned earlier, one characteristic of the ABA model is the FAA. Several studies have documented the potential factors and neuronal structures leading to this particular behavior that can be generated like a foraging behavior or to increase the internal temperature due to energy deficit (212, 215, 321–323, 339). FAA itself can also influence the pattern of food intake. Indeed, in the FRW protocol, mice display a shift in the meal initiation compared to the pair-fed group (150). One explanation, suggested by Woods (340), considered eating to be a homeostatic stressful event, because the digested nutriments that reached the blood during and after a meal markedly disrupt energy homeostasis. Thus, the combination of both events, activity and feeding, could generate a stressful energy event especially in the short term, leading to increase in corticosterone levels and resulting to delay the meal initiation. Such phenomenon could occur in the ABA

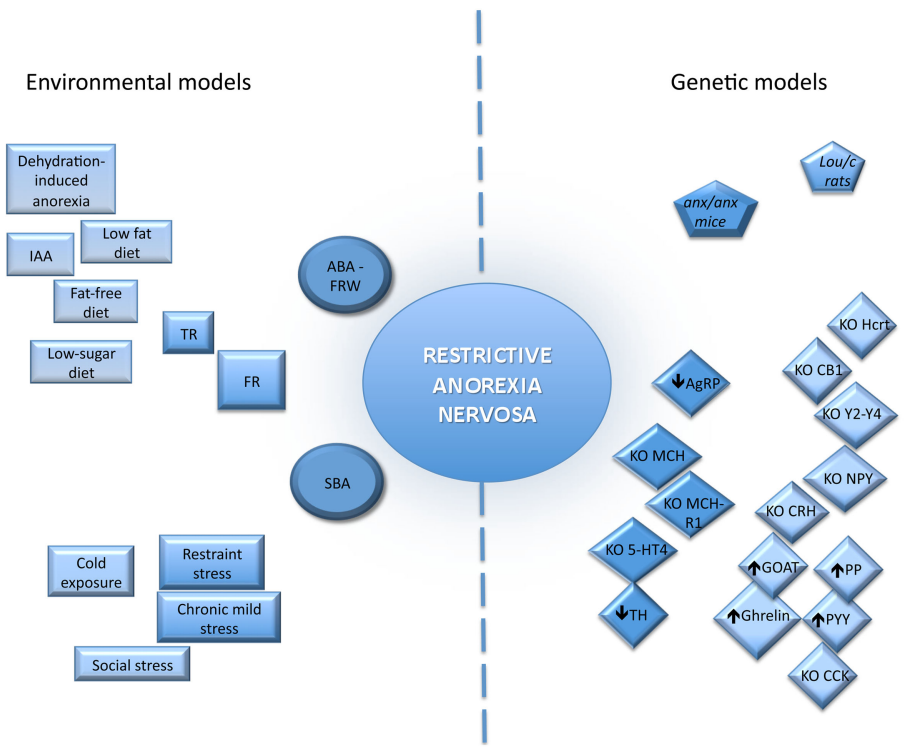

FIGURE 1 | Schematic representation of the relevance of the different animal models for anorexia nervosa, environmental and genetics, described in the review. The more the models mimic restrictive anorexia nervosa symptoms the closer they are to the center of the figure. In the environmental models, the squares correspond to models based on one inducing factors and the circles to models based on several inducing factors. In the genetic models, the pentagons point out genetic models with spontaneous mutations whereas the diamonds show the genetically engineered mice: knock out (KO), transgenic (↑), specific deletion (↓). ABA, activity based anorexia; AgRP, agouti related peptide; CB1, cannabinoid receptor type 1; CCK, cholescystokin; CRH, corticotropin releasing hormone; FR, food restriction; IAA, indispensable amino acid deficient diet; GOAT, ghrelin O-acyltransferase; Hcrt, orexin/hypocretin; MCH, melanocortin concentrating hormone; NPY, neuropeptide Y; PP, polypancreatic peptide; PYY, peptide YY; SBA, separation based anorexia; TH, tyrosine hydroxylase; TR, time-restricted feeding; 5-HT R, serotonin receptor.

model, where the pattern of food intake has never been measured in metabolic cages, as it was done for FRW mice. The "self starvation" observed might thus be due to this delay in the initiation of the meal, which is, as mentioned above, time limited. Concerning the temperature, the ABA protocol induces a decrease of body temperature (341, 342). In addition, even if a negative correlation between FAA and body temperature was observed, no causal link has been demonstrated (325). Nevertheless, it was suggested that the decrease of body temperature is one of the factors contributing to physical activity (342). When ABA rats have access to a warm platform, they decrease their running wheel activity (320, 325, 343), similarly as observed in AN patients whose excessive physical activity vary depending on the ambient temperature (272).

All the data collected with both ABA and FRW models are totally useful to dissect the different mechanisms involved in the maintenance of the AN phenotype. Combining the different approaches on the short and long term will have an indubitable benefit to study the interactions between the various peripheral and central actors whose dialogs seem strongly impaired.

Conclusion

This review aims to depict the different animal models currently used or potentially interesting to study one or several aspects of restrictive AN (**Figure 1**). The definition of a pertinent animal model of psychiatric disorder remains extremely difficult. In the case of AN, more specially the restrictive subtype, many symptoms can be mimicked in rodents like the body weight loss, the changes in energy expenditure, increased physical activity, several endocrine and neurotransmitters changes that reflects similar physiological and neurobiological mechanisms inherent to the natural and adapted regulation of feeding. In this sense, some of the currently available animal models described here answer to the "face validity," i.e., they mimic most of the symptoms of the human pathology. However, AN is usually associated with a refusal to eat. In rodents, such behavior is not natural, even if a kind of self-starvation is observed in migratory and hibernating animals. The "self-starvation" induced by some protocols does not reflect the human starvation, which is classically described to be associated with a personality trait involving neuronal inhibitory cognitive circuits. Even if self starvation is observed in some models like the well known ABA model, one may considered that the starvation is essentially due to physiological factors like temperature, dryness of the food, or even the delay in the initiation of the meal due to the intense physical activity observed before feeding. These models give certainly important information's about the physiological changes occurring at this period, but do not reflect the self-starvation observed in human, which remains to be understood. Is it only driven by cognitive inputs or is it under the influence of factors regulating the feeding homeostasis like ghrelin or leptin which receptors are distributed in numerous "non-homeostatic" brain areas? Brain imaging might help to solve this question and would permit to give more credit to what we obtained in animal models. Even if all of these models do not fully answer to criterion of "construct validity," i.e., a common etiology or similar conditions of induction, they fulfill the "predictive validity," as the different pharmacological treatments used to restore body weight and other altered functions give encouraging results. As a conclusion, it is to note that current environmental models based on a combination of several inducing factors appear to be more relevant than the other models but may be to further improve studies on AN, new models coupling genetic and environmental factors remain to create and assess.

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Membrane-initiated non-genomic signaling by estrogens in the hypothalamus: cross-talk with glucocorticoids with implications for behavior

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The estrogen receptor and glucocorticoid receptor are members of the nuclear receptor superfamily that can signal using both non-genomic and genomic transcriptional modes. Though genomic modes of signaling have been well characterized and several behaviors attributed to this signaling mechanism, the physiological significance of non-genomic modes of signaling has not been well understood. This has partly been due to the controversy regarding the identity of the membrane ER (mER) or membrane GR (mGR) that may mediate rapid, non-genomic signaling and the downstream signaling cascades that may result as a consequence of steroid ligands binding the mER or the mGR. Both estrogens and glucocorticoids exert a number of actions on the hypothalamus, including feedback. This review focuses on the various candidates for the mER or mGR in the hypothalamus and the contribution of non-genomic signaling to classical hypothalamically driven behaviors and changes in neuronal morphology. It also attempts to categorize some of the possible functions of non-genomic signaling at both the cellular level and at the organismal level that are relevant for behavior, including some behaviors that are regulated by both estrogens and glucocorticoids in a potentially synergistic manner. Lastly, it attempts to show that steroid signaling via non-genomic modes may provide the organism with rapid behavioral responses to stimuli.

Keywords: hypothalamus, spine density, membrane-initiated signaling, GPCR, estrogen receptor variants, aggression, lordosis, glucocorticoid receptor

GENOMIC AND NON-GENOMIC SIGNALING BY NUCLEAR RECEPTORS

Nuclear receptor ligands such as estrogen and glucocorticoids signal via both non-genomic and genomic pathways within cells. The genomic or transcriptional pathway is the best elucidated primarily due to the well-characterized nature of the estrogen receptor (ER)α and β and the glucocorticoid receptor (GR), all of which are members of the nuclear receptor superfamily. Once bound to their cognate ligands, these receptors act as ligand-activated transcription factors in the nucleus by binding to specific enhancer elements such as the estrogen response element (ERE) (1) and glucocorticoid response element (GRE) (2) in the promoters of genes. Both receptors have a modular structure, with a conserved DNA-binding domain, multiple transactivation domains, and a C-terminal ligand-binding domain (3, 4).

On the other hand, non-genomic signaling, first described by Szego and Davis in 1967, as the rapid increase in cAMP in the uterus occurred within 15 min of 17β-estradiol (17β-E) administration to ovariectomized mice (5). In the central nervous system (CNS), 17β-E was shown to rapidly depolarize proopiomelanocortin (POMC) hypothalamic neurons via Akt or protein kinase (PK) B, extracellular regulated kinase (ERK/MAPK), PKA, and PKC pathways (6, 7). In other tissues such as rat hippocampal neurons, phospho-cAMP response element binding protein (pCREB) increased within 1 h of 17β-E addition and this increase was blocked by inhibitors to both calmodulin kinase II (CamKII) and ERK pathways (8). In the case of corticosterone-mediated rapid actions, treatment of neurons with dexamethasone, a synthetic glucocorticoid, rapidly induced the nuclear localization of the GR (9, 10), an effect potentiated by the inhibition of p38MAPK (11). Extracts from rat hippocampal synaptoneurosomes showed a reduction in Akt and ERK phosphorylation within 30 min in response to pharmacological inhibition of the GR by RU-486 (12), suggesting that the classical nuclear receptor was required for non-genomic signaling in the hippocampus. Apart from kinase activation, dexamethasone-mediated negative feedback at the corticotropin releasing hormone (CRH) neuron was also rapid, consisting of suppression of the excitatory drive to the CRH neuron, mediated by endocannabinoids acting as a retrograde messenger to the presynaptic glutamatergic neuron (13), an effect mimicked with a membrane-limited dexamethasone conjugated to bovine serum albumin (Dex-BSA) (13). Hence, non-genomic signaling by steroid hormones is extra-nuclear signaling that is initiated by the endogenous ligand within minutes, in contrast to the hours required to detect transcriptionally regulated proteins.

Central to this concept of non-genomic signaling that is typically demonstrated by the use of membrane-limited conjugates

(14), is the idea of a receptor that initiates such signaling from the plasma membrane. However, with the exception of the membrane progesterone receptors (mPRs) that belong to the progestin and adipoQ receptor (PAQR) family, the identity of the membrane ER (mER) and membrane GR (mGR) has remained elusive (15). This review aims to describe the current candidates for the mER and the mGR that mediate rapid non-genomic signaling from the plasma cell membrane as well as focus on rapid actions that are relevant for hypothalamically driven behaviors that are dependent on estrogens but that have a glucocorticoid-regulated component. We concentrate on the hypothalamus since this is a classically steroid-responsive area of the brain and is critical for several estrogen-dependent behaviors (16). For more general reviews on rapid actions of estrogens and glucocorticoids in the CNS, including tools that are typically used to elucidate membrane-initiated non-genomic effects, the reader is referred to (7, 14, 17–19).

THE MEMBRANE ER AND GR

In the CNS, both ^{3}H-labeled 17β-E (20) and a ^{125}I-labeled membrane-impermeant conjugate where 17β-E is attached to bovine serum albumin (E2-BSA) (21) showed relatively high affinity binding to rat plasma membranes, suggesting the presence of a mER. What is a good definition for a mER or mGR? Previously, Micevych et al. (22) have suggested that ICI 182,780 antagonism, stereospecific 17β-E binding and sequence homology to the ERα and ERβ should be considered pre-requisites for a protein to be termed a mER. We propose, given the off-target effects of ICI 182,780 (23) that the definition of the mER or mGR be modified slightly to consider proteins capable of specific binding to 17β-E or to dexamethasone (in the case of the mGR), presence at/near the plasma membrane and signaling from the membrane. It should be noted that this definition would exclude the binding of proteins to 17α-E, an estrogen often used in studies of as an inactive isomer of 17β-estradiol, but that recently has been shown to increase neurogenesis and to mediate neuroprotection (24). Most studies described below show evidence of the candidate mER at the plasma membrane and its ability to rapidly within minutes modulate rapid signaling pathways such as kinase regulation or calcium flux. A few studies also demonstrate the regulation of the candidate ER at the membrane.

ERα AS THE mER

As we will focus on outputs dependent on the hypothalamus in this review, we will initially discuss the nuclear classical ERα as a candidate mER because (a) this is the most abundant ER isoform in the medial preoptic area (mPOA), ventromedial hypothalamus (VMH), and arcuate nucleus (ARH) (25–27) and (b) loss of ERα in the hypothalamus abrogated hypothalamically driven lordosis behavior in females (28) and aggressive behavior in males (29). A number of studies using 17β-E binding, electron microscopy, immunocytochemistry, and western blotting have examined the idea that a small percentage (3–5%) of the total pool of the classical ERα is present on the plasma membrane (30). Consistent with this idea, immunocytochemistry using minimal fixation in both the breast cancer MCF-7 cell line (31) and anterior pituitary GH3 cell line (32), revealed ERα at the membrane in caveolae in some, but not all cells. MCF-7 cells with ERα at the plasma membrane showed increased phospho-ERK (pERK) within 10 min of application of either 17β-E or a membrane-limited E2-peroxidase conjugate (33). In the CNS, ERα was localized to the dendrites and axon terminals in the guinea pig hypothalamus (34), while ERα was detected in axon terminals, dendritic spines, as well as in astrocytes in the CA1 using immuno-electron microscopy in the proestrous female rat (35). In the dorsal CA1 from the female rat, ERα was present in synaptic vesicles in the axon of some GABAergic basket cells; 17β-E moved these ERα-containing vesicles toward synapses within 24 h (36). In addition, some proportion of this extra-nuclear ERα at synaptosomes and vesicles was phosphorylated though the function of phosphorylation in localization of the ERα remains unknown (37). In hypothalamic neurons and astrocytes obtained from both male and female rats, a full-length 66 kDa form of the ERα and a 52 kDa variant has been detected using surface biotinylation and immunocytochemistry (38, 39). These studies demonstrate that the nuclear ERα is present at the plasma membrane in many cell types, including the hypothalamus.

TARGETING OF ERα TO THE PLASMA MEMBRANE

Though hydropathicity analysis shows that ERα may have a potential trans-membrane domain (38), the idea that ERα requires a membrane protein to tether it to the plasma membrane has been cemented primarily by the discovery of caveolin proteins that can anchor the ERα in lipid rafts and caveolae. Caveolin proteins are highly conserved structural proteins that are necessary and sufficient for the existence of caveolae, a subset of lipid rafts (40) that comprise a restricted compartment for signaling molecules associated with the plasma membrane. Initially, ERα was reported to interact with caveolin 1 (Cav-1) in MCF-7 and vascular smooth muscle cells (VSMC) (41). The caveolins in turn act as adaptor proteins to couple ERα selectively to either other proteins such as the metabotrophic glutamate receptors (mGluR) or to processes such as palmitoylation. In the hippocampus of the female rat, Cav-1 interacted with mGluR1a so that 17β-E activated downstream $G\alpha_q$ and ERK signaling. This, in turn, increased pCREB activation within 5 min of 17β-E application (42). Hence, inhibitors to phospholipase C (PLC) and ERK and mGluR1a as well as a dominant negative Cav-1 mutant decreased pCREB activation (42). On the other hand, Cav-3 tethered ERα in hippocampal neurons to mGluR2/3; this pathway activated $G\alpha_i$ signaling, leading to the inhibition of PKA and the subsequent downregulation of pCREB (42). Hence, the type of caveolin dictated the final divergent pCREB response though the physiological conditions wherein ERα may bind to Cav-1 versus Cav-3 are unknown. In hypothalamic astrocytes, the coupling of ERα to mGluR1a resulted in an increase of calcium within 2 min of 17β-E application; this was blocked both by the ERα and ERβ antagonist, ICI 182,780 and by the mGluR1a antagonist LY 367385 (43). In striatal neurons, Cav-1 facilitated the tethering of ERα to mGluR5 and subsequent $G\alpha_q$ signaling (44). Hence, caveolins may not only tether ERs into caveolae in the plasma membrane in different cell types but also subserve tissue-specific signaling to specific Gα subunits by co-opting different mGluR partners. The coupling of ERα to mGluRs may allow downstream signaling to be potentiated by rendering it sensitive to two ligands, i.e., 17β-E for the ER and glutamate for the mGluR. This is supported by the data that in hypothalamic

astrocytes, glutamate, and 17β-E combined elicited greater Ca^{+2} increases than either ligand alone (43).

In addition to the role that mGluRs play in linking ERα to Gα proteins, ERα can also directly bind Gα subunits. 17β-E inhibited cAMP production within 5 min of addition to GT1–7 immortalized GnRH neurons via ERα that is physically tethered to $G\alpha_{i3}$ (45) protein that can be detected in co-immunoprecipitation experiments. Interestingly, addition of 17β-E decreased the amount of membrane-associated $G\alpha_{i3}$ and this decrease was blocked by the ER antagonist ICI 182,780 suggesting control of the signaling cascade by the ligand, similar to classical G-protein coupled receptors (GPCRs) (45). Though ERα has been demonstrated to tether to $G\alpha_i$ in the caveolae of endothelial cells to increase the activity of nitric oxide synthase (46), this mode of regulation of nitric oxide synthase by 17β-E has not been shown in the CNS.

Apart from serving as adaptors, the binding of caveolins to the ER also increases palmitoylation of the ER, a process by which palmitate, a C16 fatty acid is added to an internal cysteine via a thioester bond. In the human ERα, a canonical palmitoylation site exists at the cysteine amino acid residue at position 447 (C447); the corresponding site in the mouse ERα is the C451 (31). Mutant ERα where the C447 site was mutated to alanine (C447A) did not bind Cav-1, was not localized at the membrane in HeLa cells and showed decreased pERK activation in response to a 10-min application of 17β-E (47). Additionally, when a non-classical palmitoylation site, S522, in the ERα was mutated, localization at the plasma membrane and Cav-1 binding to ERα was decreased (48) in Chinese hamster ovary (CHO) cells. This mutant also decreased the ability of 17β-E to increase pCREB in hippocampal neurons from the female rat (42). Other binding partners for the ERα also lead to increases in palmitoylation and subsequent membrane localization. For example, the amino acid residue cysteine at position 451 of the mouse ERα also binds the heat-shock protein 27 (hsp27), which increased the rate of palmitoylation at this site. Therefore, siRNA to hsp27 decreased palmitoylation with subsequent decrease of ERα at the plasma membrane and decreased phospho-Akt (pAkt) normally induced by a 10 min application of 17β-E (49). The C-terminal of mouse ERα (Leu512–514) is important in ERα dimerization at the plasma membrane; mutation of these residues decreased pAkt and cAMP generation within 5 min of 17β-E addition and decreased the activation of both $G\alpha_s$ and $G\alpha_q$ subunits in the CHO cell line (50) (**Figure 1**). In the CNS, it is unknown if the ERα at the membrane exists as dimers and if these dimers use caveolin proteins as adaptors. It is also unclear if caveolin binding to the ERα precedes greater palmitoylation since ERα mutations that destroy the interaction with caveolin also destroy palmitoylation. Recently, E2-BSA has been shown to transcriptionally upregulate Cav-1 via a PI3K and ERK pathway within 12 h in endothelial cells (51). Hence, 17β-E may increase non-genomic signaling both by increasing the palmitoylation of ERα within rapid time frames and within longer time frames via the transcriptional upregulation of Cav-1.

REGULATION OF ERα AT THE MEMBRANE BY 17β-E

Similar to the tissue specificity shown by ERα-mediated rapid, non-genomic signaling, ERα at the membrane is also regulated by 17β-E in both temporal and tissue-specific manner. In hypothalamic astrocytes (38) and neurons (39) and in a hypothalamic cell line, mHypoE-38 (52), 17β-E increased the amount of both the 52 and 66 kDa mER in the cell membrane within 30 min. This increase of mERα at the plasma membrane can be blocked by ICI 182,780 and the mGluR1a antagonist LY 367385 but does not occur with E2-BSA, suggesting that this is an internal, non-membrane-initiated rapid effect that is dependent on the mGluR1a receptor. In contrast, in MCF-7 cells, 17β-E decreased Cav-1 within 8 h, thus decreasing the amount of ERα at the membrane. Similarly, 17β-E

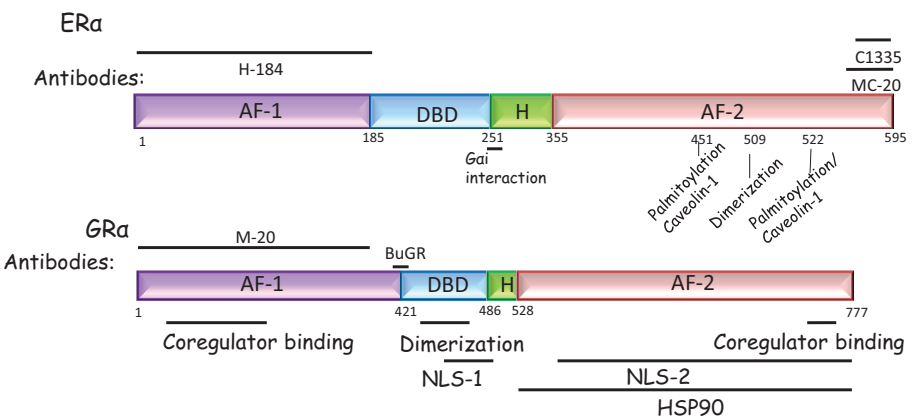

FIGURE 1 | Domain structure of the ERα and the GRα. ERα and GRα are classical intracellular nuclear receptors but may also be present on the plasma membrane. Both receptors have a N-terminal AF-1 domain, a central DNA binding domain, a hinge domain (H), and a C-terminal AF-2 domain, required to bind ligand. ERα: the antibodies most commonly used to detect ERα are the H184 (Santa Cruz Biotechnology, TX, USA) antibody raised to the N-terminal domain and the MC-20 (Santa Cruz Biotechnology, TX, USA) and C1335 (Upstate Biotechnology, NY, USA) raised to the C-terminal domain. In addition, the sites that may tether the ERα to the plasma membrane are marked. These are residues required for caveolin or $G\alpha_i$ binding or residues that are palmitoylated or required for dimerization at the membrane. GRα: the antibodies most commonly used to detect GRα are the BuGR antibody (Abcam Inc., MA, USA) and the M-20 (Santa Cruz Biotechnology, TX, USA) antibody, directed to the N-terminal domain. The NLS refers to the nuclear localization signal since nuclear localization of this receptor can occur rapidly and is a non-genomic effect.

treatment of hippocampal slices from female rats removed ERα from the synaptosomal plasma membrane by depalmitoylation within 20 min (37). In CHO cells, palmitate incorporation into ERα and association with Cav-1 decreased within 60 min of 17β-E administration, suggesting that ERα at the plasma membrane was lowered, consistent with this idea, ERK activation also decreased (47). Decreasing ERα at the membrane in these cells may allow for a decrease in non-genomic signaling and may mark a shift toward genomic transcription. In contrast, in the VSMC, 17β-E increased both Cav-1 and Cav-2 and increased ERα localization at the plasma membrane (41). Such increased ERα localization in response to the endogenous ligand may be a mechanism to sustain non-genomic signaling in some cell types.

Similar to other trans-membrane GPCRs, which may undergo desensitization via internalization in a G-protein receptor kinase 2 (GRK2)-β-arrestin-dependent manner (18), 17β-E application to cortical neuronal cultures rapidly induced GRK2 phosphorylation within 5 min and β-arrestin1 and c-Src recruitment to the ERα within 60 min. Predictably, since β-arrestin1 tagging of trans-membrane GPCRs provides a scaffold for c-Src attachment and subsequent ERK activation (53), siRNA to β-arrestin1 abrogated the 17β-E induction of pERK in these neurons (54). Though the exact partners of the ERα in this internalized signalosome are unknown, ERα internalization is initiated from the membrane since E2-BSA also promotes internalization (54). Internalization of the ERα has also been tested more directly by using a surface biotinylation technique followed by stripping the surface biotin with glutathione after 17β-E addition to cells. Hence, the continued presence of biotin in this preparation would argue that the receptor was internalized and protected against glutathione action. In hypothalamic neurons, addition of 17β-E caused internalization of both the 52 and 66 kDa forms within 30 min, with internalization of the 52 kDa form persisting for 2 h (54), demonstrating that recycling and internalization of the classical ERα as well as the 52 kDa variant occurs in a rapid time frame. An unresolved issue is the rapid decrease in total ERα protein and mRNA in the hypothalamus on 17β-E application that has been shown by several investigators (55, 56). However, it is difficult to reconcile this with the increase in plasma mERα that is seen in the hypothalamic primary neuronal and astrocytic cultures (38, 39) and in the hypothalamic cell line mHypoE-38 (52) unless ERα from other non-membrane locations decrease to a large extent; this could be a parameter to be investigated in future studies. This could also be due to the recent studies that show an increase ERα at the plasma membrane being carried out in primary cultures versus hypothalamic tissue that retain neuronal connectivity in the older studies.

DEPENDENCE OF RAPID SIGNALING ON ERα AND ERβ

In addition to direct demonstration of the ERα either at the membrane or tethering to an intrinsic membrane protein, the ER dependence of kinase activation has also been investigated. Several studies, described in this review, have used the ERα and ERβ antagonist ICI 182,780 to antagonize a rapid signaling pathway or a genetic approach that deletes either ERα or ERβ specifically in the whole animal. The availability of ERα and ERβ knockout animals, i.e., ERαKO and ERβKO, has been useful to understand the ER dependence of 17β-E-mediated non-genomic signaling *in vivo*, particularly in light of an absence of a specific antibody to the ERβ (57). In wild-type female mice, subcutaneous administration of 17β-E caused the activation of CREB and ERK within an hour, as measured by immunohistochemistry for the phosphorylated forms of these molecules. When either ERα or ERβ or both were deleted, a complex brain nuclei-specific and isoform-specific ER dependence was revealed, which may in part be due to the tissue distribution of the ER isoforms in the brain (**Table 1** below) (58). For example, in the ventrolateral VMH (vl-VMH), the loss of ERα results in the reduction of pCREB on 17β-E administration and this could be because the predominant ER isoform is the ERα and it is possible that the loss of ERα cannot be compensated by smaller amounts of ERβ (58). The use of ERαKO, ERβKO, and ERαβKO mice in this study showed that ERα is hence both necessary and sufficient for the induction of pCREB. In the mPOA, 17β-E could induce pCREB via either ERα or ERβ but required both ERα and ERβ to act in conjunction in order to induce pERK (58), demonstrating that ER isoforms can regulate rapid signaling differentially in the same tissue. The regulation in male mice is unknown and is under investigation in our laboratory.

ERα VARIANTS

In both hypothalamic neurons and astrocytes (38), a variant 52 kDa ERα (ERα-52), detected with a C-terminal antibody that also detects the full-length 66 kDa, was expressed in much higher amounts than the ERα-66 kDa form. Based on RT-PCR analysis using flanking primers to this exon in the mouse hypothalamic cell line mHypoE-38, this is believed to be an ERα that lacks exon 4 (ERαΔ4) (52). In astrocytes, only the 66 kDa form is thought to be interact with mGluR1 (43) and the interaction, signaling properties, and function, if unique, of the 52 kDa variant remain unknown. A 36 kDa variant lacking the AF-1 and AF-2 domains with a unique C-terminus was detected in human breast

Table 1 | Comparison of CREB and ERK between wild-type (WT), ERα knockout (ERαKO), ERβ knockout (ERβKO), and double ERα ERβ knockout (ERαβKO) female mouse brain.

Genotype	vlVMH		mPOA		Medial septum	
	pCREB	pERK	pCREB	pERK	pCREB	pERK
WT	++	NR	++	++	++	NR
ERαKO	–	NR	++	–	++	NR
ERβKO	++	NR	++	–	–	NR
ERαβKO	–	NR	–	–	–	NR

pCREB and pERK in 17β-E injected mice was quantitated by immunohistochemistry and compared to the levels of pCREB and pERK present in mice injected with the vehicle, in the ventrolateral VMH (vl-VMH), the medial preoptic area (mPOA) and the medial septum in female ovariectomized mice injected with 10 μg 17β-E and sacrificed an hour after injection. In WT mice, 17β-E increased pCREB in the vl-VMH and pERK and pCREB in the mPOA. ER isoform dependence varies according to the output (pCREB or pERK) measured and the nuclei. ++: upregulation compared to vehicle-treated mice. –: no upregulation compared to vehicle-treated mice. NR = data not recorded. Data are from Ref. (58).

cancer cells, coded for by a unique promoter located in intron 1 of the *Esr1*gene (59) and can be detected by a specific antibody. ERα-36 was localized to the plasma membrane, mediated rapid ERK signaling, and is induced by the selective activation of the GPR30 (discussed in Section "GPR30") by the agonist, G-1 (60). Importantly, this GPR30-selective activator, G-1, has also been demonstrated to bind and activate ERα-36 (60), suggesting that some of the responses attributed to the GPR30 might be via ERα-36. A 46 kDa variant observed in endothelial cells is also localized to the plasma membrane (61) and is composed of a splice variant missing the first 173 amino acids of ERα-66 (62). ERα-46 cannot be detected with an N-terminus antibody raised to ERα-66 such as H-184 (**Figure 1**), but should be identifiable with a C-terminus ERα antibody. A novel ER is the ER-X, an ER of 63 kDa that is expressed in the neocortex in the caveolae membrane fraction, can be identified with ERα and ERβ antibodies and is present in the ERα knockout animal (63). This ER, whose structure remains unidentified, mediated ERK signaling by both 17α-estradiol and 17β-estradiol and would not meet the criteria for a mER, based on our previous definition. The function and expression pattern of the ER-X is unknown though it was upregulated after ischemic injury (63) in the adult. If it is not present in the adult hypothalamus, it is unlikely to be a receptor that mediates normal behavior that is dependent on the hypothalamus (63). So far, neither ERα-36 nor ERα-46 has been demonstrated in the CNS, though it is possible that some antibodies directed toward the DNA-binding domain or, in the case of the 46 kDa variant, the C-terminal domain of the full-length 66 kDa ERα such as MC-20 or C1335 (**Figure 1**) may detect these forms in studies that use solely immunocytochemistry.

Gα-COUPLED mERs

The idea that the mER is a $G\alpha_q$-coupled receptor has been explored in the POMC neuron in the ARH, primarily by the laboratories of Kelly and Ronnekliev (7, 64, 65). In POMC neurons, 17β-E and E2-BSA uncoupled the inhibitory μ-opioid receptor from the G-protein coupled inward rectifying K^+ (GIRK) channel, in a PLC-, PKC-, and PKA-dependent manner (66, 67), thus decreasing hyperpolarization of these neurons and increasing neuronal excitability. Pharmacological characterization revealed that a $G\alpha_q$ receptor upregulated PLC and calcium release, which in turn activated PKC and PKA. Subsequent PKA phosphorylation of the GIRK channel uncoupled the μ-opioid receptor (7, 68) from GIRK. Though this response was specific for 17β-E versus 17α-E and was blocked by the ERα and ERβ antagonist ICI 182,780, it also occurred in ERαKO, ERβKO, and GPR30 KO mice (69), suggesting that a novel mER is coupled to $G\alpha_q$. This signaling pathway can be activated by a selective ligand, STX, which does not bind ERα, ERβ, or GPR30. Both STX and 17β-E also reduced postovariectomy weight gain, suggesting that this novel $G\alpha_q$-coupled mER is important in energy homeostasis. Leptin receptors on POMC neurons caused depolarization rapidly via the JAK/STAT and PI3K pathways that open calcium-dependent TRPC channels (70); 17β-E signaling may synergize with leptin action by increasing calcium rapidly in these neurons via this $G\alpha_q$-mER. Several reviews (7, 68, 69, 71–74) detail the studies on this STX-activated receptor.

GPR30

The GPR30, also known as GPER1, is a former orphan GPCR that was shown to bind 17β-E and increase cAMP in breast cancer SKBR3 cells via an increase in adenylyl cyclase activity (75). In the rat hypothalamus, GPR30 expression is particularly high in the PVN and SON (76) with low expression in the VMH. Cell fractionation and immunocytochemistry revealed GPR30 to be localized at the plasma membrane in both the SKBR3 ($GPR30^+$, $ER\alpha^-$, $ER\beta^-$) and MDA-MB-231 ($GPR30^-$, $ER\alpha^-$, $ER\beta^+$; GPR30 overexpression) breast cancer cell lines (77). Though GPR30 was not detected at the plasma membrane using surface biotinylation in hypothalamic astrocytes (38), GPR30 was localized to the postsynaptic density in the hippocampus and associates with PSD-95 through its C-terminal tail (78). This association with PSD-95 can localize GPR30 to the plasma membrane, independent of 17β-E. Immuno-electron microscopy analysis of rat hippocampi also revealed membrane localization with no intracellular staining (79). However, it can be detected intracellularly in normal mammary gland epithelial cells (80) and colocalized with a marker of the Golgi apparatus in primary cultures of the hippocampus (81). Also, overexpressed GPR30 in COS7 cells exhibits localization at the endoplasmic reticulum and the Golgi (82), suggesting that cell type can determine localization. In addition, HeLa cells transfected with FLAG-GPR30 show mostly staining at the membrane while cells transfected with GFP-GPR30 show staining mainly in the endoplasmic reticulum [(79) #6908], suggesting that inclusion of molecular tags may interfere with proper intracellular trafficking, possibly confounding the interpretation of experiments relying on ectopic expression of GPR30.

Studies describing the regulation of internalization of GPR30 have also yielded conflicting results. When FLAG-GPR30 was ectopically expressed in HeLa cells, receptor endocytosis was ligand dependent and internalized GPR30 occupied a diffuse cytoplasmic localization consistent with classical receptor recycling or proteasomal degradation pathways (79). In human embryonic kidney (HEK)-293 cells, GPR30 internalization was not only ligand-dependent but also occurs constitutively (83). While most GPCRs are either recycled to the plasma membrane via endosomes or degraded in lysosomes to limit excessive signaling, GPR30 rapidly accumulates in the perinuclear compartment via clathrin-coated vesicles (83). It is thought that this Rab 11-dependent accumulation is in the trans-Golgi network and degradation occurs via an ubiquitin-proteasome-mediated pathway, unlike the endosomal degradation or recycling to the surface that is typical for other GPCRs (83). Since this pathway for internalization and degradation is rapid and constitutive, Cheng et al. propose this as the reason for the difficulty in detecting GPR30 at the plasma membrane in some cell lines (83).

GPR30 can signal via both the $G\alpha_s$ and the $G_{\beta\gamma}$ subunits that are associated with the receptor upon activation. The Gβγ subunit transactivated the EGFR receptor leading to downstream activation ERK protein, while simultaneous activation of the $G\alpha_s$ subunit by 17β-E inactivated ERK signaling through activation of adenylyl cyclase and PKA, thus limiting cAMP signaling to a short time frame (23). Furthermore, although GPR30 protein was readily detectable in both the microsomal and plasma membrane subcellular fractions of breast cancer cell lines, only GPR30

in the plasma membrane fraction bound ligand and activated G-protein signaling, suggesting that only membrane-associated GPR30 protein is functional (77). However, GPR30 activation in COS7 cells initiated intracellular calcium mobilization and nuclear accumulation of PIP3 via EGFR transactivation (82) via a non-membrane-initiated signaling mechanism since calcium flux was not replicated by membrane restricted estradiol derivatives (84). These discrepant results have yet to be reconciled. A recent study reported that GPR30 could decrease cAMP that was elevated by heterologous ligands, such as forskolin, via the C-terminal PDZ domain (85). This domain could bind membrane-activated guanylate cyclases (MAGUKs), which act as adaptors for AKAP5 (PKA anchoring protein) that in turn decreased adenylate cyclase activity. This would decrease the cAMP that is elevated by 17β-E binding of the GPR30 and may serve to limit the time frame of GPR30 signaling (85). Hence, both ERK and cAMP signaling downstream of the GPR30 may be restricted to very short time frames.

THE iGR AS THE mGR

Similar to the ERα being considered a possible mER, the intracellular GR (iGR) that exists as a complex with heat-shock protein 90 (HSP90), p23, and a tetratricopeptide protein (86) in the cytoplasm has been proposed as a candidate mGR (87). Stable reduction of mGR levels in $CD14^+$ monocytes using stably transfected siRNA to iGRα suggested that both mGR and iGR were derived from the same transcript (88). GR exists in two isoforms, GRα and GRβ. GRβ, generated by alternative splicing, lacks the last 50 amino acids of the GRα carboxy terminus, and instead possesses a unique 15 amino acid sequence at its C-terminus (89). GRβ neither binds glucocorticoids nor has intrinsic transcriptional activity, but has been implicated as a dominant negative inhibitor of GRα activity through formation of a non-functional heterodimer (90). Higher concentrations of GRβ relative to GRα, therefore, result in decreased glucocorticoid sensitivity. However, levels of GRβ protein in the brain are extremely low (89) and this is not thought to be a regulator of GRα in the brain. The presence of mGRα was detected at very low levels in human lymphocytes and leukocytes using membrane-impermeable fluorescent liposomes and GR-specific antibodies (91) such as M-20 (**Figure 1**). Similar to the tethering of ERα to the plasma membrane, mGRα has been associated with Cav-1 in MCF-7 cells (92). However, while plasma membrane association of other steroid hormone receptors, including ER, is dependent on palmitoylation, mutation of the homologous sequence in GR did not affect membrane localization (93), suggesting that other mechanisms must tether mGR to the plasma membrane. In the brain, most of the rapid effects of glucocorticoids have been confined to the hippocampus and to the hypothalamus. Membrane glucocorticoid receptors were first observed in the synaptic plasma membrane fractions (SPM) of rat brain via [3H]-corticosterone binding assays (20). Hypothalamic SPM had a higher binding capacity than hippocampal or cortical SPM, which is in contrast to corticosterone binding of the iGR, which is lower in the hypothalamus and much higher in cerebral cortex and hippocampus (94). Synaptosomal fractions from rat hippocampus contained plasma membrane-associated GR (95) and GR immunoreactivity was observed at the plasma membranes and vesicle membranes of the hypothalamus (96) while in the CA1, GR was shown in the spine (97). Functionally, JNK and p38 MAPK were activated within 10 min of glucocorticoid administration in primary hippocampal neurons (98). The hypothalamo–pituitary–adrenal (HPA) axis is also subject to rapid negative feedback at the level of the hypothalamus (99). Bath application of glucocorticoids to rat hypothalamic slices caused a rapid suppression of glutamate-mediated excitatory synaptic currents onto CRH neurons that was decreased by a CB1 receptor antagonist (13). The release of endocannabinoids that bind to the CB1 receptor is dependent on corticosterone rapidly acting on the CRH neuron and is dependent on $G\alpha_s$-driven PKA activation in the CRH neuron (100). However, the release of nitric oxide (NO) that increased GABAergic inhibition (101) onto the CRH neuron was dependent on Gβγ signaling (100). Hence, in the PVN, rapid negative feedback by glucocorticoids on post-synaptic CRH neurons is exerted by a combination of suppression of presynaptic glutamatergic neurons and excitation of presynaptic GABAergic neurons. The identity of the mGR that can signal via both $G\alpha_s$ and Gβγ subunits in the CRH neuron remains unknown.

UNRESOLVED QUESTIONS: mER AND mGR

As is evident in the preceding sections, several questions about the identity of the mER and mGR remain. Though the strongest evidence exists for a post-translationally modified classical ER, there are other viable candidates for the ER (102) based both on evidence of proteins that interact with antibodies raised to the classical ER or to the continued existence of non-genomic effects in ERαKO and ERβKO mice (63). Different ER proteins might be mERs in different tissues (14) or different proteins might be mERs in the same tissue at different times to generate divergent rapid signaling outcomes within the same cell or different tissues that is congruent with incoming stimuli. It is also possible that the time frame of non-genomic signaling might be different when there are different mERs present – a GPCR such as GPR30 may activate the ERK and PKA pathways very transiently whereas ERα or ERβ at the membrane may be capable of more sustained activation. Again, this would change the response of the cell to stimuli, depending on the mER present. Second, if the mER is the ERα, the mechanism by which only a small proportion of the total ERα is targeted to the membrane is not known and strengthens the idea that different pools of ERα may exist within cells (30) with different functions. The rationale for the variable amount of ERα at the membrane in some cells versus others in cell lines is also unclear but could allow for 17β-E to employ non-genomic versus genomic signaling to different extents in different cells, which maybe at different physiological states. Third, apart from the difficulty in studying ERβ localization or interaction with other proteins due to the unavailability of a reliable antibody (57), the localization of the ERα variants is also particularly understudied, but the predominance of the 52 kDa form as opposed to the full-length 66 kDa form in the CNS (39, 52) argues for an important role of these variants in estrogen signaling in the brain, as opposed to other classical estrogen-responsive tissues such as the uterus. The rapid actions of these different ERα variants may also oppose each other in some cases (39). It is, therefore, likely that the ratio of different ERs, including mERs

expressed in each cell that determines the cellular response to 17β-E exposure. The significance of these spatially separated and functionally opposing ER populations may be to modulate the hypothalamic and hippocampal neuron response to estrogen and prevent runaway signaling. Fourth, though the GPR30 has a role to play in estrogen-mediated physiology, the acceptance of GPR30 as a mER has not been universal (103) with some investigators preferring to term it as a "collaborator" to the ERα, which is deemed to be the primary mER that signals from the membrane (30). If GPR30 or $G\alpha_q$-coupled novel proteins are the mERs in some tissues, it is also possible that they crosstalk with classical ERα or ERβ, accounting in some studies for the ICI-mediated antagonism of the effect. Though this cooperation between GPR30 and ERα has been shown to have a proliferative effect on ovarian cancer cells (104), a functional effect has not been demonstrated in the hypothalamus. In the ventral hippocampus in male mice, Hart et al. showed an injection of G-1 could increase the phosphorylation of an ERK-sensitive serine site on the ERα at position 118 within 30 min, suggesting that these two receptors could interact with each in the CNS (105). Future studies on the colocalization and the functional interaction between ERα and GPR30 in the hypothalamus will prove useful in this regard. There are far fewer studies on the mGR, when compared to the mER, and most studies investigate the possibility of the mGR being equivalent to the iGR. Surprisingly, the idea that different G-protein subunits, particularly that the Gβγ subunit can support signaling from the unidentified mGR in the CRH neuron and from the $G\alpha_q$-coupled, STX-activated mER in the POMC neuron is also seen with a traditional GPCR such as GPR30. Whether signaling from both G-protein subunits is concomitant or if one signaling pathway predominates, under certain physiological conditions, is not known.

Lastly, the model generated by the existing literature is that the mER or mGR is at the inner leaflet of the membrane, associated with intrinsic membrane proteins or scaffolds such as caveolin. However, surface biotinylation experiments imply that some part of the mER or mGR is exposed to the extracellular mileu (18). Though an abundance of hydrophobic amino acids in the ligand-binding domain of the mER predicts that this domain might insert into the plasma membrane (106), no study has demonstrated this in the CNS. While recycling of this receptor from the membrane is to be expected, the pathways (Rab-mediated or β-arrestin mediated) are possibly cell-specific and are worthy of more attention since non-genomic signaling is possibly terminated during internalization.

MORPHOLOGICAL AND BEHAVIORAL OUTPUTS IN THE CNS DEPENDENT ON NON-GENOMIC SIGNALING BY ESTROGENS AND GLUCOCORTICOIDS

Though kinase regulation and calcium flux has been shown to occur rapidly when cells are exposed to the hormone in a number of tissues and the idea of non-genomic signaling more accepted than ever before, the relevance of non-genomic signaling for behavior has remained murky. Here, we shall confine ourselves to primarily hypothalamically driven behaviors or possible neural correlates that are either rapidly induced by 17β-E or E2-BSA or that may have a non-genomic component. Finally, we will discuss the intersection of glucocorticoid rapid signaling in a 17β-E-mediated behavior.

SPINOGENESIS

Estrogen is critical in the display of female sex behavior in rodents by acting on the hypothalamus; retrograde tract tracing using the pseudorabies virus (PRV) injected into the lumbar muscles labeled the lordosis circuit, in particular, the plexus of oxytocin fibers in the vl-VMH (107). In the hypothalamus, estradiol benzoate (EB) injections to ovariectomized rats increased spine density by 48% in the vl-VMH as compared to oil injections (108). Surprisingly, only 3% of PRV-labeled neurons were $ER\alpha^+$ and these were not the neurons that showed an increase in spine density (109) upon EB administration. These data argue that the increase in spine density in the VMH may be only indirectly dependent on ERα signaling or could be due to another ER while the longer time frames used in the study do not allow us to conclude if there is a non-genomic component. A candidate could be the GPR30 receptor, which is expressed in the vl-VMH; the role of this receptor in spinogenesis in the hypothalamus is not known. Rapid effects on spinogenesis and spine morphology following GR activation have been observed in the hippocampus, but not in the hypothalamus. CA1 neurons from male rat hippocampal slices treated with dexamethasone for 1 h demonstrated a translation-independent increase in spine density, which was lost with co-application of dexamethasone with either the GR antagonist RU-486 or the NMDA receptor blocker MK-801 (95). The proportion of mushroom-shaped and thin-type spines was also increased following GR activation (95).

Does non-genomic signaling play a role in the increase in spine density by estrogens or by glucorticoids? The mGluR1a antagonist, LY367385 in the ARH decreased phosphorylated cofilin levels that are induced within an hour of EB injection and that are required for actin reorganization and spinogenesis, suggesting that at least some of the initial aspects of spinogenesis are mediated via non-genomic signaling from the ERα-mGluR1a complex at the membrane (110). Though EB can increase filopodial spines in the ARH within 4 h of treatment, mushroom shaped, more stable spines require time frames in excess of 20 h and parallels the time frames required for the full display of lordosis behavior, suggesting that rapid non-genomic signaling is insufficient for formation of stable spines (110). Stabilization of the PSD-95 protein, a scaffolding protein enriched in mushroom-shaped dendritic spines, was dependent on pAkt and also required 48 h of 17β-E treatment in differentiated NG-108 cells (111). Another model for the increase in 17β-E-mediated increase in spine density in the hypothalamus is that 17β-E stimulates PI3K activation pre-synaptically, inducing glutamate release, followed by NMDA receptor activation and ERK signaling post-synaptically, with a subsequent increase in spinophilin protein that was correlated with an increase in stable dendritic spines (112) on the post-synaptic neuron. Both ERK and PI3K signaling are implicated in the increase in spine density, though the formation of mature mushroom spines also appears to require transcription (113). Though there are no reports of glucocorticoid-mediated regulation of spine density in the hypothalamus, suppression of PKA, PKC, MAPK, or PI3K signaling completely blocked GR-mediated spinogenesis in CA1 neurons, suggesting that GR signals through convergent kinase pathways

to increase actin polymerization, which would allow for spine changes (114). In CA1 neurons, treatment with the synthetic glucocorticoid dexamethasone increased p-cofilin levels within 30 min, similar to the rapid induction seen in the ARH by EB (97).

Consistent with the idea that 17β-E-mediated spinogenesis in the VMH has a functional consequence, cytochalasin D, an actin polymerization inhibitor, blocked the formation of spines in the ARH and reduced lordosis (110) though the relevance of such spinogenesis to sex behavior in rodents (see Sex Behavior or Lordosis in Females) is unknown. The consequences of spine disruption for other behaviors such as aggressive behavior or male sex behavior (see Male Sex Behavior) are also unknown. Though most of the studies have shown effects on spine density or morphology in longer time frames that do not allow one to parse non-genomic actions from transcription, spine density on the apical dendrite in the stratum radiatum and in the stratum lacunosum moleculare of the CA1 neuron was increased within 40 min by the ERα selective agonist, propyl pyrazole triol (PPT); however, these were at high doses that were not correlated with doses of PPT that led to an improvement in social memory within 40 min of administration (115). In mature rat cortical neurons, 17β-E rapidly increased ERK and p21-activated kinase to increase dendritic spine density (116) within 30 min. Though this suggests that non-genomic signaling may be sufficient for an increase in spine density at least in the hippocampus and cortex, this has to be confirmed with experiments that utilize a transcription inhibitor. A similar rapid effect of estrogen on spine density in the hypothalamus has not been demonstrated.

SEX BEHAVIOR OR LORDOSIS IN FEMALES

Lordosis in rodents is a 17β-E-driven behavior where integration of sensory information within the limbic-hypothalamic circuit culminates in VMH projections onto neurons of the periaqueductal gray (PAG) and spinal cord motor neurons, resulting in the classical lordosis posture (16). A series of elegant experiments from the laboratory of Micevych delineated a microcircuit consisting of neuronal afferents from the ARH to the mPOA (113). In this circuit, the initial membrane-initiated signaling by 17β-E in the ARH neurons increased PKCθ (117) and released neuropeptide Y that activates β-endorphin expressing ARH neurons that project into the mPOA (118). In the mPOA, β-endorphin released from the ARH caused the internalization of μ-opioid receptors (MOR) within 30 min of E2-BSA (119) administration into the ARH, suggesting that this is a membrane-initiated non-transcriptional event. This transient inhibition of lordosis initiated by MOR internalization is removed 30 h later by progesterone (120). A number of different receptors, including ERα and mGluR1a, play a role in this membrane-initiated signaling to influence lordosis behavior. Not only does ERα and mGluR1a colocalize in about 23% of ERα-expressing neurons but deletion of ERα also blocked MOR internalization in the mPOA (121) and abolishes lordosis (122). Blocking of MOR internalization with application of an mGluR1 antagonist or Cav-1 siRNA into the ARH also, in turn, reduced lordosis (119, 123). Though this suggests that ERα and mGluR1 signal together as in the hippocampus (124), the selective mER agonist, STX in the ARH also induced MOR internalization and lordosis (125), an effect blocked by the mGluR1a antagonist. This suggests that in the ARH, both ERα and the $G\alpha_q$-mER may couple to mGluR1a and facilitate lordosis, though the mechanisms by which this occurs are unclear. Apart from these receptors, the localization of the GPR30 in the hypothalamus (126) and the ability of G-1, an agonist at the GPR30 to facilitate lordosis in female ovariectoimized mice (127) argues that GPR30 signaling is also important, at least in mice. It is unknown if the ERα, the $G\alpha_q$-mER, and the GPR30 interact or signal to each other or represent independent parallel signaling pathways that drive lordosis in estrogen primed ovariectomized female rodents, though any model must take into account the necessity of ERα in the VMH for lordosis.

Since Kow et al. demonstrated that E2-BSA alone in the VMH does not induce lordosis (128), we can presume that non-genomic signaling is insufficient but may potentiate or prime lordosis that is in itself dependent on nuclear transcription (129) via a coupled signaling pathway (130). Consistent with this idea, a mouse that possesses an ERα that cannot bind an ERE ($ER\alpha^{-/AA}$) (131) did not display lordosis behavior (132), demonstrating the importance of 17β-E-bound ERα transcriptional action at an ERE for lordosis. Also, combined E2-BSA and 17β-E administrations into the VMH increase progesterone receptor (PR) protein levels higher than either hormone alone (133), demonstrating potentiation of transcription by a membrane-limited estrogen conjugate. The reduction of ERα in the VMH using adenovirus-associated shRNA viral vectors that abolished lordosis behavior (29) shows the absolute requirement for the ERα, which could be due to its participation both as a mER and as a nuclear transcription factor in the VMH. An additional role could be the signaling initiated by ERα acting as a mER in the ARH but as a transcription factor that upregulates the PR in the VMH (134), both processes that are required for lordosis. In this scenario, membrane-initiated signaling in the ARH results in transient inhibition of lordosis so that 17β-E can transcriptionally activate genes such as the PR in the VMH that are important for the full display of lordosis that occurs 30 h later (113).

MALE SEX BEHAVIOR

In males, 17β-E given intraperitoneally at high concentrations to castrated adult rats increased some aspects of male sex behavior, i.e., genital sniffs and mounting within 15 min, a time frame commensurate with non-genomic actions (135). However, since protein synthesis inhibitors (136) and the presence of the ERE-binding mutant, the $ER\alpha^{-/AA}$ mouse abolished male sex behavior (131), it is reasonable to conclude that male sex behavior, similar to female sex behavior, has a non-genomic component that is not sufficient for the full display of the behavior. Though most of the studies investigate the role of testosterone in males (137, 138), testosterone can be converted to 17β-E by aromatase (139). In the castrated and testosterone supplemented quail that was treated with the aromatase inhibitor, vorozole, administration of 17β-E or E2-BSA intracerebroventricularly (icv) increased appetitive sexual behavior but not the final consummatory aspects of sexual behavior within 15 min (140). This appetitive sexual behavior was also blocked by vorozole, administered icv 15 min before behavioral testing (140). This implies that neuroestrogens that are rapidly generated in response to stimuli such as the sight of a female signal

rapidly to initiate appetitive sexual behavior. Though ICI 182,780 antagonized the increase in appetitive sexual behavior seen rapidly with 17β-E administration (140), the exact mER that mediates the rapid regulation of sex behavior or aromatase in the quail or the mouse is unknown. In the male mouse, consistent with the interpretation that arises from the data in the quail, 17β-E administered to aromatase knockout male mice reversed the lack of sex behavior while vorozole decreased sex behavior in C57BL/6 mice within 15 min of administration (141). Stimuli-dependent rapid regulation of aromatase could represent a mechanism to locally elevate neuroestrogens, which then increase sex behavior in male quail or in the male rodent. However, contrary to this expectation, aromatase activity decreased rapidly when male quail were exposed to female stimuli but neuroestrogen concentrations itself increased in the mPOA (142), a nuclei important for male sex behavior. However, it is important to note that the acute action of 17β-E on appetitive sexual behavior in the male quail was always preceded by initial copulation with a receptive female to establish this learned response, wherein the sight of the same female would initiate appetitive sexual behavior (143). In this case, it is possible that prior sexual experience may initiate transcriptional signaling by 17β-E, which in turn primes rapid non-genomic signaling to achieve greater efficiency in subsequent sexual interactions and optimize reproduction; this remains to be tested. Similar to female sex behavior, the non-genomic component in male sex behavior is important for the initial approach and mounting aspects of male sex behavior.

AGGRESSION IN MALES

Castration in male rodents removes both testosterone and estrogens and results in loss of aggression in response to territorial intrusion (144, 145). The importance of estrogen in male aggressive behavior is shown by the deletion of aromatase, which abolished aggression by male mice in a resident-intruder paradigm, though this is possibly also due to organizational defects since 17β-E reinstated aggression only when administered before postnatal day 7 (146). In CD-1 outbred male mice, ERα concentrations in several areas of the circuit involved in aggression such as the bed nucleus of the stria terminalis (BNST), the lateral septum (LS), and the anterior hypothalamus (AH) was higher in more aggressive mice (147). Reduction of ERα in the VMH by adenovirus mediated transfer of siRNA to ERα abolished aggressive behavior in male mice, demonstrating that ERα expression in the VMH is necessary for aggression in adult male mice (29), just as it was important for sex behavior in male mice. In California, beach mice that were castrated and supplemented with testosterone and an aromatase inhibitor so that they possessed testosterone but not estrogen, aggression increased rapidly within 15 min of injection with cyclodextrin-conjugated 17β-E (148). This suggests that testosterone alone was not sufficient to elicit aggressive behavior in a resident-intruder paradigm and that 17β-E is necessary to activate aggression. The time frame and the inability of cycloheximide (149) to decrease this aggression in the beach mouse argue that 17β-E acts non-genomically, though most studies on aggression, with the exception of those cited in this paragraph, in the rat and mouse have used time frames that do not allow us to parse a non-genomic effect from a genomic/coupled signaling effect.

What is the source of 17β-E? In rodents, aromatase is expressed in axon terminals (150–152) particularly in the hypothalamus (153) and brain 17β-E concentrations in the male rat are equal to or higher (~8 nM) than in the plasma (154). Hence, 17β-E generated at the synapse has been envisioned to function as a neuromodulator in short time frames (155), in line with the data in male birds where neuroestrogens generated at axon terminals are important in learning in songbirds (156) and in sex behavior in the quail (157, 158). In the castrated male rat, EB synergized with dihydrotestosterone (DHT) to increase aromatase activity (159). In addition, 17β-E can transcriptionally induce the aromatase gene via ERα-c-jun complex binding to AP-1 elements in the brain-specific promoter of aromatase (160). Therefore, similar to spinogenesis, the regulation of aromatase by 17β-E itself may represent another correlate where both rapid, non-genomic and slower, transcriptional mechanisms converge. The areas of the brain wherein this may occur in the rodent are not clear and are being investigated in our laboratory.

Though 17β-E can rapidly increase aggression by modulating neurotransmitter release [(145) and references therein], it may also do via regulating the HPA axis. In the white-crowned sparrow, neither brain 17β-E concentrations nor aromatase activity in nuclei involved in aggression were correlated with aggressive behavior (161) though corticosterone did increase rapidly. What is the link between glucocorticoids and aggression? Though aggression itself is stressful and leads to secretion of glucocorticoids, dominance in an aggressive encounter typically leads to lowering of the corticosterone level (162). However, pre-fight corticosterone levels have been shown to be associated with aggressiveness in fish and acute treatment with adrenocorticotrophic hormone (ACTH) increased fighting behavior in male mice (163) while acute treatment with corticosterone 2 min before an encounter increased aggressiveness in male rats (164) and risk assessment in the open field and elevated plus maze tests (165). Supporting the idea that corticosterone is important in regulation the onset of aggressive behavior, acute treatment with GR antagonists (166, 167) or prevention of glucocorticoid synthesis (164) prevented aggression when presented with social challenge. Since acute central application of corticosterone increased aggression within 7 min and protein synthesis inhibitors did not decrease it, non-genomic signaling by glucocorticoids in the CNS is most likely required to increase aggression (164). Consistent with this idea, acute corticosterone injection also decreased the magnitude of electrical stimulation to the hypothalamus required to elicit attack (168). In addition, corticosterone administration to rats in established colonies did not change aggression, demonstrating that glucocorticoids promote aggression only in ethologically relevant situations such as territorial intrusion (169). However, chronic elevated levels of glucocorticoids, such as seen in stressed or socially defeated animals decrease aggression and increased submissiveness in hamsters (170, 171), mice (172), and rats (173, 174). This is thought to be due to genomic signaling by glucocorticoids though the molecular mechanisms by which glucocorticoids facilitate or inhibit aggression are unclear (175).

Gonadal status can modify the actions of glucocorticoids on the hypothalamus or can regulate corticosterone levels itself. For example, gonadal steroids can modify glucocorticoid-mediated

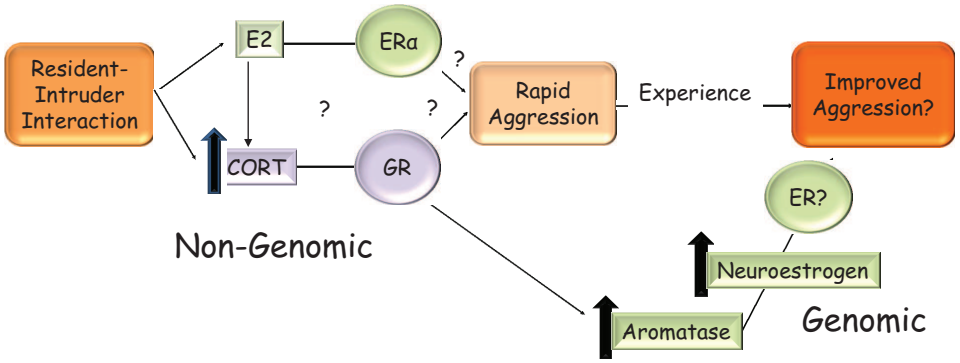

FIGURE 2 | Hypothetical model in the rodent for the rapid and sustained increase in aggression that is seen when an intruder enters a resident's home-cage. On visual and/or olfactory cues, 17β-E in the brain may increase corticosterone via an unknown mechanism. This increase in corticosterone may rapidly and non-genomically increase aggressive behavior. The mechanism by which corticosterone does so is not known but may involve the regulation of aromatase and could be GR mediated. It is not known if 17β-E's regulation of aggressive behavior is solely via corticosterone increase or these are two parallel pathways, which require ERα, as shown in the figure. Elevated corticosterone levels can increase transcription from the brain-specific aromatase promoter, which in turn increases neuroestrogens. This may contribute to shorter latencies of attack in successive bouts. The interaction of estrogen and corticosterone ensures that the resident rodent is aggressive only in the presence of a relevant external cue, i.e., intruder and a relevant internal cue, i.e., sufficient 17β-E.

negative feedback on CRH. 17β-E chronically administered to gonadectomized and adrenalectomized female rats treated with high doses of corticosterone increased CRH mRNA levels, thus counteracting negative feedback, while DHT treatment to male rats had an opposing effect and caused a further decrease in CRH levels (176). In female ovariectomized rats, icv, but not systemic, 17β-E injection increased corticosterone rapidly within 30 min, an effect mimicked with the ERα selective agonist, PPT, injected into the PVN (177). In gonadectomized male rats, CRH mRNA was higher when they were treated with EB but lower when treated with DHT, implying that estrogens can regulate the HPA axis in males also (178). A number of mechanisms may be involved in the rapid increase of aggression by glucocorticoids in response to social challenge, including the regulation of dopamine (179, 180) and serotonin (181, 182) neurotransmission. Here, we will focus on one such mechanism that is responsive to both estrogens and glucocorticoids in the hypothalamus, namely, the regulation of aromatase. Acute stressors, which increase glucocorticoids, increased neuroestrogen concentrations in the PVN and aromatase mRNA in the PVN within an hour in female rats, suggesting that corticosteroid release may also increase neuroestrogen in the hypothalamus (183). Similarly, in male quail, rapid increases in aromatase activity also occurred on restraint stress in the mPOA (184), demonstrating that corticosterone can affect aromatase using a non-genomic signaling mechanism that is yet unknown, in both birds and rodents. Furthermore, aromatase protein levels are increased in response to glucocorticoid treatment in a hypothalamic cell line via a transcriptional mechanism (185), leading to a possible increase in the amount of aromatase that may increase the efficiency of winning future bouts. These data demonstrate that 17β-E in the brain can increase plasma corticosterone, which in turn can increase aromatase activity and/or transcription in the hypothalamus leading to a positive feedback circuit that may be active under physiologically relevant stressful contexts such territorial intrusion. The relevance of this pathway

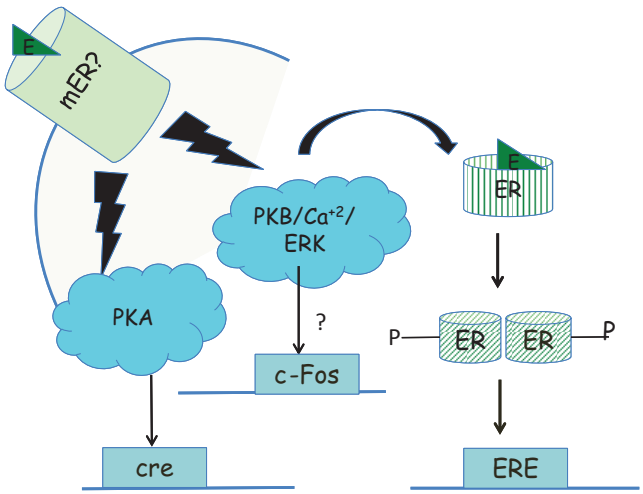

FIGURE 3 | Priming of transcription within neurons by non-genomic signaling. Non-genomic signaling, initiated at the plasma membrane by an unknown mER can activate ERK signaling, which in turn may cause phosphorylation of the ERα and increased transcription from promoters than contain EREs (130). Alternatively, ERK signaling may also lead via unknown mechanisms to increased transcription from the c-Fos promoter (190) while PKA signaling generated by the mER may lead to increased transcription from promoters that have a cAMP response element (CRE) enhancer (189).

(**Figure 2**) to aggression is under active investigation in our laboratory, with the idea that glucocorticoid control of aggressive behavior is dependent on neuroestrogen concentrations in the brain.

FUNCTIONS OF NON-GENOMIC SIGNALING

One function of non-genomic signaling within a cell could be to provide cells with another pathway by which they can respond

differently to the same endogenous ligand. For example, acute signaling by glucocorticoids increases aggression in an ethologically relevant context but chronic signaling by glucocorticoids increases submission (186). A parallel is seen with the regulation of the CRH neuron by acute or chronic glucocorticoid exposure via the regulation of synaptic inputs in the opposite direction. In the acute phase, rapid signaling by glucocorticoids increases negative feedback on the CRH neuron decreasing the firing of the neuron (187), while chronic exposure to glucocorticoids decreases negative feedback on the CRH and increases CRH mRNA, increasing HPA axis reactivity (188). A second function of membrane-initiated signaling might be to prime nuclear transcription via the phosphorylation of ERα, seen in a neuroblastoma cell line (130) (**Figure 3**), leading to greater transcription from genes with EREs in their promoters. In some cases, rapid signaling could also activate genes that do not have classical EREs in their promoters. For example, PKA signaling induced by 17β-E in the SK-N-SH neuronal cell line was required to induce the neurotensin/neuromedin gene (189), while E2-BSA in this cell line activated a reporter gene driven by a c-Fos promoter in an ERK-dependent manner (190). The idea that non-genomic signaling might prime later outputs such as behavior is also evident from the studies on sex behavior in female and male rodents. Apart from the potentiation of transcription by rapid signaling, non-genomic signaling could also decrease transcription if the decrease in transcription is ultimately important in leading to an optimal cellular response. For example, the increase in PI3K and MAPK activation that occurs within 30 min via ERα in the mHypoE-38 hypothalamic cell line is required for the long-term repression of neuropeptide Y by 17β-E (191). Third, non-genomic signaling via one pathway could also synergize with rapid signaling pathways initiated by other ligands. For instance, 17β-E-induced increases in calcium in POMC neurons augmented the activation of the calcium-sensitive TRPC channels (192) already upregulated by leptin (65). Additionally, 17β-E also transcriptionally induced PI3K p85 subunit in the hypothalamus (193, 194) and this combined with the non-genomic signaling-mediated increase in calcium potentiates the PI3K and calcium-mediated TRPC channel opening activated by leptin (70). In this case, 17β-E utilized both non-genomic, i.e., calcium increase and genomic signaling, i.e., PI3K transcriptional upregulation to synergize with the rapid signaling initiated by the leptin receptor to converge onto neuronal depolarization. Fourth, non-genomic signaling pathways may also synergize with a parallel, independent transcriptional pathway, in response to the same ligand. The rapid action of glucocorticoids in decreasing glutamatergic inputs to the CRH neuron combined with slower transcriptional downregulation of the CRH and vasopressin gene are dual pathways that achieve negative feedback

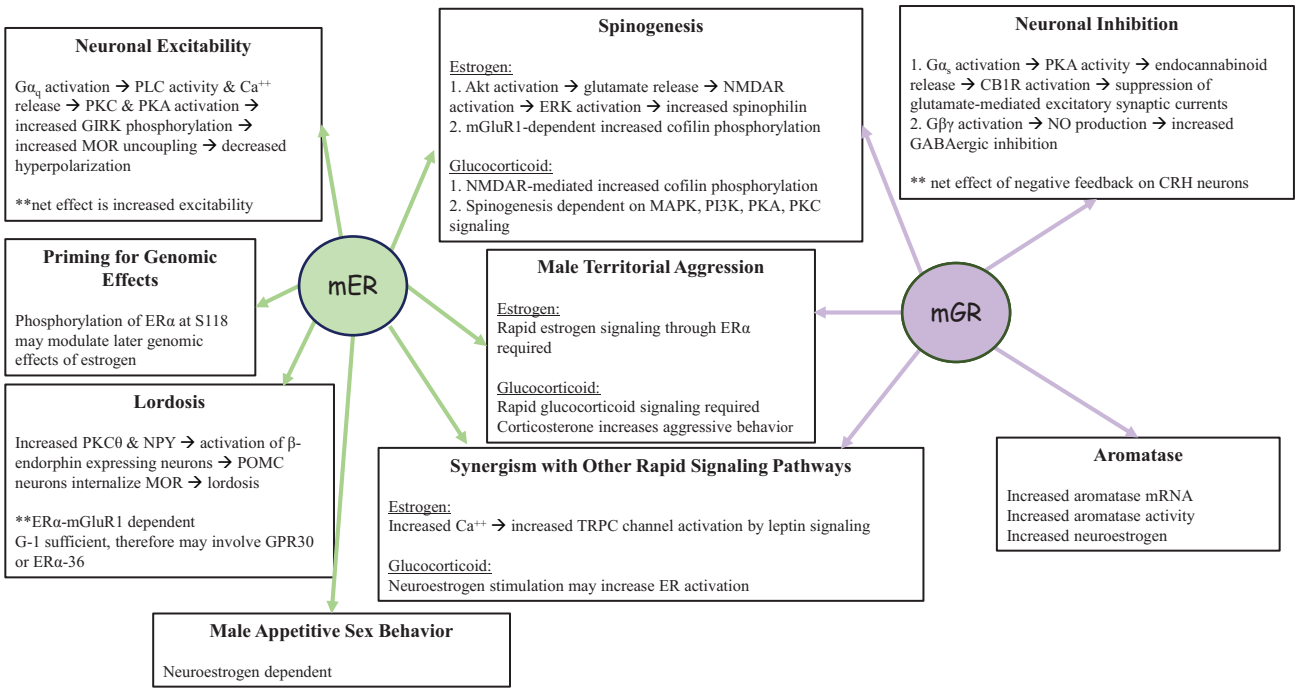

FIGURE 4 | Functions of non-genomic signaling initiated by the mER or the mGR in the CNS. Both mER and mGR contribute to the regulation of male territorial aggression and spinogenesis in neurons, as well as synergizing with other rapid signaling pathways. In the pre-opiomelanocortin (POMC) neuron shown on the left, non-genomic signaling by the mER increases neuronal excitability while rapid negative feedback by the mGR in corticotropin releasing hormone (CRH) neurons decreases neuronal excitability (shown on the right). In addition, the mER plays a role in male sexual behavior and may interact with GPR30 regulates lordosis behavior in female rodents. Glucocorticoids via the mGR also increase neuroestrogen concentrations, via aromatase activity and the increase in aromatase protein, thus providing a route by which the actions of estrogens and glucocorticoids can converge in the brain.

Table 2 | Receptors that are activated or inhibited by the natural estrogen 17β-estradiol, the natural glucocorticoid, corticosterone, a membrane-limited estrogen conjugate (E2-BSA) or glucocorticoid-conjugate (Dex-BSA), or selective agonists or antagonists.

Hormone/drug	Receptor activation	Receptor antagonism	Reference
17β-estradiol	All ERs	None	
E2-BSA	All mERs	None	
Propyl pyrazole triol (PPT)	ERα; 410-fold selectivity over ERβ. Also binds GPR30	None reported	(199)
R,R(THC)	ERα	ERβ	(200)
Diarylpropionitrile (DPN)	ERβ; 70-fold selectivity over ERα	None reported	(201)
ICI 182,780	GPR30	ERα/ERβ	(202)
G-1	GPR30 and ERα-36	None reported	(60)
G-15	ERα or ERβ; activates ERE	GPR30	(203)
G-36	None reported	GPR30	(203)
Corticosterone	All GRs Mineralocorticoid receptor	None reported	(204)
Dexamethasone	All GRs MR (30% of affinity to GR)	None reported	(204)
Dex-BSA	All mGRs	None reported	
RU-486 (mifepristone)	None reported	GR, progesterone receptor	(205)

As can be seen, several selective agonists or antagonists show cross-reactivity to other receptors for estrogens and to other receptors for mineralocorticoids and progestins, in the case of the glucorticoids. Hence, results obtained using these compounds must be carefully interpreted.
MR, mineralocorticoid receptor.

regulation in the PVN (19). Glucocorticoid-mediated rapid regulation of aromatase combined with the slower transcriptional increase or aromatase mRNA and protein is yet another example where both modes would finally increase neuroestrogens in the hypothalamus. Lastly, non-genomic signaling in one cell type may also synergize with genomic signaling in another cell type to converge onto behavioral outputs. This is demonstrated by the ability of 17β-E to elicit a rapid calcium increase in hypothalamic astrocytes that is required for the increased synthesis of neuroprogesterone (195). This release of progesterone that binds to the PR that is transcriptionally induced by the liganded ERα in hypothalamic neurons is required for lordosis behavior in rodents (195). Some of these functions of non-genomic signaling are shown in **Figure 4**.

FUTURE DIRECTIONS

Understanding the many facets of non-genomic signaling would also spotlight the role and source of the ligand. The idea that local estrogen synthesis in the brain, i.e., neuroestrogens is important in transducing environmental stimuli to behavior is fairly recent and has mostly been explored in birds (155, 156, 196). In addition, this would bring into focus the role of estrogens in males; traditionally, classical female-typical behaviors such as lordosis have received far more attention in the estrogen field. Second, many of the studies described above were done in rats whereas there are hints that there may be subtle differences in the mouse. For example, though there is very little GPR30 protein reported in the rat amygdala (76), there is high expression of GPR30 in the mouse basolateral amygdala and intra-amygdalar injection of G-1 attenuates behaviors that denote anxiety in the mouse (197). Similarly, though G-1 failed to regulate sex behavior in the female rat (198), it could do so in the female mouse (127). Though studies on rapid non-genomic signaling have become more mainstream, a number of mechanistic aspects of non-genomic signaling and coupled signaling remain unknown including the temporal nature of the shift from non-genomic signaling to genomic signaling. The role of the ERα variants and GR variants in any of these hypothalamically driven behaviors or in spinogenesis is unclear, with almost no studies on the subject. In this regard, it is worth nothing that studies using agonists or antagonists should be careful to verify if the effects are specific – for example, some of the G-1 mediated effects could be via ERα-36 and some of the ICI-mediated effects could be via GPR30 (**Table 2**).

Complementary studies using deletions of the genes in animals or preferably using site-specific or temporally specific deletion would prove useful to establish specificity. The detailed molecular pathways by which 17β-E regulates spinogenesis or behaviors in the VMH are also not well worked out, though there has been considerable progress in the last decade on the receptors that might be involved. Lastly, understanding non-genomic signaling is possibly a mechanism to convey information about external physiologically relevant stimuli rapidly to the animal to generate both acute and chronic outcomes.

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Insulin, aging, and the brain: mechanisms and implications

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There is now an impressive body of literature implicating insulin and insulin signaling in successful aging and longevity. New information from *in vivo* and *in vitro* studies concerning insulin and insulin receptors has extended our understanding of the physiological role of insulin in the brain. However, the relevance of these to aging and longevity remains to be elucidated. Here, we review advances in our understanding of the physiological role of insulin in the brain, how insulin gets into the brain, and its relevance to aging and longevity. Furthermore, we examine possible future therapeutic applications and implications of insulin in the context of available models of delayed and accelerated aging.

Keywords: insulin, insulin receptors, brain, inflammation, delayed aging, accelerated aging, longevity

INTRODUCTION

Pathways that orchestrate the responses of the organism to changes in its environment have been implicated in the genetic regulation of lifespan across different species. One of the key pathways identified by genetic analysis of long-lived *Caenorhabditis elegans* (*C. elegans*) mutants is insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS) (1, 2). In invertebrates, multiple insulin/IGF-1-like ligands signal via a common receptor, which shows homology to the mammalian insulin and IGF-1 receptors. Also in mammals, insulin/IGF-1 signaling has been linked to aging, lifespan, and longevity (3). Although in mammals, insulin and IGF-1 act predominantly via distinct receptors, there is extensive overlap and interaction in their downstream signaling cascades, making it difficult to separate effects of insulin signaling from those of IGF-1 signaling. The long-lived phenotype of FIRKO mice, which were made by selective disruption of the insulin receptor (IR) in adipose tissue, supports a role of insulin signaling in longevity (4). Moreover, many of the long-lived mouse mutants with disrupted GH/IGF-1 signaling display enhanced insulin sensitivity. In humans, a hallmark phenotype of healthy longevity is maintenance of insulin sensitivity (5, 6), which has been observed in familial human longevity (7, 8), as well as in centenarians (9–11). Insulin influences all aspects of human physiology (12, 13). Besides regulating peripheral glucose homeostasis, insulin is an important neuromodulator that contributes to neurobiological processes (14), with growing evidence that insulin supports behavioral, cellular, biochemical, and molecular functions (15). In literature, evidence linking aging and insulin signaling includes prolongation of life span in rodents via genetic mutations affecting insulin signaling pathways or via interventions that down-regulate nutrient sensing pathways such as caloric restriction. Further evidence includes data on the role of type 2 diabetes in accelerated aging syndromes, and the increased incidence of insulin resistance with age (16). In model organisms (nematodes and fruit flies), specific neural manipulations of insulin signaling have also been linked to aging and lifespan (17, 18). Insulin is produced in the brain of these organisms, making it undoubtedly a neuropeptide. In mammals and humans, IRs are highly abundant in many brain areas and nuclei, but it remains unclear if insulin is produced in the brain. Furthermore, the physiological and pathophysiological mechanisms of insulin action in the brain in relation to aging and longevity remain to be elucidated.

With the global population aging, there has been an astonishing increase in the prevalence of obesity (19), metabolic syndrome (20), type 2 diabetes (21), and neurodegenerative diseases (22). Insulin resistance is a shared feature in these diverse pathologies (13, 23–26). It therefore becomes critical to understand the role of insulin in healthy longevity, as this may be relevant to combatting age-related disorders that have been linked to disturbances in glucose metabolism. The aim of this article is to review advances in our knowledge about insulin, insulin signaling, and the brain, and to present these in the context of available models of delayed and accelerated aging. Furthermore, we will examine the links between inflammation, metabolic health, and brain health, and their effect on aging. Finally, we will review therapeutic options to enhance brain insulin action, including measures to enhance local brain insulin levels as well as measures to enhance the brain responses to insulin.

INSULIN AND THE BRAIN: A CENTURY OF DISCOVERIES

Insulin, after discovery in 1921, was initially considered a peripheral hormone and thus unable to cross the blood–brain barrier (BBB) (27). However, in 1967, Margolis and Altszuler demonstrated in dogs that the concentration of cerebrospinal fluid (CSF) insulin increased after an increase in plasma insulin (28), thus showing that insulin is able to cross the blood–CSF barrier. In 1978, Havrankova et al. demonstrated the widespread presence of IRs in the central nervous system (CNS) of the rat (29). Later that year, they further demonstrated that high levels of insulin were present in rat brain extracts, and found that the concentration of insulin in the CNS was considerably higher than its concentration in the circulation (30). They thus proposed a physiological role

for insulin in the CNS. In the 1980s, further evidence that insulin from peripheral circulation crosses the BBB thus gaining access to the brain was provided. In 1983, Dorn et al. demonstrated that the human brain contains insulin in concentrations much higher than the blood, the highest being in the hypothalamus (31). Furthermore, they showed the presence of high concentrations of insulin in the brains and spinal cords of human cadavers, mice, and rats (32). Baskin et al. demonstrated uptake in the hypothalamus of [125I]iodoinsulin after the insulin had been stereotaxically injected into a lateral cerebral ventricle. Furthermore, they detected insulin-like immunoreactivity in the periventricular, supraoptic, suprachiasmatic, arcuate, and lateral hypothalamic nuclei of the rat hypothalamus (33, 34). In 1992, Schechter et al. delineated the ontogeny of rabbit brain insulin concentration and demonstrated that insulin availability is developmentally regulated (35). In the past decade, studies of the effects of insulin in the brain have been enhanced after development of non-invasive methods of selective delivery of insulin into the brain, via the intranasal route, circumventing peripheral effects of systemic hypoglycemia (36). This has advanced our understanding of potentially therapeutic effects of enhancing insulin concentrations in the brain. Furthermore, studies in recent years have brought forward the role of insulin signaling in the hypothalamus, as a key player in regulation of hepatic glucose production and food intake (37).

BRAIN INSULIN: IS INSULIN A NEUROPEPTIDE IN HUMANS?

In the rabbit, discordance was observed between insulin concentrations in serum and CSF (35). Insulin was found to be present in high concentrations in brain micro-vessels (38), brain extracts (39), and immature nerve cell bodies (35, 40), despite that only 0.046% of peripheral insulin crosses the BBB in mice (12, 41). Moreover, brain insulin concentrations were observed to vary according to developmental stages, with peak amounts being observed during the critical phases of brain growth and development (42). Taken together, these results suggest that brain insulin availability is strictly regulated and can reach high levels in the CNS. This raises the question as to the source of brain insulin, does all of brain insulin derive from the periphery or is insulin also synthesized in the brain (**Figure 1**)?

There is unequivocal evidence for selective, regulated, time dependent, temperature sensitive, carrier mediated, and saturable insulin transport to the brain (43–50). In mice, human insulin was shown to access the CNS after crossing the BBB (51). In rabbits, insulin infused into the carotid artery was shown to have crossed the BBB into the peri-capillary space and brain parenchyma with preservation of the peptide's integrity (45). In dogs, studies using a three component mathematical model (plasma, intermediate component, and CSF) have shown that insulin delivery to the CNS fits a receptor-mediated saturable process (43). In healthy humans, during hyperinsulinemic, euglycemic clamp studies, increase in circulating insulin was demonstrated to rapidly affect brain activity, alongside rapid cerebral insulin signal transduction, independent of the systemic effects of the insulin (48).

Apart from passage through the BBB, direct access of insulin to the CSF has also been demonstrated (**Figure 1**). This alternative

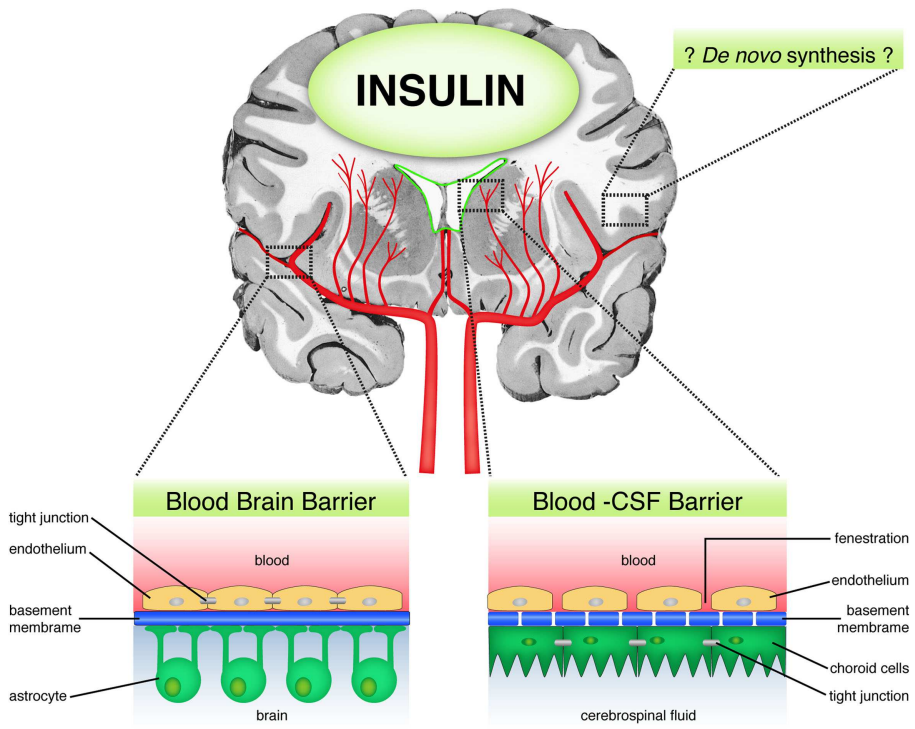

FIGURE 1 | Sources of brain insulin. Schematic diagram showing the possible sources of brain insulin. First, peripheral insulin can access the brain through the blood brain barrier (BBB) via a selective, carrier-mediated transport system. Second, insulin may diffuse through the blood–CSF barrier in circumventricular regions, which are lacking in BBB. Third, there is some limited evidence suggesting the possibility of *de novo* insulin synthesis in the brain.

route occurs through circumventricular regions, such as the area postrema, which lack a BBB (34, 52–54). Unlike the BBB that contains tight junctions, the capillaries in the circumventricular regions are porous, thereby allowing plasma solubles to diffuse freely and directly into these areas (55). The route through which insulin accesses the brain has implications for the rate of convection and diffusion in the brain, and distribution of the insulin into the brain parenchyma. Following intraventricular administration of insulin, insulin becomes distributed through the ventricular compartments and to the surface of the brain bathed by the subarachnoid space, with relatively slow rate of diffusion into the brain parenchyma, and is minimal at distances more than 1–2 mm removed from the CSF surface (55, 56). In addition, insulin delivered into the CSF undergoes relatively rapid bulk flow through the CSF flow tracks. For example, the entire CSF volume is turned over every 4–5 h following production at the choroid plexus in the human brain (55).

In model organisms, insulin is biosynthesized by neurons in the brain and it exerts both local and remote actions, including regulation of homeostasis; making it undoubtedly a neuropeptide. In humans, however, insulin is mainly produced in the pancreas, which raises the question as to whether insulin can be considered a neuropeptide in humans. Neuropeptides have been defined as "small proteinaceous substances produced and released by neurons through the regulated secretory route and acting on neural substrates" (57). Neuropeptides have been shown to have strict, cell specific expression patterns, on which the physiological or behavioral role of the peptides is based. Criteria for classification as a neuropeptide include gene expression and biosynthesis by neurons; storage, and regulated release upon demand and ability to modulate or mediate neural functioning directly through receptors (57). Although IRs are highly abundant in many brain areas and nuclei, it remains unclear if insulin is produced in the brain. Therefore, following the strict criteria for neuropeptide definition, it becomes debatable if mammalian insulin is a true neuropeptide.

Evidence in favor of insulin synthesis in the brain mostly derives from *in vitro* studies, including the study by Clarke et al. in 1986, which demonstrated the synthesis of insulin by cultured rat brain neuronal and astrocyte glial cells and their release of insulin in primary culture. The insulin release after membrane depolarization of the neurons was biphasic, in a manner similar to that of pancreatic beta cells (58). In 1990s, Schechter et al. provided both *in vivo* and *in vitro* evidence from mammalian brains supporting the *de novo* synthesis of insulin. *In vitro* evidence included the demonstration of preproinsulin I and II mRNA in neuron cell cultures of fetal rat brains (59). From *in vivo* studies, presence of preproinsulin I and II mRNAs and insulin immunoreaction was detected within the rough endoplasmic reticulum, the Golgi apparatus, cytoplasm, axon, synapsis, and dendrites of the rat fetal brain (40).

Summarily, as can be seen in **Figure 1**, whether insulin is derived from the periphery, local sources or both, insulin is present in the CNS, where it subserves many functions and contributes to neurobiological processes.

ACTIVATION OF INSULIN RECEPTORS IN THE BRAIN

As in peripheral tissues, insulin signaling in the brain occurs mainly via the IR pathway (**Figure 2**), which contains several critical nodes of interaction with other signaling pathways (60). Activation of the insulin signaling cascade starts with binding of the insulin ligand to the IR, which belongs to the family of tyrosine kinase receptors, auto phosphorylation of which is essential for their activation. Upon activation, the IR phosphorylates insulin receptor substrate (IRS) proteins. IRS proteins are also activated upon binding of the IGF-1 ligand to its cognate receptor. Thus, IRS proteins represent a critical node of conversion of the insulin and IGF-1 signaling cascades, and their crosstalk with other pathways, such as cytokine signaling. In addition to its activation of the Ras–mitogen-activated protein kinase (MAPK) pathway, activated IRS proteins serve as docking sites for the assembly and activation of, among others, phospho-inositol-3 kinase (PI3K), which generates the lipid second messenger phosphatidylinositol 3,4,5-triphosphate (PIP3). PI3K represents another critical node of crosstalk with other signaling pathways, including the c-Jun-N-terminal kinase (JNK) stress signaling pathway. Elevated levels of PIP3 activate phosphoinositide-dependent protein kinase-1

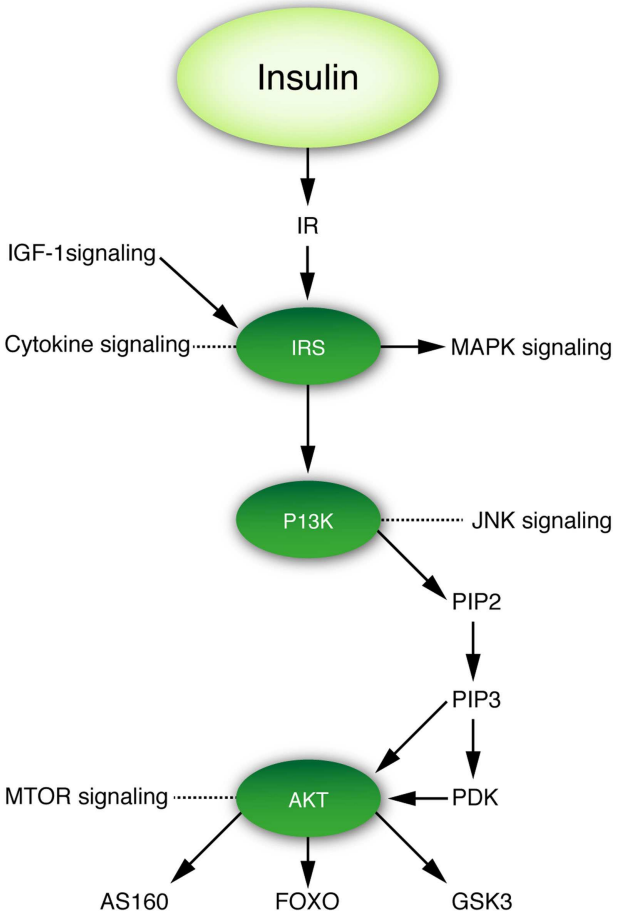

FIGURE 2 | Insulin–IRS–P13K–AKT signaling cascade and its crosstalk with other signaling pathways. Figure denotes three critical nodes in insulin signaling that are important for the interaction of insulin signaling with other pathways relevant to this review.

(PDK1) and AKT. AKT represents yet another critical node of interaction with the mammalian target of rapamycin (mTOR) nutrient signaling pathway. AKT targets include glycogen synthase kinase 3 (GSK3), Akt substrate of 160 kDa (AS160, phosphorylation of which is required for translocation of the glucose transporter GLUT4 to the plasma membrane) and forkhead transcription factors (FOXOs) (**Figure 2**). Phosphorylation of FOXOs induces their translocation from the nucleus, which causes profound changes in the transcription of key factors implicated in metabolism, cell cycle regulation, apoptosis, and resistance to oxidative stress (61).

DISTRIBUTION OF INSULIN RECEPTORS IN THE BRAIN

In higher mammals and humans, IRs are widely distributed throughout peripheral tissues, with their main function being to transport glucose into cells, inhibit glucose production and increase glucose uptake by triggering signaling pathways in the liver, muscle, and fat (62). The IR consists of a tetramer, with two alpha subunits and two beta subunits. Brain IR subunits differ structurally from peripheral IR subunits in that they have a lower molecular weight (63) and can withstand exposure to high concentrations of insulin without undergoing down-regulation (64, 65). The mammalian brain has specific IRs (29, 66), which are of two types. One is the neuronal/neuron-specific type, which is abundant in the neuron (67), while the second type is the non-neuronal/peripheral-like type, with lower density in glial cells (38, 66, 68). Insulin receptors are highly abundant in the neurons, with high protein concentrations in cell bodies and synapses, and less abundant in the glia. Brain IRs are abundant in the brain, but are highly enriched in the olfactory bulb, hypothalamus, hippocampus, cerebellum, amygdala, and cerebral cortex (12).

Growing but controversial evidence suggests that the specific regional concentrations of IR reflect different IR functions associated with particular brain regions. IR enrichment in the hypothalamus and limbic system including the hippocampus, pyriform cortex, and amygdala, areas that reciprocally connect and communicate with each other, has been proposed to be suggestive of a role in emotion and higher cognitive functions, particularly learning and memory (69, 70). Higher IR concentrations are found in the hippocampus, which is critically involved in spatial memory processing, suggesting insulin's role in learning and declarative memory (69). Evidence that synthesis of IR may be increased in these hippocampal areas as a result of learning is supported by the up-regulation and the changes in distribution patterns of IR mRNA in the hippocampus and dentate gyrus following water maze training in rats (70). Insulin is involved in the regulation of food intake, which is consistent with the high concentrations of IR in the olfactory bulb and the hypothalamus. Furthermore, the high concentration of IR in the choroid plexus suggests that it may be required for transport of peripheral insulin across the blood–CSF barrier (70).

FUNCTIONAL SIGNIFICANCE OF INSULIN IN THE BRAIN

As the most potent anabolic hormone yet identified, insulin has both metabolic and non-metabolic functions. Insulin regulates food intake, as well as glucose, lipid, and energy homeostasis and stimulates synthesis (as well as inhibition of breakdown) of glycogen, triglycerides, and most proteins. It is also involved in regulation of hedonic behavior and non-homeostatic control of intake of food and other substances via reward processing.

NON-METABOLIC FUNCTIONS

The presence of insulin and IRs in the brain indicates that the brain is a target organ for insulin. Insulin plays a key role in synaptic plasticity, apoptosis, mood, learning, reproduction, and growth (37, 71–74). Insulin and IR expression in the brain has been suggested to exert neurotrophic effects on CNS neurons (75). Insulin has been considered to support neuronal protein synthesis and cytoskeletal protein expression (75), neurite outgrowth (76, 77), migration, and differentiation in the absence of other growth factors (78, 79), and nascent synapse formation (75, 80). It promotes growth and regeneration of axonal sprouts, especially small sized sensory neurons (81), neuronal survival, circuit development, synaptic plasticity (82), and postsynaptic neurotransmitter receptor trafficking (80). Evidence in favor of insulin's role as a neurotransmitter in the CNS includes the observations that (i) insulin is present in neurons (67), (ii) neurons contain specific IRs (64), and (iii) insulin affects neuronal firing and catecholamine metabolism (83–85). Insulin also has effects on BBB function, including ability to affect the transport of other substances. Binding sites for insulin have been described at both the choroid plexus and on brain endothelial cells (86, 87). Insulin also has neuro-protective properties (88–90). Central insulin plays a role in cognitive processes such as attention, executive functioning, learning, and memory (91), and direct application of insulin to the CNS in humans has been shown to improve memory and cognition (92, 93). Thus, insulin is involved in attributes that are essential for healthy aging.

METABOLIC FUNCTIONS

The brain plays a key role in maintenance of homeostasis, or the ability to maintain vital parameters of the internal environment within narrow limits, despite fluctuations in the external environment. Metabolic homeostasis requires the integration of numerous cues reflecting energy availability by the hypothalamus and nearby brain structures, to mount a coordinated response to adapt fuel flux so as to maintain energy homeostasis. Insulin is one of the many cues informing the brain about energy status. Research on insulin signaling has primarily focused on insulin-mediated processes in the classical insulin target organs. These include glucose uptake into skeletal muscle, inhibition of glucose production by the liver, and inhibition of lipolysis in adipose tissue. However, in 1979, a role for insulin in the central regulation of energy homeostasis was suggested based on the observations that insulin levels circulate in proportion to fat mass in most mammals and that intra-cerebroventricular insulin administration results in a dose dependent reduction in food intake and body weight in monkeys (94). In line, IRs are expressed throughout the mammalian brain (29). Metabolic syndrome and diabetes have traditionally been considered as peripheral metabolic diseases. Recently, various non-invasive brain-imaging techniques have revealed structural and functional abnormalities that are associated with diabetes. Critical autonomic regulatory neurons

in the hypothalamus and brainstem are responsible for maintenance of energy homeostasis and functional changes in these areas are associated with the development of diabetes (95). It was also shown that after hepatic branch vagotomy the suppression of hepatic gluconeogenesis induced by increasing circulating insulin levels was reduced by half (96). Mechanistically, binding of insulin to the IR and activation of the PI3K pathway in hypothalamic glucose-responsive neurons, which was shown to induce their hyperpolarization by opening of ATP-dependent potassium channels (97), has been implicated in the central effects of insulin on hepatic glucose production (96). Recently, it was shown that ingestion of a glucose solution resulted in a prolonged and significant blood oxygen dependent decrease in activity in the hypothalamus of healthy subjects, but not in type 2 diabetic patients (98). Insulin is also involved in regulation of energy homeostasis via IR in the ventromedial hypothalamus and acts on the brain to suppress feeding (99). Thus, insulin acts as a satiety factor, a finding supported by the observation that the response of glucose-excited neurons in the ventrolateral and ventromedial hypothalamic nucleus to decreased glucose is blunted by insulin (100).

Taken together, these data indicate that, beside peripheral insulin resistance, reduced brain insulin action may also contribute to loss of maintenance of metabolic control. Indeed, brain specific deletion of the IR was shown to result in enhanced food intake in female mice; and in mild obesity, hyperleptinemia, insulin resistance, and hypertriglyceridemia in both male and female mice (101). In line with these findings, in rats, decreasing hypothalamic IRs caused overeating and insulin resistance and hypothalamic insulin signaling was shown to be required for inhibition of glucose production (102). High-fat diet-induced obesity is associated with reduced brain insulin transport and an impairment of insulin action when given directly into the CNS, suggesting a loss of the effectiveness of insulin in the CNS to provide feedback signaling in circumstances of chronic hyperinsulinemia (103). Upon aging, peripheral insulin resistance progressively increases, inducing compensatory chronic elevations in circulating insulin levels. Therefore, central insulin action will be discussed in the context of models of delayed and accelerated aging.

INSULIN AND THE BRAIN: MODELS OF DELAYED AGING

NEMATODE MODELS OF DELAYED AGING

There is an impressive body of literature implicating insulin/IGF-1 like ligands and insulin/IGF-1 signaling in the regulation of metabolism, development, and longevity in the roundworm *C. elegans* (104). In response to unfavorable stressful environmental conditions, *C. elegans* larvae can transiently exit the cycle of growth and development to sexual maturity by transformation into developmentally arrested, non-feeding, stress resistant, and long-lived dauer larvae (105, 106). It was found that several dauer formation defective (daf) mutants are also long-lived, possibly because these mutants display specific key features of the dauer stage while developing in sexually mature adults, such as enhanced resistance to multiple stresses due to induction of cytoprotective pathways (107). Of the many long-lived daf mutants in nematodes, the ones that are best characterized comprise the *daf-2*, *age-1 (daf 23)*, *daf-16*, and *daf-18* mutants. Cloning and sequencing of the loci affected in long-lived daf mutants has revealed that these show strong sequence homology with evolutionarily conserved components of the mammalian insulin/insulin-like growth factor-1 signal transduction cascade (108–110). For example, the *daf-2* gene that has been shown to regulate lifespan in *C. elegans*, and the related tyrosine kinase receptors InR in *Drosophila melanogaster* (*D. melanogaster*) encode components that are homologous to the mammalian insulin and insulin-like growth factor-1 receptors. In response to food or the perception of food, multiple insulin-like ligands are secreted from neurosecretory cells in the brain of *C. elegans* (111) and *D. melanogaster* (112), indicating that in these invertebrates, the CNS plays a key role in insulin signaling mediated regulation of physiology and lifespan in response to environmental cues. Moreover, more than 10 years ago, Wolkow et al. (17) demonstrated that restoration of the daf-2 pathway of insulin-like signaling in neurons alone was sufficient to restore wildtype lifespan in *C. elegans*, and thus provided further evidence as to the role of insulin in the nervous system as a central regulator of animal longevity.

MOUSE MODELS OF DELAYED AGING

In mammals, the insulin/insulin-like growth factor-1 signaling cascade exhibits some striking differences compared to the insulin/insulin-like growth factor-1 signaling cascade in invertebrates (113). These differences include the acquisition of GH as a main regulator of IGF-1 production by the liver, and the acquisition of separate receptors for insulin and IGF-1. Again, several of the existing long-lived mammalian mutants with defects in insulin/IGF-1 signaling point to a role of the CNS in the regulation of mammalian longevity. The mutations that have thus far been most consistently and most strongly associated with increases in lifespan in mice comprise the *Prop-1* mutation displayed by Ames dwarf mice (114) and the *Pit-1* mutation displayed by Snell dwarf mice (115). These two mutations confer a defect in the development of the anterior pituitary gland, which causes a lifelong combined hormonal deficiency in growth hormone, thyroid stimulating hormone, and prolactin. In these as well as other long-lived mice, longevity has been strongly correlated with enhanced insulin sensitivity (116). In addition to the Ames and Snell dwarf mice, many other mouse mutants with defects in insulin/IGF-1 signaling have been described to display a longevity phenotype, which strongly implicates the insulin/IGF-1 signaling pathway in the regulation of rodent longevity. Involvement of both insulin signaling and IGF-1 signaling in mouse longevity was suggested by the long-lived phenotypes displayed by mice with selective disruption of the IR in adipose tissue (4) and mice heterozygous for mutation of IGF-1R (1). Summarily, improved insulin control (of carbohydrate homeostasis) has been identified as one of the pathways implicated in the remarkable extension of longevity in long-lived mouse mutants (117).

HUMAN MODELS OF DELAYED AGING

Also in humans, preserved insulin sensitivity has been associated with longevity. Insulin resistance has been shown to predict the development of age-related diseases, including hypertension, coronary heart disease, stroke, cancer, and type 2 diabetes (118). In the general population, the association between aging and

decline in insulin sensitivity (119–123) has been demonstrated in several studies (**Figure 3**). Mechanisms suggested to contribute to decreased insulin sensitivity in the elderly include (i) age-related receptor and post-receptor defects in insulin action (124, 125), (ii) an age-related decrease in insulin stimulated whole body glucose oxidation (126), (iii) an age-related reduction in beta cell response to glucose (126), and (iv) impaired insulin-mediated glucose uptake, and inability to suppress hepatic glucose output (127, 128). In contrast, centenarians, which exhibit exceptional longevity, seem protected against the age-related decline in insulin sensitivity when compared to a group of advanced middle-aged individuals. (11) Of note, a methodological difficulty that is associated with the comparison of groups that differ in calendar age is potential confounding by the changes that occur in body composition and endocrine function with advancing age. Moreover, differences may exist between different birth cohorts in environmental impacts, including differences in the availability vaccinations or medications (e.g., antibiotics).

The relationship between longevity and preserved insulin action has also been observed in studies of familial longevity. In the Leiden longevity study, offspring of long-lived nonagenarian siblings, having inherited on average 50% of the genetic propensity of their long-lived parent were included together with the partners of the offspring (129), with whom they have shared the same socio-economic and geographical environment for decades and who are of a similar age. We showed that already at middle age the offspring from these long-lived siblings displayed a decreased mortality risk suggesting that there is indeed evidence for genetic enrichment for longevity (129). Moreover, human offspring of exceptionally long-lived siblings, when compared to their partners showed a remarkably lower prevalence of metabolic syndrome (130) and diabetes (131). After exclusion of diabetic patients, the offspring of exceptionally long-lived siblings displayed lower circulating levels of glucose and slightly lower circulating insulin levels (7). Using hyperinsulinemic euglycemic clamps studies, we could show that the offspring of long-lived siblings specifically displayed enhanced peripheral insulin sensitivity compared to age matched controls (8). A study using high field (7-T) MR spectroscopy of the tibialis anterior muscle indicated that the enhanced peripheral insulin sensitivity of offspring is associated with lower intramyocellular lipid content, which may be indicative of better mitochondrial capacity (132).

The mechanisms underlying the preserved insulin action in centenarians as well as in offspring of nonagenarian siblings remain unclear. However, suggested mechanisms include genetic enrichment for favorable features related to body fat and lipoprotein distribution, reduced plasma free radical concentrations, and enhanced cellular response to oxidative stress and immune function (11, 133, 134). Taken together, these results suggest that maintenance of insulin sensitivity is a key feature of healthy longevity.

INSULIN AND THE BRAIN: MODELS OF ACCELERATED AGING

OBESITY AS A MODEL FOR ACCELERATED AGING

The most common acquired factors causing insulin resistance are obesity and a sedentary lifestyle. Obesity and the associated increase in body fat are the consequences of chronic, long-term nutrient excess. In Western societies, the prevalence of obesity continues to increase and numerous studies have demonstrated an association between obesity and enhanced mortality risk (135). The relationship between obesity and excess mortality is consistent with evidence that obese individuals are at increased risk of essential hypertension, type 2 diabetes mellitus (DM2), and cardiovascular disease (CVD). It has been suggested that insulin

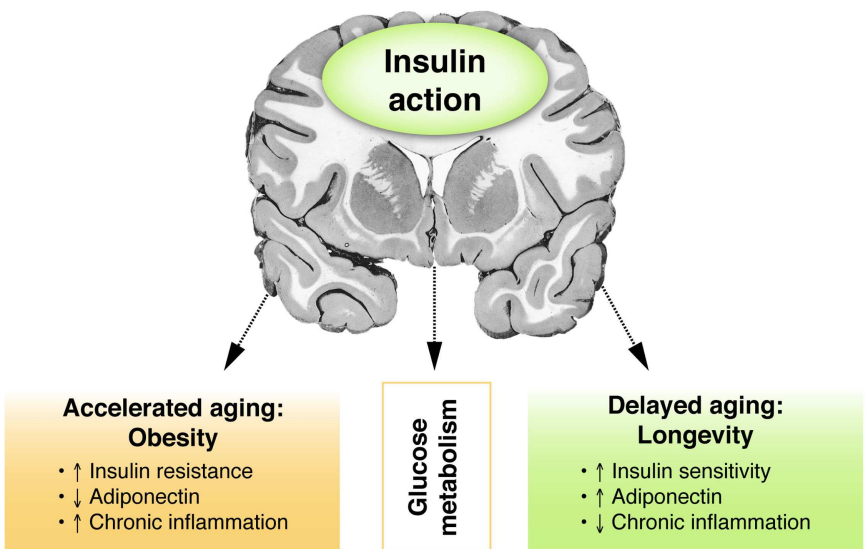

FIGURE 3 | Insulin and the brain: models of accelerated and delayed aging. Figure showing the putative relationship between central insulin action and glucose metabolism in models of accelerated or delayed aging. Obesity as a model for accelerated aging is associated with peripheral insulin resistance, decreased adiponectin levels, and enhanced chronic inflammation. Opposite features are observed in healthy longevity as a model of delayed aging.

resistance is the major contributor to clinical outcomes associated with obesity (136).

It is not known why some obese individuals develop insulin resistance while others remain insulin sensitive (137). A potential mechanism that might explain the association between excess adiposity and peripheral insulin resistance is impaired adipogenesis and reduced lipogenesis in subcutaneous fat, which would lead to enhanced deposition of fat in the visceral depots and larger sizes of visceral adipocytes (137, 138). Increased visceral adiposity is associated with enhanced secretion of inflammatory cytokines and induction of insulin resistance (139). Nutrient excess results in enhanced exposure of cells and tissues to high levels of circulatory glucose and fatty acids. These exposures can activate various intracellular inflammatory pathways and lead to mitochondrial dysfunction, reactive oxygen species (ROS), ER stress, and the associated unfolded protein response that induce resistance to both leptin and insulin (140). ROS can have both stimulatory and inhibitory effects on insulin signaling. It was shown that under normal physiological conditions, optimal activation of the IR requires redox priming by IR mediated activation of NAD(P)H oxidase (NOX) in many cell types (141). In addition, mild bursts in intracellular ROS can activate the IR receptor independent of insulin, allowing for ROS mediated ligand activation bypass of IR signaling (142). In contrast, increased levels of ROS or prolonged exposure to oxidative stress have been shown to inhibit insulin signaling and to induce insulin resistance (143). Enhanced exposure of skeletal muscle to high levels of fatty acids in circulation can result in enhanced levels of intramyocellular triglyceride storage. Because intramyocellular lipid droplets are stored in close vicinity to mitochondria, which constitute the main intracellular source of ROS, intramyocellular triglycerides are very vulnerable to oxidation. Upon peroxidation of intramyocellular triglycerides toxic lipid species are generated, including diacylglycerol (DAG), ceramide, and long-chain fatty acyl-CoA, which impair insulin signaling (143). Both enhanced influx, as a consequence of nutrient excess, and reduced efflux, as a result reduced oxidative capacity and mitochondrial dysfunction have been implicated in the accumulation of toxic intramyocellular lipids (144, 145). In support of a role of reduced efflux due to mitochondrial dysfunction, non-obese, insulin sensitive first degree relatives of patients with type 2 diabetes were shown to display impaired ability to switch to fat oxidation after high-fat intake (146), as well as higher levels of intramyocellular lipids and reduced oxidative capacity (147). These data implicate ROS and mitochondrial dysfunction in the development of insulin resistance.

It is unknown via which mechanisms insulin resistance is associated with a shortening of lifespan. If peripheral organs, such as skeletal muscle and adipose tissue become less responsive to insulin, euglycemia will be maintained by the capacity of the pancreas to hypersecrete insulin so as to overcome insulin resistance at peripheral organs. Exposure to continuous surges of hyperinsulinemia may overstimulate other tissues that have remained normally responsive to insulin, such as the liver, resulting in a pro-atherogenic lipid profile (148). Other data implicate adiponectin in the association between insulin resistance and lifespan. Adiponectin, an anti-inflammatory adipokine secreted by adipose tissue (149) was found to be negatively correlated with adipocyte size and obesity (150). Interestingly, elevated adiponectin levels have been observed in long-lived mice, such as the Ames dwarf mice (151) as well as in long-lived humans, such as centenarians (152–154). Recently, effects of adiponectin on peripheral insulin sensitivity also implicate central effects on reduction of high-fat diet-induced hypothalamic inflammation and insulin resistance (155).

INFLAMMATION AND THE BRAIN

INFLAMMATION AND AGING: INFLAMMAGING

Inflammaging is characterized by the increase in chronic, low-grade inflammation in the absence of overt infection that occurs with aging (156). Inflammaging as well as the circulatory markers that characterize this state, including C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor α (TNF-α), and interleukin 1 beta (IL1beta) are strong risk factors for many age-related diseases and mortality. It is thought that part of these circulatory factors are produced locally, after which these leak into the circulation. Different sources that contribute to the state of inflammaging include the accumulation of cellular debris and organelle components, accumulation of senescent cells, immunosenescence, changes in the gut microbiome and deregulation of the coagulation system.

Macromolecules, cells, and tissues are continuously damaged and repaired. Chronic inflammation is part of regular tissue remodeling as it facilitates tissue repair and turnover. However, a persistent inflammatory response can lead to tissue degeneration by activated leukocytes, cytokines, or collagen deposition. In literature, one key structure where links between inflammation and aging are emerging is the hypothalamus.

HYPOTHALAMIC INFLAMMATION

The hypothalamus is the seat of control of various metabolic and non-metabolic processes in the body, and is responsible for maintenance of homeostasis from early life through to aging. Besides its role in the synthesis and secretion of neurohormones, the hypothalamus regulates energy balance, stress responsiveness, as well as lipid and glucose metabolism. Diet-induced obesity has been shown to be associated with central leptin and insulin resistance (157). High-fat feeding has been shown to induce hypothalamic inflammation, which has been linked to the development of insulin resistance and obesity (157–159). In 2005, De Souza et al. demonstrated that 6 weeks of high-fat feeding induced impaired functional and molecular activation of the insulin-signaling pathway, with accompanying expression of several pro-inflammatory cytokines (IL-1β, TNFα, and IL-6) and inflammatory responsive proteins in the hypothalamus (158). Moreover, hypothalamic inflammation was shown to decrease the efficacy of central insulin administration to inhibit lipolysis, even before the onset of peripheral insulin resistance in white adipose tissue (160). Recently, a series of experiments in mice has demonstrated that hypothalamic inflammation occurs rapidly after high-fat feeding and is mediated by hyper activation of hypothalamic microglia, which was associated with gliosis in the ARC nucleus and eventual reduction in the number of POMC neurons, which are key in the regulation of energy homeostasis and adiposity (161).

Microglia are resident macrophages that play an important role in the clearance of cell debris via phagocytosis and the release of pro-inflammatory cytokines to recruit other immune responsive cells to the sites of injury in the CNS, including blood-borne macrophages. It is pivotal for tissue homeostasis and repair that the initial inflammatory immune response is followed by an active phase of resolution of inflammation and scar tissue. Recently, it has been shown that after insult, monocyte-derived M2-like macrophages are recruited to the site of injury and that these have an essential role as inflammation-resolving cells in recovery from acute CNS injury. The anti-inflammatory activity displayed by M2-like macrophages, notably their IL10 expression, is required for regulation of the activated microglia (162, 163). In addition, their expression of matrix degrading enzymes favors axonal regrowth by degradation of the glial scar (164). CNS specific T cells facilitate recruitment of blood-borne M2-like macrophages to the CNS through the choroid plexus within the blood–CSF barrier (165). Age-related Th2 inflammation is associated with chronically elevated IL4 levels, which can disrupt choroid plexus barrier functions and thus prevent the resolution of pro-inflammatory processes and induce a state of CNS inflammaging.

THERAPEUTIC MEASURES AND FUTURE PROSPECTS

Since brain insulin has been linked with aging, two possible mechanisms can be proffered for enhancing brain insulin action (**Figure 4**). Enhanced insulin efficacy might occur through measures aimed at minimizing inflammation; and enhanced delivery might be promoted to the brain areas that are crucial for healthy longevity.

Inflammation, including that occurring in the hypothalamus, has been linked to age-related decline in insulin sensitivity. It has been shown that hypothalamic microglia hyperactivation is regulated by metabolic hormones [leptin, glucagon-like peptide 1 (GLP-1)] and diet but not by body weight *per se* (166). Inflammaging may be treatable and preventable through changes in lifestyle. Interventions that are currently applied to reduce the state of low-grade chronic inflammaging include low dosing of aspirin or statins, weight loss, and exercise. Notably, a lower intake of calories and food that is rich in saturated fat and carbohydrates has been shown to reduce inflammaging (167). In mice, it was shown that hypothalamic inflammation can be resolved by central administration of omega3 and omega9 fatty acids after which body weight regulation and food intake were normalized (168). Physical exercise is known to be protective against numerous diseases and reduction of inflammation has been implicated in the health benefits conferred by exercise (169). Recently, in mice, exercise has also been shown to protect against hypothalamic inflammation induced by high-fat diet (170). Future research may focus on hypothalamic microglia as relevant targets for prevention and treatment of metabolic disorders.

The strong blood glucose lowering effects of intravenously administered insulin have hampered research on the role of insulin in the brain. These hypoglycemic effects can be circumvented by intranasal administration of insulin, which is an innovative way to enhance insulin concentration in the brain without affecting insulin concentration in the circulation (171). Intranasal administration of insulin was shown to be safe and effective in numerous studies in healthy humans and in patients with metabolic disease or cognitive impairment (172). Sub-chronic intranasal insulin application in humans was shown to decrease food intake and weight gain (92) in healthy young men, and to improve declarative memory and mood (173). Moreover, sub-chronic intranasal insulin application in humans was also shown to decrease HPA activation in response to a social stress test. It was shown that insulin may also influence meal-induced thermogenesis and postprandial insulin levels (174). Future research may focus on unraveling the effects of intranasal insulin on other aspects of energy and glucose metabolism in different age groups.

CONCLUSION

Insulin is the most powerful anabolic hormone discovered to date. Besides the well-established action of insulin in peripheral organs, such as liver, muscle, and adipose tissue, it is becoming

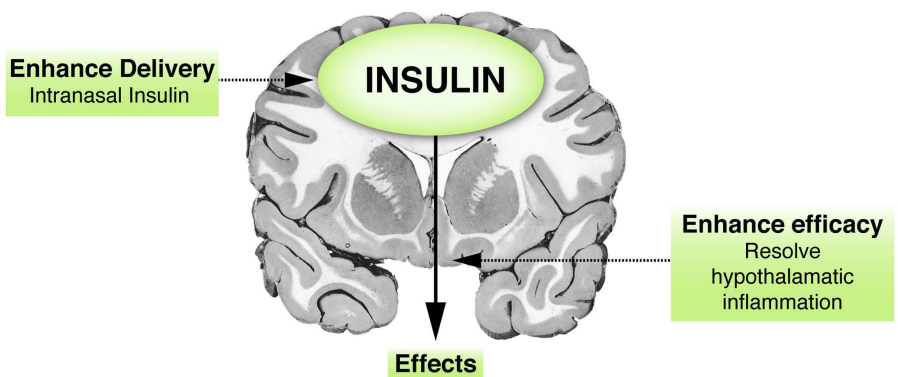

FIGURE 4 | Insulin and the brain: therapeutic implications. Hypothetical figure presenting two possibilities of enhancing brain insulin action. First, a way of increasing insulin concentrations in the brain is via enhanced delivery, such as delivery via the intranasal route, which has been shown to have some beneficial effects. Second, insulin action could probably also be augmented by enhancing its efficacy, for example, via resolution of brain (hypothalamic) inflammation.

increasingly clear that insulin affects important features of glucose metabolism via central mechanisms. Insulin signaling has been linked to longevity in organisms ranging from nematodes to mammals. While insulin is clearly a neuropeptide in nematodes, it is not yet clear how central insulin contributes to the differences in glucose metabolism that are observed in the context of conditions that are associated with accelerated aging, such as obesity, and delayed aging, such as healthy human longevity. However, novel data indicate that obesity is associated with reduced brain insulin action. Potential mechanisms that contribute to deficits in brain insulin action are impaired transport of insulin from the periphery to the brain and reduced brain insulin sensitivity due to hypothalamic inflammation. In contrast, we speculate that healthy longevity is associated with preserved brain insulin action, and discuss potential ways of enhancing brain insulin action in old age. Given the increasing prevalence of population aging, improving brain insulin action may represent an important therapeutic option to facilitate health in old age.

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Neuroendocrine role for VGF

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The *vgf* gene (non-acronymic) is highly conserved and was identified on the basis of its rapid induction *in vitro* by nerve growth factor, although can also be induced by brain-derived neurotrophic factor, and glial-derived growth factor. The VGF gene gives rise to a 68 kDa precursor polypeptide, which is induced robustly, relatively selectively and is synthesized exclusively in neuronal and neuroendocrine cells. Post-translational processing by neuroendocrine specific prohormone convertases in these cells results in the production of a number of smaller peptides. The VGF gene and peptides are widely expressed throughout the brain, particularly in the hypothalamus and hippocampus, in peripheral tissues including the pituitary gland, the adrenal glands, and the pancreas, and in the gastrointestinal tract in both the myenteric plexus and in endocrine cells. VGF peptides have been associated with a number of neuroendocrine roles, and in this review, we aim to describe these roles to highlight the importance of VGF as therapeutic target for a number of disorders, particularly those associated with energy metabolism, pain, reproduction, and cognition.

Keywords: VGF, energy homeostasis, pain, cognition, reproduction

INTRODUCTION

VGF (non-acronymic) is a neurotrophin-induced gene, which was first identified as VGF8a, NGF33.1, and a2 on the basis of its rapid induction in PC12 cells treated with nerve growth factor (NGF) (1–3). Subsequent studies demonstrated that VGF is similarly upregulated by numerous neurotrophins, including brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), in neuronal targets such as cortical or hippocampal neurons (4). However, VGF mRNA levels are only marginally increased by other growth factors including epidermal growth factor (EGF), fibroblast growth factor (FGF), interleukin-6 (IL-6), and insulin, despite the capacity of these proteins to robustly induce transcription of other immediate early genes in the PC12 cell line (3, 5, 6).

The VGF polypeptide, which is robustly and exclusively synthesized in neuronal and neuroendocrine cells (1, 3, 7, 8), is processed by the prohormone convertases (PC), PC1/3 and PC2 (9). VGF derived peptides with specific neuronal bioactivities include TLQP-62, TLQP-21, HHPD-41, AQEE-30, AQEE-11, LQEQ-19, and neuroendocrine regulatory peptides-1 and -2 (NERP-1 and -2; 9–11). Studies have shown that TLQP-62 and AQEE-30 increase the firing rate of hippocampal neurons, induce neurogenesis, and have anti-depressive properties (4, 12, 13), whereas HHPD-41, AQEE-30, AQEE-11, and LQEQ-19 stimulate sympathetic outflow and facilitate penile erection in rats (14–16); and TLQP-21 and NERP-2 regulate energy balance (17–20). Furthermore, TLQP-21 regulates contractile activity in the gastrointestinal tract, has analgesic properties, reduces neuronal apoptosis *in vitro* and decreases rodent blood pressure (21–23) and NERP-1 and -2 regulate water homeostasis and suppress vasopressin release (11, 20, 24). Here, we review the regulation of VGF and the neuroendocrine role of its derived peptides.

THE TRANSCRIPTIONAL REGULATION OF VGF

IN VITRO

The gene itself is highly conserved among mammalian species in respect to the coding region and the promoter sequence (25). The VGF promoter region contains a CCAAT box, various specificity protein 1 (SP-1), and activating protein 2 (AP-2) sites and a silencer element similar to the one involved in tissue-specific expression of neuronal genes (3, 25). Furthermore, it contains a cyclic AMP response element (CRE), which is embedded within a 14bp palindromic sequence, mutations of which abolish NGF and cAMP responses (6). VGF expression in response to neurotrophins that requires the combined actions of several regulator complexes; in addition to the CRE, the CCAAT box was shown to be important for NGF induction (26), possibly in association with the activity of a large complex containing a CRE binding protein (CREB), mammalian achaete-scute homolog-1 (MASH-1), and p300 (27).

IN VIVO

A genomic fragment extending from 800-bp 5' to the transcriptional start site and including the first 700-bp of 5'-untranslated sequence results in reporter gene expression in a tissue-restricted pattern similar to that of the endogenous VGF gene (28). Interestingly, this region of the promoter contains a putative silencer element that is located 400-bp 5' to the transcriptional start site, which prevents expression in non-neuronal cell lines (25). VGF mRNA in the hypothalamus alters in response to feeding/fasting (14, 15, 20), salt loading (29), adrenalectomy (30), and seasonal rhythms (31). Furthermore, VGF mRNA varies in the pituitary during the estrous cycle (32) and in the suprachiasmatic nucleus (SCN) according to circadian rhythmicity (33); while gastric damage increases VGF mRNA in the nucleus tractus solitarius (NTS) and dorsomedial nucleus of the vagus (34). VGF mRNA is also

modulated in other diverse conditions, which have been well described elsewhere (35).

THE STRUCTURE AND PROCESSING OF THE VGF POLYPEPTIDE

VGF is a 68 kDa polypeptide comprising 615 (human) or 617 (mouse/rat) amino acids with a typical secretory leader sequence of 22 amino acids at the N-terminal of VGF, which promotes translocation to the endoplasmic reticulum (ER) (36). Subsequent sequencing of the polypeptide in the mouse, horse, and bovine has confirmed extensive sequence conservation with approximately >85% identity (35). The most prominent VGF-derived peptides have apparent molecular masses of 20 (NAPP-129) and 10 kDa (TLQP-62), respectively (9) (**Figure 1**). However, the mouse and human sequences contain a minimum of 10 conserved regions of basic amino acid residues, which represent potential PC cleavage sites (37) (**Figure 2**). Indeed cleavage at the Arg-Pro-Arg$_{555}$ sequence in the rat has been shown to give rise to the TLQP peptides (9). It is possible, however, that the number and function of VGF derived peptides are greater than currently known (38). The extensive review by Ferri et al. (35) describes this in more detail.

DISTRIBUTION OF VGF AND ITS DERIVED PEPTIDES

VGF mRNA

VGF mRNA is widely expressed throughout the nervous system. During embryogenesis VGF mRNA is expressed in distinct neurotrophin-responsive targets in the central and peripheral nervous system (CNS and PNS, respectively) in the rat (39, 40). At birth, VGF mRNA is expressed in neurons throughout the brain and in peripheral endocrine and neuroendocrine tissues. While in the adult brain VGF mRNA has the highest expression in the hypothalamus and the granular layer of the cerebellum, it is also expressed in a number of other brain areas including the main and accessory olfactory bulbs, hippocampus, cortex, basal ganglia, thalamus, amygdala, midbrain, and the brainstem. Within the hypothalamus, the highest concentrations of VGF mRNA have been found in the ventromedial hypothalamus, in particular the arcuate nucleus (ARC), as well as in the SCN (7, 39, 41). VGF mRNA expression in the mouse is similar to the rat (14).

VGF PEPTIDES

VGF and its derived peptides are found in dense core vesicles and are released in response to depolarizing signals from neuronal and neuroendocrine cells through the regulated secretory pathway (10, 42, 43). Antibodies raised to synthetic peptides corresponding to the C- or N-termini of potential or actual cleavage products have been utilized to study VGF derived peptide distribution. In animal tissues, VGF immunoreactivity was restricted to central and peripheral neurons (41, 44), as well as to endocrine cells of the pituitary, adrenal medulla, gut, and pancreas (44). The highest concentrations of VGF immunoreactivity have correspondingly been found in the medial hypothalamus, particularly in the ARC, in the SCN, and in the parvocellular and magnocellular cells of the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) (41). Weak immunoreactivity was also detected in the hippocampus, amygdala, thalamus, and cerebral cortex (41). VGF immunoreactivity was also displayed in the female rat in the pars distalis, mainly with C-terminal antibodies (32). This, however, disappeared in accordance with the estrous peak of luteinizing hormone (LH) secretion, along with an induction of VGF mRNA in the pituitary. Additionally, VGF-derived peptides are prominent in the adult spinal cord, in α- and γ-motor neurons of the ventral horn and in the dorsal horn neurons, as well as cells of the inner nuclear and ganglion cell layers of the retina (45). In the PNS, both sympathetic ganglia and dorsal root ganglia of primary sensory neurons are important sites of localization of VGF-derived

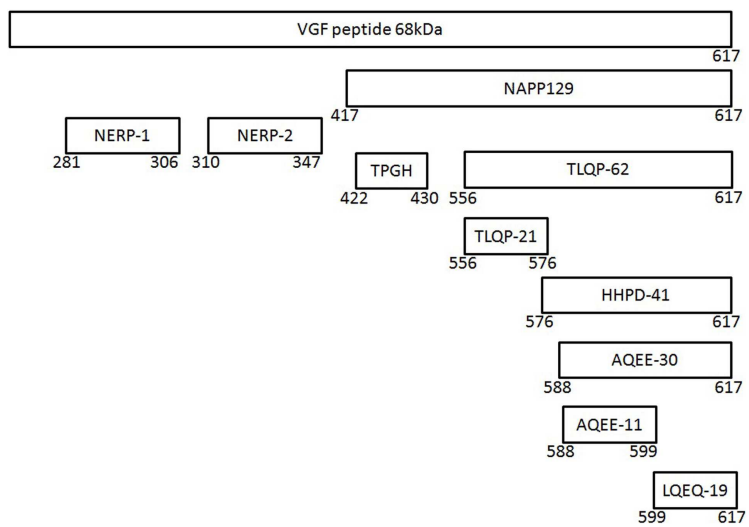

FIGURE 1 | The *vgf* gene and its derived peptides. The VGF polypeptide is the precursor of several biologically active peptides, which are released and play a role in intercellular communication. The gene contains a number of specific sequences, which are highly conserved between the species and these represent potential cleavage sites for the convertases of the kexin/subtilisin-like series proteinases family, namely prohormone convertases-1/3 and -2.

FIGURE 2 | Comparison of the human and mouse VGF polypeptide sequences. * indicates the position which have a single, fully conserved residue. ":" indicates conservation between groups of strongly similar properties scoring >0.5 in the Gonnect PAM 250 matrix. " . " indicates conservation between groups of weakly similar properties scoring <0.5 in the Gonnect PAM 250 matrix. Clusters of basic amino acids, which represent potential cleavage sites, are boxed. Sequence identity was >85%.

peptides (40). This is comparable to the expression of neuropeptide Y (NPY), ghrelin, and cholecystokinin (CCK), all of which regulate feeding and in some cases, gastrointestinal motility (46, 47). VGF-derived peptides are also present in mouse brown adipose tissue (BAT), where they are reduced in response to a high fat diet (HFD) (48).

VGF RECEPTORS

Of all the VGF derived peptides, TLQP-21 has had the most interest (17, 19, 23, 49–52). Previously, TLQP-21 was shown to bind to adipocyte membranes in a saturable manner, (53) and atomic force microscopy of living cells revealed the existence of a single class of binding sites for TLQP-21 (54). Taken together these results suggested a cell surface receptor for TLQP-21. Two possible receptors have recently been identified for TLQP-21. Chen et al. (55) identified gC1qR, showing that TLQP-21 activated rat macrophages through gC1qR, which then caused mechanical hypersensitivity in rats. gC1qR protein was expressed by both brain and spinal cord derived microglia (55) and is indispensable for adipogenesis and insulin signaling (56). Furthermore, obese mice fed a HFD

demonstrated increased density of TLQP-21 binding in adipose tissues (54). However, neither TLQP-62 nor LQEQ-19 elicited a response in their experimental model, both of which had been previously implicated in pain processing (22, 57). This supports the hypothesis of different receptors for the VGF derived peptides. Hannedouche et al. (58) reported the complement receptor, C3A receptor-1 (C3AR1), as a receptor for TLQP-21, which mediated activity for TLQP-21 in two different rodent cell lines. C3AR1 was originally thought to be restricted to the innate immune response, its role limited to the complement cascade. However, it has subsequently been shown to have a role in cancer (59), neurogenesis (60), and hormone release from the pituitary gland (61). However, C3AR1$^{-/-}$ mice are transiently resistant to diet-induced obesity (DIO) and are protected against HFD-induced insulin resistance (62). The discovery of these receptors will help identify the mechanisms by which TLQP-21 and possible other derived peptide may modulate its actions.

PHYSIOLOGICAL ROLES OF VGF GENE AND DERIVED PEPTIDES

ENERGY BALANCE

The high expression of VGF in the hypothalamus and the change in expression of the *vgf* gene in the ARC following acute altered energy balance first suggested the importance of VGF in the regulation of energy balance (14, 20). Indeed fasting has been shown to increase VGF mRNA expression, while administration of leptin prevents the fasting induced increase in VGF mRNA (15). These changes in VGF can be observed in models of chronic energy imbalance; VGF mRNA levels resemble that of fasted wild-type mice in the ARC of the leptin deficient *ob/ob* mouse and in the leptin resistance *db/db* mouse (15). It is well known that the ARC has two neuronal populations that respond to the fed and fasted state as well as to leptin signaling, the pro-opiomelanocortin (POMC) and neuropeptide Y (NPY) neurons (63). VGF immunoreactivity has been shown to be co-localized with both these neuronal populations in the ARC, however, expression is modulated with energy state. In the *ad libitum* fed state and re-fed animals, VGF mRNA is co-localized with POMC (15, 64). On the other hand, fasting increases co-localization of VGF in the NPY neurons (64).

Energy balance and lack of functional VGF

The function of VGF and its extension derived peptides was first assessed through the development of mice lacking a functional copy of the *vgf* gene (VGF$^{-/-}$) via homologous recombination (14). At birth, the homozygous VGF$^{-/-}$ mice are indistinguishable from either their heterozygous or wild-type littermates. No defects in development were detected in either the CNS or the PNS. However, in the weeks following birth, the VGF$^{-/-}$ mice were visibly smaller than their wild-type littermates and adults were found to weigh 50-70% less due to a 50% reduction in adiposity compared to wild-type littermates (14). Consistent with the reduction in adiposity, leptin levels, serum glucose and insulin levels, and liver glycogen were reduced (48). The mice consumed considerably more calories per gram body weight, but this increase in food intake was not sufficient to maintain the same body weight as wild-type mice. The VGF$^{-/-}$ mice utilized twice as much oxygen at rest and displayed increased locomotor activity compared to wild-type littermates (14). Overall, the major change in VGF$^{-/-}$ mice is an increase in energy consumption; indeed *vgf* gene deletion did not block obesity via monosodium glutamate administration (15) suggesting that the thermogenic pathways resulting in the VGF$^{-/-}$ phenotype are blocked. These initial observations led Hahm et al. (14) to suggest that VGF may play a non-redundant role in the regulation of energy homeostasis and antagonism of the gene may constitute a basis for the treatment of obesity. Furthermore, *vgf* gene deletion blocked the development of obesity as a result of a HFD, gold thioglucose treatment, as well as in the *agouti* mouse, and suggesting that VGF functions in outflow pathways regulating energy expenditure downstream of the hypothalamic melanocortin receptors (15).

Energy balance and VGF-derived peptides

Thus from the phenotype of the VGF$^{-/-}$ mice one might predict that VGF promotes an anabolic drive. Surprisingly, this view has not been supported by subsequent studies in mice and Siberian hamsters. Chronic intracerebroventricular (ICV) infusion of TLQP-21 in mice fed a normal lab chow resulted in a small increase in resting energy expenditure and rectal temperature (17). The changes in metabolic parameters were mirrored by increased epinephrine content in BAT, upregulation of BAT β2-adrenergic receptor (AR), uncoupling protein 1 (UCP-1) mRNA, higher expression of peroxisome proliferator-activated receptor-δ (PPAR-δ), and β3-AR in white adipose tissue (WAT). However, hypothalamic expressions of agouti-related protein (AgRP), NPY, α-melanocyte-stimulating hormone (α-MSH), POMC, and corticotrophin-releasing hormone (CRH) were unchanged (17). In mice, switched to a HFD treatment with TLQP-21 halted the expected increase in body weight and WAT, attenuated rises in leptin, and normalized ghrelin levels (17). In rats, ICV infusion of TLQP-21 significantly decreased gastric emptying, an effect that was blocked by ICV infusion of indomethacin, which blocks prostaglandin release (65).

A similar catabolic effect was noted in Siberian hamsters, a seasonal model of energy balance. Not only is VGF mRNA significantly increased in the winter weight-loss state in the dorsal medial posterior arcuate nucleus (dmpArc) (31) but ICV infusion of TLQP-21 at the onset of the dark phase was found to significantly and dose dependently decrease food intake and body weight (19). However, there was no effect on energy expenditure as Siberian hamsters pair-fed to the treated group lost a similar amount of body weight (19). Weight loss was, therefore, attributable to reduced caloric intake rather than energy expenditure.

One of the possible explanations for this contradiction between the functional *in vivo* studies and the VGF$^{-/-}$ mice, where all the VGF peptides have been ablated, is that some of these peptides may have opposing roles in energy balance. Interestingly, Bartolomucci et al. (66) have suggested that HHPD-41 increased food intake following ICV infusion, and more recently ICV infusion of NERP-2 in rats has been shown to increase food intake, body temperature, oxygen consumption, and locomotor activity (20). Furthermore, intravenous administration of NERP-2 significantly augmented glucose stimulated insulin secretion in anesthetized rats or following intraperitoneal injection to conscious mice (67). Thus VGF may have a biphasic role in the regulation of energy

balance and further characterization of the other VGF-derived peptides is required.

Energy balance and circadian rhythm

It is well known that food intake and energy metabolism in mammals are regulated by their circadian clock, and food intake is one such signal that can entrain the circadian clock (68). As previously described, VGF is expressed in the SCN, the circadian pacemaker in animals, while the E-box contained in the *vgf* gene promoter region is similar to the many clock genes such as the *per* gene (33). Therefore, it is not unexpected that the *vgf* gene exhibits circadian rhythm in the SCN even under constant dark conditions, while VGF mRNA levels are increased in response to light simulation in the SCN when light would be expected to cause a phase shift in locomotor rhythms (33). Indeed $VGF^{-/-}$ mice can maintain circadian rhythm of wheel running in constant darkness, however, the period length was found to be slightly but significantly shorter than wild-type littermates (14). Thus, this raises the question could the metabolic phenotype of the $VGF^{-/-}$ mice be attributed, in part, to the disruption of the circadian system.

VGF AND WATER BALANCE

Water deprivation and salt loading in rats increases VGF mRNA levels in both the SON and PVN, along with vasopressin mRNA (29). ICV injection of NERP-1 and NERP-2 suppresses hypertonic saline or angiotensin II induced increases in plasma vasopressin in rats (69). Additionally, ICV infusion of NERP-1 and -2 attenuated the increase in vasopressin as a result of water deprivation in rats, an effect which was reversed following immunoneutralisation by ICV infusion of anti-NERP-1 and -2 antibodies (69). Taken together, these data suggest that NERP-1 and -2 may be involved in the central control of body fluid balance.

VGF AND REPRODUCTION

The role of VGF signaling in reproduction was inferred from the observation that VGF gene deletion resulted in infertility in both male and female mice (14). In male $VGF^{-/-}$ mice, the onset of puberty and sexual maturation was delayed, and the weights of the testes, albeit having mobile spermatozoa in the lumen, were significantly lower than those of wild-type littermates (14). While in the female $VGF^{-/-}$ mice histological examination revealed no mature follicles or corpus lutea, and the ovaries, ovidut, and uteri weighed 30% less than those of the wild-type littermates (14). However, transplanting ovaries from $VGF^{-/-}$ mice into ovariectomized wild-type females restored fertility, suggesting that the reproductive deficits of $VGF^{-/-}$ mice were not the result of pathology but arose from deficits in the hypothalamic-pituitary-gonadal axis (14). However, Ferri et al. (32) showed that VGF gene expression varied during estrous; there was an increase in VGF mRNA and VGF peptide/s degranulation, suggesting perturbation of anterior pituitary function.

It is common knowledge that alterations in energy metabolism and fat stores can affect reproductive function. $VGF^{-/-}$ mice have reduced leptin and altered energy status, therefore, it could be suggested that the deficit may be due to gonadotropin releasing hormone (GnRH) synthesis or secretion. However, while GnRH levels are not affected, LH and follicle-stimulating hormone (FSH) mRNA levels were reduced in $VGF^{-/-}$ mice (14) suggesting decreased GnRH secretion. Indeed it has been shown that central administration of TLQP-21 in female rats during the pubertal transition advanced the timing of vaginal opening and increased the number of animals with signs of ovulation (70). These effects of TLQP-21 may be via stimulation of the GnRH release, as TLQP-21 has been shown to induce LH secretion *in vitro* (71). Furthermore, Pinilla et al. (71) have shown that chronic administration of TLQP-21 was able to prevent the hypogonadotropic state induced by food deprivation.

There is further evidence of VGF peptides and a possible role in the regulation of reproduction. While HHPD-41, AQEE-30, and LQEQ-19 have been shown to induce penile erection in rats following infusion into the PVN in a dose dependent manner, NERP-1 has a pro-erectile effect when injected into the lateral ventricles or the ARC of rats (72). The effect on penile erection is thought to be via nitric oxide mediated activation of oxytocinergic pathways (16).

VGF AND PAIN

VGF is a gene commonly upregulated in sensory neurons in clinically relevant models of neuropathic pain, namely, varicella zoster infection, HIV-associated neuropathy, and peripheral nerve trauma (55). Furthermore, VGF has been shown to be upregulated in the dorsal root ganglia and spinal cord in a number of neuropathic and inflammatory pain models (22, 57, 73–76). In these areas, VGF is co-localized with substance P, calcitonin gene related peptide, TrkA, and P2 × 3 (22, 51). A functional role for VGF-derived peptides has been identified in pain pathways. Indeed intrathecal infusion of TLQP-62 results in cold behavioral hypersensitivity in rats; while injection of TLQP-21 into the hind paw of mice resulted in hypersensitivity in both control animals and the formalin model of inflammatory pain (51) as well as inducing thermal hyperalgesia in the warm-water immersion tail-withdrawal test (77). Additionally, both LQEQ-19 and AQEE-30 have been shown to induce p38 MAP kinase phosphorylation in spinal microglia (22), suggesting that VGF-derived peptides have pro-nociceptive and hyperalgesic functions.

VGF AND MEMORY AND LEARNING

As previously stated VGF mRNA is expressed in the hippocampus, and it has been shown that VGF transcription is accompanied by translation within 3 hours of BDNF exposure in hippocampal slices *in vitro* (4). Additionally, VGF mRNA has been shown to be upregulated by activities, such as memory and learning (8), while $VGF^{-/-}$ mice have demonstrated impaired hippocampal-dependent spatial learning and contextual fear conditioning tasks (78). Indeed more recently, TLQP-62 has been shown to induce transient potentiation in hippocampal slices (78), enhance synaptic activity (4), and increase neurogenesis in early phase neural progenitor cells in the adult hippocampus (12, 79), as well as shown to have effect on cognitive mechanism (80), thus suggesting that VGF may be important in memory processes.

To further support this notion, proteomic studies have demonstrated a reduction in VGF-derived peptides in the cerebrospinal fluid of patients affected by Alzheimer's disease (AD) (81–83). Similarly, there was a reduction in VGF-derived peptides in the

parietal cortex of AD patients (84) and a reduction in TPGH and NERP-1 in the parietal cortex of Parkinson's disease patients (84).

VGF AND DEPRESSION

VGF protein expression is reduced in both the learned helplessness and forced swim test depression paradigms (85), while VGF is increased by antidepressant drugs and voluntary exercise (12). Exercise regulates VGF mRNA and protein expression in the rodent hippocampus and induces an antidepressant response; an opposing phenotype is observed in the heterozygous $VGF^{-/+}$ mouse (86). Recently, inhibition of phosphodiesterase-4 or -5 was shown to result in increases in cAMP, activating CREB, BDNF, and VGF, which produces antidepressant-like effects on behavior in mice (87). Similarly, microinjection of TLQP-62 into the hippocampal CA1 regions demonstrated antidepressant-like behavioral effects in mice (88), possibly via a BDNF-dependent mechanism (78).

CONCLUSION

The evidence presented in this review indicates that the gene and gene product have a key neuroendocrine role and that VGF or its derived peptides may act as biomarkers or therapeutic targets in a number of disorders such as obesity, dementia, depression, and pain. The mechanisms by which VGF and its derived peptides are involved remains to be identified, however, the discovery of the new receptors will help advancements in this area both *in vitro* and *in vivo*.

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Role of serotonin in fish reproduction

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The neuroendocrine mechanism regulates reproduction through the hypothalamo-pituitary-gonadal (HPG) axis which is evolutionarily conserved in vertebrates. The HPG axis is regulated by a variety of internal as well as external factors. Serotonin, a monoamine neurotransmitter, is involved in a wide range of reproductive functions. In mammals, serotonin regulates sexual behaviors, gonadotropin release and gonadotropin-release hormone (GnRH) secretion. However, the serotonin system in teleost may also play unique role in the control of reproduction as the mechanism of reproductive control in teleosts is not always the same as in the mammalian models. In fish, the serotonin system is also regulated by natural environmental factors as well as chemical substances. In particular, selective serotonin reuptake inhibitors (SSRIs) are commonly detected as pharmaceutical contaminants in the natural environment. Those factors may influence fish reproductive functions via the serotonin system. This review summarizes the functional significance of serotonin in the teleosts reproduction.

Keywords: teleost fish, 5-HT, GnRH, gonadotropins, pituitary, SSRI antidepressants

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Introduction

Reproduction is a biological process that results in the production of new individual. The nervous and the endocrine system work together (neuroendocrine) to control vertebrate reproduction. The neuroendocrine mechanism regulates reproduction through the hypothalamo-pituitary-gonadal (HPG) axis which is evolutionarily conserved in vertebrates. The hypothalamus is the major site responsible for the production of neuropeptide, gonadotropin-releasing hormone (GnRH) in the brain of vertebrates. In vertebrates, reproductive and sexual functions are mainly controlled by the pulsatile secretion of GnRH from the hypothalamus (Knobil, 1979; Pozor et al., 1991; Dellovade et al., 1998; Bancroft, 2005). GnRH binds to its cognate receptors located on the pituitary gonadotropes to regulate the synthesis and release of gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (McCann and Ojeda, 1996; McCann et al., 2002). These gonadotropins control gonadal development and maturation, and stimulating steroidogenesis and spermatogenesis in male testes and folliculogenesis and oogenesis in female ovaries (Pierce and Parsons, 1981; Orth, 1984; Bousfield et al., 1994). Furthermore, kisspeptin, the peptide product of *KISS1*/*Kiss1* gene and its cognate receptor (GPR54 = kisspeptin receptor) has been recognized as a potent regulator of GnRH release in mammals (Tena-Sempere, 2006; Roseweir and Millar, 2009). Those reproductive neuroendocrine signaling pathways are evolutionarily highly conserved in mammals and non-mammalian vertebrates. However, mechanism of reproductive control in non-mammalian vertebrates is not always the same as in mammalian models (Zohar et al., 2010). For example, in teleost fish, the pituitary gland is directly innervated by neurosecretory fibers and lacka hypothalamo-pituitary portal system of the median eminence (Peter et al., 1990). Many teleost species possess at least two or three GnRH types (GnRH1, GnRH2, and GnRH3) (White et al., 1995) or multiple GnRH neuronal populations in the brain (Parhar, 2002). Recent studies have revealed

the presence of two types of kisspeptin encoding genes (*kiss1* and *kiss2*) and two forms of kisspeptin receptor genes (*kissr1* and *kissr2*) in teleosts (Lee et al., 2009; Akazome et al., 2010; Um et al., 2010; Tena-Sempere et al., 2012; Gopurappilly et al., 2013). The multiplicity of neuroendocrine signaling pathways in teleosts are probably due to a gene duplication event (Lethimonier et al., 2004; Um et al., 2010), but several evidences have suggested their unique roles and functional significance in the variety of reproductive strategies in teleosts (Peter et al., 1990; White et al., 1995; Parhar, 2002; Lethimonier et al., 2004; Um et al., 2010; Zohar et al., 2010).

In vertebrates, the HPG axis is regulated by a variety of internal and external factors. For example, one of the endogenous key factors controlling reproductive processes are sex steroids feedback mechanism exerted by the gonads to the hypothalamus and pituitary (Fink, 1979). In addition to gonadal steroids, several factors such as stress, nutrition, and neurotransmitters are involved in the control of the HPG axis, in particular modulation of gonadotropin release (Gallo, 1980; Genazzani et al., 2000; Zohar et al., 2010). Neurotransmitters such as monoamine, amino acids and peptides are involved in the neuroendocrine control of reproduction (Gallo, 1980; Nock and Feder, 1982). In mammals, serotonin (5-hydroxytryptamine), a monoamine neurotransmitter is involved in a wide range of reproductive functions such as GnRH secretion, gonadotropin release, gonadal maturation and socio-sexual behaviors. On the other hand, serotonin system can be modulated by reproductive factors. In mammals, ovarian steroids such as progesterone and estrogen regulates the content of serotonin in the brain (Pecins-Thompson et al., 1996). In several mammalian species, serotonergic neurons are colocalized with estrogen receptor beta (Gundlah et al., 2001, 2005). These results indicate that serotonin and reproductive endocrine signaling pathways are closely associated. The functional interactions between serotonin and reproductive functions have also been demonstrated in teleosts (Somoza et al., 1988; Khan and Thomas, 1992). However, the serotonin system in teleost may play a unique role in the control of reproduction because of the variety of neuroendocrine signaling. This review summarizes the functional significance of serotonin in the teleosts reproduction.

Serotonin System in Teleost

Organization of Serotonin System

The organization of serotonin in the central nervous system is evolutionarily well conserved in the vertebrates (Lillesaar, 2011). In the brain of teleosts, three major serotonergic neural groups exist: (i) pretectal population, (ii) posterior tuberculum/hypothalamic populations, and (iii) raphe populations (Kah and Chambolle, 1983; Ekström and Van Veen, 1984; Frankenhuis-van den Heuvel and Nieuwenhuys, 1984; Margolis-Kazan et al., 1985; Johnston et al., 1990; Corio et al., 1991; Ekström et al., 1992; Batten et al., 1993; RodrìGuez-Gómez et al., 2000; Lillesaar, 2011). In addition, serotonin-positive cells are also present in the pineal gland, area postrema, medulla oblongata and spinal cord in the brain of teleosts (Lillesaar, 2011). In teleost, serotonergic fibers from the brain directly project to the pituitary (Kah and Chambolle, 1983; Corio et al., 1991; Khan and Thomas, 1993; RodrìGuez-Gómez et al., 2000). In some teleosts species, serotonin-immunoreactive cells also present in the pituitary (Kah and Chambolle, 1983; Ekström and Van Veen, 1984; Margolis-Kazan et al., 1985; RodrìGuez-Gómez et al., 2000).

In mammals, serotonin is synthesized from the essential amino acid, L-tryptophan with help of catalysis by two enzymes: tryptophan hydroxylase (TPH) and amino acid decarboxylase (Fitzpatrick, 1999), whereas knowledge about mechanism of the control of brain serotonin synthesis in teleosts is still limited (Höglund et al., 2005). However, teleosts fish also preserve the molecules that are involved in homeostasis of serotonin such as TPH, serotonin transporter (SERT), which reuptakes serotonin into the presynaptic serotonergic nerve terminals to recycle serotonin (Murphy et al., 1998), and monoamine oxidase (MAO), the enzyme for degradation of serotonin (Bortolato et al., 2010).

Most teleosts have two TPH genes (*tph1* and *tph2*), two SERT genes (*slc6a4a* and *slc6a4b*) but only one type of MAO gene (*mao*) (Chen et al., 1994; Setini et al., 2005; Norton et al., 2008; Rahman and Thomas, 2009). In some teleosts, such as zebrafish, stickleback and medaka, there are three genes (*tph1a*, *tph1b,* and *tph2*) encoding TPH (Lillesaar, 2011). In the brain of zebrafish, *tph1a* is present in the posterior tuberculum and hypothalamus, and also in the pineal organ, in amacrine cells of the retina, and *tph1b* is transiently expressed in a preoptic cell cluster during late embryonic stages (Bellipanni et al., 2002), and *tph2* is mainly expressed in serotonergic neurons of the raphe nuclei (superior raphe and inferior raphe) (Lillesaar, 2011) (**Figure 1**). In some teleosts, TPH is expressed in the pituitary (Boularand et al., 1998; Rahman and Thomas, 2009), indicating that serotonin may be locally produced in the pituitary. In the zebrafish, *slc6a4a* is expressed in the superior raphe and pretectal diencephalic cluster, and *slc6a4b* is seen only in the paraventricular organ and caudal zone of periventricular hypothalamus (Wang et al., 2006; Norton et al., 2008). In the serotonergic raphe nuclei, serotonergic neurons in the superior raphe project to the forebrain and midbrain, and the serotonergic cells in the inferior raphe project to hindbrain-spinal cord region in the teleosts brain (Lillesaar, 2011).

Serotonin Receptors

In teleosts, serotonin receptors have been identified and characterized in several species such as zebrafish, European flounder (*Platichthys flesus*), Gulf toadfish (*Opsanus beta*), and puffer fish (Yamaguchi and Brenner, 1997; Lu et al., 2007; Best and Alderton, 2008; Mager et al., 2012). Additionally, *in silico* analysis have predicted gene sequences encoding serotonin receptors in several other species such as the tilapia (*Oreochromis niloticus*), cichlid fish (*Haplochromis burtoni*), southern platyfish (*Xiphophorus maculatus*), and rainbow trout (*Oncorhynchus mykiss*). In the zebrafish, three serotonin receptors subtypes (5-HT1, 5-HT2, and 5-HT7) have been identified, among which three subgroups of 5-HT1 (5-HT1aa, 5-HT1ab, 5-HT1bd) and two subgroups of 5-HT2 (5-HT2A and 5-HT2C) have been identified (Norton et al., 2008; Schneider et al., 2012). In

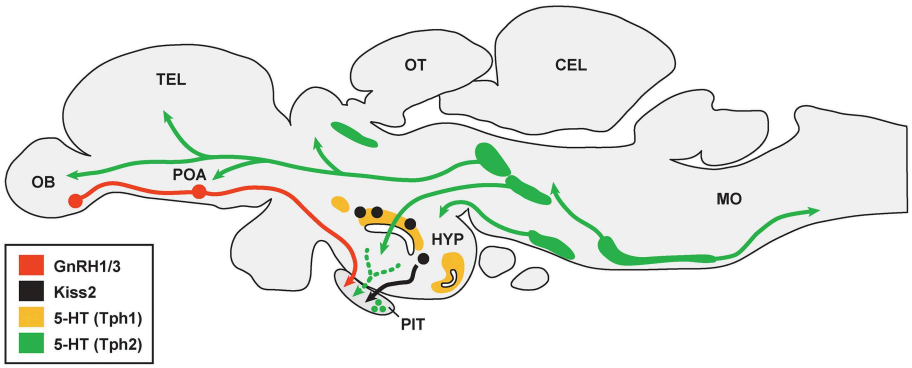

FIGURE 1 | Schematic drawing illustrating association between serotonergic cell populations with GnRH and kisspeptin neurons in the brain of teleosts. There are multiple serotonergic (5-HT) cell populations that express either Tph1 (area shaded with *yellow*) or Tph2 (area shaded with *green*). 5-HT fibers may project to gonadotropin-releasing hormone (GnRH1 and GnRH3) neurons (shown in *red*) in the olfactory bulb (OB) and preoptic area (POA), while it is unknown whether 5-HT fibers are directly associated with kisspeptin (Kiss2) neurons (*black*) in the hypothalamus (HYP). 5-HT fibers and cells are also present in the pituitary (PIT), which may associate with GnRH and Kiss2 fibers in the pituitary. TEL, telencephalon; OT, optic tectum; CEL, cerebellum; MO, medulla oblongata. The organization of serotonergic projections were adopted from Lillesaar (2011) and Gaspar and Lillesaar (2012).

the brain of zebrafish, 5-HTr1aa and 5-HTr1ab are mainly expressed in the preoptic area and hypothalamus, and 5-HTr1bd is expressed in the hypothalamus (Norton et al., 2008). In the Gulf toadfish, 5-HT2A is widely expressed in the brain including the telencephalon, midbrain, cerebellum, hindbrain and in the pituitary (Mager et al., 2012). In the zebrafish, 5-HT2C is expressed in the telencephalon, diencephalon, rhombencephalon, and spinal cord (Schneider et al., 2012).

Serotonin receptors are also expressed in peripheral tissues including gonadal tissues in teleosts. In the zebrafish, 5-HT2C receptor gene is expressed in the ovary (Schneider et al., 2012). In the toadfish, 5-HT2A is expressed in the ovary and testes (Mager et al., 2012).

Serotonin in Teleost Reproduction

GnRH Release

Serotonin modulates fish reproductive function via multiple pathways including through central (preoptic-hypothalamic area and pituitary) and peripheral (gonads) actions. In the hypothalamus, GnRH neurons play major role in the control of vertebrate reproduction. Immunohistochemical study in the Atlantic croaker have demonstrated close association of serotonin fibers with olfactory bulbular and hypothalamic GnRH neurons (Khan and Thomas, 1993). However, in the Atlantic croaker, central administration of serotonin has no effect on preoptic GnRH1 mRNA levels (Thomas et al., 2007), indicating that serotonin may stimulate GnRH release but not synthesis. Indeed, serotonin stimulates GnRH release from the hypothalamus of the seabream and goldfish (Yu et al., 1991; Senthilkumaran et al., 2001). In the zebrafish, expression of serotonin receptors are seen in several brain regions containing GnRH neurons (Norton et al., 2008), which suggests possible co-expression of serotonin receptors in GnRH neurons as in mammals (Bhattarai et al., 2013).

Kisspeptin, a ligand for G-protein coupled receptor GPR54, has recently emerged as a key player for GnRH release (Tena-Sempere, 2006; Gopurappilly et al., 2013). However, no report has described the involvement of serotonin in the regulation of the kisspeptin system in any vertebrates to date.

Gonadotropin Release

In Atlantic croaker increasing serotonin concentrations are associated with levels of gonadotropin release from the pituitary (Khan and Thomas, 1994). In several teleost species, serotonin stimulates release of gonadotropin *in vivo* and *in vitro* (Somoza et al., 1988; Somoza and Peter, 1991; Khan and Thomas, 1992). *In vitro* and *in vivo* studies in teleosts have shown the involvement of 5-HT1 or 5-HT1 receptor subtypes in stimulating gonadotropin secretion (Somoza and Peter, 1991; Khan and Thomas, 1994; Wong et al., 1998). These studies suggest that serotonin plays a prominent role in gonadotropin secretion in teleosts as demonstrated in mammals.

In the Atlantic croaker, serotonin combination with GnRH stimulates LH secretion (Wong et al., 1998). In the goldfish, serotonin stimulates release of GnRH from the cultured brain preoptic-anterior hypothalamic region and pituitary fragments (Yu et al., 1991). However, a recent *in vivo* study in Prussian carp (*Carassius gibelio* Bloch) demonstrated that serotonin alone had no influence on the spontaneous LH release, but the additive effects of serotonin was observed when GnRH analog was co-administered (Sokolowska-Mikolajczyk et al., 2015). These observations indicate functional interaction between serotonin and GnRH system in teleosts. However, an *in vitro* study in the red seabream demonstrated that serotonin stimulates the release of GnRH from the hypothalamus but not from the pituitary of immature fish (Senthilkumaran et al., 2001). Therefore, in teleosts, the mode of action of serotonin on gonadotropin release could be changed reproductive-stage dependently. Additionally, serotonin is also known to modulate growth hormone (GH)

release in goldfish (Somoza and Peter, 1991; Wong et al., 1998). In the goldfish, GnRH-stimulated GH secretion is interfered by serotonin with PKC and Ca^{2+} signaling pathways in pituitary cells (Yu et al., 2008). Those signaling pathways could also be involved in GnRH-primed gonadotropin secretion in teleosts.

Gonadal Maturation

In addition to its central action on the reproductive axis, serotonin directly acts on gonads. In the Gulf killifish (*Fundulus grandis*), 10 days of daily injection of serotonin precursor with dopamine precursor increases gonadosomatic index in male (Emata et al., 1985). An *in vitro* study in the Japanese medaka (*Oryzias latipes*) has shown stimulatory effect of serotonin on oocyte maturation in a dose-dependent manner, which is modulated via stimulation of the synthesis of estrogen and the maturation-inducing steroids (MIS: 17α,20β-dihydroxy-4-pregnen-3-one) by the granulosa cells (Iwamatsu et al., 1993) On the contrary, in the mummichog (*Fundulus heteroclitus*), serotonin inhibits oocyte maturation, especially oocyte meiosis (Cerdá et al., 1995, 1997, 1998).

Although the expression of serotonin receptors in the testis has not been reported in teleosts, in freshwater catfish (*Channa punctatus* Bloch), MAO activity has been noted in the testis (Katti and Sathyanesan, 1986), and MOA activity and serotonin contents in testis represents correlative changes with testicular maturation (Joshi and Sathyanesan, 1980). These results suggest that locally produced serotonin may participate in testicular maturation.

Social and Reproductive Behaviors

The role of serotonin in social behavior has been well demonstrated in fish (Winberg and Nilsson, 1993), while no report has demonstrated the involvement of serotonin in sexual behavior. As social status and reproductive activity are closely related, alteration of serotonin during different social status may directly influence reproductive activities. In teleosts fish, serotonin plays primary inhibitory role in aggressive behavior (Munro, 1986; Adams et al., 1996; Winberg et al., 2001; Perreault et al., 2003). In the fighting fish *Betta splendens*, serotonin decreases aggression via 5-HT1A receptors (Clotfelter et al., 2007). On the contrary, higher levels of serotonin metabolite are found in the brain of subordinate compared with dominant fish (Winberg and Lepage, 1998; Lorenzi et al., 2009). In a cichlid fish *Astatotilapia burtoni*, subordinate males have higher serotonergic turnover and higher expression of two serotonin receptor genes (5-HT1A and 2A) in the telencephalon (Loveland et al., 2014), indicating a correlation between social status and the serotonin system. In the Arctic charr (*Salvelinus alpinus L.*), higher brain serotonergic levels and activity is socially induced in subordinates (Winberg et al., 1991, 1992).

Modulation of Serotonin Activity

Gonadal Steroids

In teleosts, serotonin levels in the brain and pituitary are modulated by reproductive cycles and gonadal steroids (Subhedar et al., 1997; Hernandez-Rauda and Aldegunde, 2002b). In the tilapia, estrogen alters the brain serotonin content during the early brain development stage, which is mediated by decreasing TPH activity and increasing MAO activity (Tsai and Wang, 1999). In the adult male marine yellow snapper (*Lutjanus argentiventris*), serotonin levels in the telencephalon reach the peak during the prespawning period, and are lowest during the spawning period (Hernandez-Rauda and Aldegunde, 2002a). Furthermore, blocking serotonin synthesis alters brain aromatase activity during the critical period of sexual differentiation in the tilapia (Tsai et al., 2000), suggesting possible involvement of serotonin in brain sex determination.

Endocrine Disruptors

Endocrine disruptors such as polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) can modulate serotonergic activity (Stephanou et al., 1998; Gesto et al., 2006; Clotfelter et al., 2010; Rahman et al., 2011). Some of these endocrine disruptors have a significant influence on fish reproductive function through the serotonin system. For example, PAHs such as naphthalene and benzo[α]pyrene disrupt the reproductive axis in teleosts (Hose et al., 1981; Yarahmadi et al., 2013). PCB inhibits serotonergic and TPH activity as well as disrupts GnRH and gonadotropin secretion in the Atlantic croaker (Khan and Thomas, 2000, 2006). Similarly, para-chlorophenylalanine (PCPA) reduces hypothalamic serotonin levels and impairs GnRH and LH secretion in the Atlantic croaker (Khan and Thomas, 2001). These results suggest that the serotonin system is one of the major targets for neuroendocrine disruption, which may lead to inhibition of reproductive functions.

Environmental and Social Factors

In teleosts, the brain serotonergic activity displays diurnal or seasonal variations (Khan and Joy, 1988; Senthilkumaran and Joy, 1993), which may have significant effects on the reproductive functions. In teleosts, serotonin concentrations in the brain are higher in the morning than evening (Fingerman, 1976; Khan and Joy, 1988). In the *Channa punctatus*, there are diurnal variations in the serotonin content (Khan and Joy, 1988) as well as MAO activity in the hypothalamus (Khan and Joy, 1987, 1988), suggesting diurnal variation of the hypothalamic serotonin levels. Seasonal variation of hypothalamic serotonin content has also been noted in the catfish, *Heteropneustes fossilis* (Senthilkumaran and Joy, 1994). These seasonal changes in serotonin levels could also be due to environmental factors such as water temperature and photoperiod. In the tilapia, the hypothalamic serotonin content is lower in fish exposed to higher water temperature than those in lower temperature group (Tsai and Wang, 1997). In contrast, expression of serotonin receptors (5-HT1A and 1D) in the brain are increased by low temperature in the tilapia during the sexual differentiation (Wang and Tsai, 2006). In several fish species, photoperiods alter hypothalamic serotonin content and turnover (Olcese et al., 1980; Senthilkumaran and Joy, 1994), which can be modulated by melatonin levels (Joy and Khan, 1991). In the goldfish, pinealectomy and melatonin administration have a significant effect on hypothalamic serotonin content and serotonergic activity (Olcese et al., 1981). These results indicate environmental

factors may influence reproductive functions via diurnal and seasonal change of serotonin activity.

In the protogynous fish, Hawaiian saddleback wrasse (*Thalassoma duperrey*), serotonin inhibits both initiation and completion of sex reversal (Larson et al., 2003a). Furthermore, in the same fish, serotonin levels in the brain are altered by socially induced sex reversal, which could be associated with territorial acquisition (Larson et al., 2003b). These results suggest that serotonin is also regulated by social behaviors.

Selective Serotonin Reuptake Inhibitor (SSRI)

Selective serotonin reuptake inhibitors (SSRIs) are widely used as antidepressants in the treatment of major depressive disorder and anxiety disorders (Lesch, 2001; Homberg et al., 2010). SSRIs have been detected as pharmaceutical contaminants in surface waters and sewage effluents (Kreke and Dietrich, 2008; Oakes et al., 2010) as well as in fish brain tissue (Schultz et al., 2010) owing to their widespread and increasing rates of administration. SSRIs block the presynaptic SERT and prevent the clearance of synaptic serotonin, which causes an elevation of extracellular serotonin concentrations (Tollefson and Rosenbaum, 1995). Chronic exposure to SSRIs cause significant decrease of serotonin content in the fish brain (Gaworecki and Klaine, 2008; Winder et al., 2009; Bisesi et al., 2014), which can influence the neuroendocrine control of reproductive function. Among the SSRIs, fluoxetine (also known as PROZAC) has been widely used to investigate the serotonergic modulation of the teleosts endocrine system (Somoza and Peter, 1991; Kreke and Dietrich, 2008). In female fish, fluoxetine treatment significantly reduces egg production and ovarian levels of estrogen, and gene expression levels of aromatase, FSH- and LH-receptors (Lister et al., 2009; Forsatkar et al., 2014). Conversely, fluoxetine has stimulatory effects on GnRH and LH release in some fish species (Somoza et al., 1988; Yu et al., 1991).

SSRIs also have influence on not only endocrine system, but also behaviors. In male fathead minnows (*Pimephales promelas*), exposure to sertraline, a SSRI decreases shelter-seeking behavior, suggesting that sertraline elicits an anxiolytic effect (Valenti et al., 2012). In hybrid striped bass (Morone saxatilis × M. chrysops), fluoxetine exposures decrease in ability of fish to capture prey (Gaworecki and Klaine, 2008). In the bluehead wrasse (*Thalassoma bifasciatum*), fluoxetine treatment decreases territorial aggression. However, in male *B. splendens*, there was no effect of chronic intramuscular injections of fluoxetine on aggressive behavior (Clotfelter et al., 2007). These observations suggest that environmental SSRIs may have significant impact on reproductive capability of fish via behavioral disruption.

A variety of influences of SSRIs on fish reproduction could be due to different doses, administrations, duration of SSRI treatments and physiological, reproductive status and sex of fish treated and species differences (Sumpter et al., 2014). However, it is still unclear how SSRIs act on the HPG axis via the serotonin system. In addition, most antidepressant drugs are specifically designed for humans (mammals), but not for fish. Therefore, the effects of these drugs may not be specific in teleosts. In fish, SSRIs are suggested to interact with and inhibit some P450 isozymes that are responsible for steroid metabolism (Kreke and Dietrich, 2008), which might have effect on the reproductive neuroendocrine control.

Summary

Serotonin is one of the classic neurotransmitter and the structure of its related molecules such as TPH and SERT, and their brain organization are highly conserved in mammalian and non-mammalian vertebrates, suggesting functional conservation of the role of serotonin system in vertebrate reproduction. Several physiological studies have demonstrated the role of serotonin in a variety of reproductive functions including the control of GnRH release, LH release, gonadal maturation, and socio-sexual behaviors in teleosts (**Figure 2**). However, the serotonin system in teleost may also play unique role in the control of reproduction as the mechanism of reproductive control in teleosts is not always the same as in the mammalian models (Xiong et al., 1994; Zohar et al., 2010). For example,

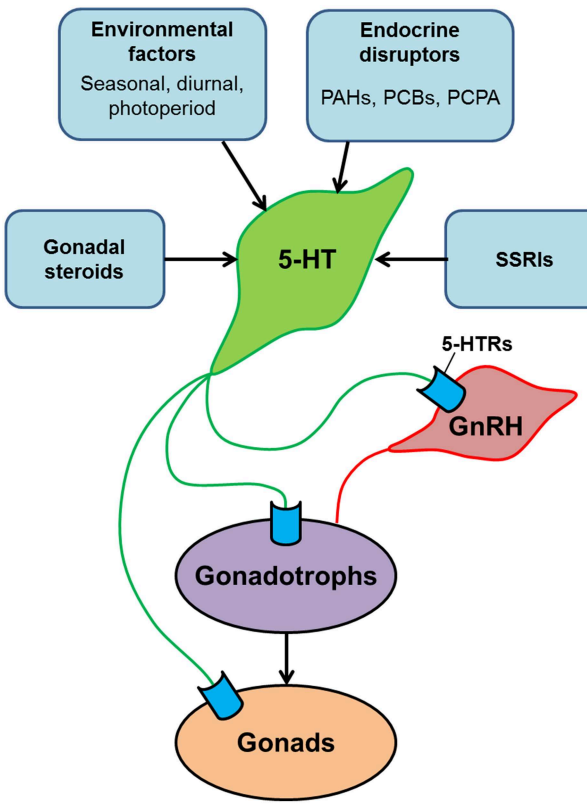

FIGURE 2 | Schematic model illustrating the serotonergic action on the hypothalamus-pituitary-gonadal axis of teleosts. Serotonin (5-HT) modulates the reproductive system at multiple levels: the hypothalamus (via GnRH neurons), the pituitary (via gonadotrophs) and the gonads. 5-HT system is modulated by several factors such as gonadal steroids, environmental factors and social cues. In addition, central 5-HT system is also influenced by chemical substances such as endocrine disrupters and selective serotonin reuptake inhibitors (SSRIs), which exist in surface waters and sewage effluents as contaminants. Exposure of fish to those chemical substances may have significant impacts on reproductive functions.

in some fish, there are serotoninergic cell populations in the hypothalamus and in the pituitary, which indicates the presence of multiple pathways of gonadotropin control by the serotonin system. In fish, the serotonin system is also regulated by natural environmental factors as well as chemical substances. In particular, SSRIs are commonly detected as pharmaceutical contaminants in the natural environment (Brooks et al., 2005; Corcoran et al., 2010). Several research articles demonstrate that acute and chronic exposure to SSRIs induces a variety of change in physiological and behavioral parameters in fish. However, environmental SSRIs could act on fish reproductive system via multiple pathways, the detail mechanisms underlying the effect of SSRIs on fish serotonin system and reproductive neuroendocrine system need to be examined to evaluate the potential influence of the SSRIs on fish reproductive functions.

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