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RESEARCH TOPICS

ESTROGENIC CONTROL OF HYPOTHALAMIC GnRH NEURONS

Topic Editor
Henryk Urbanski

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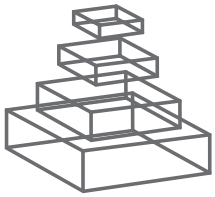

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ESTROGENIC CONTROL OF HYPOTHALAMIC GnRH NEURONS

Topic Editor:
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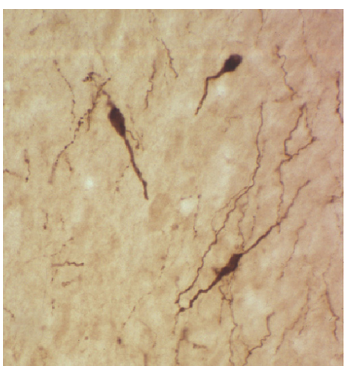

GnRH-1 neurons in the rhesus macaque hypothalamus. Image by Henryk Urbanski.

In female mammals, sexual maturation is initiated by a change in the release pattern of pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and a consequential increase in the secretion of ovarian sex-steroids, estradiol and progesterone. Orchestrating these endocrine changes is a diffuse population of hypothalamic neurons that produce the neuropeptide, gonadotropin-releasing hormone (GnRH). Although the development and functional integration of GnRH neurons with the rest of the central nervous system is still poorly understood, recent progress has been made on several fronts. For example, there is now evidence that some mammalian species express more than one molecular form of GnRH (GnRH-I and GnRH-II), and that the two corresponding GnRH neuronal sub-populations may play different roles in the regulation of reproductive function and behavior. Moreover, through the use of transgenic animal models, neuronal fiber tracing, gene expression profiling, and electrophysiologcal recordings, new insights have been gained into the mechanisms that regulate GnRH release. The focus of this Research Topic is on the positive and negative actions that estrogens exert within the mammalian hypothalamus, especially on the reproductive neuroendocrine axis around the time of the preovulatory LH surge. Our hope is that a deeper understanding of the endocrine interactions between the hypothalamus, pituitary gland, and gonads will provide a solid foundation upon which to develop more effective therapies for pubertal disorders, infertility, and menopause.

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EDITORIAL
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Introduction to special topic—estrogenic control of hypothalamic GnRH neurons

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In female mammals, sexual maturation is initiated by a change in the release pattern of pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and a consequential increase in the secretion of ovarian sex-steroids, estradiol and progesterone. Orchestrating these endocrine changes is a diffuse population of hypothalamic neurons that produce the neuropeptide, gonadotropin-releasing hormone (GnRH). Although the development and functional integration of GnRH neurons with the rest of the central nervous system is still poorly understood, recent progress has been made on several fronts. For example, there is now evidence that some mammalian species express more than one molecular form of GnRH (GnRH-I and GnRH-II), and that the two corresponding GnRH neuronal sub-populations may play different roles in the regulation of reproductive function and behavior. Moreover, through the use of transgenic animal models, neuronal fiber tracing, gene expression profiling, and electrophysiologcal recordings, new insights have been gained into the mechanisms that regulate GnRH release. This volume brings together 10 articles that reflect current thinking about the role of GnRH in mediating estrogenic feedback to the neuroendocrine reproductive axis. The main focus is on the negative and positive actions that estrogens exert within the hypothalamus of mammals, especially around the time of the preovulatory LH surge. However, the articles also present findings from other vertebrate classes, and so provide an intersting comparative perspective. Taken together, the knowledge presented in this volume represents a foundation upon which to develop more effective therapies for pubertal disorders, infertility, and menopause.

The first article (Sower and Baron, 2011) demonstrates the expression of estrogen receptors within the lamprey hypothalamus, suggesting potential feedback interactions between esatradiol and GnRH neurons. Phylogenetically, lampreys are positioned as basal vertebrates, and like many other non-mammalian vertebrates they express multiple forms of GnRH. Consequently, studies in lampreys provide us with important insights into the molecular evolution of estrogenic-GnRH interactions.

A cluster of three articles then focus on hypothalamic neuropeptides that exert a major influences on the secretion of GnRH, and which are thought to mediate the feedback effects of sex-steroids on the reproductive axis. For example, studies in rodents have clearly shown the importance of kisspeptin to both pulsatile and surge modes of GnRH release (Navarro, 2012), while human studies have observed sexual dimorphism in kisspeptin neurons during aging (Hrabovszky et al., 2011). Again, an interesting evolutionary perspective is provided by comparing the steroid sensitivity of kisspeptin neurons in various non-mammalian vertebrates (Kanda and Oka, 2012).

Although the exact mechanism by which estrogens interact with the reproductive neuroendocrine axis is unclear, our general understanding of this issue is neatly summarized by a series of three reviews (Micevych and Sinchak, 2011; Kenealy and Terasawa, 2012; Radovick et al., 2012). Of particular interest is the finding that sex-steroids exert their feedback not only through classic estrogen receptors located in the nucleus, but also more rapidly through receptors located in the cell membrane.

Various physiological functions show adaptations for life in an environment that undergoes marked changes across the 24-h day. For example, in many mammals the timing of the preovulatory LH surge occurs at a very specific time of day. Two articles discuss the interactions between the circadian timing system and estrogen-sensitive neural circuits that regulate GnRH secretion and the preovulatory surge (Sun et al., 2012; Williams and Kriegsfeld, 2012).

Finally, a novel hypothesis is proposed to explain the conundrum of how estrogens appear to exert both negative and positive feedback actions on the reproductive axis (Urbanski, 2012). Data is presented from non-human primate studies to suggest that negative and positive estrogen feedback are two completely separate mechanisms, which are mediated, respectively, by two distinct GnRH neuronal populations. This hypothesis challenges the common assumption that has dominated the field for the past four decades, namely that individual GnRH neurons are relatively homogenous and can mediate both negative and positive estrogen feedback.

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The interrelationship of estrogen receptor and GnRH in a basal vertebrate, the sea lamprey

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The hypothalamic-pituitary system is considered to be a vertebrate innovation and seminal event that emerged prior to or during the differentiation of the ancestral agnathans. Lampreys are the earliest evolved vertebrates for which there is a demonstrated neuroendocrine system. Lampreys have three hypothalamic gonadotropin-releasing hormones (GnRHs; lGnRH-I, -II, and -III) and two and possibly three pituitary GnRH receptors involved in mediating reproductive processes. Estradiol is considered to be a major reproductive steroid in both male and female lampreys. The purpose of this study was to investigate estrogen receptor (ER) expression in the lamprey brain in adult sea lampreys. Expression of ER mRNA was confirmed in the adult lamprey brain using RT-PCR. Using digoxigenin (DIG)-labeled probes, ER expression was shown to yield moderate, but distinct reaction products in specific neuronal nuclei of the lamprey brain, including the olfactory lobe, hypothalamus, habenular area, and hindbrain. Expression of ER in the hypothalamic area of the brain provides evidence of potential interaction between estradiol and GnRH(s), and is consistent with previous evidence showing estrogen feedback on GnRH in adult lamprey brain. Earlier studies have reported that there is a close distribution of glutamic acid decarboxylase (GAD; GABA-synthesizing enzyme) and lamprey GnRH in the preoptic region in adult lampreys. The establishment of a direct estradiol–kisspeptin–GABA–GnRH interaction in lamprey has yet to be determined and will require future functional and co-localization studies. The phylogenetic position of lampreys as a basal vertebrate allows lampreys to be a basis for understanding the molecular evolution of the neuroendocrine system that arose in the vertebrates.

Keywords: estrogen receptor, lamprey, GnRH, in situ hybridization, lamprey hypothalamus, estradiol, basal vertebrate, agnathan

INTRODUCTION

Modern vertebrates are classified into two major groups, the gnathostomes (jawed vertebrates) and the agnathans (jawless vertebrates). The agnathans are classified into two groups, myxinoids (hagfish) and petromyzonids (lampreys), while the gnathostomes constitute all other living vertebrates including the bony and cartilaginous fishes and the tetrapods. The hypothalamic-pituitary system is considered to be a vertebrate innovation and seminal event that emerged prior to or during the differentiation of the ancestral agnathans (Sower et al., 2009). Lampreys are the earliest evolved vertebrates for which there is a demonstrated neuroendocrine system. Lampreys have three hypothalamic gonadotropin-releasing hormone (GnRHs; lGnRH-I, -II, and -III) and two and possibly three pituitary GnRH receptors involved in mediating reproductive processes (Sower et al., 2009; Hall et al., 2011).

In lampreys, there is a general pattern of GnRH distribution in the anterior-preoptic-neurohypophysial tract to the neurohypophysis of adult lampreys as determined by several immunohistochemical (IHC) studies using specific GnRH antisera to each of the lamprey GnRHs and also by *in situ* hybridization studies using specific GnRH probes (Crim et al., 1979a,b; Nozaki and Kobayashi, 1979; King et al., 1988; Nozaki et al., 1994; Reed et al., 2002; Kavanaugh et al., 2008). Data showing GnRH immunoreactive (ir) neurons in adult lampreys using IHC showed cells restricted to a single bilateral dense arc along the third ventricle in the rostral hypothalamus and preoptic area. Of all vertebrates, only the agnathan and teleosts lack a portal vascular system (median eminence) for transferring neurohormones from the hypothalamus to the adenohypophysis (Gorbman, 1965). Studies were done to experimentally examine the functional anatomical relationship between the hypothalamus and adenohypophysis in sea lamprey (Nozaki et al., 1994). It was shown that neurosecretory peptides like GnRH diffuse from the brain (neurohypophysis) to the adenohypophysis, and thus regulate its secretory activity in lampreys. Thus, there is evidence of normal occurrence of GnRH in a part of the lamprey brain homologous with that brain region in later evolved vertebrates in which GnRH localization forms part of a neuroendocrine mechanism for gonadotropin secretion. In other words, neurosecretory peptides like GnRH are able to diffuse from the brain to the pituitary to regulate its secretory activity. In addition, Crim (1981) and King et al. (1988) showed that GnRH neurons project into the third ventricle. These authors proposed an additional route of GnRH via secretion into the third

ventricle and transported by tanycytes to the adenohypophysis (King et al., 1988).

There are several important brain neurohormones/factors that have been shown to stimulate/modulate GnRH and/or gonadotropin synthesis and/or release in vertebrates. In some teleosts, those neurohormones/factors include dopamine, neuropeptide Y, gamma-aminobutyric acid (GABA), and more recently gonadotropin-inhibitory hormone (GnIH) and kisspeptin (KiSS; Kah and Dufour, 2010). In lampreys, GABA and neuropeptide Y have been shown to be involved with brain GnRH and reproduction (Conlon et al., 1994; Root et al., 2004, 2005). Glutamic acid decarboxylase (GAD; GABA-synthesizing enzyme) mRNA was expressed in four distinct cell populations in the lamprey brain, ranging from the telencephalon and diencephalon of the forebrain to the mesencephalon and rhombencephalon of the midbrain and hindbrain in adult lamprey (Root et al., 2005). The close distribution of GAD and lamprey GnRH in the preoptic region supports the hypothesis that GABA might act on the reproductive axis through the feedback on GnRH neurons (Reed et al., 2002; Root et al., 2005).

Data supporting estrogen feedback on GnRH has been shown in lamprey. Sower (1997) observed that adult female lamprey injected with microencapsulated E2 experienced an elevation in GnRH concentrations, and activity of E2 in the hypothalamic region is supported *in vitro* autoradiography studies demonstrating binding of E2 in that area (Kim et al., 1980, 1981). However, it is unclear if the E2–GnRH interaction in lamprey occurs through a direct or an indirect mechanism. Within the preoptic anterior hypothalamus, co-localization studies of GABA and GnRH in lamprey have indicated GABA and GnRH-containing cells are closely associated (Reed et al., 2002; Root et al., 2005), supporting the possibility of a GABA-mediated (indirect) mechanism of E2–GnRH interaction in lamprey.

With the cloning of an estrogen receptor (ER), it is now possible to examine the expression of the receptor in the adult lamprey brain (Thornton, 2001). In contrast to teleost fish that have three types of ER (Hawkins et al., 2000; Menuet et al., 2002), lampreys apparently only have one ER (Thornton, 2001). Earlier studies in mammals suggested that the mechanism of E2 action on GnRH is largely indirect, and neurons of the anteroventral periventricular brain region that express the neurotransmitter, gamma amino butyric acid (GABA), are believed to be responsible for facilitating interaction between E2 and GnRH (Petersen et al., 2003). The discovery of kisspeptin and subsequent studies now suggest that steroid feedback may also be occurring on GnRH neurons via kisspeptin and its receptor (Roa et al., 2009).

The proximity of GnRH to GABAergic neurons and the effects of GABA/muscimol on GnRH support a role for GABA in the regulation of lamprey GnRH. However, such studies do not necessarily imply E2 involvement in GABA–GnRH interaction. Until recently, it was not clear that a classical ER existed in lamprey. Thornton's (2001) discovery and cloning of a lamprey provides an opportunity to enhance our understanding of the relationships between GABA, GnRH, and E2 in lamprey. Therefore, in the present study, we examined the expression patterns of ER in the adult sea lamprey, *Petromyzon marinus*, brain in relation to GnRH and GABA.

MATERIALS AND METHODS

COLLECTION AND SAMPLING

Adult female sea lampreys were collected from the fish ladder on the Cocheco River in Dover, New Hampshire. Collection of lamprey occurred in May and June during two successive seasons during the lamprey's upstream spawning migration from the ocean to coastal rivers. The lampreys were transported to the freshwater fish hatchery at the University of New Hampshire and maintained in an artificial spawning channel supplied with flow-through water from a nearby stream-fed reservoir at an ambient temperature range of 13–20°C, under natural photoperiod following the University of New Hampshire Institutional Animal Care and Use guidelines.

RT-PCR

Expression of ER was examined in the lamprey brain using RT-PCR, both for the purpose of verifying expression and to generate cDNA template for the synthesis of *in situ* hybridization probes. Expression was also assessed in ovary, testis, and muscle tissue for the purpose of comparison with expression in the brain. One adult, female lamprey was killed by decapitation. Tissues for were snap frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from ∼100 mg of each tissue using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Gene specific primers were selected against the partial cDNA sequence of ER cloned from the sea lamprey by Thornton (2001), Accession #AY028456, using Primer3[1] and were obtained from Integrated DNA technology (Coralville, IA, USA).

Estrogen receptor left primer (first set): 5′-CCTCGTGCACAGAG TTCT-3′
Estrogen receptor right primer (first set): 5′-GTAGCGATCCGGA GCTGA-3′
Estrogen receptor left primer (second set: 5′-GACATGTTCGACA TGCTGCT-3′
Estrogen receptor right primer (second set): 5′-AGCGGGATCAC ATTCTTACG-3′

RT-PCR reactions were prepared (16.75 μl H20, 2.5 μl buffer, 1.5 μl $MgCl_2$, 0.5 μl dNTPs, 0.25 μl Taq Polymerase, 500 nM primer concentration, and 1 μl template). RT-PCR reactions were performed using an Eppendorf Thermal Cycler under the following conditions: 48°C/1 min; (95°C/15 s; 60.1°C/1 min; 72°C/1 min) × 35 cycles; 72°C/5 min; 10°C/hold. The reaction products were analyzed on 1% agarose gels electrophoresed at 90 V for 1 h and stained with ethidium bromide for purposes of visualization.

For the purpose of verifying the sequence identity of candidate ER-bands, RT-PCR products were first gel purified as described in the QIAEX II Gel Purification Kit© (QUIGEN, Valencia, CA, USA). Purified products were then inserted into the pGEM-T-Easy vector (Promega, Madison, WI, USA) and subsequently transformed into *E. coli* JM109 cells (Promega). Ligations and transformations were performed following the protocol described in the pGEM-T-Easy Vector System (Promega). Overnight cultures were used

[1] http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

for plasmid preparation with the Wizard Plus Miniprep system (Promega), following the manufacturer's protocol. Purified plasmid was then sent for sequencing at the University of Utah's Health Science Center[2].

RNA PROBE SYNTHESIS

Templates for probe synthesis were prepared in plasmids as described above. Purified plasmid containing the ER insert was then digested overnight at 37°C with either *Sal*I or *Nco*I [2 μg BSA and 1× Buffer D (Promega)] restriction enzymes producing singly digested linearized plasmid. Digestions were analyzed by 1% agarose gel electrophoresis to confirm presence of expected clones. Riboprobes were synthesized using the SP6 Riboprobe Synthesis Kit (Promega), and either ^{35}S- or Digoxigenin (DIG)-labeled UTP as previously described (Rubin et al., 1997). This protocol was slightly modified in that the transcription reactions were allowed to continue overnight instead of 2 h.

EVALUATION OF RNA PROBE YIELD

The yield of DIG-labeled probes was evaluated by spectrometric analysis of total RNA and a modified DIG Northern blot protocol (Allen et al., 2000). For the Northern blot, in the place of total RNA, full length probe template cDNA used for each riboprobe synthesis was cross-linked to a nylon membrane and hybridized with the different anti-sense and sense riboprobes. Briefly, template cDNA was denatured for 2 min at 100°C, spotted onto nylon membrane and UV cross-linked. Hybridization solution (50% dextran sulfate, 4 × SSC, 1 × Denhardt's, 1 mg/ml yeast tRNA, 10 mM DTT) was prepared and mixed with 0.5 mg/ml DIG-labeled RNA diluted in DEPC water. Probes were applied to each template cDNA and allowed to hybridize overnight at 55°C. Following hybridization, membranes were washed twice in 2 × SSC for 5 min each at RT, 1 × SSC for 15 min at 55°C, and Maleate Buffer (0.1 M maleic acid, 0.3% Tween, 0.15 M NaCl; pH 7.5) for 2 min at RT. Membranes were blocked in Blocking Buffer for 30 min at RT and then incubated in anti-DIG-alkaline phosphatase (AP; Roche) antibody (1:5000) for 30 min at RT. Membranes were washed twice in Maleate Buffer for 15 min each at RT, and then incubated in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate [NBT/BCIP (Roche) 1:50 diluted in 100 mM Tris–HCl, 100 mM NaCl, 50 mM Mg_2Cl_2; pH 9.5)] in darkness for 3 h at RT.

For ^{35}S-labeled probes, radioactivity in the waste products from the LiCl precipitation and washing of probes was measured and compared against the activity of the purified probes. Based on this relationship, the % incorporation of ^{35}S-UTP was calculated. And based on the % of label incorporated, product yield from the synthesis reactions was calculated.

IN SITU HYBRIDIZATION

Forty-seven adult, female lamprey were killed by decapitation. Brains were dissected, immersed in O.C.T. Compound (Miles Inc. Elkhart, IN), and then frozen on dry ice. Sagittal tissue sections of 16–18 μm were cut the day of dissection on a cryostat (Reichert-Jung, Leica Instruments, Heidelberg, Germany) at −12°C. Sections were then mounted onto Vectabond (Vector Laboratories, Burlingame, CA, USA) coated slides and immediately moved into tissue preparation steps of day 1 of the *in situ* hybridization protocol. Tissue sections were incubated with either anti-sense (odd numbered slides) or sense (even numbered slides) riboprobes for lamprey ER. The application and signal development of DIG-labeled probes followed the protocol previously described by Rubin et al. (1997), and characterized for use in lamprey by Root et al. (2005).

The application and signal development of ^{35}S-labeled probes followed the protocol characterized by Root et al. (2005), with the exception of signal development, which followed the protocol described by Hrabovszky et al. (2004). The concentration of probe in the hybridization solution, the concentration of antibody applied, and the stringency of the washes were all varied to optimize the results. Following signal development, both ^{35}S and DIG-labeled tissue sections were viewed under light microscopy using an Olympus BH2 microscope, and digital photographs were captured with a Nikon Coolpix 5000 camera. A detailed visual assessment was made of signal deposition for each slide. In addition, images were also collected at Tufts Medical School using a Zeiss Axioscope and Spot RT color camera (Diagnostic Instruments, Inc.), and submitted for analysis by Optimus™analysis software, which quantitatively compared the signal intensity between sense and anti-sense slides.

RESULTS

RT-PCR

The first set of primers for lamprey ER yielded specific transcripts in ovarian tissue, but not in brain, testis, or muscle tissue (results not shown). In contrast, the second set primers for lamprey ER yielded specific transcripts in all tissues examined, with strongest expression in ovarian and muscle tissues (**Figure 1**).

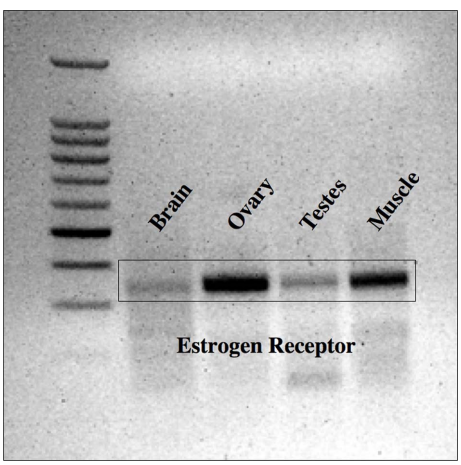

FIGURE 1 | Expression of ER in lamprey tissues. Lamprey ER was most strongly expressed in the ovarian and muscle tissues, but was also expressed in the testis and brain. 100 kb ladder from Promega (Madison, WI, USA).

[2]http://hci-w1.hci.utah.edu/OrderTracking/index.html

Notably, the expression of ER in the target tissue (brain) was exceptionally weak. Megalign™analysis software was used to compare sequenced PCR products with the published sequence for lamprey ER. Specifically, the transcript cloned from the first set of primers (ER1 = 407 bp total length) had 100% identity with the published sequence for lamprey ER (Thornton, 2001). In contrast, the transcript cloned from second set of primers (ER2 = 341 bp total length) contained a single deletion at position 285, relative to the published sequence. The sequence confirms the analyzed RT-PCR product is from the ER gene; as to the single base-pair difference noted – while presumably changing the reading frame – it is most likely an artifact. A pseudogene would be expected to be much more different over the 341-bps sequenced, thus was not pursued further.

EVALUATION OF RNA PROBE YIELD

The fidelity of DIG-labeled probes was supported through Dot Blot analysis that revealed binding of ER1, ER2, and LIII (GnRH-III) to respective cDNAs (**Figure 2**). In addition, probes yield was determined to high, as determined by absorption at 260 nm. For ^{35}S-labeled probes, a high amount of label was determined to have been incorporated into all probes, generating a high yield of product that was also consistent between probes (data not shown).

IN SITU HYBRIDIZATION

A total of 44 lamprey brains were used for DIG-labeled *in situ* hybridization experiments. DIG-labeled, anti-sense ER probes demonstrated moderate, yet distinct reaction product in specific neuronal nuclei across many regions of the lamprey brain, including the olfactory lobe, hypothalamus, habenular area, and hindbrain. Major brain regions were scored for signal intensity on every slide. Patterns of staining were consistent between all brains examined. Slides were subjected to image analysis performed at Tufts University, and in most cases, indicated that staining on sense slides was significantly less robust than that on anti-sense slides. In addition, similar results were observed in both sagittal (**Figure 3**) and transverse tissue sections (**Figure 4**).

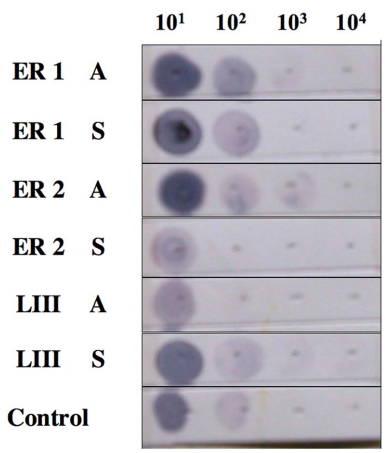

FIGURE 2 | Fidelity of estrogen receptor and GnRH-III (DIG-labeled) probes. Probe fidelity was supported through Dot Blot analysis that revealed binding of ER1, ER2, and LIII (GnRH-III) to respective cDNAs.

For ^{35}S-labeled tissue sections, a total of three lamprey brains were used, and for samples in which the high stringency wash was omitted, results were similar to those seem using DIG-labeled probes. However, while anti-sense slides tended to be more heavily stained than sense slides, a high degree of background staining prevented a detailed analysis (**Figure 5**). In contrast, for ^{35}S-labeled tissue sections exposed to 10 min of a high stringency wash, no signal was observed (results not shown). The limited range of stringencies attempted (0 and 10 min) using ^{35}S-labeled probes limits the derivative power of these results.

DISCUSSION

In this study, ER was shown to be expressed in the lamprey brain by RT-PCR and DIG-labeled *in situ* hybridization. Specifically, *in situ* hybridization indicated that ER is expressed in the olfactory bulb, hypothalamus, habenular area, midbrain, and hindbrain of the lamprey. As stated earlier, in lampreys, there is a general pattern of GnRH distribution in the anterior-preoptic-neurohypophysial tract to the neurohypophysis of adult lampreys (Crim et al.,

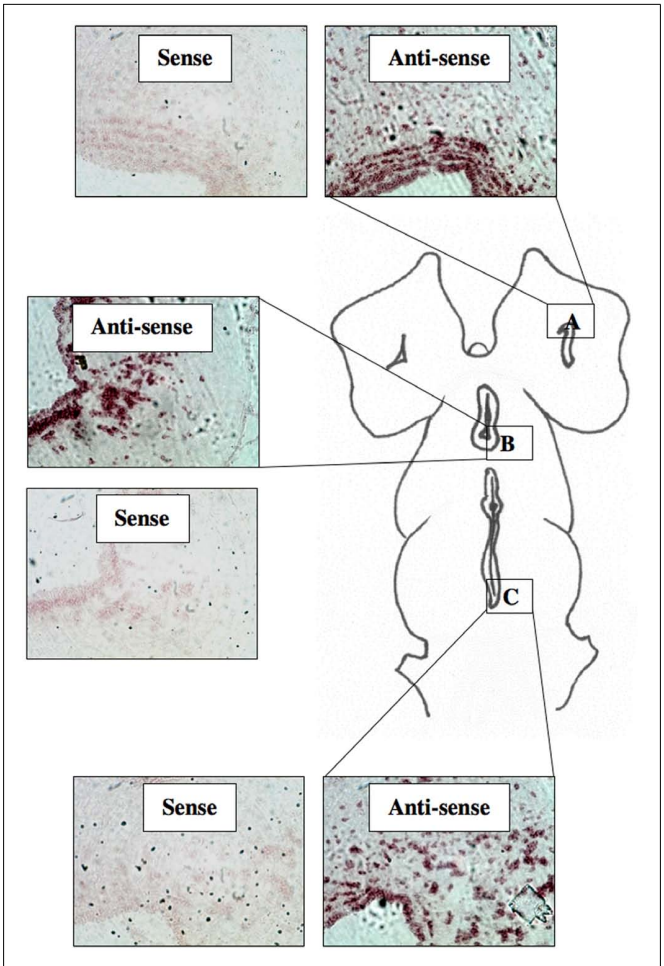

FIGURE 3 | DIG-labeled *In situ* hybridization for lamprey ER. Light micrographs of sagittal tissue sections showing reaction product using probe ER2 within the olfactory bulb **(A)**, the hypothalamus **(B)**, and the midbrain **(C)**. (200× mag).

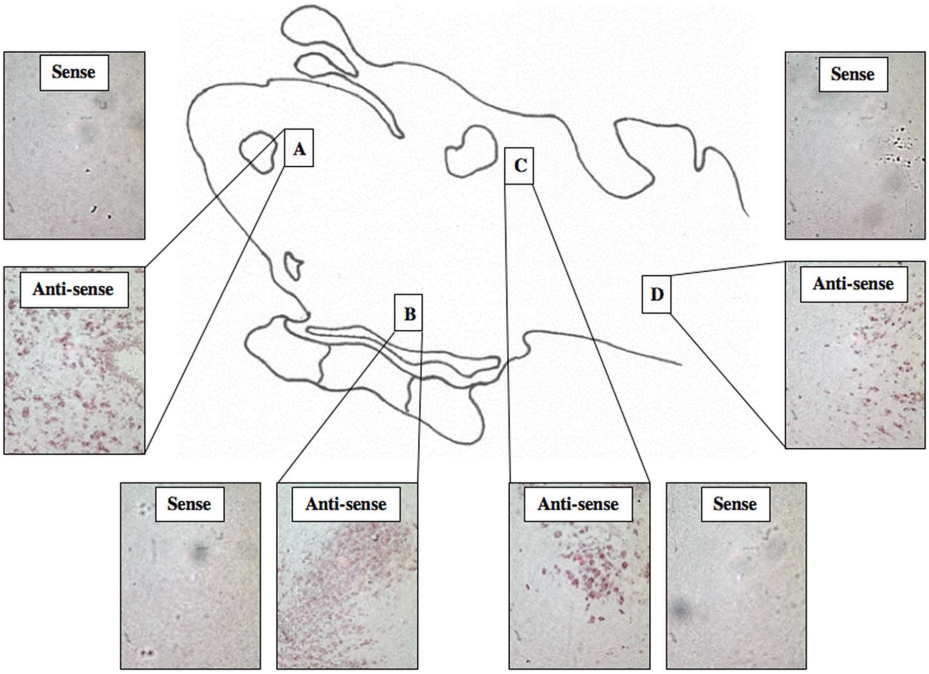

FIGURE 4 | DIG-Labeled *In situ* hybridization for lamprey ER. Light micrographs of transverse tissue sections showing reaction product using probe ER2 within the olfactory bulb **(A)**, the hypothalamus **(B)**, the midbrain **(C)**, and the hindbrain **(D)**. (200× mag).

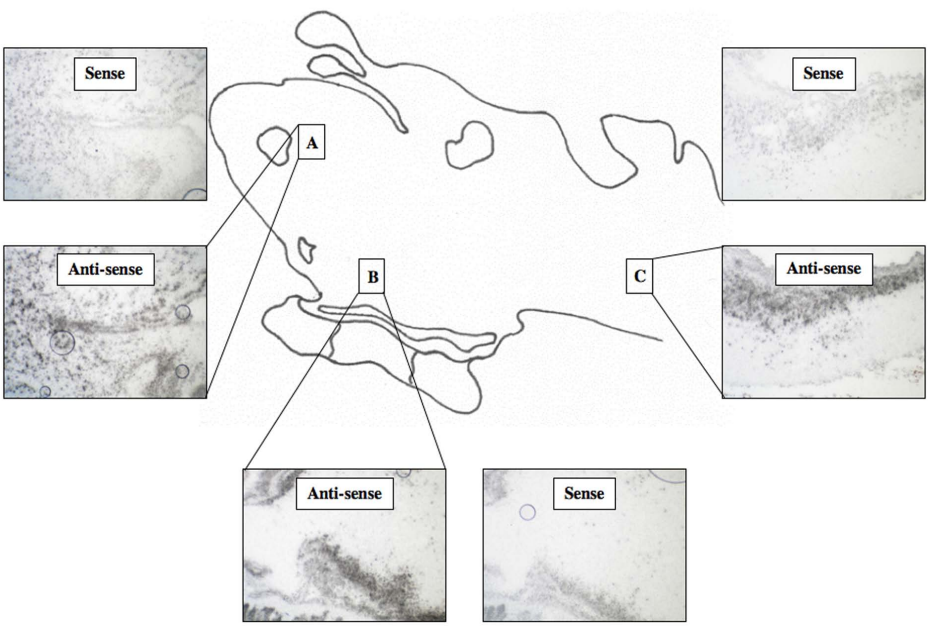

FIGURE 5 | 35 S-labeled *In situ* hybridization for lamprey ER. Light micrographs of sagittal tissue sections showing reaction product within the olfactory bulb **(A)**, the hypothalamus **(B)**, and the hindbrain **(C)**. (200× mag).

1979a,b; Nozaki and Kobayashi, 1979; Nozaki and Gorbman, 1984; King et al., 1988; Reed et al., 2002; Kavanaugh et al., 2008). Expression of ER in the hypothalamic area of the brain supports the possibility of interaction between E2 and GnRH, and is consistent with previous evidence that E2 feedback is occurring indirectly or directly via GnRH neurons (Sower, 1987).

The current study revealed much broader expression of ER than that of previous studies in lamprey. Autoradiographic studies using ^{3}H-labeled E2 previously documented binding only within the hypothalamic region and regions immediately adjacent the hypothalamus (Kim et al., 1980, 1981). In the current study, staining was observed in and around the hypothalamus, but also within the olfactory lobe, habenular area, and hindbrain. In vertebrates other than lamprey, expression of ER occurs widely in the brain (Shima et al., 2003), and it may be that the distribution of ER in the lamprey brain more closely parallels that of other vertebrates than was previously thought. The discrepancy between autoradiographic and *in situ* hybridization studies may have occurred due to the presence of another as yet unknown ER isoform in lamprey or due to non-specific binding. There is increasing evidence suggesting that ERs are capable of activation by means other than E2 (Blaustein, 2004), therefore *in situ* hybridization may reveal regions of ER expression that function independently of E2, and are otherwise undetectable by autoradiographic methods using ^{3}H-labeled E2.

The pattern of ER expression observed in this study is similar to that of ER expression previously observed in the brains of teleost fish (Menuet et al., 2003; Pellegrini et al., 2005). Interaction between E2 and GnRH has been demonstrated in teleosts (Trudeau et al., 1993), and the absence of ER expression in the GnRH neurons of teleosts suggests that E2–GnRH interaction in this vertebrate class occurs indirectly (Navas et al., 1995). While lamprey have a single identified ER (Thornton, 2001), teleost fish have three types of ER (Hawkins et al., 2000; Menuet et al., 2002). These ERs are differentially expressed in the brain (Hawkins et al., 2000; Pellegrini et al., 2005), and also appear to have distinct functions in the neuroendocrine control of reproduction and behavior (Hawkins et al., 2005). In comparison to teleosts, the possession of only a single ER by lamprey may suggest alternative signaling pathways for E2 in the lamprey brain.

The current study identified ER expression within the same hypothalamic region where GnRH expression occurs, but did not offer any insight into how E2 may interact with GnRH in lamprey. A key question remains as to whether E2 acts directly on GnRH-expressing neurons, or indirectly through a mechanism utilizing GABA or kisspeptin. Root et al. (2005) identified GABA-expressing neurons in close proximity to neurons expressing lamprey GnRH, supporting the possibility of an indirect action of E2 on GnRH. However, the critical evidence needed in order to establish the direct and/or indirect nature of E2–GnRH interaction in lamprey lies in the determination of cellular co-localization between GABA, GnRH, kisspeptin, and ER. Kisspeptin and its receptor is another system that may also be involved in feedback studies. The Kiss1/GpR54 system was discovered and shown to be the central gatekeeper in the regulation of GnRH and puberty in mammals (Seminara et al., 2003; Seminara and Crowley, 2008). In mammals, the kisspeptin system acts in regulating many aspects of reproduction functions including the mediation of steroid feedback (Roa et al., 2009). The identification and function of kisspeptin(s) and respective receptors in lampreys has not yet been elucidated. Future studies will be required to examine the interrelationship between the kisspeptin, GnRH, GABA, and steroid feedback systems and to determine whether there is another ER in lampreys.

Estradiol is considered to be a major reproductive hormone in both male and female lampreys (Sower and Kawauchi, 2010). The role of estradiol in reproduction is supported by the cloning of an estrogen-like receptor in sea lamprey (Thornton, 2001). In sea and Japanese river (*Lethenteron camtschaticum*) lampreys, estradiol concentrations increased during spermiation (Fukayama and Takahashi, 1985; Sower et al., 1985; Fahien and Sower, 1990) and decreased during ovulation (Sower et al., 1985; Bolduc and Sower, 1992). In the first reported study examining sex steroid profiles in the Pacific lamprey (*Entosphenus tridentatus*) during overwintering and sexual maturation, estradiol levels were usually higher in males than in females and increases coincided with the development of secondary sex characteristics (Mesa et al., 2010). In another study, there were higher plasma concentrations of estradiol in females compared to males and in both sexes, plasma estradiol significantly increased as the season progressed correlating with a temperature increase that is in general agreement with these earlier studies (Sower and Kawauchi, 2010). In males, higher estradiol concentrations corresponded to males that have mature sperm as shown in maturing lampreys (Fukayama and Takahashi, 1985; Sower et al., 1985; Linville et al., 1987) and are consistent with the presence of an ER in the male testes (Ho et al., 1987). While estradiol is considered to be a major steroid involved in reproductive processes, the precise function(s) of estradiol and its corresponding receptor(s) in both male and female lampreys need to be elucidated. There are still many questions remaining as to the type of steroids that are synthesized and their respective functions (reviewed in Bryan et al., 2008). For example, there is growing evidence that all lampreys produce gonadal steroids that are different from those of other vertebrates by possessing an additional hydroxyl group at the C15 position (Bryan et al., 2006, 2008).

In conclusion, the current study identifies for the first time expression of ER in the lamprey brain using both RT-PCR and *in situ* hybridization. A broad expression of ER was observed using *in situ* hybridization, including expression in the olfactory lobe, hypothalamus, habenular area, and hindbrain. Expression of ER in the hypothalamic region supports the possibility of an E2 feedback mechanism the GnRH system, similar to that observed in other vertebrate groups. By demonstrating ER expression in the lamprey brain using *in situ* hybridization, the current study provides a means for further investigation of possible mechanisms of interaction between E2 and GnRH in lamprey and other critical neurohormone systems.

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New insights into the control of pulsatile GnRH release: the role of Kiss1/neurokinin B neurons

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Gonadotropin-releasing hormone (GnRH) is the ultimate output signal of an intricate network of neuroendocrine factors that, acting on the pituitary, trigger gonadotropin release. In turn, gonadotropins exert their trophic action on the gonads to stimulate the synthesis of sex steroids thus completing the gonadotropic axis through feedback regulatory mechanisms of GnRH release. These feedback loops are predominantly inhibitory in both sexes, leading to tonic pulsatile release of GnRH from puberty onward. However, in the female, rising levels of estradiol along the estrous cycle evoke an additional positive feedback that prompts a surge-like pattern of GnRH release prior to ovulation. Kisspeptins, secreted from hypothalamic Kiss1 neurons, are poised as major conduits to regulate this dual secretory pathway. Kiss1 neurons are diverse in origin, nature, and function, convening distinct neuronal populations in two main hypothalamic nuclei: the arcuate nucleus (ARC) and the anteroventral periventricular nucleus. Recent studies from our group and others point out Kiss1 neurons in the ARC as the plausible generator of GnRH pulses through a system of pulsatile kisspeptin release shaped by the coordinated action of neurokinin B (NKB) and dynorphin A (Dyn) that are co-expressed in Kiss1 neurons (so-called KNDy neurons). In this review, we aim to document the recent findings and working models directed toward the identification of the Kiss1-dependent mechanisms of GnRH release through a synoptic overview of the state-of-the-art in the field.

Keywords: GnRH, kisspeptin, neurokinin B, estradiol, KNDy

INTRODUCTION

Reproductive function is an exquisitely regulated process that ensures perpetuation of the species. A plethora of central and peripheral signals interplay to modulate the coordinated action of the hypothalamus, pituitary, and gonads, which form the gonadotropic (aka HPG) axis. The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) is the ultimate central elicitor that conveys these regulatory signals down to the pituitary level to dictate the specific pattern of gonadotropin release (Fink, 2000). GnRH secretion varies significantly along the animal's life, being subjected to circadian and ultradian regulation, i.e., seasonal and developmental changes. For instance, GnRH secretion is enhanced at the time of puberty onset (Harris and Levine, 2003), leading to high frequency pulses that induce the awakening of the reproductive function in both males and females and are maintained from that period throughout the animal's lifespan. To note, GnRH displays a bimodal pattern of release: episodic versus surge-like (Maeda et al., 2010), where gonadal steroids play a crucial role. In this context, both sexes are subjected to the inhibitory action of these gonadal steroids through negative feedback mechanisms at the hypothalamic and pituitary level, which determine the tonic mode of GnRH secretion. In addition, adult females also exhibit a positive, estradiol-dependent, feedback of GnRH release as a unique surge-like peak that evokes ovulation by means of the consequent surge of luteinizing hormone (LH) release (Moenter et al., 1991). We are only starting to decipher the mechanisms underlying this dual regulatory process of GnRH release (Glidewell-Kenney et al., 2007), which is currently a subject of intense research in the field.

Focusing on the intermittent release of GnRH, the mechanism that dictates the amplitude and frequency of each pulse remains also poorly understood, being a matter of intense debate among neuroendocrinologists. Hence, in this review, we aim to offer a novel insight into a plausible mechanism of GnRH pulse generation through a comprehensive compilation of the latest advances in the field focusing on the interaction between kisspeptins and neurokinin B (NKB).

NEUROANATOMICAL DISTRIBUTION OF GnRH NEURONS

Gonadotropin-releasing hormone neurons present a scattered distribution in the brain, which has limited the thorough characterization of the physiological properties of this neuronal group. GnRH neurons originate in the olfactory placode. From embryonic day 9.5 onward (in rodents), they initiate a migratory process from this location to distinct brain areas that vary depending on the species (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). In rodents, mature GnRH cell bodies are present in the medial septum, preoptic area, and the anterior hypothalamic areas (Herbison,

2006). To note, a recent study has evidenced that the anatomical distribution of these neurons depends upon their birthdate, with neurons that undergo differentiation earlier in the embryonic life populating the rostral areas of the brain (Jasoni et al., 2009). GnRH neurons, regardless of the location of their cell bodies, project to the median eminence in a process guided by yet unknown factors from the medio-basal hypothalamus (Gibson et al., 2000). Therein, GnRH is secreted into the hypophyseal portal system to stimulate gonadotropin release.

CENTRAL CONTROL OF REPRODUCTIVE FUNCTION: THE ROLE OF Kiss1 NEURONS

Reproduction is an extremely energy costly process for the organism and, as such, is subjected to very tight regulatory mechanisms (Hill et al., 2008). As mentioned above, GnRH is the main factor that integrates the complex array of central and peripheral cues onto pituitary gonadotropes. However, GnRH neurons are not directly receptive to most of the major regulators of reproduction, e.g., metabolic cues, such as leptin, or gonadal factors such as sex steroids. In this context, Kiss1 neurons have been extensively documented in the last few years as major gatekeepers of reproductive function. Inactivating mutations in either *KISS1* or *KISS1R* genes (encoding kisspeptins and the Kiss1 receptor – formerly known as GPR54, respectively) lead to hypogonadotropic hypogonadism and infertility in both humans and mice (De Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003). Importantly, the vast majority of GnRH neurons express the Kiss1 receptor (Han et al., 2005) and Kiss1 neurons are sensitive to sex steroids as evidenced by the presence of estrogen receptor (ER)-α, ERβ, and androgen receptor (Smith et al., 2005a,b). Thus, Kiss1 neurons have been described to play a role as a common funnel to transmit essential information for reproductive viability down to the seemingly irresponsive GnRH neurons by means of kisspeptin release (Navarro and Tena-Sempere, 2011). To note, the population of Kiss1 neurons is sexually differentiated, being predominantly present at the arcuate nucleus (ARC) in both sexes (Gottsch et al., 2004), where they mediate the negative feedback of sex steroids upon GnRH release (Smith et al., 2005a) and the anteroventral periventricular area (AVPV/PeN) with greater density in females than in males, specially in rodents, where Kiss1 neurons are poised as elicitors of the preovulatory GnRH/LH surge through positive feedback mechanisms (Smith et al., 2005a; **Figure 1**). Importantly, the mechanism underlying this differential activation of Kiss1 neurons in the ARC versus the AVPV relies on the ability of estradiol to activate *Kiss1* transcription via estrogen response element (ERE)-independent pathways in the ARC, whereas its activation in the AVPV is mediated by the binding of the ER to the EREs in the *Kiss1* promoter, so-called "classical" route of ER action (Gottsch et al., 2009).

In this vein, in the absence of the inhibitory drive of sex steroids, GnRH shows a strictly episodic pattern of release in males (Caraty and Locatelli, 1988; Tilbrook and Clarke, 2001) and during most of the ovarian cycle in females (Levine and Ramirez, 1982), suggesting that similar mechanisms for pulse generation might exist in both sexes. Noteworthy, intermittent hormone release is a feature present in many neuroendocrine systems, including GnRH release, however, determining whether the final pattern of release is dictated by the neuron itself or through a synchronized circuitry that operates upon them, remains largely controversial.

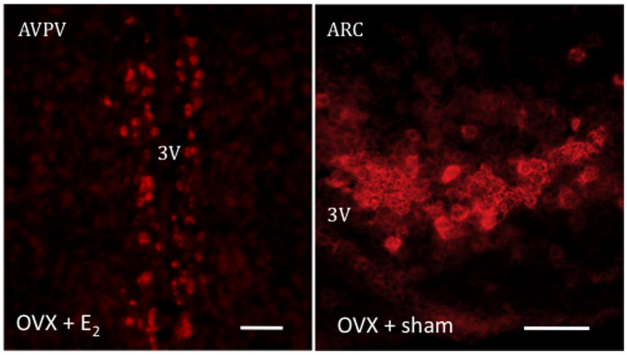

FIGURE 1 | Representative photomicrographs depicting an *in situ* hybridization assay for *Kiss1* mRNA, labeled using digoxigenin coupled with vector red. The expression of *Kiss1* mRNA is stimulated in the presence of E2 in the AVPV or its absence in the ARC. Scale bar 50 μm.

GnRH NEURONS AS PULSE GENERATORS

Over the last two decades, a number of studies have suggested that GnRH neurons themselves present a pattern of firing bursts that could, indeed, account for the pulsatile release of this decapeptide. First, the GnRH cell line, GT1, exhibits spontaneous pulses of neuronal activation and GnRH secretion in the absence of any other cell line (Martinez De La Escalera et al., 1992; Bosma, 1993). Second, embryonic GnRH cells obtained from the nasal placode of rats displayed a pulsatile fashion of GnRH release (Funabashi et al., 2000).

This rhythmic pattern of GnRH release seems to be Ca^{2+}-dependent (Krsmanovic et al., 1992). Indeed, cultured embryonic GnRH cells from monkeys exhibit synchronized Ca^{2+} oscillations every 50–70 min, which is similar to the interval of GnRH pulses observed *in vivo* (Terasawa et al., 1999). Moreover, this process is stimulated by cAMP (Krsmanovic et al., 2001) in a mechanism mediated by specific A-kinase anchoring proteins (AKAPs; Chen et al., 2010). However, a recent study by Frattarelli et al. (2011) indicates that in GT1-7 cells, only 25% of GnRH and cAMP pulses coincide, suggesting that while cAMP may positively regulate GnRH secretion, it does not participate in the mechanism of pulsatile GnRH release. Another remarkable feature of GnRH neurons is their ability to synchronize the release of GnRH with time in culture through gap junction coupling (Bose et al., 2010). This, together with the fact that GnRH neurons do express the GnRH receptor and present auto-feedback loops in approximately 50% of them (Han et al., 2010) may suggest the presence of auto-regulatory pathways. Nonetheless, these mechanisms remain to be validated *in vivo* since the close proximity of GnRH neurons in culture is not present in the brain where, as mentioned above, GnRH neurons occupy a disperse distribution that may compromise direct interactions between them.

Despite these evidences signifying the presence of an internal pacemaker, the inability of GnRH neurons to modify its pattern of release under critical physiologic conditions for the regulation

of the reproductive axis such us puberty onset, variation in the sex steroid milieu, etc., makes the presence of additional regulatory mechanisms – able to respond to a wider array of regulators, mandatory.

GnRH PULSE GENERATOR OUTSIDE GnRH NEURONS

We could postulate that GnRH neurons, as pacemaker neurons, would need the coordinated interaction of an ensemble of regulatory factors that led to the fine-tuning of GnRH secretion. Indeed, mounting data support this contention. The presence of a master GnRH pulse generator has been localized within the medio-basal hypothalamus, pointing to the ARC as the most likely candidate. First, female rhesus monkeys bearing bilateral lesions in the medio-basal hypothalamus showed absence of endogenous GnRH/LH pulses (Pohl et al., 1983). Second, hypothalamic explants of monkeys and rats devoid of GnRH cell bodies exhibit GnRH pulses (Purnelle et al., 1997; Woller et al., 2010). Third, despite some discrepancies in the field, endogenous pulses of GT1-7 cells seem to present a cadence of 21–26 min (Bosma, 1993; Moenter et al., 2003) while *in vivo*, GnRH pulses present 40–60 min intervals depending on the species (Han et al., 2010). Fourth, electrophysiological recordings of multi-unic activity (MUA) volleys located in the ARC, invariably resemble LH pulses in different species (Goubillon et al., 1995; Ohkura et al., 2009). Fifth, as mentioned previously, GnRH neurons themselves do not express ER alpha (Hrabovszky et al., 2000). Therefore, they are not able to properly respond to changes in the sex steroid milieu, which constitutes a major regulatory drive to the gonadotropic axis. Indeed, in the absence of negative feedback of sex steroids after gonadectomy, or menopause in primates, GnRH pulses are significantly increased (Gore et al., 2004). Altogether, there are sufficient evidences to locate the primary generator of GnRH pulses in the ARC. However, we cannot rule out that other mechanisms may play a role in regulating GnRH discharges and that they could be placed in different brain regions, e.g., inputs coming from the suprachiasmatic nucleus (SCN) through vasoactive intestinal polypeptide (VIP; Krajnak et al., 1998; Gerhold et al., 2005) or GABAergic and glutamatergic interactions (Chu and Moenter, 2005) among those possible pathways.

Kiss1 NEURONS AS HOLDERS OF THE GnRH PULSE GENERATOR

The ARC presents a complex array of neuronal networks that participate in the control of reproduction and/or metabolism. Among those, the subpopulation of Kiss1 neurons located in this nucleus could serve the function of GnRH pulse generator based on the following findings: (a) GnRH neurons express the Kiss1 receptor and are activated by kisspeptins (Han et al., 2005); (b) Kiss1 neurons from the ARC contact GnRH terminals in the median eminence of rats and sheep (Matsuyama et al., 2011; Uenoyama et al., 2011); (c) kisspeptin release to the portal system in monkeys is pulsatile (Keen et al., 2008); (d) MUA volleys in goats, that closely reproduce LH pulses, are recorded from the surroundings of Kiss1 neurons and are not affected after exogenous kisspeptin treatment unlike LH pulses (Ohkura et al., 2009); (e) the number of *Kiss1*-expressing neurons in the ARC of lambs is greater in animals exhibiting higher frequency of LH pulses (Redmond et al., 2011); (f) the increase in GnRH neuronal firing observed in menopausal primates due to the absence of sex steroids (Gore et al., 2004) correlates with the higher level of *Kiss1* expression in the infundibular nucleus of post-menopausal women and primates (Rometo et al., 2007; Eghlidi et al., 2010; Hrabovszky et al., 2011). To note, earlier reports documented that the pulsatile release of GnRH is blunted after prolonged periods of estradiol withdrawal, e.g., older post-menopausal women (Hall et al., 2000), which may reflect the potential decrease in *Kiss1* expression in the ARC produced by aging, as was recently documented in old acyclic rats (Downs and Wise, 2011) although, intriguingly, it does not seem to be the case in post-menopausal women where kisspeptin immunoreactivity remains elevated well beyond menopause (Hrabovszky et al., 2011), suggesting possible species differences in the regulation of *Kiss1* expression in aged individuals; (g) the administration of a kisspeptin antagonist into the ARC – but not the POA, strongly suppresses LH pulses in rats (Li et al., 2009); (h) the administration of intravenous kisspeptin pulses to agonadal juvenile monkeys elicits a sustained train of GnRH discharges (Plant et al., 2006). In addition, (i) an elegant study by Gottsch et al. (2011) has recently demonstrated by whole-cell patch recordings that Kiss1 neurons, isolated from *Kiss1*-CreGFP mice, present spontaneous activity as well as h- and T-type Ca^{2+} currents (**Figure 2**), which are typical features of pacemaker neurons. However, a note of caution should be added regarding possible species differences in the generation of GnRH pulses. Thus, a recent study by Chan et al. (2011) demonstrates that iv administration of kisspeptin to adult men not only stimulates LH release but delays the occurrence of the next LH pulse by an interval similar to that of normal interpulse intervals, which would suggest a reset of the GnRH pulse generator.

THE ROLE OF NEUROKININ B IN THE GENERATION OF KISSPEPTIN PULSES

Neurokinin B has recently emerged as a critical player in the central control of reproductive function. In 2009, human genetic studies showed that patients bearing inactivating mutations in the genes encoding NKB or its receptor (neurokinin 3 receptor, NK3R), *TAC3* and *TACR3* respectively in humans, exhibit hypogonadotropic hypogonadism and infertility (Topaloglu et al., 2009). These findings have been also (partially) recapitulated in *Tacr3* null mice (Yang et al., 2012) indicating that the NKB/NK3R system plays a role in the control of gonadotropin secretion in different species. Initial reports denoted an inhibitory action of an NKB agonist, senktide, on LH release in rodents (Sandoval-Guzman and Rance, 2004; Navarro et al., 2009), however, we have recently documented high sensitivity of the NKB/NK3R system to circulating levels of sex steroids, which allows LH release under the appropriate steroid milieu in prepubertal and adult female rats (Navarro et al., 2011a, 2012). In addition, to date, a stimulatory role of NKB on LH release has also been reported in mice, monkeys, goats, and sheep (Billings et al., 2010; Ramaswamy et al., 2010; Wakabayashi et al., 2010; Navarro et al., 2011b). Recently, though, a report by Kinsey-Jones and colleagues showed otherwise. In their study, senktide exerted a decrease in LH release in ovariectomized (OVX) and estradiol-replaced female rats (Kinsey-Jones et al., 2012). Notwithstanding, their estradiol replacement seemingly failed to decrease LH release after ovariectomy, which is a required

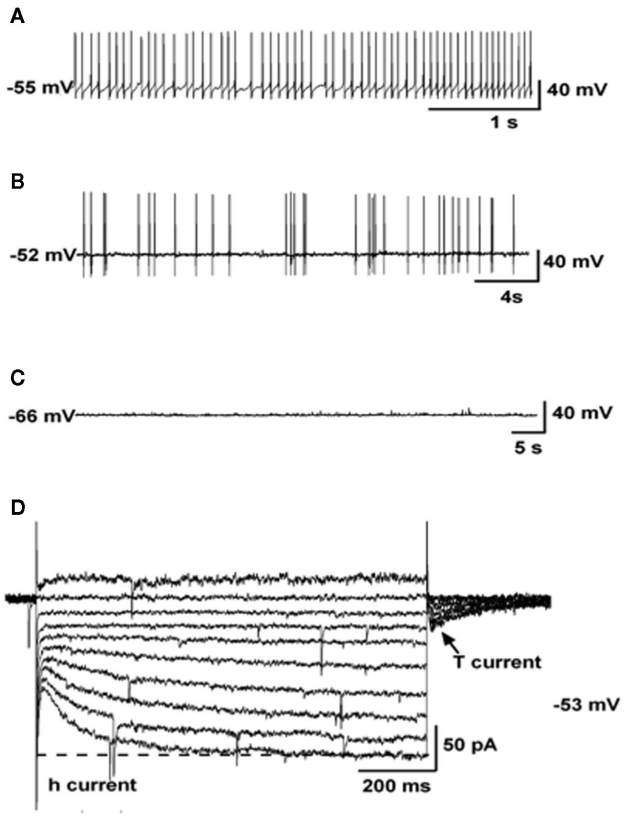

FIGURE 2 | Electrophysiological characteristics of arcuate Kiss1 neurons in oil-treated OVX *Kiss1-CreGFP* mice using whole-cell patch recording. Kiss1 neurons in the ARC (of the female) rest at -63.8 ± 2.3 mV ($n = 20$). **(A–C)**, Representative traces of action potentials recorded from arcuate Kiss1 neurons showing tonic **(A)**, irregular **(B)**, and silent **(C)** firing patterns. **(D)**, Ensemble of currents in response to depolarizing/hyperpolarizing steps from −50 to −140 mV illustrating the expression of a hyperpolarization-activated cation current (h-current) and a T-type Ca^{2+} current (*arrow*) in a representative Kiss1 neuron. $V_{hold} = -60$ mV. Adapted with permission from Gottsch et al. (2011).

proof of the proper action of the negative feedback of estradiol and might explain this *a priori* controversy. Therefore, assuming that these animals reproduced an OVX+ sham model and considering the stimulation of LH release that they also observed in intact diestrus rats, these results would actually be in keeping with our previous study in female rats (Navarro et al., 2011a). Regarding its anatomical distribution, the genes encoding NKB in rodents (*Tac2*) and NK3R (*Tacr3*) are enormously spread out throughout the entire brain (Warden and Young, 1988; Shughrue et al., 1996; Navarro et al., 2011a), where they have been proposed to mediate a number of physiological processes (Pantaleo et al., 2010); however, regarding the areas known to hold neuromodulators of reproductive function, we could highlight the ARC, ventromedial (VMN), and paraventricular (PVN) nuclei as well as the lateral hypothalamic area (LHA; Navarro et al., 2011a). Importantly, not only NKB is expressed in the ARC but virtually all Kiss1 neurons in this nucleus (but not in the AVPV/PeN) co-express NKB – and Dynorphin A (Goodman et al., 2007; Navarro et al., 2009; Wakabayashi et al., 2010), which has served the scientific community to rename these neurons as KNDy neurons (Lehman et al., 2010). Moreover, KNDy neurons do express NK3R (Burke et al., 2006; Navarro et al., 2009) facilitating autosynaptic loops within the dense network of NKB/kisspeptin fibers present in the ARC as documented in rodents (Burke et al., 2006; Krajewski et al., 2010; Yeo and Herbison, 2011). Indeed, the following pieces of evidence support the contention that NKB acts upstream of, or immediately on, Kiss1 neurons in the ARC to exert its regulatory action on reproductive function: (a) central administration of the NKB agonist senktide to gonadectomized and estradiol-replaced female rats induces *C-fos* mRNA expression in Kiss1 neurons (Navarro et al., 2011a); (b) senktide and NKB profoundly depolarize Kiss1 neurons measured by whole-cell patch recording, phenomenon that is prevented by the NKB antagonist SB222200 (Navarro et al., 2011b); (c) animals bearing a non-functional Kiss1 receptor (*Kiss1r* KO mice) do not respond to the central administration of senktide in terms of LH release while wild-type controls do (García-Galiano et al., 2012); (d) central administration of NKB to goats induces a clear increase in the frequency and amplitude of MUA volleys in the ARC, which were invariably mirrored by LH pulses (Wakabayashi et al., 2010; **Figure 3**), yet the administration of kisspeptin modified LH pulses without modifying MUA volleys (Ohkura et al., 2009); (e) juvenile monkeys showing blunted LH responses to senktide due to NK3R desensitization still respond to kisspeptin, however, those subjected to KISS1R desensitization showed virtual absence of LH response to senktide, although admittedly, a small residual response to senktide was observed in this model suggesting a likely (minor) kisspeptin-independent action of senktide on GnRH release in the monkey (Ramaswamy et al., 2010, 2011). Altogether, these data make the case for a very likely action of NKB upon Kiss1 neurons, however, we cannot rule out the action of this peptide on additional brain areas. In this vein, preliminary data showed expression of NK3R in GnRH terminals of rats (Krajewski et al., 2005; Burke et al., 2006) and *Tacr3* mRNA in GnRH neurons in the mouse (Todman et al., 2005) as well as in the immortalized GT1-7 cells – a model of differentiated GnRH neurons (Glidewell-Kenney et al., 2010). However, these results are controversial. On the one hand, a call of caution needs to be exercised when using immortalized cell lines, which may not faithfully resemble *in vivo* models and, on the other hand, the latter data in mice and rats seem to be rebutted by recent studies documenting the absence of NK3R immunolocalization and *Tacr3* mRNA in GnRH neurons of sheep (Amstalden et al., 2009) and mice (Navarro et al., 2011b), respectively. Furthermore, the lack – or residual expression – of NK3R in GnRH neurons is supported by (1) the very illustrative studies of desensitization of NK3R versus Kiss1r in the agonadal juvenile monkey mentioned above, where kisspeptin-desensitized monkeys displayed a robust suppression (albeit not complete) of the LH response to senktide (Ramaswamy et al., 2011) and (2) the lack of action potentials in GnRH–GFP neurons subjected to whole-cell recordings after senktide treatment in the mouse (Navarro et al., 2011b). It is worth mentioning that this study shows lack of activity at the GnRH cell body level but does not rule out the presence of NK3R at the level of GnRH terminals. In this sense, (3) Corander et al. (2010) showed that, unlike kisspeptin, addition of NKB to hypothalamic explants from male rats devoid

of GnRH cell bodies did not evoke any effect on GnRH release, which, initially, would preclude a direct action at the level of these terminals.

In any case, the number of NKB neurons in the ARC of male mice seems to be twice that of the Kiss1 neurons (Navarro et al., 2011b). This, taken together with the fact that NKB fibers, at least in mice and rats, project to neighboring hypothalamic areas of the ARC – including bilateral projections within the ARC and to the median eminence, periventricular nucleus, BNST, dorsomedial nucleus, lateral hypothalamus, and rostral preoptic area (Burke et al., 2006; Krajewski et al., 2010; Yeo and Herbison, 2011) – suggests the existence of possible, yet unknown, additional regulatory pathways.

In this intricate machinery of co-transmitters of kisspeptin, the role of dynorphin remains far less characterized. Dynorphin has been long recognized as an inhibitor of gonadotropin release in a number of species (Schulz et al., 1981; Kinoshita et al., 1982). Importantly, based on recent studies in the goat, the role of dynorphin seems to lay upstream of the Kiss1 neuron. Thus, central administration of the dynorphin receptor (*Kappa* opiod receptor, KOR) agonist, U50488, strongly suppresses MUA volleys in the ARC and LH pulses (Wakabayashi et al., 2010; **Figure 3**). In this same direction, the administration of a KOR antagonist, nor-BNI, increases the frequency of MUA volleys that is mirrored by the same increase in LH pulses (Wakabayashi et al., 2010; **Figure 3**). Therefore, these data suggest that dynorphin participates to shut down Kiss1 neurons, hence blocking kisspeptin–GnRH–LH release. Nonetheless, in this scenario, how (and where) dynorphin plays its role remains controversial. It is clear that the action of dynorphin eventually turns back to Kiss1 neurons through direct (autosynaptic contact) or indirect (intermediate neurons) mechanisms. Latest evidences seem to support the latter alternative. We have recently documented that Kiss1 neurons in the ARC of male mice are virtually devoid of KOR (less than 6%; Navarro et al., 2011b), while females displayed a slightly greater percentage of colocalization (∼20%; Navarro et al., 2009), indicating the possible existence of a marginal subset of Kiss1 neurons in the ARC expressing KOR. In any case, even if KOR is present in a reduced number of Kiss1 neurons, the primary place of action of dynorphin seems to reside outside and upstream of Kiss1 neurons (**Figure 4**) since GnRH neurons are also devoid of KOR (Mitchell et al., 1997; Sannella and Petersen, 1997); however, the identity of this neuronal population – that must project back to Kiss1 neurons, remains a mystery.

Altogether, there is compelling evidence to infer that kisspeptin release is pulsatile and that those pulses correlate with GnRH/LH pulses, which, presumably, are dependent on the former. Consequently, we have proposed a model whereby KNDy neurons, in the absence of the inhibitory drive of sex steroids, would activate the NKB pathway and release this neuropeptide on the same KNDy neuron through recurrent collaterals as well as to nearby NKDy neurons (**Figure 4**). This feature might be of crucial importance for the proper functioning of this model, playing a critical role in the synchronization of KNDy neurons. In this sense, in order to present unique GnRH pulses, the population of KNDy neurons must evoke a unique kisspeptin pulse, which is initiated by a spreading NKB activating wave across the ARC. This possibility is in keeping with the dense network of NKB fibers surrounding KNDy neurons in the ARC, as commented above (Burke et al.,

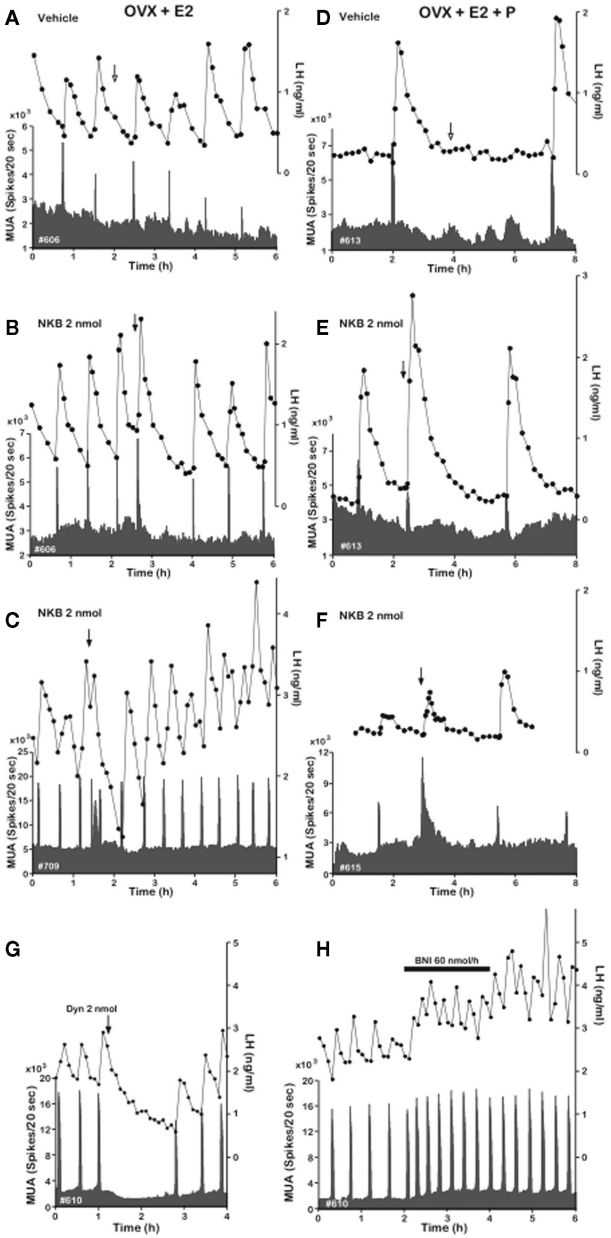

FIGURE 3 | Effect of NKB on MUA and plasma LH in OVX-plus-E_2- and OVX-plus-E_2-plus-P-treated goats. Representative profiles of MUA and plasma LH concentrations in OVX-plus-E_2 **(A–C)** and OVX-plus-E_2-plus-P **(D–F)** goats that received an intracerebroventricular injection of vehicle **(A,D)** or 2 nmol **(B,C,E,F)** of NKB are shown. Also, effects of Dyn and nor-BNI on the MUA and plasma LH in the OVX goat are included. **(G)**, Representative profiles of MUA and plasma LH concentrations in one animal that received an intracerebroventricular injection of 2 nmol of Dyn are shown. The arrow indicates timing of injection. **(H)**, Representative profiles of MUA and plasma LH concentrations in one animal that received an intracerebroventricular infusion of nor-BNI at a rate of 60 nmol 600 μl^{-1} h^{-1} for 2 h are shown. The bar indicates the period of infusion. Adapted with permission from Wakabayashi et al. (2010).

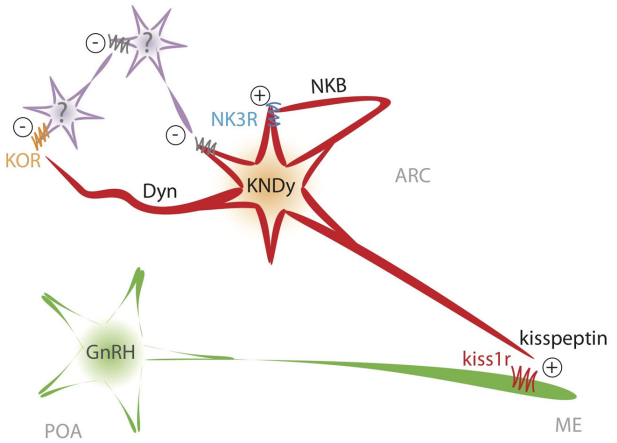

FIGURE 4 | Schematic representation of the proposed model of interaction between NKB and kisspeptin in the ARC to shape GnRH/LH pulses.

2006; Krajewski et al., 2010; Yeo and Herbison, 2011). Next, the activation of the NKB receptor, NK3R, initiates the process of kisspeptin and dynorphin secretion. Kisspeptin would then reach the kisspeptin receptor at the GnRH terminals to induce GnRH release to the median eminence (**Figure 4**). To note, there is no direct evidence of the presence of Kiss1r at the level of GnRH terminals, however, this could be inferred based on the observation that kisspeptin can elicit GnRH secretion from explants of the mediobasal hypothalamus, which contains few, if any, GnRH cells bodies (Irwig et al., 2004; Thompson et al., 2004; Castellano et al., 2006; D'Anglemont De Tassigny et al., 2008) and the close apposition of kisspeptin fibers at the GnRH terminals (Matsuyama et al., 2011; Uenoyama et al., 2011). At the same time, dynorphin, acting mainly on yet unknown intermediate neurons, feeds back to Kiss1 neurons through inhibitory signals – dependent on G_i/G_0 protein coupling (Schoffelmeer et al., 1988) – that eventually shut down NKB and kisspeptin release. In this situation, the cease in NKB secretion terminates the release of dynorphin and, therefore, the action of the inhibitory drive. As a consequence, NKDy neurons would become readily available to re-start NKB secretion, then leading to a new pulse of kisspeptin (**Figure 4**). However, one could be tempted to hypothesize that KNDy neurons are, most likely, not able to discriminate when to secrete one neurotransmitter or another after a process of neuronal activation and, therefore, all these factors (kisspeptin, NKB, and dynorphin) could be released at the same time after a common burst comprising all KNDy neurons. If this were true, it could mean a critical limitation of the model. Although this is merely speculative, current data indicates that this limitation could, at least partially, be overcome because of the restricted sites of action of each of those factors in the context of the generation of GnRH pulses. (a) Kisspeptin would act only on GnRH neurons, since KNDy neurons themselves in the ARC are devoid of Kiss1 receptor (D'Anglemont De Tassigny et al., 2008) and therefore do not respond to kisspeptin (Navarro et al., 2011b); (b) NKB would act mainly on KNDy neurons since GnRH neurons (at least in mice and sheep) do not express detectable levels of NK3R (Billings et al., 2010; Navarro et al., 2011b); (c) GnRH neurons do not express KOR (Mitchell et al., 1997; Sannella and Petersen, 1997) and KNDy neurons might do it only in a low percentage (Navarro et al., 2009, 2011b), then indicating that dynorphin must act upon intermediate neurons, increasing the turn around time of the inhibitory signal onto KNDy neurons (**Figure 4**). This would lead to a short delay period after the stimulation of NKB, therefore allowing for the appearance of a kisspeptin pulse.

CONCLUSION AND FUTURE PERSPECTIVES

During the last few years, the data supporting the pivotal role of kisspeptin in the regulation of GnRH release in a wide range of species is so consistent that we can ensure this phenomenon without a doubt. In this manuscript, we have offered a comprehensive review of the latest advances in the physiology and mechanism of action of Kiss1 neurons in the ARC, adding an intriguing new role to this neuronal group: serve as the GnRH pulse generator, whose characterization has remained elusive to neuroendocrinologists for decades. As a note of caution, we do not position this mechanism as the only center governing GnRH release. There are, indeed, evidences to believe that in the absence of Kiss1 signaling there is a residual, but significant, stimulatory drive onto GnRH neurons from an unknown source (Chan et al., 2009). Thus, it is feasible that a more complex network of neuronal circuits interplays to exert their regulation on GnRH release under specific physiologic conditions, ensuring the appropriate response of the gonadotropic axis to those circumstances. For this reason, we do believe that the tandem Kisspeptin/NKB is essential for the tonic episodic release of GnRH while yet there are significant open questions in this model that await to be addressed in the upcoming years. For example, it would be of great interest to decipher what the genuine role of dynorphin in this model is and what interneurons it is acting on. In addition, it is known that GnRH neurons exhibit a prolonged period of activation after a single kisspeptin pulse (Han et al., 2005), therefore, there must be a factor that actively block kisspeptin's action on GnRH neurons after every pulse, whose nature is to date unidentified.

In sum, kisspeptin and NKB play a fundamental role in the tonic release of GnRH; however, further research is needed to offer a more detailed insight into the exact process of generation of GnRH pulses.

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Sexual dimorphism of kisspeptin and neurokinin B immunoreactive neurons in the infundibular nucleus of aged men and women

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†The authors dedicate this article to the memory of our colleague Dr. László Sarkadi who has recently passed away.

The secretory output of gonadotropin-releasing hormone (GnRH) neurons is critically influenced by peptidergic neurons synthesizing kisspeptins (KP) and neurokinin B (NKB) in the hypothalamic infundibular nucleus (Inf). These cells mediate negative feedback effects of sex steroids on the reproductive axis. While negative feedback is lost in postmenopausal women, it is partly preserved by the sustained testosterone secretion in aged men. We hypothesized that the different reproductive physiology of aged men and women is reflected in morphological differences of KP and NKB neurons. This sexual dimorphism was studied with immunohistochemistry in hypothalamic sections of aged human male (≥50 years) and female (>55 years) subjects. KP and NKB cell bodies of the Inf were larger in females. The number of KP cell bodies, the density of KP fibers, and the incidence of their contacts on GnRH neurons were much higher in aged women compared with men. The number of NKB cell bodies was only slightly higher in women and there was no sexual dimorphism in the regional density of NKB fibers and the incidence of their appositions onto GnRH cells. The incidences of NKB cell bodies, fibers, and appositions onto GnRH neurons exceeded several-fold those of KP-IR elements in men. More NKB than KP inputs to GnRH cells were also present in women. Immunofluorescent studies identified only partial overlap between KP and NKB axons. KP and NKB were colocalized in higher percentages of afferents to GnRH neurons in women compared with men. Most of these sex differences might be explained with the lack of estrogen negative feedback in aged women, whereas testosterone can continue to suppress KP, and to a lesser extent, NKB synthesis in men. Overall, sex differences in reproductive physiology of aged humans were reflected in the dramatic sexual dimorphism of the KP system, with significantly higher incidences of KP-IR neurons, fibers and inputs to GnRH neurons in aged females vs. males.

Keywords: immunohistochemistry, infundibulum, reproduction, gonadotropin-releasing hormone

INTRODUCTION

Neurons synthesizing gonadotropin-releasing hormone-I (GnRH) represent the final common pathway of the hypothalamus in the neuroendocrine regulation of reproduction. The pulsatile pattern of GnRH secretion into the hypophysial portal circulation is shaped by a sex steroid-sensitive neuronal circuitry that acts upstream from GnRH cells (Christian and Moenter, 2010). In both males (Tilbrook and Clarke, 2001) and females (Moenter et al., 2009), gonadal steroid hormones exert homeostatic negative feedback on GnRH release via this upstream circuitry. In females, elevated estradiol in the late follicular phase of the reproductive cycle causes a switch from negative to positive feedback to induce a surge of GnRH from the hypothalamus. The subsequent surge of luteinizing hormone from the adenohypophysis triggers ovulation (Christian and Moenter, 2010).

Much research effort over the past decades has been invested in the identification of the neuronal circuitry that mediates the negative and positive feedback effects of gonadal steroid hormones to the GnRH neuronal system. Hypothalamic neurons synthesizing kisspeptins and NKB are recently recognized key players of this neuronal network. Loss-of-function mutations of the genes encoding kisspeptin receptor (KISS1R; de Roux et al., 2003; Seminara et al., 2003; Semple et al., 2005), NKB (Topaloglu et al., 2009), or the receptor for NKB (NK3; Guran et al., 2009; Topaloglu et al., 2009) cause hypogonadotropic hypogonadism in the human. Kisspeptins regulate reproduction via direct stimulatory actions on GnRH neurons. Accordingly, GnRH

neurons receive kisspeptin-immunoreactive (IR) afferent contacts (Kinoshita et al., 2005; Clarkson and Herbison, 2006; Ramaswamy et al., 2008; Smith et al., 2008; Hrabovszky et al., 2010), express KISS1R mRNA (Irwig et al., 2004; Han et al., 2005; Messager et al., 2005) and respond to kisspeptins with depolarization (Han et al., 2005; Dumalska et al., 2008; Pielecka-Fortuna et al., 2008) and cFos expression (Irwig et al., 2004; Matsui et al., 2004; Kauffman et al., 2007). Similarly to kisspeptins, NKB acts upstream from the GnRH neuron. Neurokinin B-IR axons form frequent intranuclear contacts with other NKB-IR neurons that express the NKB receptor (NK3) in the arcuate nucleus (ARC; Burke et al., 2006; Navarro et al., 2009; Amstalden et al., 2010). In addition, the possibility exists that NKB influences GnRH secretion from the hypothalamic median eminence where GnRH-IR axons are apposed to NKB-IR axons (Krajewski et al., 2005; Ciofi et al., 2006) and express NK3 immunoreactivity (Krajewski et al., 2005) in the rat.

As reviewed recently (Lehman et al., 2010b), kisspeptin synthesizing neurons in various mammalian species have been localized to two major anatomical sites, the preoptic region and the ARC. Neurokinin B neurons have a wider distribution. A role in reproduction is likely attributable to NKB neurons of the ARC which also synthesize kisspeptins and dynorphins in several species (Goodman et al., 2007; Navarro et al., 2009; Cheng et al., 2010).

The kisspeptin cell population of the preoptic region exhibits conspicuous anatomical variations among species. In laboratory rodents, they form a compact nucleus in the rostral periventricular area of the third ventricle (RP3V; Gottsch et al., 2004; Smith et al., 2005a, 2006b; also referred to as kisspeptin neurons of the anteroventral periventricular nucleus; AVPV) and exhibit a robust sexual dimorphism. They occur in higher numbers (Clarkson and Herbison, 2006; Adachi et al., 2007; Kauffman et al., 2007; Ansel et al., 2010) and provide input to a higher percentage of GnRH neurons (Clarkson and Herbison, 2006) in females compared with males. There is strong evidence suggesting that in rodents, the kisspeptin cell population of the RP3V is critically involved in positive estrogen feedback to GnRH neurons (Adachi et al., 2007; Herbison, 2008). Kisspeptin synthesizing neurons are more scattered and occur in lower numbers in the preoptic region of the sheep (Franceschini et al., 2006; Goodman et al., 2007), the monkey (Smith et al., 2010), and the human (Hrabovszky et al., 2010). The issue of whether preoptic kisspeptin neurons of these species are functionally homologous to kisspeptin neurons of the rodent RP3V is debated, given that positive estrogen feedback in sheep (Caraty et al., 1998) and primates (Krey et al., 1975; Hess et al., 1977; Knobil, 1980) is thought to take place in the ARC (infundibular nucleus; Inf, in humans).

The second kisspeptin synthesizing cell group can be consistently detected within the ARC in all animal species studied (Gottsch et al., 2004; Irwig et al., 2004; Han et al., 2005; Kinoshita et al., 2005; Shahab et al., 2005; Smith et al., 2005a, 2007, 2008, 2009, 2010; Clarkson and Herbison, 2006; Estrada et al., 2006; Franceschini et al., 2006; Revel et al., 2006; Adachi et al., 2007; Goodman et al., 2007; Greives et al., 2007; Mason et al., 2007; Rometo et al., 2007; Decourt et al., 2008; Ramaswamy et al., 2008, 2010; Clarkson et al., 2009; Kauffman, 2009; Magee et al., 2009; Ohkura et al., 2009; Takase et al., 2009; Ansel et al., 2010; Cheng et al., 2010; Desroziers et al., 2010; Wakabayashi et al., 2010) and in the analogous Inf of the human (Rometo et al., 2007; Hrabovszky et al., 2010). Unlike the preoptic kisspeptin cell population, these kisspeptin neurons also synthesize NKB in sheep (Goodman et al., 2007; Cheng et al., 2010), goat (Wakabayashi et al., 2010), mice (Navarro et al., 2009), monkey (Ramaswamy et al., 2010), and human (Hrabovszky et al., 2010). The recently introduced terminology of KNDy neurons (Cheng et al., 2010) refers to the co-synthesis of dynorphins by the majority of these kisspeptin/NKB cells at least in the sheep (Goodman et al., 2007; Cheng et al., 2010), the goat (Wakabayashi et al., 2010), the mouse (Navarro et al., 2009), and the rat (Burke et al., 2006). Evidence from studies of sheep suggests that KNDy neurons of the ARC play an important role in conveying the negative feedback effects of sexual steroids onto GnRH neurons (Smith et al., 2007), and possibly, also the positive feedback effects of estrogens (Lehman et al., 2010a) in this species. Thus, KNDy neurons appear to constitute an important component of the GnRH pulse generator (Navarro et al., 2009; Wakabayashi et al., 2010).

The sex-specific spatial and temporal patterns of hypothalamic kisspeptin expression strongly depend on the activational effects of sexual steroid hormones. Large subsets of kisspeptin neurons in both the preoptic region and the ARC contain receptors for estradiol, testosterone, and progesterone in various species (Smith et al., 2005a,b, 2006b, 2007; Franceschini et al., 2006; Adachi et al., 2007; Clarkson et al., 2008; Cheng et al., 2010). In rodents, androgens and estrogens upregulate kisspeptin expression in the RP3V (Smith et al., 2005a,b, 2006a; Adachi et al., 2007; Kauffman et al., 2007) at the site of positive estrogen feedback. In contrast, kisspeptin expression in the ARC/Inf is regulated negatively by sex steroid hormones in rodents and other species (Smith et al., 2005a,b, 2006a; Adachi et al., 2007; Kauffman et al., 2007; Kim et al., 2009; Eghlidi et al., 2010) and so is NKB expression at this site (Rance and Young, 1991; Abel et al., 1999; Danzer et al., 1999; Dellovade and Merchenthaler, 2004; Eghlidi et al., 2010).

The sex-dependent pattern of hypothalamic kisspeptin expression also depends on the organizational effects of testosterone exposure in males, in addition to the circulating levels of sex steroid hormones. There is a neonatally determined robust sexual dimorphism of the RP3V in adult rats (Kauffman et al., 2007) and mice (Clarkson and Herbison, 2006), with higher cell numbers in females compared with males. Prenatal testosterone exposure accounts for a similar sexual dimorphism of kisspeptin neurons in the preoptic area of the sheep (Cheng et al., 2010).

In contrast, no apparent organizational effects of the perinatal testosterone exposure have been observed on the abundance of kisspeptin neurons in the rodent ARC which contains similar kisspeptin cell numbers in intact males and in diestrous females, or in gonadectomized males and females receiving the same estrogen or testosterone regimen (Kauffman et al., 2007). No sex difference appears to exist in the number of NKB neurons either. However, in the rat a sexual dimorphism that develops under the organizational effects of sex steroids has been reported in the projection fields of NKB-IR axons (Ciofi et al., 2006). Unlike in rodents, the ARC of the female sheep contains higher NKB (Goubillon et al., 2000) and kisspeptin (Cheng et al., 2010) cell numbers, compared with males. Moreover, in our previous work we have also identified higher kisspeptin neuron and fiber densities in the Inf of women vs. men (Hrabovszky et al., 2010); it requires clarification to what

extent this sexual dimorphism reflects the organizational effects of sex steroids during development or the difference in the adult hormonal status between men and women.

Reproductive aging is accompanied by sex-specific neuromorphological alterations of the Inf. While gonadal functions in aging men can be well-preserved throughout life (Araujo and Wittert, 2011), the negative feedback response of the reproductive axis to testosterone shows a declining trend (Veldhuis et al., 2010). This reduced feedback may be correlated with a mild neuronal hypertrophy in the Inf of aged men (Rance et al., 1993). Reproductive aging is more dramatic in postmenopausal women after the depletion of ovarian follicles, leading to the loss of circulating estrogen and causing the reduction of negative estrogen feedback (Rance, 2009). Comparison of histological samples from pre- and postmenopausal women revealed profound anatomical changes in the Inf where negative feedback is thought to take place (Rance, 2009). Accordingly, *in situ* hybridization studies identified the postmenopausal hypertrophy of neurons that express estrogen receptor alpha (Rance et al., 1990), substance P (Rance and Young, 1991), NKB (Rance and Young, 1991), kisspeptins (Rometo et al., 2007), and prodynorphin (Rometo and Rance, 2008). These morphometric alterations were also associated with increased NKB (Rance and Young, 1991) and kisspeptin (Rometo et al., 2007) and decreased prodynorphin mRNA expression (Rometo and Rance, 2008) at this site.

In the present study we address several immunohistochemical correlates of the functional differences that characterize the kisspeptin and NKB neuronal systems of the Inf in aged men and women. The comparative analysis of *post mortem* histological samples from intact human male and female subjects reveals sex differences that likely represent the combined organizational and activational effects of sex steroids. The following parameters will be analyzed and compared:

(i) *Perikaryon size of kisspeptin-IR and preproNKB-IR neurons*
Previous morphometric studies established a robust postmenopausal hypertrophy of NKB (Rance and Young, 1991) and kisspeptin (Rometo et al., 2007) mRNA expressing neurons that were identified with *in situ* hybridization. A mild neuronal hypertrophy also occurs with aging in unidentified neurons of the Inf in men (Rance et al., 1993). Considering the negative feedback effect of the continued testosterone exposure, we predict smaller kisspeptin-IR and NKB-IR neuronal perikarya in aged men vs. women. In the present study, we measured and compared the profile area of kisspeptin-IR and NKB-IR neurons between aged male ($\geq$ 50 years) and postmenopausal female ($\geq$ 55 years) subjects.

(ii) *Incidence of kisspeptin-IR and preproNKB-IR cell bodies in the Inf*
A recent immunohistochemical study from our laboratory detected significantly more kisspeptin-IR cell bodies in the Inf of women vs. men of various ages (Hrabovszky et al., 2010). Here we repeated this study on histological samples derived from aged men and postmenopausal women. We extended the quantitative analysis to preproNKB-IR neurons that show a substantial overlap with kisspeptin-IR neurons in human females (Hrabovszky et al., 2010).

(iii) *Regional density of kisspeptin-IR and preproNKB-IR fibers*
Our previous immunohistochemical study also revealed more kisspeptin-IR axonal profiles in the Inf and other hypothalamic regions of women compared with men (Hrabovszky et al., 2010). Here we used computerized image analysis to compare the regional densities of kisspeptin-IR and preproNKB-IR fibers between aged men and women.

(iv) *Frequency of kisspeptin-IR and preproNKB-IR appositions onto GnRH-IR neurons*
A sexual dimorphism has been observed in the innervation pattern of GnRH neurons in mice, in that a higher percentage of rostral preoptic GnRH neurons receive kisspeptin-IR appositions in females compared with males (Clarkson and Herbison, 2006). In our previous immunohistochemical study of human hypothalami we provided morphological indication that kisspeptin-IR axons communicate with GnRH-IR neurons via axo-somatic, axo-dendritic, and axo-axonal interactions (Hrabovszky et al., 2010). Here we addressed a sex difference in the incidences of axo-somatic and axo-dendritic appositions of kisspeptin-IR and preproNKB-IR fibers onto GnRH-IR neurons of the Inf.

(v) *Colocalization of kisspeptins and preproNKB-IR in afferents to GnRH-IR neurons*
In a previous dual-immunofluorescent study we demonstrated a partial colocalization of kisspeptin and preproNKB immunoreactivities in female human subjects (Hrabovszky et al., 2010). The fraction of kisspeptin-IR axons with NKB signal (56% in the Inf and 17% in the periventricular nucleus) and reversely, was surprisingly low, considering the good matches of kisspeptin, NKB, and dynorphin labeling in the ARC of the female sheep (Goodman et al., 2007) and mice (Navarro et al., 2009). Here we carried out triple-immunofluorescent studies coupled with confocal microscopy to determine whether kisspeptin and preproNKB immunoreactivities are colocalized in afferent contacts on GnRH neurons.

MATERIALS AND METHODS

HUMAN SUBJECTS

Human hypothalamic tissue samples from nine male subjects above 50 years of age (50, 50, 52, 53, 59, 62, 66, 66, 67 years) and from seven postmenopausal female subjects above 55 years of age (57, 57, 58, 63, 64, 69, 70 years) were obtained at autopsy from the Forensic Medicine Department of the University of Debrecen, with the permission of the Regional Committee of Science and Research Ethics (DEOEC RKEB/IKEB: 3183-2010). Selection criteria included sudden causes of death, lack of history of neurological and endocrine disorders and *post mortem* intervals between 12 and 48 h.

TISSUE PREPARATION FOR IMMUNOHISTOCHEMISTRY

Following dissection, the hypothalamic tissue blocks were rinsed briefly with running tap water and then, immersion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS; pH 7.4) for 7–14 days at 4°C. Following fixation, the hypothalami were trimmed further to include the optic chiasma rostrally, the mammillary bodies caudally, and the anterior commissure dorsally

(Hrabovszky et al., 2007, 2010). Sagittal cuts were made 2 cm lateral from midsagittal plane on both sides and then, the blocks were cut in halves and infiltrated with 20% sucrose for 5 days at 4°C. The right hemihypothalami were placed in a freezing mold, surrounded with Jung tissue freezing medium (Leica Microsystems, Nussloch Gmbh, Germany; diluted 1:1 with 0.9% sodium chloride solution), snap-frozen on powdered dry ice, and sectioned serially at 30 μm with a Leica SM 2000R freezing microtome (Leica Microsystems) parallel to the plane of the lamina terminalis. The sections were stored permanently in anti-freeze solution (30% ethylene glycol; 25% glycerol; 0.05 M phosphate buffer; pH 7.4) at −20°C.

TISSUE PRETREATMENTS

Prior to immunohistochemistry, the sections were rinsed in PBS and pretreated with a mixture of 0.5% H_2O_2 and 0.2% Triton ×-100 for 30 min. Then, antigen retrieval was carried out by using a 0.1 M citrate buffer wash at 80°C for 30 min. In dual-immunofluorescent experiments, the sections were also pretreated with Sudan black to reduce tissue autofluorescence from lipofuscin deposits, as described earlier (Mihaly et al., 2002; Hrabovszky et al., 2010).

DETECTION OF KISSPEPTIN AND NEUROKININ B SYNTHESIZING NEURONS

To detect kisspeptin immunoreactivity, every 24th hemihypothalamic section from the infundibular nucleus of each human individual was incubated in a sheep polyclonal antiserum (GQ2; 1:100,000) against human kisspeptin-54. This antiserum recognizes human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and it shows virtually no cross-reactivity (<0.01%) with other related human RF amide peptide, including prolactin releasing peptide, neuropeptide FF, neuropeptide AF, and RF amide-related peptides (RFRP1, RFRP2, RFRP3; Dhillo et al., 2005). The GQ2 antibodies were used successfully in previous immunohistochemical experiments to study the distribution of kisspeptin neurons and their connectivity to GnRH cells in the rhesus monkey (Ramaswamy et al., 2008) and the human (Hrabovszky et al., 2010). Incubation in the GQ2 primary antisera (48 h at 4°C) was followed by biotinylated secondary antibodies (biotin-SP–antisheep IgG; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:500) and the ABC Elite reagent (Vector, Burlingame, CA, USA; 1:1000) for 60 min each. The peroxidase signal was visualized with nickel-intensified diaminobenzidine chromogen and then, post-intensified with silver–gold (Liposits et al., 1984).

To detect NKB synthesizing neurons, a second series of sections was incubated with a previously characterized rabbit polyclonal antiserum (IS-682, 1:100,000) against the C-terminal 28 amino acids of human preproNKB (Hrabovszky et al., 2010), followed by working dilutions of biotinylated secondary antibodies (biotin-SP–antirabbit IgG; Jackson ImmunoResearch Laboratories; 1:500; 1 h) and the ABC reagent (1:1000; 1 h). The signal was visualized with silver–gold-intensified Ni-DAB, as in case of kisspeptin-IR neurons.

DETECTION OF GnRH NEURONS

Following the detection of kisspeptin or NKB immunoreactivity, GnRH neurons were detected with a new guinea pig antiserum to GnRH (#1018; 1:50,000). The primary antibodies were reacted with biotin-SP–anti-guinea pig IgG (Jackson ImmunoResearch; 1:500; 1 h) and ABC (1:1000; 1 h) and then, immunoreactivity was visualized with DAB chromogen alone.

To generate the #1018 antiserum, an immunization antigen was prepared by conjugating 4 mg mammalian GnRH to 25 mg bovine thyroglobulin with 12 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in 4 ml 100 mM MES buffer (pH 4.7). Unconjugated peptides were removed from the antigen solution by dialysis against 0.1 M phosphate buffered saline (PBS, pH 7.6; 3 × 8 h). Two adult female guinea pigs were immunized intradermally (i.d.) with 10 μg antigen in 500 μl solution. For initial immunization, 10 μg antigen complex in 250 μl PBS was emulsified with an equal volume of Freund's complete adjuvant (Sigma). Subsequent boosts with Freund's incomplete adjuvant were administered at 2-week intervals. Antisera were sampled from the ear 1 week after each booster injection and tested at various dilutions on tissue sections of the mouse preoptic area (for GnRH cell bodies) and median eminence (for GnRH axons). Upon achievement of the appropriate serum titer (3 months after the beginning of immunization), the animals were exsanguinated. The blood was allowed to clot and serum was collected. Sodium azide was added at 0.1% and antiserum aliquots were transferred to −20°C for long-term storage. Antibody samples were tested with immunohistochemistry on paraformaldehyde-fixed mouse and human tissue sections. For peroxidase-based detection of GnRH, various dilutions of the primary antibodies were made with 2% normal horse serum in PBS and applied to the sections for 16–48 h. The primary antibodies were reacted with biotinylated secondary antibodies (biotin-SP–anti-guinea pig IgG; Jackson ImmunoResearch Laboratories; 1:500) and the ABC Elite reagent for 60 min each. The peroxidase signal was visualized with Ni-DAB chromogen. As one positive control for the specificity of GnRH cell body labeling, the immunofluorescent detection of GnRH was performed on preoptic sections of GnRH-GFP transgenic mice in which GnRH neurons exhibit green fluorescence (Suter et al., 2000). As a second positive control also applicable to human test sections, dual-immunofluorescent visualization of GnRH immunoreactivity was carried out with one of the new guinea pig antisera (#1018 or #1035), in combination with a reference rabbit GnRH antiserum (LR1; 1: 5000) that was kindly provided by Dr. R. A. Benoit and used in previous publications from different laboratories, including our own (Hrabovszky et al., 2007, 2010). In both control experiments, incubation with guinea pig GnRH antisera was followed by donkey anti-guinea pig-Cy3 (1: 1000; Jackson ImmunoResearch) for 5 h. The LR1 antibody was reacted with donkey anti-rabbit–FITC (1:200) for 5 h.

TRIPLE-IMMUNOFLUORESCENT VISUALIZATION OF PREPRONKB, KISSPEPTINS, AND GnRH

A series of sections was used to study the colocalization pattern of NKB and kisspeptin in neuronal afferents to GnRH neurons. Incubation in a cocktail of primary antibodies (rabbit anti-preproNKB, 1:1000; sheep anti-kisspeptin, 1:1000; guinea pig anti-GnRH, 1:3000) for 48 h at 4°C was followed by a cocktail of fluorochrome-conjugated secondary antibodies (all

raised in donkey; anti-rabbit–FITC, 1:250; anti-sheep–Cy3, 1:1000; anti-guinea pig–AMCA, 1:100; Jackson ImmunoResearch) for 5 h.

SECTION MOUNTING AND COVERSLIPPING

Sections dual-immunostained with the combination of silver–gold-intensified Ni-DAB and DAB chromogens were mounted on microscope slides from Elvanol, air-dried, dehydrated with 95% (5 min), followed by 100% (2 × 5 min) ethanol, cleared with xylene (2 × 5 min), and coverslipped with DPX mounting medium (Fluka Chemie; Buchs, Switzerland). Representative microscopic images were prepared with an AxioCam MRc 5 digital camera mounted on a Zeiss AxioImager M1 microscope using the AxioVision 4.6 software (Carl Zeiss, Göttingen, Germany). Sections processed for immunofluorescent experiments were mounted from 0.1 M Tris–HCl buffer (pH 7.6) and coverslipped with the aqueous mounting medium Mowiol.

ANALYSES AND STATISTICS

To exclude any bias, the immunostained samples were randomized, coded, and analyzed by investigators blind to the origin of samples. All statistical comparisons were carried out with one-way ANOVA followed by Newman–Keuls *post hoc* test using the Statistica 8.0 software package (StatSoft, Inc., Tulsa, USA). Immunohistochemical samples from five to nine male and five to seven female subjects were included in each experiment and statistical group.

Experiment 1. Studies of the perikaryon size of kisspeptin-IR and preproNKB-IR neurons

To determine the average size of kisspeptin-IR and peproNKB-IR cell bodies, 10–30 solitary neurons, which showed no overlap with one another, were identified in digital images of the Inf from each individual. To exclude immunoreactive neuronal processes from the area analyzed, the tissue area surrounding each immunolabeled neuron was erased using the Adobe Photoshop CS software. The digital images of selected cell bodies were compiled into TIF files and opened for area/cell body analysis with the Image J software (public domain at http://rsb.info.nih.gov/ij/download/src/). A threshold was determined and set to only highlight the labeled cell bodies in all specimens. The signal areas were measured this way and then, converted to square micrometer using appropriate calibration. For each human subject the mean profile area of labeled perikarya was derived from an average of 10–30 labeled cells.

Experiment 2. Studies of the incidence of kisspeptin-IR and preproNKB-IR cell bodies in the Inf

As validated previously (Hrabovszky et al., 2010), kisspeptin-IR and preproNKB-IR cell bodies were counted in the Inf at 100× magnification in a 0.25-mm^2 counting area, with the aid of a 5 × 5 ocular grid. Each human individual was finally characterized with the highest number of immunoreactive cell bodies per 0.25 mm^2 counting area, as determined from two to six sections.

Experiment 3. Studies of the regional density of kisspeptin-IR and preproNKB-IR fibers

Digital images were captured in regions of the Inf that contained the bulk of kisspeptin-IR and preproNKB-IR cell bodies. The files were opened with the Adobe Photoshop CS software. The immunolabeled cell bodies were erased ("eraser tool") from the photomicrographs. The remaining images were compiled into TIF files and opened with the Image J software. The regional fiber density in each photograph was defined as the area occupied by immunoreactive fibers/total area. For each subject, the mean fiber density was derived from one to three digital images.

Experiment 4. Studies of the frequency of kisspeptin-IR and preproNKB-IR appositions onto GnRH-IR neurons

One or two sections were selected from each individual to analyze the number of axonal contacts along the outlines of GnRH neurons and their dendrites. The counting was carried out using a 100× oil immersion objective. A contact was defined using stringent criteria that were applied consistently, as follows. The axon and the GnRH profile had to be in the same focus plane without any visible intervening gap, and dubious instances of partial overlap were not considered. For each subject, both the mean number of contacts/GnRH soma and the mean number of contacts/100 μm GnRH dendrite were measured.

Experiment 5. Colocalization studies of kisspeptins and preproNKB in neuronal afferents to GnRH neurons

One section from the triple-immunofluorescent specimens of the Inf was selected from each individual to analyze single- and double-labeled kisspeptin-IR and preproNKB-IR neuronal appositions onto GnRH neurons. Multiple stacks of optical slices (512 × 512 pixels, z-steps 0.6 μm) were obtained by scanning GnRH neurons in the Inf and their kisspeptin-IR and preproNKB-IR contacts using a 60× oil immersion objective and a Radiance 2100 confocal microscope (Bio-Rad Laboratories, Hemel Hempstead, UK). The three fluorochromes were detected with the following laser lines and filters: 488 nm for FITC, 543 nm for CY3 and Alexa594, 405 nm for AMCA, with dichroic/emission filters 560 nm/500–540 nm for FITC, 650 nm/560–610 nm for CY3 and Alexa594, 500 nm/420–480 nm for AMCA. The separately recorded green, red, and blue channels were merged and displayed with the Laser Vox software (Bio-Rad) running on an IBM-compatible personal computer. Appositions were validated if no gap was visible between the juxtaposed profiles in at least one optical slice. A total of 394 kisspeptin-IR contacts and 337 preproNKB-IR contacts (mixed axo-dendritic and axo-somatic) were analyzed to count the percent ratios of double-labeled neuronal appositions onto the cell bodies and the dendrites of GnRH-IR neurons. The colocalization percentages were determined in five male and six female individuals and compared.

RESULTS

EXPERIMENT 1. PERIKARYON SIZE OF KISSPEPTIN-IR AND PREPRONKB-IR NEURONS

Kisspeptin-IR cell bodies ($P = 0.01$) as well as preproNKB-IR cell bodies ($P = 0.002$) were hypertrophied and their profile area was significantly larger in the Inf of aged women (284.2 ± 27.3 μm^2 for kisspeptin-IR and 298.1 ± 19.7 μm^2 for preproNKB-IR neurons) in comparison with men (154.8 ± 19.2 μm^2 for kisspeptin-IR and 190.4 ± 20.4 μm^2 for preproNKB-IR neurons; **Figures 1C,D,G,H** and **2**).

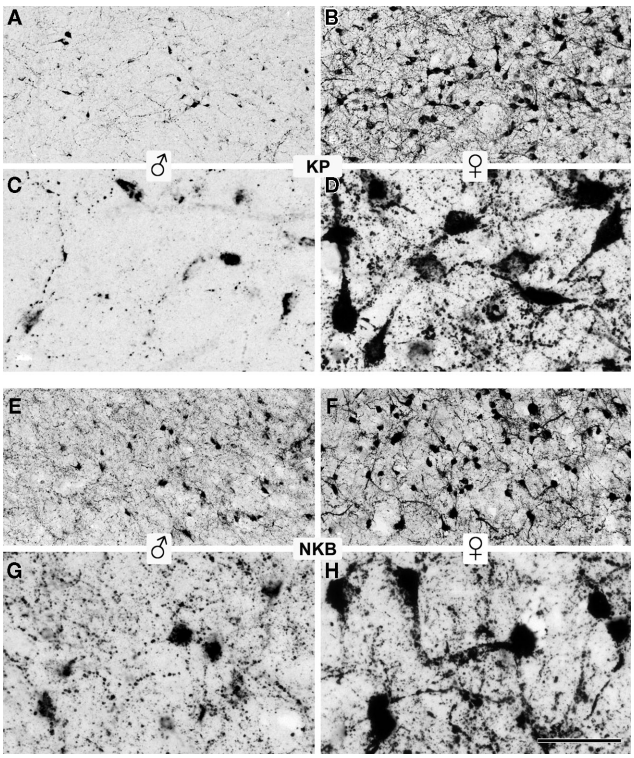

FIGURE 1 | Sexual dimorphism of kisspeptin-IR and preproNKB-IR neuronal elements in the Inf of aged human subjects. Kisspeptin **(A–D)** and preproNKB **(E–H)** immunoreactivities were visualized using the silver–gold-intensified Ni-DAB chromogen. The photomicrographs were taken from the Inf of 59-year-old male **(A,C)** and 62-year-old male **(E,G)** and 66-year-old **(B,D,F,H)** female individuals. Both kisspeptin-IR **(D)** and preproNKB-IR **(H)** cell bodies are larger in postmenopausal women compared with aged men (**C** and **G**, respectively). Neuronal hypertrophy reflects lack of estrogen negative feedback in postmenopausal women. Kisspeptin neurons also show other robust sex differences in that the number of KP-IR cell bodies and the density of kisspeptin-IR fibers are much higher in aged women **(B)** compared with men **(A)**. The number of preproNKB-IR cell bodies is also higher in women **(F)** compared with men **(E)**, but the difference is less dramatic than in case of kisspeptin-IR cell bodies. No obvious sexual dimorphism exists in the regional density of NKB fibers **(E–H)**. For quantitative comparisons, see **Figures 2–4**. Scale bar = 200 μm in **(A,B,E,F)** and 50 μm in **(C,D,G,H)**.

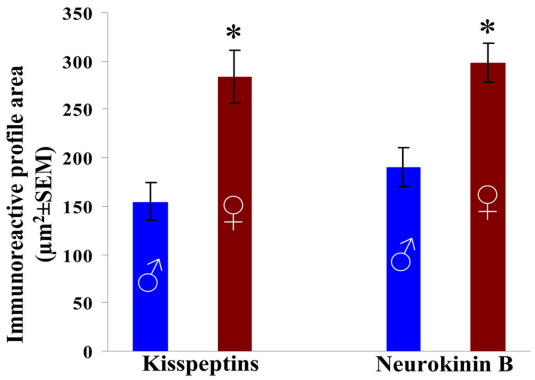

FIGURE 2 | Size of kisspeptin-IR and preproNKB-IR perikarya in the Inf of aged men and women. The mean surface area covered by the silver–gold-intensified Ni-DAB chromogen was used as an index of perikaryon size. The area of immunolabeled cell bodies differs between aged men and women in case of both peptides. The difference likely reflects a robust neuronal hypertrophy in postmenopausal females. $^{*}P < 0.05$.

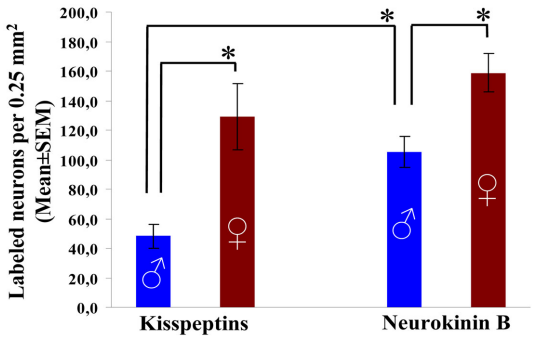

FIGURE 3 | Regional abundances of kisspeptin-IR and preproNKB-IR neuronal perikarya in the Inf of aged men and women. The maximal number of immunoreactive cell bodies per 0.25 mm² counting frame (1–6 per subject) was determined with the aid of an ocular frame and used as the index of regional neuron density. The abundance of kisspeptin neurons determined this way is much higher in aged women compared with aged men, whereas the number of NKB cell bodies is only slightly (but significantly) higher in females. Note also that men, unlike women, contain 2.2-times as many preproNKB-IR as kisspeptin-IR cell bodies. $^{*}P < 0.05$.

EXPERIMENT 2. INCIDENCE OF KISSPEPTIN-IR AND PREPRONKB-IR CELL BODIES IN THE Inf

Quantitative analysis of the labeled cell bodies (using the maximal number of labeled cell bodies per 0.25 mm^2 counting frame for each male and female individual) revealed the following differences

In males, preproNKB-IR cell bodies showed a significantly higher incidence compared with kisspeptin-IR cell bodies ($P = 0.0005$). PreproNKB-IR neurons outnumbered kisspeptin-IR neurons by 120% (**Figures 1A,E** and **3**).

In females, the mean incidence of preproNKB-IR cell bodies was only 23% higher than that of kisspeptin-IR perikarya. This subtle difference was not statistically significant ($P = 0.28$; **Figures 1B,F** and **3**).

Kisspeptin-IR cell bodies showed a 170% higher mean density (incidence of cell bodies per 0.25 mm^2 counting frame) in females compared with males. This robust sex difference was statistically significant ($P = 0.002$; **Figures 1A,B** and **3**).

PreproNKB-IR cell bodies showed a 51% higher mean incidence in females vs. males. This numerical sex difference was much less conspicuous than for kisspeptin-IR cell bodies, but statistically significant ($P = 0.006$; **Figures 1E,F** and **3**).

EXPERIMENT 3. REGIONAL DENSITY OF KISSPEPTIN-IR AND PREPRONKB-IR FIBERS

The Inf of aged men exhibited a few kisspeptin-IR fibers only, in contrast with dense fiber networks in the Inf of postmenopausal women (**Figures 1A–D**). PreproNKB-IR axons did not seem to

show this robust sex difference (**Figures 1E–H**). Quantitative analysis of labeled axons (area covered by immunohistochemical signal per total area) established the following differences:

In the Inf of males, the mean density of preproNKB-IR axons was 180% higher than that of kisspeptin-IR fibers. The difference was statistically significant ($P = 0.004$; **Figures 1A,C,E,G** and **4**).

In the Inf of females, the densities of preproNKB-IR and kisspeptin-IR fibers did not differ statistically ($P = 0.41$; **Figures 1B,D,F,H** and **4**).

Kisspeptin-IR fibers showed a robust sexual dimorphism, with a 161% higher density in females vs. males. This difference was statistically significant ($P = 0.02$; **Figures 1A–D** and **4**).

PreproNKB-IR fibers showed only 8.4% higher mean density in females than in males and the sexes did not differ statistically ($P = 0.60$; **Figures 1E–H** and **4**), in contrast with the dramatic and significant sexual dimorphism of the kisspeptin-IR fiber network.

EXPERIMENT 4. FREQUENCY OF KISSPEPTIN-IR AND PREPRONKB-IR APPOSITIONS ONTO GnRH-IR NEURONS

Both guinea pigs (#1018 and #1035) that were immunized against the mammalian GnRH raised antibodies that reacted specifically with GnRH neurons in mouse and human tissue sections (**Figure 5**). Using peroxidase-based immunohistochemistry, IR neurons exhibited the typical fusiform shape and distribution pattern of GnRH neurons in the medial preoptic area of mice (**Figure 5A**). In immunofluorescent experiments on tissue sections from GnRH–GFP transgenic mice, the immunoreactive cell bodies also exhibited the green fluorescence of GFP (**Figures 5B–E**). The combined use of the new guinea pig antisera and the LR1 reference rabbit antiserum on human test sections provided an additional evidence for labeling specificity (**Figures 5F–I**). The optimal dilutions of the new antisera were 1:50,000–1:200,000 for peroxidase-based immunohistochemistry and 1:3,000–1:10,000 for immunofluorescence using fluorochrome-conjugated secondary antibodies.

Sections double-labeled for kisspeptins and GnRH or preproNKB and GnRH used silver–gold-intensified Ni-DAB and DAB chromogens, in combination. The high-power light microscopic analysis of these sections confirmed our previous observation (Hrabovszky et al., 2010) that kisspeptin-IR axons establish axo-somatic and axo-dendritic contacts on GnRH neurons of the Inf (**Figures 6A–D**), in addition to axo-axonal contacts in the infundibular stalk that were not analyzed here. In the present study, we observed similar axo-somatic, axo-dendritic, and axo-axonal (not shown) appositions between preproNKB-IR axons and GnRH-IR neurons (**Figures 6E–H**).

The semiquantitative analysis of kisspeptin-IR contacts on GnRH-IR cell bodies and dendrites revealed a significantly heavier kisspeptin-IR input to the cell bodies ($P = 0.03$) and the dendrites ($P = 0.021$) of GnRH-IR neurons in women compared with men (**Figure 7**).

In both sexes, GnRH-IR cell bodies and dendrites received significantly heavier preproNKB-IR input than kisspeptin-IR input (male cell bodies: $P = 0.002$; male dendrites: $P = 0.0002$; female cell bodies: $P = 0.02$; female dendrites: $P = 0.02$; **Figure 7**).

In contrast with the dramatic sexual dimorphism of kisspeptin-IR inputs, no significant sex difference was observed in the incidence of preproNKB-IR axo-somatic and axo-dendritic juxtapositions to GnRH-IR neurons (axo-dendritic contacts: $P = 0.24$; axo-somatic contacts: $P = 0.36$; **Figure 7**). However, there was a trend for somewhat more preproNKB-IR contacts in females.

EXPERIMENT 5. COLOCALIZATION OF KISSPEPTINS AND PREPRONKB IN NEURONAL AFFERENTS TO GnRH NEURONS

The triple-immunofluorescent specimens contained numerous kisspeptin-IR and preproNKB-IR fibers. The sensitivity of the approach was not sufficient to visualize high numbers of kisspeptin-IR and preproNKB-IR cell bodies. The axonal kisspeptin and preproNKB immunolabeling showed a partial overlap only and GnRH neurons were most frequently contacted by single-labeled axons both in males and females (**Figure 8**). The semiquantitative analysis of afferent contacts onto GnRH-IR cell bodies and dendrites (**Figure 9**) established that $8.8 \pm 5.5\%$ of preproNKB-IR afferents in males also contained kisspeptin immunoreactivity. The ratio of double-labeled afferents was $31.3 \pm 4.9\%$ in females ($P = 0.01$). Similarly, the percentage of kisspeptin-IR contacts that colocalized preproNKB signal was significantly higher ($P = 0.033$) in females ($25.8 \pm 2.4\%$) vs. males ($10.2 \pm 4.6\%$).

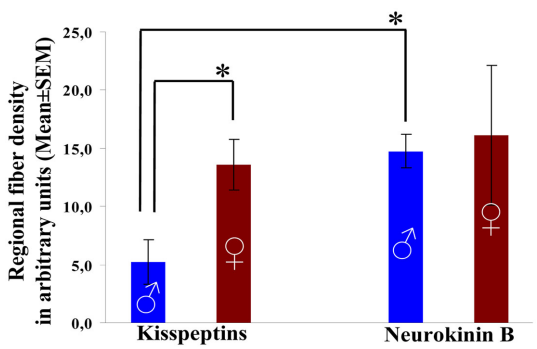

FIGURE 4 | Density of kisspeptin-IR and preproNKB-IR fibers in the Inf of aged men and women. The area covered by immunoreactive fibers (divided by the total area analyzed) was determined with the Image J software in digital photographs of the Inf and used as an index of regional fiber density (presented in arbitrary units). Areas of the photomicrographs that were occupied by labeled cell bodies and their proximal dendrites were erased using the Adobe Photoshop software and excluded from the analysis. The density of preproNKB-IR fibers defined this way was 2.8-fold as high as the density of kisspeptin-IR fibers in aged males, but not different in aged females. The density of kisspeptin-IR fibers showed a dramatic sexual dimorphism and was 2.5-fold higher in aged females vs. males. The mean density of preproNKB-IR fibers did not differ significantly between sexes. $^*P < 0.05$.

DISCUSSION

SEX DIFFERENCE IN PERIKARYON SIZE OF KISSPEPTIN AND PREPRONKB NEURONS

The studies of a putative sex difference in the perikaryon size of kisspeptin-IR and preproNKB-IR neurons (mean cross-sectional area of immunoreactive neurons) relied on previous work indicating that the profile area of neurons in the Inf is significantly greater in postmenopausal women than in aged men (Rance

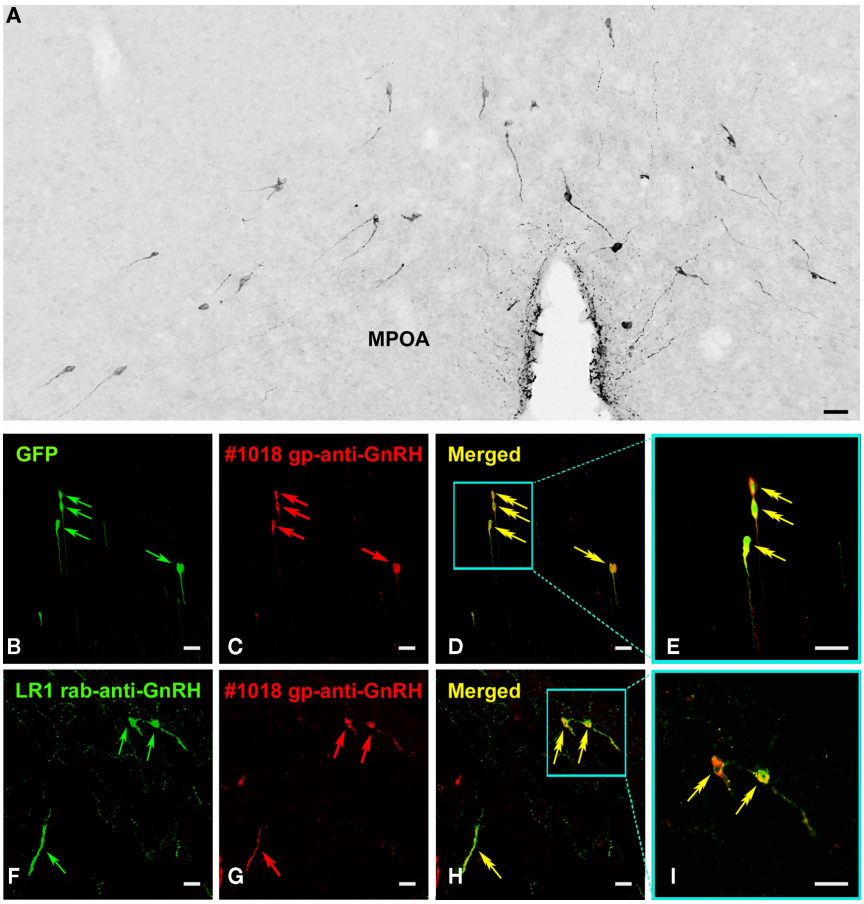

FIGURE 5 | Specificity test results using a new guinea pig GnRH antiserum. Using a new polyclonal GnRH antiserum (#1018) for peroxidase-based immunohistochemistry, the Ni-DAB chromogen reveals GnRH-IR neurons and their fibers in the medial preoptic area (MPOA) of mice. The labeled neurons exhibit characteristic fusiform shapes **(A)**. In positive control experiments, the distribution of the immunofluorescent GnRH signal in labeled cell bodies [red arrows in **(C)**] shows a good agreement with the native green fluorescence of GnRH–GFP neurons [green arrows in **(B)**] of transgenic mice. Double arrows in **(D)** [merged from **(B,C)**] and **(E)** [high-power image of framed region in **(D)**] point to double-labeled neurons. The dual-immunofluorescent visualization of GnRH-IR neurons in the human hypothalamic infundibular nucleus provides additional evidence that a reference rabbit antiserum [LR1; **(F)**] and the new guinea pig antiserum **(G)** recognize the same neurons. Double arrows in **(H)** [merged from **(F,G)**] and **(I)** [high-power image of framed region in **(H)**] point to the dual-labeled GnRH cell bodies and their processes. Scale bars = 50 μm.

et al., 1993). This morphometric sex difference is thought to result primarily from postmenopausal alterations of the female Inf. In 1966, Sheehan and Kovacs reported neuronal hypertrophy in a subregion of the Inf termed subventricular nucleus. The hypertrophied neurons contained enlarged nuclei and nucleoli and a prominent Nissl substance. They occurred in female individuals above 50 years of age and similarly, in women with post-partum hypopituitarism (Sheehan and Kovacs, 1966). Sheehan (1967) concluded that neuronal hypertrophy is a consequence of ovarian failure. A series of *in situ* hybridization studies from Rance and co-workers established later that the hypertrophied neurons express estrogen receptor-α (Rance et al., 1990), substance P (Rance and Young, 1991), NKB (Rance and Young, 1991), kisspeptin (Rometo et al., 2007), and prodynorphin (Rometo and Rance, 2008) mRNAs. It was concluded that the neuronal hypertrophy is not a compensatory response to neuronal cell death, because the number of neurons in the Inf was similar in pre- and postmenopausal women (Abel and Rance, 2000). Ovariectomy was also capable of inducing the hypertrophy of kisspeptin mRNA expressing neurons in the Inf of monkeys (Rometo et al., 2007), providing experimental evidence to the concept that the enlargement of neuronal perikarya is a consequence of the loss of estrogen.

The morphometric analyses of kisspeptin-IR and preproNKB-IR neurons in our present study revealed a robust sex difference in the size (mean profile area) of kisspeptin-IR as well as preproNKB-IR neurons in the Inf of aged men and women. The mean cross-sectional area we determined for postmenopausally hypertrophied neurons ($284.2 \pm 27.3\ \mu m^2$ for kisspeptin-IR and $298.1 \pm 19.7\ \mu m^2$ for preproNKB-IR neurons) agrees well with the profile area of kisspeptin mRNA expressing hypertrophied neurons ($280.9 \pm 17.3\ \mu m^2$), as measured previously in toluidine blue-stained samples (Rometo et al., 2007). Moreover, profile area values we determined for aged

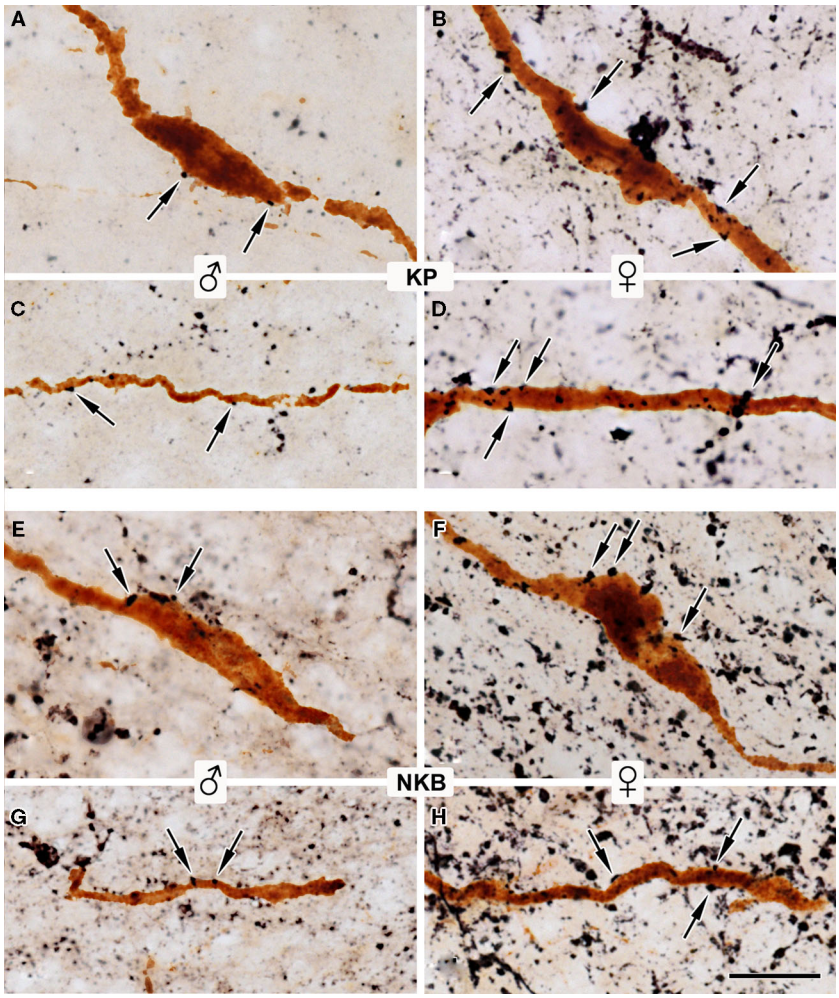

FIGURE 6 | Identification of kisspeptin-IR and preproNKB-IR afferent contacts on GnRH-IR neurons of the Inf. Sections double-labeled for kisspeptins (KP) and GnRH **(A–D)** or preproNKB (NKB) and GnRH **(E–H)** with the combined use of silver– gold-intensified Ni-DAB and DAB chromogens demonstrate that kisspeptin-IR axons establish axo-somatic **(A,B)** and axo-dendritic **(C,D)** contacts (arrows) on GnRH neurons of the Inf. Similar contacts can also be observed in high numbers between preproNKB-IR axons and GnRH neurons [arrows in **(E–H)**]. The kisspeptin-IR input is significantly heavier on GnRH-IR neurons from women vs. men [compare **(B)** to **(A)** and **(D)** to **(C)**]. In both sexes, GnRH-IR cell bodies and dendrites receive more preproNKB-IR **(E–H)** than kisspeptin-IR **(A–D)** input. In contrast with the dramatic sexual dimorphism of kisspeptin-IR inputs, no significant sex difference can be observed in the incidence of preproNKB-IR axo-somatic **(E,F)** and axo-dendritic **(G,H)** juxtapositions to GnRH-IR neurons. [**(A,C)**: 59-year-old male; **(E,G)**: 52-year-old male; **(B,D)**: 57-year-old female; **(F,H)**: 64-year-old female]. For quantitative comparisons, see **Figure 7**. Scale bar = 20 μm.

men ($154.8 \pm 19.2\,\mu m^2$ for kisspeptin-IR and $190.4 \pm 20.4\,\mu m^2$ for preproNKB-IR neurons) are also comparable to the mean cross-sectional area of toluidine blue-stained hypertrophied neurons in the Inf of older men ($176.6 \pm 1.7\,\mu m^2$; Rance et al., 1993), in spite of differences in the applied histochemical approaches.

It is interesting to note that an aging-related neuronal hypertrophy has also been noticed in the male Inf, although it did not reach the magnitude of postmenopausal hypertrophy (Rance et al., 1993). The phenotype of hypertrophied neurons in the male Inf requires clarification. Given that sex steroid levels only decline modestly and exhibit considerable individual variations among aging males (Araujo and Wittert, 2011), the mild increase in the size of neurons in the Inf of aging men may not be directly linked to a decrease of sex steroid levels, but instead, may partly reflect an age-related decrease in the sensitivity of the male reproductive axis to androgen. This would be in accordance with the reported age-related decline of androgen negative feedback (Veldhuis et al., 2010).

The explanation for the sex difference we report here for the immunoreactive profile areas of kisspeptin-IR and preproNKB-IR neurons represents a sex difference in aging-related neuronal hypertrophy. In women, this reaches a higher magnitude due to ovarian failure after menopause, whereas in men the presence of testosterone continues to inhibit the synthetic activity of kisspeptin and preproNKB neurons in the Inf.

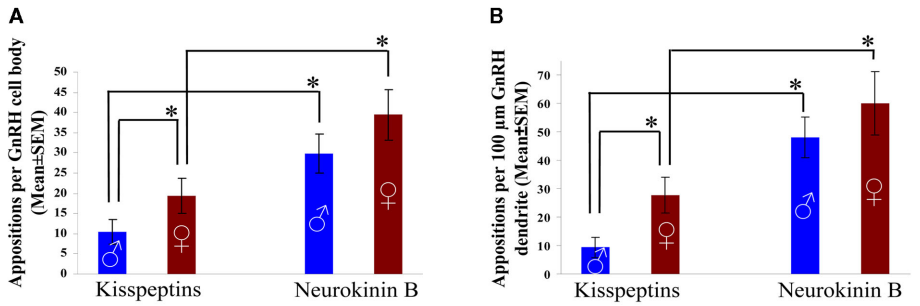

FIGURE 7 | Incidence of kisspeptin-IR and preproNKB-IR contacts on GnRH-IR neurons of the infundibular nucleus. High-power light microscopic analysis of sections, dual-immunolabeled with the combined use of silver– gold-intensified Ni-DAB and DAB chromogens (see **Figure 6**), was carried out to determine the relative frequencies of kisspeptin-IR (left column pairs) and preproNKB-IR (right column pairs) neuronal appositions onto the somata **(A)** and dendrites **(B)** of GnRH-IR neurons. The counts were obtained from all GnRH-IR cell bodies and dendrites that were identified in 1–3 sections of the Inf from each of eight male and six female individuals. The innervation patterns are highly similar on GnRH cell bodies **(A)** and dendrites **(B)**. Note that the number of preproNKB-IR contacts is significantly higher than the number of kisspeptin-IR contacts on both the somatic **(A)** and the dendritic **(B)** membranes and in both males and females. The kisspeptin-IR input to GnRH cell bodies **(A)** and dendrites **(B)** is highly sexually dimorphic. The mean incidence of kisspeptin-IR contacts is 88.2% higher on GnRH-IR cell bodies and 177.4% higher on GnRH-IR dendrites in postmenopausal females vs. aged males. In contrast, there is no statistical difference between the two sexes in the incidences of preproNKB-IR contacts, although the means are somewhat higher in females. $^{*}P < 0.05$.

HIGHER RELATIVE LEVELS OF PREPRONKB VS. KISSPEPTIN IMMUNOLABELING

An important observation in this study was the heavier preproNKB than kisspeptin immunolabeling in the Inf, in particular, in aged men. This difference represents a biological rather than technical phenomenon, given that both kisspeptin and NKB immunoreactivities showed obvious sex-dependent patterns instead of random variations across tissue specimens. The observation that the Inf contained 2.2-times as many preproNKB-IR as kisspeptin-IR cell bodies, 2.8-times as high preproNKB-IR as KP-IR fiber densities and 3–5-times as many preproNKB-IR as KP-IR neuronal contacts on GnRH neurons, indicate that a large subset of NKB neurons do not synthesize detectable amounts of kisspeptins in the Inf of aged men. This conclusion gains additional support from immunofluorescent results which confirmed a substantial level of mismatch between kisspeptin-IR and preproNKB-IR axons in the Inf, including higher numbers of single-labeled than double-labeled preproNKB-IR as well as kisspeptin-IR afferent contacts onto GnRH neurons. The sensitivity of the immunofluorescent detection method did not allow us to determine the exact percentages of overlap between kisspeptin and preproNKB-synthesizing neuronal cell bodies in the two sexes.

The dominance of preproNKB over kisspeptin labeling was also present in postmenopausal females, although the differences did not reach the same magnitudes as in men. Notably, samples from postmenopausal women showed only 1.2-times as many preproNKB-IR as kisspeptin-IR neurons, only 1.6–2.2-times as many preproNKB-IR as kisspeptin-IR afferent contacts on GnRH neurons, and no significant difference in the overall density of preproNKB-IR vs. kisspeptin-IR fibers in the Inf.

The partial segregation of the kisspeptin from the NKB immunolabeling in our study, with the dominance of the latter, reveals a difference from the female sheep (Goodman et al., 2007) and rodents (Navarro et al., 2009) where the majority of kisspeptin neurons in the ARC also contain NKB (and dynorphins). Of note, a more recent study of male mice identified about twice as many NKB mRNA expressing as kisspeptin mRNA expressing neurons in the ARC (Navarro et al., 2011). Together with the previous report on females (Navarro et al., 2009), this observation indicates a sex difference in the extent of coexpression. This possible sexual dimorphism is reminiscent to our observation that NKB cell numbers exceed kisspeptin cell numbers by more in aged human males than in females. A different colocalization pattern has been observed recently in the ARC of adults in castrated male monkey model in which no NKB neurons without kisspeptin labeling were observed and only 40–60% of kisspeptin-IR neurons contained preproNKB immunoreactivity (Ramaswamy et al., 2010).

Two possibilities may explain the sex difference in relative levels of preproNKB vs. kisspeptin immunolabeling in our study. First, the putative organizational effects of a developmental sex steroid exposure may generate a lower degree of colocalization in males than in females which may be maintained throughout life. Second, the different levels of negative sex steroid feedback between aged men and women may differentially influence the expression patterns of the two peptides. The dramatically low kisspeptin levels in aged men may reflect the higher reactivity of the *KISS1* gene to the suppressive effects of testosterone. Kisspeptin (Rometo et al., 2007; Kim et al., 2009; Eghlidi et al., 2010) as well as NKB (Rance and Bruce, 1994; Eghlidi et al., 2010) mRNAs have lower premenopausal than postmenopausal levels in primates, indicating that sex steroids negatively regulate the expression of both neuropeptides. These data are also in accordance with the negative regulation of kisspeptin and NKB expression by sex steroids in various animal species (Rance and Young, 1991; Abel et al., 1999; Danzer et al., 1999; Dellovade and Merchenthaler, 2004; Smith et al., 2005a,b, 2006a; Adachi et al., 2007; Kauffman et al., 2007; Kim et al., 2009; Navarro et al., 2009; Eghlidi et al., 2010). In our immunohistochemical studies of aged women, we observed high levels of kisspeptin immunoreactivities that approached the levels of preproNKB-IR labeling. From this we

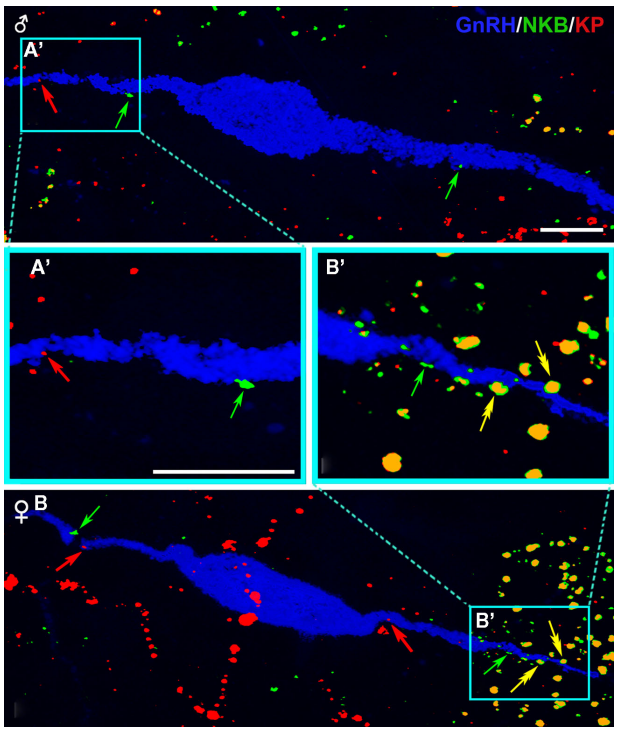

FIGURE 8 | Detection of preproNKB and kisspeptin immunoreactivities in neuronal appositions onto GnRH neurons of the Inf. The simultaneous immunofluorescent detection of preproNKB (green color), kisspeptins (red color), and GnRH (blue color), followed by confocal analysis revealed direct appositions of single- and double-immunolabeled (yellow color) axons onto GnRH-IR cell bodies and dendrites. Note in stacks of optical slices that preproNKB/kisspeptin dual-IR appositions (yellow) are more typical in postmenopausal female [**(B)** and high-power image of the framed dendritic region **(B')**] than in aged male [**(A)** and high-power inset **(A')**] subjects. Red and green arrows indicate afferent contacts that are single-labeled for kisspeptins and preproNKB, respectively, whereas yellow double-arrows indicate double-labeled appositions. For quantitative results of sex comparisons, see **Figure 9**. Scale bars = 10 μm [Bars in **(A,A')** also refer to **(B,B')**, respectively].

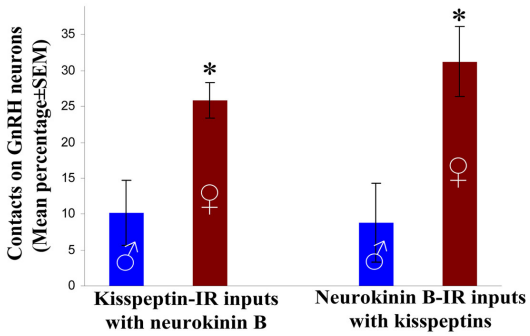

FIGURE 9 | Sex difference in the colocalization patterns of kisspeptins and preproNKB in neuronal afferents to GnRH neurons. As revealed on triple-immunofluorescent specimens, GnRH neurons receive kisspeptin-IR, preproNKB-IR, and double-labeled neuronal contacts. The percentage of kisspeptin-IR contacts that colocalized preproNKB signal is significantly higher in females (25.8 ± 2.4%) vs. males (10.2 ± 4.6%). Similarly, while only 8.8 ± 5.5% of preproNKB-IR afferents in males contain kisspeptin immunoreactivity, the extent of this colocalization is 31.3 ± 4.9% in females. $^{*}P < 0.05$.

propose that the postmenopausal disruption of negative estrogen feedback exerts more dramatic effects on *KISS1* compared with preproNKB expression.

It is interesting to note that preproNKB-IR appositions occurred more frequently than kisspeptin-IR contacts on GnRH neurons. There was no sex difference in the incidence of preproNKB-IR contacts on GnRH neurons, although NKB neurons were present in higher numbers in the Inf of aged women vs. men. It is possible that preproNKB-IR systems outside the Inf, that are not sexually dimorphic, contribute significantly to these afferent contacts. The putative origins of such inputs may include NKB neurons in the periventricular nucleus and the bed nucleus of the stria terminalis (Hrabovszky et al., 2010). It is also possible that the number of preproNKB-synthesizing neurons is similar in the Inf of the two sexes, but different peptide synthesis and release dynamics cause a sex differences in the number of detectable cell bodies, without affecting preproNKB immunoreactivity in fibers and their terminals on GnRH neurons.

SEX DIFFERENCES IN KISSPEPTIN IMMUNOREACTIVITY

Kisspeptin is a strong stimulator of luteinizing hormone release in both women and men (Dhillo et al., 2005, 2007), with a sexual dimorphism in kisspeptin responsiveness of the reproductive axis. While systemic kisspeptin-10 administration stimulates gonadotropin secretion in adult men, it only elicits luteinizing hormone and follicle stimulating hormone secretion in the preovulatory phase, but not during most of the follicular phase, of the menstrual cycle in women (Jayasena et al., 2011).

Kisspeptins represent the main output signal from Kisspeptin/NKB/Dynorphin (KNDy) neurons in a recently proposed model of the GnRH pulse generator (Navarro et al., 2009). Kisspeptin neurons in the human can influence GnRH neurons via axo-somatic, axo-dendritic, and axo-axonal communication (Hrabovszky et al., 2010). Our immunohistochemical results revealed dramatic sex differences in the number of kisspeptin-IR cell bodies, the density of kisspeptin-IR fibers, the incidence of kisspeptin-IR contacts on GnRH neurons and the percent degrees of kisspeptin/NKB colocalization in neuronal afferents to GnRH neurons. All these parameters were significantly higher in aged women compared with aged men, which is compatible with a higher kisspeptin-mediated direct excitatory drive on the reproductive axis in postmenopausal women in comparison with aged men.

It remains to be established if developmental exposure to sex steroids contributes to any extent to the sexual dimorphism of the hypothalamic kisspeptin system. Such organizational effects exist in the ARC of the sheep which contains higher numbers of kisspeptin neurons in females vs. males (Cheng et al., 2010). Most measurable parameters of the sexual dimorphism we report in the present study may be explained by the increased expression of kisspeptin mRNA and the increased number of neurons expressing kisspeptin in women after menopause (Rometo et al., 2007). Similarly, the different incidence of kisspeptin-IR neuronal contacts on GnRH cells and the different colocalization percentages in this inputs between the two sexes may also reflect a different level

of biosynthesis which may cause subthreshold levels of kisspeptin in a subset of kisspeptin axons, in particular, in males. It requires clarification if the sex difference in the number of kisspeptin-IR neuronal appositions onto GnRH neurons reflects the numerical difference in synaptic inputs or the lower detectability of kisspeptin in a subset of kisspeptin/NKB afferent axons in aged men. The former hypothesis gains support from the observation that the number of excitatory and inhibitory synapses in the ARC can be dynamically regulated by sex steroids (Parducz et al., 2002; Csakvari et al., 2007). The second possibility would be compatible with high levels of kisspeptin immunoreactivity in postmenopausal females which coincided with a higher percentage of NKB-IR contacts that contained kisspeptin in our postmenopausal female samples. Our immunohistochemical observation that the kisspeptin-IR innervation of human GnRH neurons is heavier in women is in accordance with the previous findings in mice which revealed a higher percentage of GnRH neurons that received kisspeptin-IR afferent contacts in the rostral preoptic area of females (Clarkson and Herbison, 2006).

Future studies addressing sex differences between young female and male individuals will be critically important to interpret some observations of the present study. Because kisspeptin and NKB neurons in both young men and women (for most of the menstrual cycle) are under negative sex steroid feedback, their putative sex difference may reflect organizational effects of sex steroids during early development, rather than the different activational effects of the actual sex steroid exposure. Also, it will be similarly important to identify age effects by comparing staining patterns of young to aged subjects of the same sex. Unfortunately, histological samples for such comparative immunohistochemical analyses are currently unavailable in our laboratories.

SEX DIFFERENCES IN PREPRONKB IMMUNOREACTIVITY

Similarly to kisspeptins, NKB also acts upstream from the GnRH neuron. In rats, NKB might directly influence GnRH secretion from the hypothalamic median eminence where GnRH-IR axons are apposed to NKB-IR axons (Krajewski et al., 2005; Ciofi et al., 2006) and express NK3 (Krajewski et al., 2005). Our present study revealed frequent axo-dendritic and axo-somatic contacts between preproNKB-IR axons and GnRH neurons of the human. It requires clarification if NKB can postsynaptically influence GnRH secretion at these contact sites, given that NK3-IR or NK3 mRNA has not been detected in a majority of the cell bodies of GnRH neurons, in rats (Krajewski et al., 2005), ewe (Amstalden et al., 2010), and mice (Navarro et al., 2011). A more likely site of NKB action is on other NKB synthesizing neurons of the ARC/Inf which express NK3 and establish frequent contacts with one another (Burke et al., 2006; Navarro et al., 2009; Amstalden et al., 2010). A recent model of the GnRH pulse generator proposed an intranuclear interplay between three colocalized neuropeptides, kisspeptins, NKB, and dynorphins, which uses NK3 and the dynorphin receptor KOR, but not the kisspeptin receptor (Navarro et al., 2009). Our present study only revealed a significant sex difference in the incidence of prepoNKB-IR cell bodies but not in the density of preproNKB-IR fibers and inputs to GnRH neurons. We have to note that sex differences of NKB immunoreactivity were relatively subtle, in comparison with the robust sexual dimorphism of kisspeptin immunoreactivity. If NKB subserves basic mechanisms of pulsatility in the Inf, the NKB-dependent signaling appears to be relatively well-preserved in aged males as well as females. On the other hand, the existing sex differences of the NKB system may be physiologically important in the generation of the different pulsatility patterns between aged males and females.

As we also discussed for kisspeptin, sex differences in preproNKB immunoreactivity can either be partly or entirely due to the different sex steroid exposure of preproNKB neurons in aged males and females. Notably, NKB neurons of the rat (Burke et al., 2006) and the sheep (Goubillon et al., 2000) contain estrogen receptor-α and estrogen treatment reduces preproNKB mRNA expression in the ARC of the rat (Danzer et al., 1999), mouse (Dellovade and Merchenthaler, 2004), sheep (Pillon et al., 2003), and monkeys (Abel et al., 1999). Long-term, but not short-term, ovariectomy also increased preproNKB mRNA expression in the ARC of monkeys, similarly to the postmenopausal increase observed in old females (Eghlidi et al., 2010).

Our results that preproNKB-IR neurons outnumbered kisspeptin-IR neurons by 120% in aged males suggest that many NKB neurons do not synthesize detectable amounts of kisspeptins. We have also observed many single-labeled kisspeptin-IR and preproNKB-IR axons in the Inf, which serves as further support for a substantial amount of segregation of kisspeptin and NKB synthesis in the human Inf. These observations are in contrast with the high degree of match between these neuropeptides in sheep (Goodman et al., 2007) and rodents (Navarro et al., 2009) and also indicate that the presence of the other putative neuromessengers of the GnRH pulse generator in kisspeptin and NKB neurons of the human Inf need to be carefully readdressed to reveal potential species differences. Future colocalization experiments will be required to establish the putative coexpression of dynorphins, NK3, and the dynorphin receptor KOR in human kisspeptin and NKB neurons. It is worth of note in this context that although prodynorphin expression has been detected in hypertrophied neurons in the Inf of postmenopausal women with *in situ* hybridization (Rometo and Rance, 2008) and in the ARC of monkeys with real-time PCR (Eghlidi et al., 2010), our preliminary immunohistochemical studies found little evidence for dynorphin A and dynorphin B immunoreactivities in the Inf of *post mortem* tissue samples. The regulation of prodynorphin expression in the ARC/Inf of different species is also somewhat controversial. The number of neurons expressing prodynorphin was reduced in postmenopausal women (Rometo and Rance, 2008), showed no significant postmenopausal change in monkeys (Eghlidi et al., 2010) and was upregulated in the absence of sex steroids (in parallel with KISS1 and preproNKB gene expression) in mice (Navarro et al., 2009).

THE MAIN OBSERVATIONS OF OUR PRESENT STUDY ARE SUMMARIZED BELOW

(i) KP- and NKB-immunoreactive (IR) cell bodies of the Inf are larger in aged human females than in males, which reflect a postmenopausal loss of estrogen negative feedback.
(ii) The number of KP-IR cell bodies, the density of KP-IR fibers, and the incidence of their contacts on GnRH neurons are much higher in aged women compared with men.

(iii) The number of NKB cell bodies is also only slightly higher in the Inf of women compared with men, whereas there is no significant sexual dimorphism in the regional density of NKB fibers and the incidence of their appositions onto GnRH cells.

(iv) The incidences of NKB-IR cell bodies, fibers, and appositions onto GnRH neurons exceed several-fold those of KP-IR elements in men. More NKB than KP inputs to GnRH cells are also present in women, whereas NKB and KP immunoreactivities do not differ in the other parameters.

(v) Only partial overlap exists between KP-IR and NKB-IR axons. KP is present in a higher percentage of NKB-IR afferents to GnRH neurons in women (31%) compared with men (9%) and the percentage of KP-IR contacts co-containing NKB is also higher in females (26%) than in males (10%).

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Evolutionary insights into the steroid sensitive *kiss1* and *kiss2* neurons in the vertebrate brain

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Kisspeptin was originally found as a peptide product of *Kiss1* gene and is now supposed to be an essential central regulator of reproduction in mammals. However, there is now a growing body of evidence to suggest that *kiss2*, the paralogous gene for *kiss1*, evolved in parallel during vertebrate lineage, and the *kiss2* product also activates the GPR54 (kisspeptin receptor) signaling pathways. Therefore, it is now widely accepted that both *kiss1* and *kiss2* are the kisspeptin genes. Interestingly, either *kiss1* or *kiss2* or both have been lost during evolution in many vertebrate species, and the functional significance of *kiss1* or *kiss2* for the central regulation of reproduction is suggested to vary according to the species. Here, we argue that the steroid sensitivity of the *kiss1* or *kiss2* neurons has been well conserved during evolution among tetrapods and teleosts, and thus it may be the key to understanding the functional homologies of certain populations of kisspeptin (*kiss1* or *kiss2*) neurons among different species of vertebrates. In the present review, we will first introduce recent advances in the study of steroid sensitive *kiss1* and *kiss2* systems in vertebrates and effects of peptide administrations *in vivo*. By comparing the similarities and differences between *kiss1* and *kiss2* of neuronal localization and sensitivity to gonadal steroids in various tetrapods and teleosts, we discuss the evolution of kisspeptin neuronal systems after gene duplication of ancestral kisspeptin genes to give rise to *kiss1* and *kiss2*.

Keywords: kisspeptin, *kiss1*, *kiss2*, evolution, estrogen, steroid feedback, GnRH

INTRODUCTION

Recent studies have shown that kisspeptin plays an essential role in reproductive functions in mammals. Kisspeptin attracts particular attention, since previous reports have shown that the lack of kisspeptin receptors gene, *GPR54*, in both mice and humans, or of the ligand gene (*Kiss1*) in mice results in reproductive dysfunctions. More interestingly, the kisspeptin neurons have been shown to express estrogen receptor alpha (ERα), while the GnRH neurons express kisspeptin receptors, and kisspeptin depolarizes GnRH1 neurons in mice. These results suggest that they are not only involved in the sex steroid feedback but also are possible candidate for the "missing link" in the gonadotropin feedback control (Tena-Sempere, 2005; Smith, 2008).

On the other hand, fewer studies exist on the non-mammalian kisspeptin, and somewhat contradictory results among different species appear to confuse general conclusions about the functions of kisspeptin, especially in teleosts. This may be because of the fact that not a few studies in teleost kisspeptin have used pharmacological methods such as intracerebroventricular (ICV) or peripheral administration of kisspeptins. We should consider that the physiological functions of each kisspeptin neuron population may not be assessed only by such analyses, because the pharmacological administration may activate unexpected signaling pathways apart from the actual kisspeptin neuron networks. Therefore, the detailed information on the anatomy of the axonal projections and on the physiology and distribution of receptors are necessary before the administration studies. On the other hand, the kisspeptin neurons in certain brain nuclei show steroid sensitivity in all the animal species thus far examined across teleosts and tetrapods. Therefore, we may be able to correlate the functional properties of the different populations of kisspeptin neurons in various species by using the sex steroid sensitivity as more physiological criteria.

On the other hand, it has been generally accepted in evolutionary biology that genes duplicated from a single gene in the ancestral vertebrate undergo sub-functionalization, neo-functionalization, or non-functionalization (Ohno, 1970). Recent literature on the kisspeptin of non-mammalian species suggests that the *kiss1* and *kiss2* systems may have undergone such evolutionary processes. To understand the parallel evolution of these genes in the kisspeptin neuronal systems, we here propose that the steroid sensitivity helps to identify the functionally equivalent neuronal populations among different species, because the steroid sensitivity appears to be the evolutionarily well conserved feature of certain populations of the kisspeptin neurons. As will be argued below, the non-mammalian kisspeptin systems show a wealth of diversity of gene expression (*kiss1* and/or *kiss2*) pattern in the brain and dynamic changes in expression according to the sex steroid milieu. Therefore, in spite of some kind of confusion in the kisspeptin studies of non-mammalian species, the biological study of *kiss1* and *kiss2*, and of neurons that express these genes will provide us interesting insights into the general features of the kisspeptin systems in vertebrates. In addition to be an interesting model to understand the general evolutionary mechanisms of paralogous

genes, the study of kisspeptin systems in non-mammalian vertebrates may lead us to find novel functions of kisspeptins, which may have been overlooked in previous studies using limited groups of mammalian species, which have globally lost *Kiss2*.

In the present review, we will introduce recent findings about *kiss1* and *kiss2* in vertebrates, mainly mammals and teleosts, and discuss their functions from various aspects, including cellular localizations, steroid sensitivity, and receptor distributions. Although there are a small number of studies that have been performed in non-mammalian tetrapods and vertebrates that emerged prior to the divergence of tetrapod from teleosts, we propose some general hypotheses about the evolution of *kiss1* and *kiss2* in the vertebrate lineage by comparing the two distinct groups, teleosts and tetrapods (see **Table 1** for nomenclature of kisspeptin genes and peptides)[1].

KISS1 AND KISS2

KISS1 AND *KISS2* ARE THE SISTER GENES: WHOLE GENOME DUPLICATION IN THE ANCESTRAL VERTEBRATE

In 2008, *kiss1* gene was isolated in non-mammalian species (Kanda et al., 2008; van Aerle et al., 2008). Then in 2009, *kiss2* was cloned from teleosts (Kitahashi et al., 2009) and amphibians (Lee et al., 2009) as the gene responsible for the peptide that showed a similar amino acid sequence to Kiss1. Because some ligand–receptor interaction studies showed that both Kiss2 and Kiss1 activate the kisspeptin receptor signaling pathways in *Xenopus tropicalis*, zebrafish (Lee et al., 2009), bullfrog (Moon et al., 2009), and goldfish (Li et al., 2009), Kiss2 has been recognized as one of the "kisspeptin" peptides.

Felip et al. (2009) performed a sophisticated synteny analysis of *kiss1* and *kiss2* genes in vertebrate species. In their report, it was strongly suggested that *kiss1* and *kiss2* are duplicated together with some surrounding genes such as *golt1a/b*, *plekha5/6*, *pik3c2b/cg*, and *etnk1/2*. They also discovered that only one co-ortholog for each pair was found in a chordate (*Ciona*), suggesting that these genes including *kiss1*/*kiss2* resulted from a gene duplication event that occurred at least "after" the divergence of urochordates and vertebrates.

Concerning the duplication of genes in general, it is strongly suggested that the common ancestors of the present vertebrates underwent whole genome duplication (WGD) twice (2R hypothesis; Ohno, 1970) that is evidenced by results of recent genome sequencing in vertebrate and urochordate species (Putnam et al., 2008). In addition, the ancestors of teleosts are suggested to have undergone one additional WGD [third-round (3R)-WGD; reviewed in Sato and Nishida, 2010]. Here, because amphibians and lampreys have both *kiss1* and *kiss2*, these homologs are considered to have duplicated in the ancestral vertebrate before the emergence of lamprey, as suggested by Felip et al. (2009). During evolution, some species such as the puffer fish, stickleback (Felip et al., 2009; Kitahashi et al., 2009; Li et al., 2009; Shahjahan et al., 2010; Yang et al., 2010), and some perciform fish (Felip et al., 2009; Mechaly et al., 2011) seem to have lost the *kiss1* gene. On the other hand, in tetrapods, most mammals have lost *Kiss2* during evolution. Because the platypus possesses both *Kiss1* and *Kiss2*, the loss of *Kiss2* must have occurred at least after the divergence of monotreme and other mammals. On the other hand, because the opossum is reported to lack *Kiss2* in its genome database (Felip et al., 2009), we may predict that the loss of *Kiss2* in the mammalian lineage occurred before the divergence of marsupials and placentarians (**Figure 1**).

Recent studies that are based on the genome sequence data suggested that most of the duplicated genes are subsequently lost rapidly after duplication (Brunet et al., 2006; Sato et al., 2009). Thus, for the teleost specific 3R-WGD, it is suggested that both *kiss1* and *kiss2* duplicated once again to give rise to four genes, and two of them were likely lost immediately in the early teleost lineage.

Taken together, this conservative organization of loci that contain *kiss1*/*kiss2* observed widely in vertebrates strongly suggest that *kiss1* and *kiss2* genes were duplicated at the locus level, and as Um et al. (2010) suggested, this duplication probably occurred in two rounds of WGD (1R-WGD and 2R-WGD; reviewed in Sato and Nishida, 2010) event (**Figure 1**).

KISS1 AND KISS2 ACTIVATE KISSPEPTIN RECEPTOR, GPR54

After the identification of Kiss1 and Kiss2, several ligand–receptor interaction studies have shown that both Kiss1 and Kiss2 activate the kisspeptin receptor signaling pathways in goldfish (Li et al., 2009), zebrafish, *Xenopus* (Lee et al., 2009), orange spotted grouper (Shi et al., 2010), and bullfrog (Moon et al., 2009), suggesting that Kiss1 and Kiss2 bind to the same kisspeptin receptor in vertebrates. Although the activation of the PKC or the cAMP pathway by Kiss1 and Kiss2 is slightly different in each species, it is generally accepted that both Kiss1 and Kiss2 are ligands for GPR54 in vertebrates. Thus, both Kiss1 and Kiss2, the peptide products of sister genes, can function as kisspeptins.

The kisspeptin receptor in mammals has been referred to either as GPR54 (Seminara et al., 2003) or Kiss1r (Gottsch et al., 2009). In many species studied so far, it has often been shown that more than one ligand and more than one receptor for kisspeptin bind to one another promiscuously (Lee et al., 2009; Li et al., 2009). Since recent studies also show the promiscuity of kisspeptin and other RF amide peptides (Lyubimov et al., 2010), it may be also possible that certain peptides other than Kiss1 and Kiss2 activate the kisspeptin receptor signaling pathways. Thus, although we once proposed a systematic nomenclature for kisspeptin receptor (Akazome et al., 2010), we refer to the kisspeptin receptors as GPR54-1 and 2 as proposed in Lee et al. (2009) in the present review.

[1]According to the Zebrafish Information Network, ZFIN; http://zfin.org/zf info/nomen.html, we will italicize gene names, such as *kiss1* and *kiss2*, and Romanize protein and peptide name, such as Kiss1 and Kiss2 (for details, see **Table 1**). We will call the receptor for kisspeptins as "GPR54" in the present review. This is because a recent study has reported on the promiscuous nature of ligands and receptors for RF amide families, including kisspeptin (Lyubimov et al., 2010), and the terms, kissr or Kissr, may lead to misunderstandings about the ligand receptor relationships.

Table 1 | Terminology of gene and protein names for kisspeptin.

	Primates	Non-primate mammals	Non-mammalian vertebrates
gene/mRNA	*KISS1*	*Kiss1*	*kiss1*
Protein	KISS1	Kiss1	Kiss1

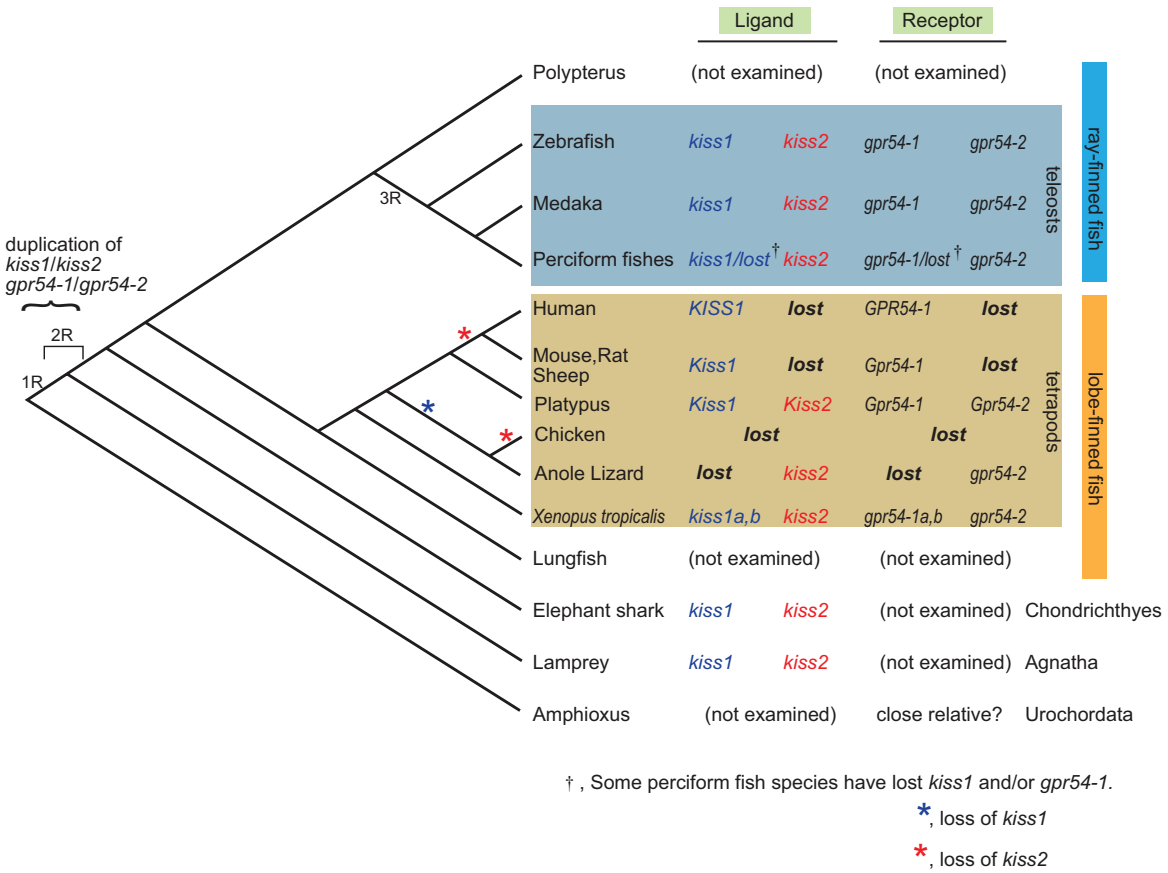

FIGURE 1 | Summary of the subtypes of ligand and receptor genes for kisspeptin systems in the genome along the vertebrate lineage. *kiss1* and *kiss2* are supposed to be duplicated before the emergence of lamprey, and probably due to the whole genome duplication of the ancestral vertebrate. It is supposed that *kiss2* and *gpr54-2* were lost in marsupial and placental mammals after the divergence from the monotreme during mammalian evolution. It should be noted that some teleost species have lost *gpr54-1*, but no teleosts have lost *gpr54-2*, suggesting the significance of *gpr54-2* in teleosts, which is opposite to the case in the mammalian lineage. It is also consistent with the higher level of expression of *gpr54-2* compared to that of *gpr54-1* in teleost brains. The loss of *kiss1* (blue) or *kiss2* (red) is indicated by asterisks.

KISSPEPTIN ADMINISTRATION STUDY OF KISSPEPTIN FUNCTIONS

In mammals, many studies have shown that peripheral or ICV injection of kisspeptin evokes LH secretion in rodents (Gottsch et al., 2004; Irwig et al., 2004; Matsui et al., 2004; Navarro et al., 2004, 2005a,b), sheep (Messager et al., 2005), monkey (Shahab et al., 2005; Plant et al., 2006; Seminara et al., 2006), and human (Dhillo et al., 2005). Moreover, it has been reported that the kisspeptin stimulates GnRH neurons both directly and indirectly via interneurons (Han et al., 2005; Dumalska et al., 2008; Liu et al., 2008; Pielecka-Fortuna et al., 2008; Zhang et al., 2008; Pielecka-Fortuna and Moenter, 2010). However, the number of studies in animals that possess both Kiss1 and Kiss2 is limited. In such animals, there is no evidence for direct kisspeptin GnRH regulation on GnRH release, and the results of peripheral administration of Kiss1 and Kiss2 decapeptides are not consistent with one another. Administration of the core decapeptide of Kiss2, but not of Kiss1, significantly increased LHβ and FSHβ subunit mRNA expression in the zebrafish pituitary (Kitahashi et al., 2009). Kiss2 also induced LH and FSH secretion in sea bass with higher potency than Kiss1 decapeptide (Felip et al., 2009). In contrast, intraperitoneal administration of Kiss2 peptide in goldfish did not increase serum LH levels, although the administration of Kiss1 peptide did (Li et al., 2009). Thus, there appears to be a difference amongst species in the relative potencies of Kiss1 and Kiss2 for facilitating LH and FSH secretion and in the time of LH/FSH rise after administration of kisspeptins. Therefore, the induction of LH/FSH secretion by kisspeptins (Kiss1 or Kiss2) in non-mammalian vertebrates needs more experimental evidence to be accepted as a general notion. Moreover, because peripheral administration does not reflect the actual axonal projection and the receptor distribution of kisspeptin neurons in the brain, cellular-level studies, such as electrophysiology, are necessary to determine the action sites of kisspeptins and the possible interactions between kisspeptin and GnRH neurons. Therefore, electrophysiological examination of effects of either Kiss1 or Kiss2 on GnRH neurons in species that possess both genes is important for the understanding of such peptidergic systems that arose from the gene duplication.

INVOLVEMENT OF KISSPEPTIN NEURONS IN THE STEROID FEEDBACK SYSTEM

In the rodent anteroventral periventricular nucleus (AVPV) and sheep preoptic area (POA), *kiss1* mRNA expression in Kiss1 neurons are upregulated, whereas those in the arcuate nucleus (ARC) are downregulated, by gonadal steroids (Irwig et al., 2004;Smith et al., 2005a,b, 2007, 2008; Revel et al., 2006; Adachi et al., 2007; Ansel et al., 2010). Although there is a discrepancy that ewe ARC Kiss1 show higher expression in the breeding season in spite of the negative regulation of gonadal steroids (Estrada et al., 2006; Smith, 2009), it is generally accepted that AVPV/POA Kiss1 neurons are positively regulated, and ARC Kiss1 neurons are negatively regulated by the gonadal steroids. These properties are widely recognized in mammalian species.

In mice, it has been shown that GnRH neurons do not express ERα (reviewed in Herbison and Pape, 2001), which are essential for normal reproductive functions (Couse et al., 2003; Dorling et al., 2003; Wintermantel et al., 2006). Thus, Herbison concludes that estrogen acts indirectly on GnRH neurons to bring about their activation (Herbison, 2008). This missing link in mice was found to be explained by steroid sensitive kisspeptin neurons, because kisspeptin directly depolarizes GnRH neurons in mice (Han et al., 2005; Dumalska et al., 2008; Liu et al., 2008; Pielecka-Fortuna et al., 2008; Zhang et al., 2008; Pielecka-Fortuna and Moenter, 2010), which is expected to facilitate firing activities and then GnRH release. Thus, the role of kisspeptin neurons in steroid feedback is one of the most interesting topics in the study of kisspeptin neuron systems.

In non-mammalian vertebrates, the localization of *kiss1*/*kiss2* neurons is reported in medaka (Kanda et al., 2008; Mitani et al., 2010), zebrafish (Kitahashi et al., 2009), *Xenopus* (Lee et al., 2009), seabream (Shimizu et al., 2012), and puffer fish (Kanda et al., 2010). Among them, medaka and goldfish are the only species whose steroid sensitivity has been examined experimentally. Therefore, recent data shown by us in medaka will be described below. In addition, we will also describe results in the goldfish, which gives us insights into the evolutionary aspects of *kiss1*/*kiss2* genes.

GONADAL STEROIDS UPREGULATE *KISS1* EXPRESSION IN MEDAKA NVT NEURONS

In situ hybridization studies have shown the localization of *kiss1* and *kiss2* neurons in the medaka brain (Kanda et al., 2008; Kitahashi et al., 2009; Mitani et al., 2010). The *kiss1* neurons are localized in the hypothalamic nuclei, nucleus ventralis tuberis (NVT) and nucleus posterioris periventricularis (NPPv), as well as in an extrahypothalamic nucleus, habenula. On the other hand, the *kiss2* neurons are localized in nucleus recessus lateralis (NRL). Among these *kiss1* and *kiss2* neurons, only the *kiss1* neurons in NVT show prominent steroid sensitivity in their kisspeptin gene expression. Ovariectomy (OVX) dramatically reduced *kiss1* expression in NVT neurons, which were recovered by subsequent estrogen replacement. Because our unpublished data showed that 11 ketotestosterone, a non-aromatizable androgen, did not recover this decrease at all, the steroid feedback activity seems to be mediated by estrogen receptor. It was also shown that NVT *kiss1* neurons are the only kisspeptin neuron population that shows expressional variations according to the breeding states. Moreover, double insitu hybridization analysis has shown that *kiss1* neurons in NVT express ERα. In addition to ERα, NVT *kiss1* neurons in medaka express ERβ as well (Mitani et al., 2010), which is similar to *kiss1* neurons in mice (Smith et al., 2005a).

Interestingly, in addition to steroid sensitivity, NVT *kiss1* neurons also show sexual dimorphism in number (Kanda et al., 2008). In contrast to the sexual dimorphism found in rodents, in which females have more AVPV *Kiss1* neurons than males (Clarkson and Herbison, 2006; Smith et al., 2006; Adachi et al., 2007; Kauffman et al., 2007), male medaka show significantly more NVT *kiss1* neurons than females. Comparative analyses in much more vertebrate species should be necessary for understanding the organization of sexual dimorphism and the functional significance of male- or female-predominant expressions.

POA *KISS2* NEURONS IN GOLDFISH SHOW STEROID SENSITIVITY LIKE POA *KISS1* NEURONS IN MAMMALS

In zebrafish, localization of *kiss1* and *kiss2* neurons was analyzed by *in situ* hybridization (Kitahashi et al., 2009; Servili et al., 2011). The *kiss1* neurons in the zebrafish brain are distributed in the habenula and periventricular hypothalamus, while the *kiss2* neurons are distributed in the posterior tuberal nucleus, the periventricular hypothalamic nucleus, and parvocellular preoptic nucleus (Kitahashi et al., 2009; Servili et al., 2011). In the juvenile zebrafish, it was demonstrated that estradiol administration increases the mRNA expression of *kiss1*, *kiss2*, and *gpr54-2* in the brain (Servili et al., 2011). Among them, Servili et al. focused on *kiss2* neurons in the dorsal hypothalamus (Hd), caudal hypothalamus (Hc), and anterior tuberal nucleus (ATN), and demonstrated that all of them showed higher *kiss2* expression after estradiol administration in juvenile fish. Because it may not be physiological to administrate estrogen to juveniles, it may be rather difficult to interpret these results. In spite of this, it is intriguing to investigate the homologous relationships of these neurons to the medaka steroid sensitive *kiss1* neurons in NVT and steroid insensitive *kiss2* neurons in NRL. However, as the hypothalamic structures vary even among teleosts, and no clear experimental evidence for the nucleus-specific steroid sensitivity in adults has been shown in any fish except medaka, further examination of the effects of gonadal steroids on the teleost kisspeptin neurons using ovariectomy should be necessary.

Therefore, we recently performed *kiss1* and *kiss2* *in situ* hybridization in the goldfish, because the goldfish is rather easily amenable to ovariectomy, and it belongs to the same Cypriniformes as the zebrafish. We found a prominent expression of *kiss2* in POA, unlike results in zebrafish, and found that the POA *kiss2* neurons show clear steroid sensitivity (Kanda et al., 2012). In the adult goldfish in the breeding condition, the *kiss1* neurons are localized in the habenula, whereas the *kiss2* neurons are located in nucleus lateralis tuberis (NLT), NRL, and POA. Among these neurons, the POA *kiss2* neurons decreased in number after OVX, and the reduction was recovered by estrogen implant. It strongly suggests that POA *kiss2* neurons are upregulated by ovarian estrogen, which is similar to the AVPV/POA *kiss1* neurons in mammals. The discussion on the homology and the evolutionary hypothesis derived there from will be described in detail later in this review.

PUFFER FISH, WHICH POSSESS ONLY *KISS2*, SHOW *KISS2* EXPRESSION IN POA AND HYPOTHALAMUS

From the genome database analysis, puffer fish are supposed to possess only *kiss2* expressing neurons, because they have lost *kiss1* at the genome level. Shahjahan et al. (2010) took advantage of the seasonally breeding grass puffer and showed changes in the expression levels of *kiss2*/*gpr54-2* genes together with the gonadosomatic index (GSI) during the spawning period. Here, because of the absence of *kiss1* neurons, the *kiss2* neurons in certain brain area are supposed to subserve the kisspeptin functions in this species.

Recently, we analyzed the localization of *kiss2* neurons in juvenile green puffer fish by *in situ* hybridization and found that they are expressed in the hypothalamic nucleus NRL and the POA (Kanda et al., 2010), which is similar to the results in the zebrafish (Kitahashi et al., 2009; Servili et al., 2011). Unfortunately, it is technically difficult to raise green puffer to breeding conditions, and future studies using puffer fish that are capable of breeding to full maturity in aquarium tanks will be interesting.

In addition to this observation, it was recently shown that *kiss2* expression is increased during the pre-spawning season (late spermatogenesis stage in male, and early vitellogenesis stage in female) in club mackerel (Selvaraj et al., 2010). Further expression analyses in some teleost species may lead us to find some general expressional variations of kisspeptin genes in the seasonal breeders.

SPECIES DIFFERENCE IN THE FUNCTIONS OF THE SISTER GENES, *KISS1* AND *KISS2*

There are many species differences in the functions of *kiss1* and *kiss2* neurons in vertebrates. The most extreme example lies between *kiss1*-lacking puffer fish and *KISS2*-lacking human. Even within the teleost species that express both *kiss1* and *kiss2*, there are obvious species differences. For example, in medaka, only the NVT *kiss1* neurons show steroid sensitivity, whereas there is no such *kiss1* neuron in the hypothalamus of zebrafish, and, instead, many *kiss2* neurons are localized in the hypothalamus.

Medaka *kiss2* neurons are localized in NRL, where *kiss2* neurons are also localized in zebrafish. Although Servili et al. (2011) proposed a possibility of functional similarity between the steroid sensitive medaka NVT *kiss1* neurons and some of the zebrafish *kiss2* neurons in the ventral hypothalamus, the zebrafish *kiss2* neurons appear to contain neurons equivalent to the medaka *kiss2* neurons and some other populations of neurons. It should be interesting to search for experimental evidence for such homologies. *In situ* hybridization and immunohistochemistry using some other fish species may give us clues to further understanding of the functional homology and evolution of these sister gene-expressing neuron systems. Experimental analysis on the effects of gonadal steroids by gonadectomy in various non-mammalian species should be very helpful to discuss true functional or morphological homologies, because the steroid sensitivity well characterizes the property of each nucleus, but such studies have been performed only in a small number of species such as medaka and goldfish.

EVOLUTION OF *KISS1* AND *KISS2* NEURONS IN EACH NUCLEUS – A WORKING HYPOTHESIS

As described above, *kiss1* and *kiss2* in the present vertebrate species are suggested to be the sister genes, which originate from the gene duplication event in the ancestral vertebrate. Furthermore, it is highly possible that they were duplicated during the genome-wide duplication events. Because these sister genes possess family genes in their loci, they are considered to have duplicated at least at the locus level, regardless of whether the WGD event made these sister genes or not. Thus, just after the duplication, *kiss1* and *kiss2* must have had completely the same sequence in their open reading frame as well as the regulatory sequence, and they must have been co-expressed in the same location at first. During evolution, one or even both of them was silenced in some species, and their location of expression and function diverged. Moreover, as seen between medaka and zebrafish/goldfish, the general functions or relative functional importance of *kiss1* and *kiss2* for the central regulation of reproduction are different among species; their functions are considered to have diverged among species during evolution (Kanda et al., 2012).

Interestingly, the inversion of the importance of *kiss1* and *kiss2* for the central regulation of reproduction occur rather commonly among different species (see the previous section). On the other hand, this phenomenon never occurred during the evolution of the hypophysiotropic GnRH system; the Cyprinids and Salmonids have lost *gnrh1*, but this lack seems to have been functionally compensated by the remaining genes (Okubo and Nagahama, 2008). We suppose that the functional conservativeness of the GnRH systems may be due to the fact that loss of the *gnrh* function, especially the hypophysiotropic one, would lead to severe reproductive dysfunctions (Cattanach et al., 1977) or to a failure of normal sexual maturation (Wu et al., 2006).

We have been routinely performing in situ hybridization of *kiss1* and *kiss2* genes (Kanda et al., 2008; Mitani et al., 2010) and find it more difficult to detect them compared to *gnrh2* or *gnrh3* (Gopinath et al., 2004; Okubo et al., 2006; Palevitch et al., 2007), which empirically suggests the lower level of expression of *kiss1*/*kiss2*, especially during the developmental stages. Moreover, the reported lack of *gpr54* expression in the hypophysiotropic GnRH1 neurons in some teleosts (Grone et al., 2010; and our unpublished observation) suggests that the physiological functions and their mechanisms are somewhat more diverged in the vertebrate *kiss1*/*kiss2* systems, compared to the rather conservative GnRH systems. Thus, unlike the GnRH systems, in which the inversion of physiological functions between the hypothalamic hypophysiotropic (GnRH1) and extrahypothalamic neuromodulatory GnRH systems (GnRH2/GnRH3) has never been reported, the *kiss1* and *kiss2* systems are the ones having rather promiscuous ligand–receptor relationships and are supposed to be more adaptive during evolution; they appear to have avoided extreme selection pressure. Thus, the understanding of the evolution of *kiss1* and *kiss2* neuron systems may lead to a model for the study of general evolutionary mechanism of peptidergic neurons in the absence of strong selection pressure.

EVOLUTION OF STEROID SENSITIVE KISSPEPTIN NEURONS IN POA/AVPV

The distribution of *kiss1* and *kiss2* neurons in representative animal species and in the presumptive common ancestral animals are shown in **Figure 2**. It has been already suggested that POA Kiss1 neurons in sheep, AVPV Kiss1 neurons in rodents, and PeN Kiss1 neurons in pig are homologous in mammals (Smith, 2009; Tomikawa et al., 2010). These forebrain kisspeptin neurons in mammals, which show steroid sensitivity, are Kiss1 neurons, although they have not been examined in monotreme, which have both *Kiss1* and *Kiss2* genes. On the other hand, the goldfish steroid sensitive POA kisspeptin neurons express *kiss2* mRNA (Kanda et al., 2012). This may sound a little bit strange at first sight. However, the results in *Xenopus laevis* may give us a hint for the evolutionary mechanism. The *Xenopus* POA kisspeptin neurons express *kiss2* mRNA (Lee et al., 2009). Thus, all the vertebrate forebrain populations of kisspeptin neurons studied so far, except for mammals, are Kiss2 neurons. We therefore hypothesize that both ancestral teleosts and ancestral tetrapods expressed *kiss2* in POA. We further hypothesize that in mammals *Kiss1* is expressed in the neurons that are homologous to *Xenopus* POA *kiss2* neurons; this conversion of *Kiss2* to *Kiss1* may have occurred because of the loss of *Kiss2* after the divergence of the ancestor of the present mammals from the monotreme. Thus, we assume that, during mammalian evolution, the loss of *Kiss2* gene triggered the expression of *Kiss1* in mammalian POA. An alternative possibility is that *Kiss1* neurons emerged in the POA before they lost *Kiss2*.

However, the overlapped functions tend to be lost rapidly during evolution in general. We suppose that the loss of *Kiss2* triggered the expression of *Kiss1* in the same neurons because of the similar regulatory sequence between *Kiss1* and *Kiss2*. In other words, the loss of *Kiss2* might have canceled the expressional inhibition of *Kiss1* in POA kisspeptin neurons. Such a phenomenon is called the "genetic robustness" and has been only examined in the duplicated genes of *C. elegans* and yeast (Gu et al., 2003; Conant and Wagner, 2004), or has been observed in human genetic disease (Hsiao and Vitkup, 2008). In these studies, it was shown that the duplicated genes, which have a similar copy as the sister genes, tend to cause genetic disease less frequently compared to the singleton genes. Thus, it is supposed that closely related genes, such as sister genes, often compensate for the gene loss, and we suppose that *Kiss2* gene loss and emergence of *Kiss1* neurons in mammalian lineage coincided with each other. To our knowledge, there has been no report on the occurrence of such "genetic robustness" in the highly complicated neuronal systems of vertebrates. Although it is difficult to use monotreme for experimental use, it is intriguing to study the localization of monotreme *Kiss1* and *Kiss2* neurons. Thus, the evolutionary studies of *kiss1* and *kiss2* genes should serve as a good model system for the study of evolution of sub-functionalized sister genes in the central nervous system in general.

STEROID SENSITIVE AND INSENSITIVE HYPOTHALAMIC KISSPEPTIN NEURONS

In mammals, the hypothalamic Kiss1 neurons in ARC are steroid sensitive as described above. Because OVX increases the *Kiss1*

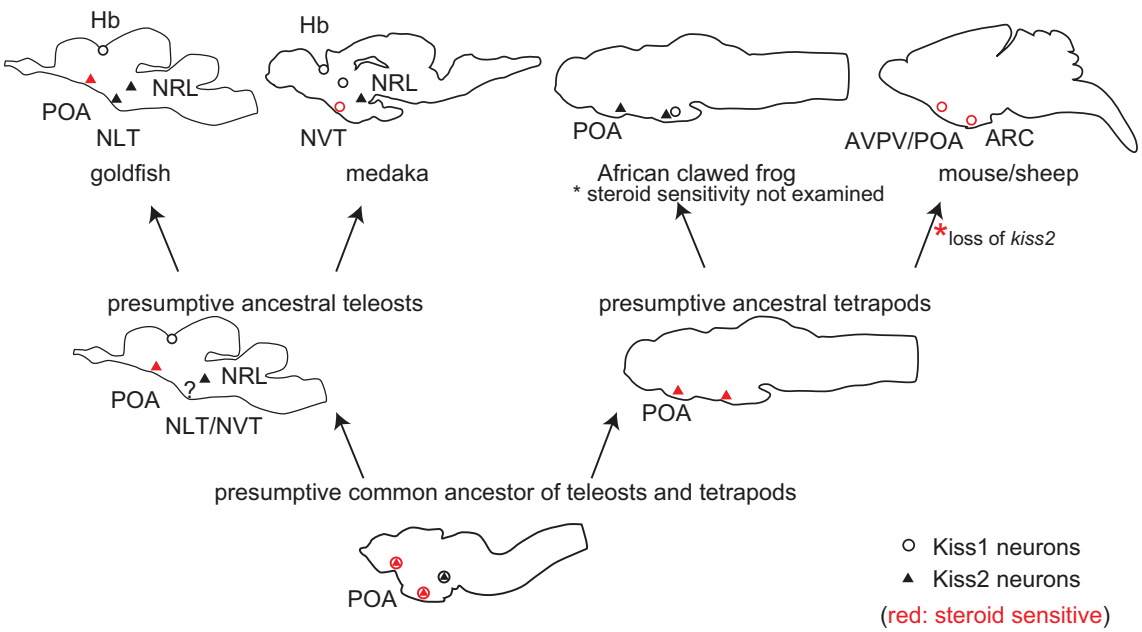

FIGURE 2 | Schematic illustrations for the distribution of *kiss1* and *kiss2* neurons in vertebrate brains, including some hypotheses. Open circles indicate *kiss1*, and filled triangles indicate *kiss2* neurons. Circles/triangles in red are *kiss1*/*kiss2* neurons that are steroid sensitive. Because *kiss1* and *kiss2* are duplicated sister genes, they are considered to have been co-expressed in the same neurons in the common ancestor of teleosts and tetrapods. Considering the fact that both amphibians and teleosts express *kiss2* in POA, the ancestral teleosts and ancestral tetrapods are supposed to have expressed *kiss2*. Because *Kiss2* was lost in the mammalian lineage, we hypothesize that *Kiss1* began to be expressed where *Kiss2* used to be expressed, to compensate for the loss of *Kiss2* during mammalian evolution.

expression, and estrogen or testosterone nullifies this increase in ARC, the ARC Kiss1 neurons are considered to be negatively regulated by ovarian steroids.

In species other than mammals, the only report about the gonadal steroid regulation on the hypothalamic kisspeptin neurons is on medaka Kiss1 neurons (Kanda et al., 2008; Mitani et al., 2010). Here, unlike the mammalian ARC Kiss1 neurons, their expression is enhanced, instead of inhibited, by ovarian steroids. Thus, one may argue against the homology of these nuclei in teleosts and mammals. We should note, however, that positive or negative regulation can be rather easily inverted by the composition of co-expressed transcription factors, such as Sp1 and Sp3 (Li et al., 2007). Thus, the difference in positive/negative regulation itself should not disprove the homology. Moreover, NVT also contains some other types of neurons involved in the homeostatic regulation like the mammalian ARC, supporting the evidence for the possible homology of these nuclei. However, because the teleosts lack the median eminence, the characteristic projection of ARC Kiss1 neurons to the median eminence, which is usually observed in mammals, cannot be observed. Instead, the medaka NVT Kiss1 neurons are shown to project to the POA. Experimental analysis of steroid sensitivity and the axonal projections of the hypothalamic Kiss1 neurons in other animals are necessary for the understanding of the evolutionary origin and possible homology of the hypothalamic Kiss1 neurons in other animals are necessary for the understanding of the evolutionary origin and possible homology of the hypothalamic Kiss1 neurons.

In medaka, gene expression of the NRL *kiss2* neurons was shown to be independent of breeding conditions), and these *kiss2* neurons were not shown to express ERα (Mitani et al., 2010). Thus, the neurons that are homologous to the steroid insensitive teleost hypothalamic (NRL) *kiss2* neurons may be absent in mammals. Further comparative studies among various species of teleosts and amphibians may solve the problem of whether steroid insensitive hypothalamic *kiss2* neurons are specific to the teleosts or just lost in mammals.

TELEOST-SPECIFIC EXPRESSION OF *KISS1* IN HABENULA

The kisspeptin neurons in the habenula have been reported only in teleosts (Kitahashi et al., 2009; Mitani et al., 2010; Servili et al., 2011), and they express *kiss1* in all those species. The projection of habenular neurons have been well studied by classical neuroanatomical experiments as well as by using recent molecular genetic techniques (Aizawa et al., 2005; Gamse et al., 2005). Consistent with results of these studies of habenular projections, the habenular Kiss1 neurons in zebrafish (Servili et al., 2011) and medaka (Kanda et al., unpublished data) appears to project to the interpeduncular nucleus (IPN) via fasciculus retroflexus. As *gpr54-2*, one subtype of *gpr54* genes in teleosts, is reported to be expressed in IPN, and *gpr54-1*, another subtype, is co-expressed in habenula in zebrafish, Servili et al. (2011) suggested the existence of the autocrine regulation of habenula (via *gpr54-1*) and the target neurons in IPN (via *gpr54-2*). Because there is no other report on the existence of *kiss1* neurons in the tetrapod habenula, it remains to be known whether the habenular *kiss1* was originally expressed in the ancestral vertebrate and subsequently lost in the tetrapod lineage, or solely began to be expressed in the teleost lineage. In order to answer this question, expression analysis of *kiss1* in phylogenetically significant species such as lungfish and polypterus are ongoing in our laboratory.

CONCLUSION

In this review article, we reviewed recent findings of kisspeptin neurons in vertebrates, by comparing studies in mammalian species and non-mammalian species, mainly teleosts. To date, only a handful of studies have shown kisspeptin neurons' functional significance in the central regulation of reproduction in species other than mammals. Because kisspeptin receptor *gpr54-2* as well as *gpr54-1* are expressed in POA and hypothalamus, but not in GnRH1 neurons in some species, kisspeptin's novel function other than the central regulation of reproduction (Kadokawa et al., 2008; Szawka et al., 2010; Yang et al., 2010; Luque et al., 2011) will also be an interesting topic in the future studies.

As reviewed above, the steroid sensitivity of kisspeptin neurons has been reported in a wide variety of species including non-mammalian vertebrates. Although the mechanisms are not well studied in the non-mammalian species, the steroid sensing feature and the related functions are highly conserved throughout vertebrates. Although the natural selection of functions of paralogous *kiss1* and *kiss2* genes are complicated, the study of evolutionary process of these sister genes may give clues to understand the evolution of the central nervous system after genome duplication in general. Here, the kisspeptin neuron system may be not only the regulator of reproductive/homeostatic functions in vertebrates but also the pioneer toward further understanding of the evolution of the central nervous system functions.

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The neurosteroid progesterone underlies estrogen positive feedback of the LH surge

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Our understanding the steroid regulation of neural function has rapidly evolved in the past decades. Not long ago the prevailing thoughts were that peripheral steroid hormones carried information to the brain which passively responded to these steroids. These steroid actions were slow, taking hours to days to be realized because they regulated gene expression. Over the past three decades, discoveries of new steroid receptors, rapid membrane-initiated signaling mechanisms, and *de novo* neurosteroidogenesis have shed new light on the complexity of steroids actions within the nervous system. Sexual differentiation of the brain during development occurs predominately through timed steroid-mediated expression of proteins and long term epigenetic modifications. In contrast across the estrous cycle, estradiol release from developing ovarian follicles initially increases slowly and then at proestrus increases rapidly. This pattern of estradiol release acts through both classical genomic mechanisms and rapid membrane-initiated signaling in the brain to coordinate reproductive behavior and physiology. This review focuses on recently discovered estrogen receptor-α membrane signaling mechanisms that estradiol utilizes during estrogen positive feedback to stimulate *de novo* progesterone synthesis within the hypothalamus to trigger the luteinizing hormone (LH) surge important for ovulation and estrous cyclicity. The activation of these signaling pathways appears to be coordinated by the rising and waning of estradiol throughout the estrous cycle and integral to the negative and positive feedback mechanisms of estradiol. This differential responsiveness is part of the timing mechanism triggering the LH surge.

Keywords: estrogen receptor, ERαΔ4, membrane estradiol signaling, receptor trafficking

INTRODUCTION

For decades the prevailing concept was that the brain passively responded to steroids originating from the periphery. These hormones were thought to mediate feedback mechanisms and regulate reproductive circuits. Although the brain does respond directly to ovarian and adrenal steroids, over the years this straight-forward notion has been drastically modified. The nervous system does not just passively respond to peripheral steroids. It actively metabolizes peripheral steroids to biologically active steroids that regulate numerous brain functions. For example, circulating testosterone can be aromatized to estradiol, or reduced to dihydrotestosterone (DHT) and further to 3β- or 3α-diols that are important in regulating behavior, negative feedback, and sexual differentiation (Lavaque et al., 2006a; Higo et al., 2009). Further, peripheral progesterone can be metabolized to allopregnanolone (3β-hydroxy-5α-pregnan-20-one). Many of these neurally converted metabolites are ligands for nuclear receptors that are transcription factors that modulate gene expression (Roselli et al., 2009). In contrast, other steroid metabolites allosterically bind to and modulate membrane receptor activity. The most notable of this type is allopregnanolone, which activates the $GABA_A$ receptor (Majewska et al., 1986; Puia et al., 1990; Hosie et al., 2006; Herd et al., 2007; Mitchell et al., 2008).

A surprising discovery was that nervous tissue synthesizes steroids *de novo* to regulate neural functions and behavior (Baulieu, 1981, 1991, 1998; Mellon, 1994; Schumacher et al., 2003). The steroids synthesized in the nervous system are referred to as neurosteroids (Baulieu, 1981, 1991), and are produced in astrocytes, oligodendrocytes, and neurons. The proteins and enzymes required for *de novo* steroidogenesis of estradiol from cholesterol are present in the brain. As in the ovary, multiple cell types cooperate in steroidogenesis. The most prevalent neurosteroids are pregnenolone, progesterone, and allopregnanolone (Corpechot et al., 1993), which are synthesized in astrocytes (Zwain and Yen, 1999).

Neurosteroidogenesis studies localized steroidogenic enzymes, enzymatic activity, and transporter proteins in expected and unexpected regions of the nervous system (Follesa et al., 2000; Wehrenberg et al., 2001; Lavaque et al., 2006b). Because of their highly localized synthesis and relatively low levels compared to circulating steroids, measuring, and determining the roles of neurosteroids in physiological, behavioral, and feedback systems has been difficult. In peripheral nerves, neurosteroids are implicated in myelination (Schumacher et al., 2000, 2003, 2004), and have therapeutic actions in the treatment of epilepsy and traumatic brain injury (Dubrovsky, 2005; Morrow, 2007; Reddy and Rogawski, 2009).

Recently, roles for neurosteroids in reproductive physiology have been defined. Immortalized murine gonadotropin releasing hormone (GnRH) neurons, GT1-1 cells, converted progesterone to allopregnanolone, and stimulated the release of GnRH by activating the $GABA_A$ receptor (el-Etr et al., 1995). Another important milestone for understanding the physiology of neurosteroids was demonstrating that the actions of peripheral steroids are integrated with the actions of neurosteroids. This review focuses on this integration controlling ovulation.

ROLE OF NEUROPROGESTERONE AND ITS METABOLITES IN FEMALE REPRODUCTION

Ovulation, a critical event in mammalian reproduction, is initiated by elevated levels of estradiol released by maturing ovarian follicles which act on the hypothalamus and pituitary. This event, known as estrogen positive feedback, produces a surge of GnRH from the hypothalamus that stimulates the secretion of luteinizing hormone (LH) from the pituitary. A puzzling aspect of estrogen positive feedback is that estrogens which are inhibitory to hormone release from the hypothalamus and pituitary gland during most of the cycle (negative feedback) now stimulate these tissues to induce a surge of hormone release, especially the neural network that controls the GnRH neurons (Chazal et al., 1974). Sequential estradiol and progesterone stimulation of the hypothalamus is essential for estrogen positive feedback to culminate in the LH surge (Chappell and Levine, 2000; Micevych et al., 2003, 2008; Micevych and Sinchak, 2008b). A key step in positive feedback is the estradiol-induced expression of hypothalamic progesterone receptors (PRs; Brom and Schwartz, 1968; Ferin et al., 1969; Labhsetwar, 1970; Rao and Mahesh, 1986; Mahesh and Brann, 1998b). Specifically, transcription and activation of PR-A in the hypothalamus are obligatory events in the induction of the GnRH and LH surges in estradiol-primed, ovariectomized (OVX) rats (Chappell and Levine, 2000).

For several years, it was not clear how PR-A was activated. One proposed mechanism was ligand-independent activation of PR that did not require progesterone (Mani et al., 1994, 1996; Mani, 2006). In place of progesterone, dopamine acting through the DA_1 receptor would activate PR. Another hypothesized mechanism was that pre-ovulatory progesterone of adrenal origin activated PR. Both the ovary and the adrenal cortex, highly steroidogenic organs, are capable of producing progesterone needed for the LH surge (Mahesh and Brann, 1998a). However, evidence for a peripheral source of progesterone is lacking. A significant rise in progesterone has not been consistently seen in the systemic circulation prior to the LH surge indicating that the progesterone required for the LH surge may not be synthesized peripherally (Feder et al., 1971; Kalra and Kalra, 1974; Smith et al., 1975). Consistent with this idea is that neither the adrenals nor the ovaries are necessary for an estrogen-induced LH surge (Mann et al., 1976; Sridaran and Blake, 1980). Indeed, OVX and adrenalectomized (ADX) rats primed with only 17β-estradiol have a robust LH surge (Mann et al., 1976; Micevych et al., 2003). However, inhibiting the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) that converts pregnenolone to progesterone in ADX/OVX rats prevents estradiol induction of the LH surge, indicating that progesterone is needed for estrogen positive feedback (Micevych et al., 2003).

Since estradiol induces an LH surge in OVX/ADX rats and blocking progesterone signaling inhibits estradiol-induced LH surges, we hypothesized that the source of this progesterone was the brain. The steroidogenic capacity of the brain has been well-established (Baulieu, 1981, 1991; Corpechot et al., 1981; Le Goascogne et al., 1987; Jung-Testas et al., 1989a,b; Mellon and Deschepper, 1993; Mellon, 1994; Guennoun et al., 1995; Koenig et al., 1995; Sanne and Krueger, 1995). Our investigations demonstrate that the enzymes and carrier proteins are present and that estradiol regulates them in a dose and time dependent manner that is congruent with estradiol levels across the estrous cycle. PCR analysis confirmed that hypothalamic tissue *in vivo* expresses mRNA for cytochrome P-450 side-chain cleavage (P450scc) and 3β-HSD, as well as the cholesterol transport protein steroidogenic acute regulatory protein (StAR) – all of the proteins needed to synthesize progesterone from cholesterol (Soma et al., 2005). Like PR, estradiol primes the neurosteroidogenic pathways. Estradiol increased hypothalamic expression 3β-HSD with a time course that would allow for neuroprogesterone synthesis to occur hours prior to the LH surge and act through estradiol-induced PRs (Soma et al., 2005). The importance of the steroidogenesis in the brain for the LH surge and estrous cycle was demonstrated in gonadally intact cycling rats. On the morning of proestrus, steroidogenesis in the hypothalamus was blocked by infusing aminoglutethimide (AGT), a P-450 side-chain cleavage (P450scc) enzyme inhibitor into the lateral ventricle. Although peripheral levels of estradiol in vehicle and AGT treated rats were similar, the estrous cycle of AGT female was arrested in proestrus and levels of progesterone in the hypothalamus and circulation remained low compared to controls. In AGT treated rats, the uterus was swollen and fluid filled, the ovaries had numerous developing follicles but no forming corpus luteum – all indications that peripheral steroidogenesis was intact. In spite of the typical proestrus rise in circulating estradiol, blocking neurosteroidogenesis prevented the LH surge, ovulation, and luteinization and arrested progression of the estrous cycle (Micevych and Sinchak, 2008a; **Figure 1**). After several days the effects of AGT wore off and the rats exhibited vaginal cytology of estrus, indicative of the LH surge, and resumed their regular 4–5 day estrous cycles. These data strongly suggest that estradiol stimulates hypothalamic neuroprogesterone synthesis, which is essential in mediating the positive feedback regulation of the LH surge.

It is always interesting to speculate how results in model species, especially rodents, illuminate the mechanism regulating the LH surge and ovulation in women. Typically, circulating levels of progesterone are low to undetectable in most primates and it is only after ovulation and luteinization that circulating levels progesterone rise. In primates, as in rodents, pre-ovulatory progesterone regulates the LH surge. In women, although estradiol alone can induce a LH surge (Leyendecker et al., 1972; Monroe et al., 1972; Yen and Tsai, 1972), treatment with the PR antagonist, RU 486, or inhibitors of progesterone synthesis attenuate the LH surge and prevent ovulation (Collins and Hodgen, 1986; Liu et al., 1987; Shoupe et al., 1987; Batista et al., 1992; Croxatto et al., 1993). These responses in women and non-human primates are consistent with our findings that estrogen positive feedback induces a hypothalamic source of pre-ovulatory progesterone that

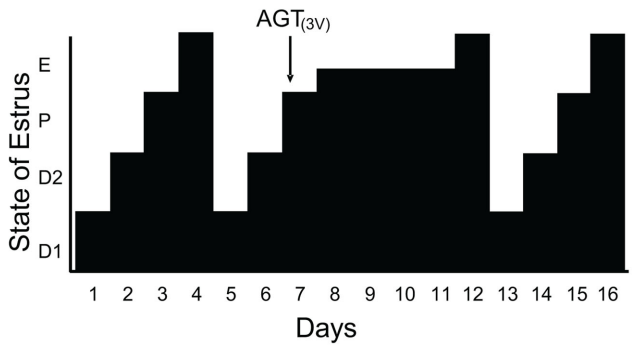

FIGURE 1 | Blocking neuroprogesterone synthesis alters the pattern of estrous cycles in gonadally intact rats. The 4-day rat estrous cycle is diagrammatically presented. Each day of the cycle is indicated on the ordinate: D1, diestrous day 1; D2, diestrous day 2; P, proestrus; and E, estrus. Neuroprogesterone synthesis was blocked by infusion of a P450scc inhibitor, aminoglutethimide (AGT), into the third ventricle (3V) via an implanted cannula. All animals treated with DMSO (5%, vehicle $n = 14$; data not shown) had normal 4 day estrous cycles as determined by vaginal cytology. In contrast, 11/14 AGT treated rats (0800 hours on proestrus, indicated by the arrow) had disrupted estrous cycles with delayed onset of estrous as determined by vaginal cytology (from Micevych and Sinchak, 2008a).

activates neural networks that regulate GnRH neuronal activity triggering the LH surge. In numerous species progesterone also positively regulates pituitary responsiveness to gonadotropes during estrogen positive feedback (Liu and Yen, 1983; Messinis and Templeton, 1990). In line with this idea, women treated with RU 486 exhibit an attenuated release of LH in response to exogenous GnRH (Kazem et al., 1996). Together these results indicate that both central and pituitary sites of progesterone action to facilitate estrogen positive feedback. It is likely that the neuroprogesterone synthesized in the hypothalamus could access the pituitary via the hypothalamo-pituitary portal circulation to modulate coordinate pituitary responsiveness with neural portion of the feedback loop.

ESTRADIOL ACTS ON ASTROCYTES

Astrocytes are responsive to various neuroactive messengers (Hosli and Hosli, 1992; Hirst et al., 1998; Chaban et al., 2004; Oka et al., 2006). Among these modulators of astrocyte function is estradiol which profoundly influences their morphology and function (Mong and Blutstein, 2006), sexual differentiation (McCarthy et al., 2002), and steroidogenesis (Sinchak et al., 2003; Micevych et al., 2007). Astrocytes in turn regulate numerous hypothalamic processes including regulation of releasing factors (Cavarretta et al., 1999; Galbiati et al., 2002; Zwain et al., 2002; Cashion et al., 2003; Mahesh et al., 2006) and synthesis of neurosteroids (Akwa et al., 1993; Jung-Testas et al., 1999; Zwain and Yen, 1999; Zwain et al., 2002). Astrocytes have all the steroidogenic enzymes required to convert cholesterol directly to progesterone (P450scc and 3β-HSD), and the transport proteins StAR and peripheral benzodiazepine receptor/translocator protein (PBR/TSPO; Schumacher et al., 2004), and are the main source of neuroprogesterone produced within the hypothalamus (Zwain and Yen, 1999; Micevych et al., 2003, 2007, 2008). As with granulosa cells of the ovary, astrocytes express estrogen receptor-α ((ERα) and ERβ, providing a potential avenue for estradiol regulation of neuroprogesterone steroidogenesis (Azcoitia et al., 1999; Garcia-Segura et al., 1999; Buchanan et al., 2000; Chaban et al., 2004; Pawlak et al., 2005; Quesada et al., 2007).

Both intracellular and membrane-associated ERs have been found in astrocytes. However, the regulation of progesterone synthesis in astrocytes is mediated through estradiol membrane signaling (EMS) that has been described in neurons and peripheral tissues (Szego and Davis, 1967; Kelly et al., 1976; Lagrange et al., 1995; Razandi et al., 1999; Filardo et al., 2000; Ivanova et al., 2001; Wade et al., 2001; Qiu et al., 2003; Chaban et al., 2004; Acconcia et al., 2005; Pawlak et al., 2005; Revankar et al., 2005; Thomas et al., 2005; Mhyre and Dorsa, 2006; Pedram et al., 2006, 2007; Bondar et al., 2009; Hirahara et al., 2009; Kuo et al., 2009). As in neurons, estradiol can influence cell signaling in astrocytes, which express ERα and ERβ both intracellularly and on the plasma membrane (Garcia-Segura et al., 1999; Chaban et al., 2004; Pawlak et al., 2005; Quesada et al., 2007). Activation of the membrane ER (mER) with estradiol or a membrane impermeable construct (estradiol-coupled to bovine serum albumin, E-6-BSA) initiates a rapid intracellular calcium concentration ($[Ca^{2+}]_i$) increase via activation of the phospholipase C/inositol trisphosphate (PLC/IP_3) pathway that releases intracellular stores of calcium from the smooth endoplasmic reticulum in neurons and astrocytes (Beyer and Raab, 1998; Chaban et al., 2004; Micevych et al., 2007). This rise in $[Ca^{2+}]_i$ stimulates the *de novo* synthesis of progesterone in post-pubertal hypothalamic astrocytes within 5 min (Micevych et al., 2003, 2007; Kuo et al., 2010). The calcium hypothesis was tested by thapsigargin, a potent Ca^{2+}-ATPase inhibitor that rapidly releases IP_3-sensitive Ca^{2+} stores from the smooth endoplasmic reticulum. This thapsigargin-induced massive release of Ca^{2+} was similar in magnitude to estradiol stimulation and within minutes resulted in progesterone synthesis (Micevych et al., 2007). Likewise, subnanomolar doses of estradiol were sufficient to induce rapid $[Ca^{2+}]_i$ release and progesterone synthesis in cultured hypothalamic astrocytes within minutes (Kuo et al., 2009). While the estradiol increase in $[Ca^{2+}]_i$ was dose-dependent, the synthesis of progesterone displayed a "step function" responding to physiological levels of estradiol that are reached during the proestrus surge (Butcher et al., 1974; Hawkins et al., 1975; Shaikh and Shaikh, 1975). The threshold response to estradiol is consistent with the idea that stimulation of neuroprogesterone synthesis is part of an "off-on-off" mechanism regulating the transition from estrogen negative feedback to estrogen positive feedback, and back again – at least in rodents (Kuo et al., 2010). Thus, as estradiol rises with developing ovarian follicles, it gradually increases levels of $[Ca^{2+}]_i$ in astrocytes; however, only when estradiol levels peak, consistent with mature follicles, does the $[Ca^{2+}]_i$ reach a critical threshold for stimulating progesterone synthesis. This response prevents hypothalamic progesterone levels from rising too early, resulting in a premature LH surge before ovarian follicles are fully mature and ready to ovulate.

MEMBRANE ESTROGEN RECEPTOR SIGNALING

Understanding the timing and mechanisms involved in estradiol signaling has been made more difficult by discoveries of several

proteins that act as membrane-associated ERs: ERα, ERβ, ER-X (Toran-Allerand, 2000; Toran-Allerand et al., 2002), STX-activated protein called mER-Gαq (Qiu et al., 2003, 2006), and GPR30 (Filardo et al., 2000, 2002; Revankar et al., 2005; Thomas et al., 2005). Although neurosteroidogenesis was activated by E-6-BSA, it was blocked by the ERα and ERβ antagonist, ICI 182,780, suggesting that this rapid signaling was mediated by these receptors (Chaban et al., 2004; Micevych et al., 2007). In the context of reproductive neuroendocrinology, the evidence is most robust for ERα as the critical membrane-associated ER. Mouse astrocytes from ERαKO mice had a significantly attenuated estradiol-induced $[Ca^{2+}]_i$ response (Kuo et al., 2010), which was mirrored *in vivo*. Priming OVX ERαKO mice with estradiol failed to significantly increase hypothalamic progesterone levels as observed in OVX wild-type mice (Kuo et al., 2010). Most dramatically, ERα was shown to be vital for estrogen positive feedback when mice in which the ERα was deleted from neurons failed to have a LH surge and ovulate (Wintermantel et al., 2006).

Classic nuclear receptors ERα (and ERβ) have been shown to associate with the plasma membrane through a variety of methods. ER over expression demonstrated that a population of these nuclear proteins are targeted to the plasma membrane (Razandi et al., 1999) where they have been localized with immunohistochemistry, western blotting, and surface biotinylation (Lagrange et al., 1995, 1996; Razandi et al., 1999; Ivanova et al., 2001; Wade et al., 2001; Chaban et al., 2004; Acconcia et al., 2005; Pawlak et al., 2005; Mhyre and Dorsa, 2006; Pedram et al., 2006; Bondar et al., 2009; Dominguez et al., 2009; Hirahara et al., 2009; Kuo et al., 2009; Sakuma et al., 2009; Dominguez and Micevych, 2010).

Membrane ERs were demonstrated in hypothalamic astrocytes and neurons using membrane-impermeable constructs such as E-6-BSA-FITC (1,3,5(10)-estratrien-3,17α-diol-6-one-bovine serum albumin-fluorescein isothiocyanate) and E-6-biotin (1,3,5(10)-estratrien-3,17α-diol-6-one-6-carboxymethloxime-NH-propyl-biotin; Dominguez et al., 2009; Micevych and Dominguez, 2009; Micevych et al., 2010). EMS activates G protein-dependent cell signaling cascades (Hammes and Levin, 2007), including activation of the mitogen-activated protein kinase (MAPK) pathway, activation of protein kinase C (PKC), increasing $[Ca^{2+}]_i$, and phosphorylation of cAMP-responsive element binding protein (CREB; Dewing et al., 2007, 2008; reviewed by Kelly and Ronnekleiv, 2008). An issue with ERα mediating EMS is that ERα is a transcription factor and does not have the canonical structure of a G protein-coupled receptor. An elegant solution was the proposed mechanism in which ligand-activated mERα or mERβ transactivated metabotropic glutamate receptors (mGluRs) to stimulate PLC/IP_3 – MAPK pathways leading to the activation of CREB (Boulware et al., 2005; Dewing et al., 2007). These signaling cascades have been implicated in the estradiol activation of lordosis behavior through neuropeptide expression and receptor activation (Watters and Dorsa, 1998). In astrocytes the interaction of mERα and mGluR1a was necessary to mediate estradiol signaling at the membrane. The increase in $[Ca^{2+}]_i$ and progesterone synthesis were blocked with the mGluR1a antagonist LY 367,385 (Kuo et al., 2009, 2010). This physiologic evidence was reinforced by the observation that ERα and mGluR1a co-immunoprecipitate in hypothalamic astrocytes (Kuo et al., 2009, 2010). Significantly, ERβ did not co-immunoprecipitate with mGluR1a, supporting the idea that of ERα is the mER mediating estradiol induction of progesterone synthesis in hypothalamic astrocytes.

In these *in vitro* experiments, we observed an interesting phenomenon. Although glutamate was not needed for estradiol transactivation of mGluR1a, activation of mGluR1a augmented the estradiol response (Kuo et al., 2009, 2010). These results demonstrated that estradiol would be maximally effective at stimulating astrocytes (and progesterone synthesis) when extracellular glutamate was elevated implying that estradiol signaling is strengthened in areas undergoing excitatory neural activity involving glutamate. Under such circumstances, astrocytes are a site of neural–hormonal integration. Indeed, middle-aged females exhibit reduced excitation of GnRH neurons and attenuated LH surges under estrogen positive feedback conditions, in part, due to decreased glutamate neurotransmission in the medial preoptic area, demonstrating the importance of local modulatory effects on estrogen positive feedback, the LH surge, and reproductive function (Neal-Perry et al., 2005, 2009). Further, it appears that menopause may be induced by the loss of estradiol-induced neuroprogesterone synthesis. As rats enter menopause, estradiol levels increase to intermediate level, though not reaching the estrogen positive feedback levels. However, the elevated estradiol levels are maintained for an extended period of time. The rat is in an extended proestrus state, similar to that induced by AGT infused into the lateral ventricle. Treating the menopausal rats with progesterone induces the LH surge, indicating that the brain is not synthesizing progesterone in response to elevated estradiol but remains responsive to progesterone (Lu et al., 1994; Mills et al., 2002). Interestingly, these females are in constant behavioral estrus and the LH surge can be induced by copulating with a male. Thus, female rats appear to become reflex ovulators as they reach their final stages of reproduction. Then after several months of acyclicity, exogenous progesterone can no longer induce the LH surge because estradiol is incapable of inducing PR expression as well as neuroprogesterone synthesis (Mills et al., 2002).

If ERα is also responsible for EMS, does it have the characteristics of other membrane receptors: present on the surface, trafficked to the membrane, and internalized after stimulation with its ligand? Surface biotinylation studies with hypothalamic astrocytes demonstrated that ERα is located on the cell surface, trafficked to the membrane, and internalized by estradiol treatment. Moreover, the trafficking and internalization are is dependent upon mGluR1a, and blocked by mGluR1a antagonism with LY 367,385 (Bondar et al., 2009). Similarly in hypothalamic neurons, estradiol treatment significantly increased the internalization of mGluR1a in parallel with ERα, and that the trafficking is dependent on caveolin (Boulware et al., 2007; Dominguez and Micevych, 2010) further supporting the idea of an ERα-mGluR1a signaling unit for EMS (Dominguez and Micevych, 2010).

These experiments revealed that estradiol regulates its own membrane-initiated signaling. Such autoregulation of signaling, for example helps to explain observations *in vivo* of the transient effect of EMS that affect sexual receptivity and progesterone synthesis (Sinchak and Micevych, 2001, 2003; Micevych et al., 2003; Dewing et al., 2007; Kuo et al., 2009, 2010). Estradiol rapidly, within 5 min, induces mERα trafficking to the plasma membrane

(Bondar et al., 2009). Peak mERα levels were achieved by 30 min. Once on the membrane, estradiol activates ERα and induces internalization. mERα trafficking and internalization is blocked by either the ER antagonist ICI 182,780, or the mGluR1a antagonist LY 367,385, verifying ERα-mGluR1a signaling (Bondar et al., 2009). As expected, trafficking to the membrane and internalization occurred in parallel, with the peak of internalization also at 30 min. A possible mechanism for insertion is the fusion of mERα–mGluR1a containing vesicles with the cell membrane. This idea is supported by the presence of ERα immunoreactive vesicles in the hippocampus and pituitary (Gonzalez et al., 2008). Internalization, like that of other membrane receptors is probably mediated by clathrin-coated endocytic vesicles, which retrieve ligand-bound receptors (Micevych and Dominguez, 2009). Once receptors are internalized, the vesicles fuse with early endosomes and release their ligand. At this juncture, the receptors can either be recycled to the cell surface or degraded (Sinchak and Micevych, 2003; Micevych and Dewing, 2011). Degradation wins out over recycling after a continuous 1–2 h exposure to estradiol, which leads to a reduction of both surface mERα and internalized mERα. The loss of mERα (i.e., down regulation) is a mechanism by which estradiol can temporally limit EMS (Bondar et al., 2009; Dominguez and Micevych, 2010).

ANOTHER mERα?

An unexpected finding of the biotinylation studies was the presence of a 52- to 55-kDa ERα immunoreactive membrane protein. This protein was also found in hypothalamic neuronal membranes and is the product of an alternatively spliced ERα mRNA in which exon 4 is deleted (ERαΔ4; Dominguez et al., submitted). Initially, Crews and colleagues reported such an ERα mRNA (Skipper et al., 1993). They reported that ERαΔ4 was very abundant in the brain. Since then, ERαΔ4 mRNA has been reported in the brain, pituitary, and breast (Fuqua et al., 1992; Bollig and Miksicek, 2000; Deecher et al., 2003; Perlman et al., 2005). We confirmed the presence of the ERαΔ4 mRNA. The resulting protein is missing the nuclear localization signal helping to explain the large quantity seen in the membrane (Pasqualini et al., 2001; Bondar et al., 2009; Dominguez and Micevych, 2010; Roepke et al., 2010). The function of the ERαΔ4 is unclear since the deletion of exon 4 affects the ligand binding domain, calling into question its estradiol dependence for signaling. Further, only the full-length 66 kDa ERα co-immunoprecipitated with mGluR1a, which in astrocytes is needed for the estradiol-induced $[Ca^{2+}]_i$ release and progesterone synthesis (Kuo et al., 2009, 2010).

OTHER ERs

In addition to mERα, several other candidate mERs have been proposed (Filardo et al., 2000; Toran-Allerand, 2000; Qiu et al., 2003). GPR30 is a G protein-coupled receptor that activates adenylyl cyclase in breast cancer cells lacking ERα and ERβ (Filardo et al., 2000, 2002; Revankar et al., 2005; Thomas et al., 2005). FLAG- and hemagglutinin-tagged GPR30 have been reported at the plasma membrane (Funakoshi et al., 2006; Filardo et al., 2007), but GPR30 could not be identified at the plasma membrane or labeled with surface biotinylation in native astrocytes or neurons (Gorosito et al., 2008; Bondar et al., 2009; Kuo et al., 2010). The GPR30 agonist G-1, a substituted dihydroquinoline (Bologa et al., 2006), stimulated $[Ca^{2+}]_i$ release and progesterone synthesis (Kuo et al., 2010); however, G-1 had a dose response curve that was different from estradiol or PPT, the selective ERα agonist. Further, since G-1 ability to stimulate $[Ca^{2+}]_i$ release was not blocked by antagonizing mGluR1a (Kuo et al., 2010). This also indicates that GPR30 uses a different signaling mechanism compared to estradiol. The lack of interaction between GPR30 and mGluR1a was confirmed by the absence of co-immunoprecipitation between these proteins (Kuo et al., 2010). While it is difficult to understand the discrepancy with GPR30 localization on the plasma membrane, our results can be interpreted to support the observation of estradiol activation of intracellular GPR30 on the endoplasmic reticulum (Revankar et al., 2005). Activation of GPR30 may directly induce the release of intracellular stores of Ca^{2+}, which in turn stimulates progesterone synthesis.

Another candidate receptor is a membrane-associated binding protein that is $G\alpha_q$-coupled and activated by estradiol as well as STX, a diphenylacrylamide selective estrogen receptor modulator (SERM; Qiu et al., 2003). STX activity is retained in double ERα/ERβ knock-out mice, but blocked with ICI 182,780 (Qiu et al., 2006). STX does not activate ERα or ERβ and has a six order of magnitude lower binding affinity compared with estradiol for these receptors (Qiu et al., 2003). Unfortunately, this mER-$G\alpha_q$ has much lower affinity for estradiol (∼20-fold) compared with STX (Qiu et al., 2006). However, this STX-activated protein also activates PLC (Qiu et al., 2003), a signaling pathway similar to that activated by ERα-mGluR1a. In hypothalamic astrocytes, STX increased $[Ca^{2+}]_i$ and progesterone synthesis through transactivation of mGluR1a (Kuo et al., 2010). It has been suggested that STX signals through GPR30, such that small interfering RNA directed against GPR30 abolished the STX-induced transcription (Lin et al., 2009). However, estradiol has been reported to activate the mER-$G\alpha_q$ signaling pathway in GPR30 knock-out mice (Qiu et al., 2008). In our hands, STX and G-1 produced distinctly different responses in hypothalamic astrocytes (Kuo et al., 2010). Although estradiol and STX responses are blocked by mGluR1a antagonism and activate the same PLC pathway, these actions are mediated through different receptors since STX stimulation of Ca^{2+} release remains in astrocytes from ERαKO mice, where estradiol was ineffective (Kuo et al., 2010). Since ERαKO mice do not demonstrate estrogen positive feedback and lack sexual receptivity due to the lack of estradiol signaling, the STX response in ERαKO mice is not consistent with a STX-related signaling mechanism for induction of the LH surge or receptivity in female reproduction (Rissman et al., 1997; Ogawa et al., 1998; Wintermantel et al., 2006; Kuo et al., 2010). Therefore, the physiological relevance of STX in reproduction remains to be elucidated, but STX may have a future as a therapeutic agent since it does not have uterotropic actions (Roepke et al., 2010).

Lastly, ER-X has been proposed as an ER during development and following injury, especially in the cortex (Toran-Allerand, 2000; Toran-Allerand et al., 2002). This ER is not inhibited by ICI 182,780, but is activated by 17α-estradiol. ER-X is unique in that it is neither blocked by ICI 182,780 nor is it stereospecific. In fact, it is preferentially activated by 17α-estradiol (Toran-Allerand et al., 2002). However, in astrocytes the estradiol action on $[Ca^{2+}]_i$ is

stereospecific (Chaban et al., 2004), and the 17β-estradiol-induced $[Ca^{2+}]_i$ release and progesterone synthesis are inhibited by ICI 182,780, which is not consistent with an ER-X mediated action (Micevych et al., 2007; Kuo et al., 2009).

In summary, several putative mERs are involved in regulation of $[Ca^{2+}]_i$ and progesterone synthesis in hypothalamic astrocytes (Kuo et al., 2010). ERα and the STX-activated Gαq-mER had a similar pharmacology including their dependence on of mGluR1a to initiate cell signaling. From a reproductive vantage point as well as evidence from wild-type and ERKO hypothalamic astrocytes, ERα appears to be the primary mER responsible for the rapid cell signaling that leads to an increase in hypothalamic neuroprogesterone (Micevych et al., 2007; Bondar et al., 2009; Kuo et al., 2009, 2010).

IS THE LH SURGE STIMULATED BY PROGESTERONE OR A METABOLITE?

Overall, there is firm evidence that estradiol stimulates the synthesis of progesterone in the hypothalamus. Progesterone in the brain can be rapidly converted to dihydroprogesterone by neuronal and glial 5α-reductases and further to allopregnenolone by astrocytic 3α-hydroxysteroid oxide reductase (Corpechot et al., 1993; Mensah-Nyagan et al., 1999). As mentioned earlier, allopregnenolone primarily interacts with the $GABA_A$ receptor. Classically, the GABA receptors are inhibitory. One hypothesis suggests that GnRH neurons have greater intracellular vs. extracellular chloride concentration, which results in an efflux of chloride rather than an influx – depolarizing the neurons and stimulating GnRH release (DeFazio and Moenter, 2002; Sullivan and Moenter, 2003). Indifferent strains of GT1 neurons, allopregnenolone in one case stimulated the release of GnRH (el Etr et al., 1995) and in another did not affect GnRH release (Sleiter et al., 2009). Interestingly, in the latter study progesterone did inhibit GnRH release through an action on membrane PRs: mPRα and mPRβ. The *in vivo* data are equally inconsistent. *In vivo*, allopregnenolone inhibited LH release in OVX, estradiol + progesterone primed rats, an effect that was reversed in the presence of bicuculline (Laconi and Cabrera, 2002). However, bicuculline facilitated GnRH release from acute hypothalamic slices (Giuliani et al., 2001). Further confusing the situation is the observation that progesterone can enhance LH secretion independently of $GABA_A$ receptor involvement (Brann et al., 1990). Thus, the role of progesterone in mediating estrogen positive feedback is well-established (Kim and Ramirez, 1982; Drouva et al., 1985; Ke and Ramirez, 1987), but evidence for a role of allopregnenolone is not as robust.

CONCLUSION

The quest to understand the CNS control of the LH surge and the mechanism of estrogen positive feedback has yielded novel insights about the role of astrocytes, progesterone, and EMS. Specifically, estradiol acting through EMS in astrocytes to induce progesterone synthesis must now be considered when describing the mechanism for estrogen positive feedback in reproductive physiology (**Figure 2**). Preliminary studies suggest that estradiol produced in the ovary also induces the synthesis of PR-A in kisspeptin (KISS) neurons of the GnRH-control network. KISS neurons, of the anteroventral periventricular nucleus (AVPV) and rostral extent of the periventricular nucleus along the III ventricle, project directly to GnRH neurons (Clarkson and Herbison, 2006; Herbison, 2007). KISS is a potent stimulator of GnRH neurons (Messager et al., 2005; d'Anglemont de Tassigny et al., 2008; Herbison, 2008; Liu et al., 2008; Clarkson et al., 2010). Local astrocytes, stimulated by peaking estradiol concentrations, increase progesterone synthesis that activates the PR in KISS neurons to stimulate GnRH release (**Figure 2**). While a role for membrane PRs has not been elucidated in positive feedback, evidence from GT1 cells suggests that neuroprogesterone from astrocytes can also stimulate membrane PRs, perhaps on native GnRH neurons.

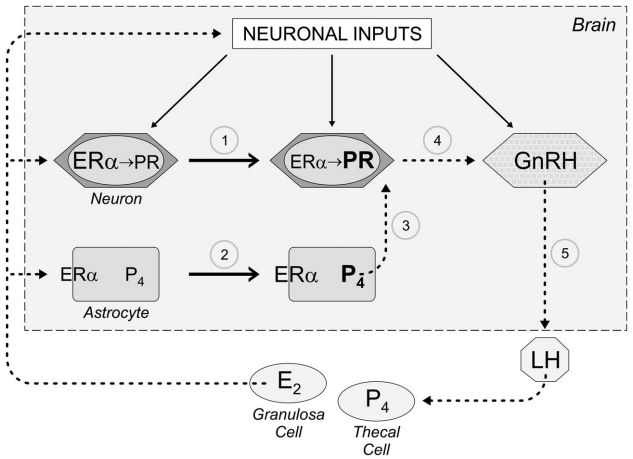

FIGURE 2 | Schematic diagram describing the role of astrocytes, which we propose mediate estrogen positive feedback of the LH surge. As follicles develop in the ovary, granulosa cells under the influence of follicle stimulating hormone (FSH) begin producing estradiol (E_2). While this level of E_2 is inhibitory to the neural network regulating the release of GnRH (negative feedback), this E_2 induces the expression of progesterone receptors in hypothalamic neurons (1) through activation of nuclear estrogen receptor-α (ERα). When circulating levels of E_2 peak on proestrus (estrogen positive feedback), E_2 now binds to ERα on the membrane of astrocytes that transactivates metabotropic glutamate receptor 1a (mGluR1a) increasing cytoplasmic calcium by releasing intracellular calcium stores (positive feedback), which stimulate the synthesis of progesterone (P_4; 2). This *de novo* synthesized P_4 diffuses from the astrocyte to bind E_2-induced PR in nearby neurons (3). We suspect that these neurons use kisspeptin as a transmitter and activate GnRH neurons (4) causing the surge release of GnRH (5) that stimulates LH release form pituitary gonadotropes. The LH then induces ovulation and luteinization of the follicle in which theca lutein cells synthesize P_4.

These studies bring into sharp focus that understanding brain function should also involve transmitters regulated at the point of their synthesis. These include the known endocannabinoids, prostaglandins, nitric oxide, and the neurosteroids. In terms estrogen positive feedback regulation of the LH surge and ovulation, neuroprogesterone rather than the metabolite, allopregnenolone that binds to the $GABA_A$ receptor, appears to be key.

Finally, astrocytes are another point of convergence and integration of hormonal and transmitter signaling (**Figure 2**). Estradiol, from the periphery binds to mER, which transactivates a mGluR1a receptor to initiate cell signaling that results in a release of intracellular stores Ca^{2+} and a stimulation of progesterone

synthesis. Glutamate is not needed for the estradiol-induced $[Ca^{2+}]_i$ increase, but in the presence of glutamate, the efficacy of estradiol is increased. Interestingly, in spite of the necessity of ERα for reproduction, including estrogen positive feedback, STX appears to activate a novel protein that mobilizes Ca^{2+}, stimulates progesterone synthesis but whose actions are blocked by antagonism of the mGluR1a. This suggests a model for EMS in which mERs, be they ERα, ERβ, or the STX-activated Gαq-mER need to transactivate mGluRs. This holds the promise of novel therapeutic approaches for any number of pathologies influenced by estradiol.

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Rapid direct action of estradiol in GnRH neurons: findings and implications

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Estradiol plays a pivotal role in the control of gonadotropin-releasing hormone (GnRH) neuronal function and female reproduction. While positive and negative feedback actions of estradiol that enhance and suppress release of GnRH and LH are primarily mediated through estrogen receptor alpha located in interneurons, a series of recent studies in our laboratory indicate that rapid excitatory actions of estradiol also directly modify GnRH neuronal activity. We observed this phenomenon in cultured primate GnRH neurons, but similar rapid direct actions of estradiol are also described in cultured GnRH neurons and green fluorescent protein-labeled GnRH neurons of mice. Importantly, rapid direct action of estradiol in GnRH neurons is mediated through membrane or membrane associated receptors, such as GPR30, STX-sensitive receptors, and ERβ. In this review, possible implications of this rapid estradiol action in GnRH neurons are discussed.

Keywords: GnRH neurons, rapid action of estradiol, membrane estrogen receptors, GPR30, primates

INTRODUCTION

Estradiol (E_2) is a vital regulator of female reproduction. In addition to trophic effects of E_2 on breast, ovarian, and uterine tissue, E_2 plays a key role in regulating the function of gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus and gonadotrophs in the pituitary throughout the female reproductive cycle. E_2 released from the ovary induces positive and negative feedback effects on GnRH neurons in the hypothalamus. Historically, it has been viewed that E_2 controls the GnRH neuronal system through interneurons or glial cells (Herbison, 2006), because earlier studies with autoradiography combined with immunocytochemistry showed that GnRH neurons do not contain nuclear estrogen receptors (ER; Shivers et al., 1983), whereas interneurons, such as those synthesizing neuropeptide Y (NPY), catecholamines, glutamate, GABA, and kisspeptin express nuclear ER (Stumpf and Jennes, 1984; Leranth et al., 1992; Smith et al., 2005; Franceschini et al., 2006).

After the discovery of ERβ (Kuiper et al., 1996), several studies found that ERβ colocalizes in GnRH neurons in several species including mice, rats, sheep, and humans (Hrabovszky et al., 2000, 2001, 2007; Herbison and Pape, 2001; Sharifi et al., 2002; Skinner and Dufourny, 2005). More recently, direct action of E_2 on GnRH neurons has been shown by several groups including our own (Abrahám et al., 2003; Temple et al., 2004; Abe and Terasawa, 2005; Abe et al., 2008; Chu et al., 2009; Noel et al., 2009; Sun et al., 2010). Direct action of E_2 is rapid, mediated through receptors associated with the plasma membrane, and seen in many types of neurons (Terasawa et al., 2009). In this article, we will review the mechanism of rapid, direct action of E_2 on GnRH neuronal activity and possible implications of direct E_2 action, focusing on our studies in non-human primates.

MODELS FOR STUDYING DIRECT E_2 ACTION IN GnRH NEURONS

The number of GnRH neurons in the brain is small and they are diffusely distributed in the preoptic area and basal hypothalamus. Thus, to study cellular and molecular mechanisms of E_2 action in GnRH neurons we need to utilize a relatively simple population of GnRH neurons that can be directly visualized. To date three *in vitro* models have been described (Terasawa, 2001): (1) GT-1 and GN11 cell lines (Mellon et al., 1990; Radovick et al., 1991), (2) cultured GnRH neurons derived from the nasal placode regions in monkeys, sheep, rats, and mice (Terasawa et al., 1993; Daikoku and Koide, 1994; Fueshko and Wray, 1994; Duittoz et al., 1997), and (3) sliced preoptic-hypothalamic green fluorescent protein (GFP) labeled mouse GnRH neurons (Spergel et al., 1999; Suter et al., 2000) or mouse and guinea pig GnRH neurons identified with single cell RT-PCR or immunocytochemistry (Lagrange et al., 1995; Zhang et al., 2007). In these models, demonstration of pulsatile GnRH release or periodic activity recorded from GnRH neurons is essential, because GnRH neurons release the decapeptide hormone into the pituitary portal circulation in a pulsatile manner (Knobil, 1980; Gearing and Terasawa, 1988; Kokoris et al., 1988) and this pulsatility is crucial for the maintenance of normal reproductive function (Knobil, 1980). Importantly, the pulse frequency of GnRH release or periodic activity in a model should reflect the species origin of these GnRH neurons. In the GT-1 cells and cultured GnRH neurons, pulsatile release of GnRH peptide with species specific frequency (i.e., 20–30 min in mouse and

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium; E_2, estradiol; E2-BSA, E2-17 hemisuccinate bovine serum albumin; EDC, E_2 dendrimer conjugate; ER, estrogen receptor; GFP, green fluorescent protein; GnRH, gonadotropin-releasing hormone; GPCR, G protein coupled receptor; GPR30, G protein coupled receptor 30; NPY, neuropeptide Y; PCOS, polycystic ovary syndrome; STX-R, membrane ER sensitive to STX.

rats, 40–60 min in sheep and monkeys) has been shown (Krsmanovic et al., 1992; Martinez de la Escalera et al., 1992; Wetsel et al., 1992; Terasawa et al., 1999a; Duittoz and Batailler, 2000; Funabashi et al., 2000; Constantin et al., 2009). In GFP-labeled mouse GnRH neurons and GT-1 cells, periodic burst firing activity similar to GnRH pulses has also been reported (Costantin and Charles, 1999; Nunemaker et al., 2003). Among these three models, we have been using cultured GnRH neurons derived from the embryonic nasal placode of rhesus monkey fetuses, which are obtained from timemated pregnancies (Terasawa et al., 1993). These GnRH neurons exhibit a spontaneous oscillatory pattern of $[Ca^{2+}]_i$ levels with variable peak amplitude and duration of each oscillation unique to each cell (Terasawa et al., 1999b). On average, the interpulse interval between $[Ca^{2+}]_i$ oscillations is 8 min with synchronization of $[Ca^{2+}]_i$ oscillations among GnRH neurons occurring at ∼60 min intervals (Terasawa et al., 1999b) and release GnRH peptide also at ∼60 min intervals (Terasawa et al., 1999a). Using our cultured primate GnRH neuron model we have reported several important discoveries (see Terasawa et al., 2009; Terasawa et al., 2010). Based on comprehensive comparisons between the embryonic GnRH neuron model (in both monkeys and mice) and GFP-labeled mice GnRH neuron model, Jasoni et al. (2010) and Constantin (2011) conclude that physiological characteristics of the two models are quite similar.

E_2 RAPIDLY STIMULATES FIRING ACTIVITY, INTRACELLULAR CALCIUM OSCILLATIONS, AND GnRH RELEASE

To determine if E_2 causes direct action in primate GnRH neurons, we first examined the effects of E_2 on firing activity. Application of E_2 (1 nM) to cultured primate GnRH neurons for 10 min, induces a 250% increase in action potential firing frequency, an increase in the number of action potentials per burst, and an increase in burst duration (Abe and Terasawa, 2005). E_2, however, did not change the timing of bursts (interburst interval) nor did it alter the cluster pattern, suggesting that E_2 modulates overall firing intensity, but not the firing pattern.

Release of GnRH is also modulated by E_2. In primate GnRH neurons, exposure to 1 nM E_2 for 20 min results in a rapid increase of GnRH peptide release, which is initiated within 10 min of E_2 application and lasts for 40 min (Noel et al., 2009). In GT-1 cells, it has been reported that exposure to a picomolar dose of E_2 for 4 h suppresses the frequency of GnRH release (Navarro et al., 2003). However, the mechanism of E_2 action between these two studies may differ, as the E_2 exposure time in the study of GT-1 cells is much longer and may lead to nuclear receptor mediated genomic action.

E_2 causes potent stimulatory effects on $[Ca^{2+}]_i$ oscillations. A 10 min exposure to E_2 at 1 nM induces a 180–200% increase in the frequency of $[Ca^{2+}]_i$ oscillations, returning to baseline levels 40–60 min after initiation of E_2 treatment (Abe et al., 2008). E_2 also increases the number of activated cells from 30 to 70%. Additionally, E_2 stimulates the average number of synchronized $[Ca^{2+}]_i$ oscillations from 1 synchronization event/hour in control samples to ∼2.7 events/hour in E_2 treated samples (Abe et al., 2008). Similar E_2 effects on $[Ca^{2+}]_i$ oscillations have also been reported in cultured mouse GnRH neurons (Temple et al., 2004) and we also confirmed similar E_2 effects in additional studies (Noel et al., 2009; Kenealy et al., 2011a,b). Importantly, tetrodotoxin does not change the pattern of the E_2-induced $[Ca^{2+}]_i$ oscillations (Abe et al., 2008), consistent with this E_2 action causing direct effects on GnRH neurons and not through interneurons.

RAPID STIMULATORY E_2 ACTION IN GnRH NEURONS IS A MEMBRANE-INITIATED MECHANISM

In order to assess the mechanism of rapid E_2 action, we have examined the effects of a plasma membrane impermeable form of E_2, E_2-17 hemisuccinate BSA (E_2-BSA). E_2-BSA (1 nM) increases the frequencies of firing activity (Abe and Terasawa, 2005) and $[Ca^{2+}]_i$ oscillations (Noel et al., 2009), and stimulates GnRH release (Noel et al., 2009), similar to the increase observed with E_2, suggesting that rapid action of E_2 occurs at the cell membrane. Moreover, exposure of GnRH neurons to the nuclear impermeable E_2 dendrimer conjugate (EDC, 1 nM), described by Harrington et al. (2006), also causes an increased frequency of $[Ca^{2+}]_i$ oscillations and elevated GnRH release (Noel et al., 2009), indicating that E_2 causes rapid effects without entering the nucleus. These observations indicate that rapid excitatory E_2 action is a membrane-initiated mechanism, and does not require genomic action of E_2. However, the amplitude and duration of the EDC- and E_2-BSA-induced GnRH release are smaller and shorter (Noel et al., 2009), indicating the presence of multiple mechanisms of E_2 action (see below).

E_2 ACTION IS NOT MEDIATED BY ERα OR ERβ

In general, E_2 action through genomic changes occurs in the order of an hour to several hours or even days. In contrast, the effects elicited by E_2 in GnRH neurons described above, i.e., the increase in firing activity, $[Ca^{2+}]_i$ oscillations, and GnRH release, occurs at a time scale of seconds to minutes. Therefore, it is speculated that the mechanism of rapid E_2 action differs from nuclear ER mediated genomic action.

To identify the type of ER mediating rapid E_2 action in GnRH neurons, we first examined the role of ERα or ERβ using the ER antagonist, ICI182,780. To our surprise, ICI182,780 blocks neither the E_2-induced $[Ca^{2+}]_i$ oscillations nor synchronization (Abe et al., 2008). Moreover, ICI182,780 did not influence the E_2-induced release of GnRH peptide (Noel et al., 2009). Considering the results of rapid direct E_2 action through ERβ in mouse GnRH neurons (Abrahám et al., 2003; Temple et al., 2004; Chu et al., 2009), we further examined E_2's effects in GnRH neurons, in which ERα and ERβ were respectively deleted by an siRNA knockdown approach. The results show that knockdown of ERα and ERβ do not interfere with the E_2-induced changes in $[Ca^{2+}]_i$ oscillations nor synchronization (Kenealy et al., 2011a), confirming our findings with ICI182,780 on $[Ca^{2+}]_i$ oscillations. Collectively, these findings suggest that ERα and ERβ are neither involved in E_2-induced $[Ca^{2+}]_i$ oscillations nor rapid release of GnRH peptide in primate GnRH neurons.

RAPID E_2 ACTION IS MEDIATED BY MULTIPLE RECEPTORS

In addition to ERα and ERβ, several types of ERs, such as ER-X (Toran-Allerand et al., 2002), G protein coupled receptor 30 (GPR30, Thomas et al., 2010), and membrane ER sensitive to the diphenylacrylamide compound STX (STX-R, Qiu et al., 2003) participate in membrane-initiated E_2 action. Interestingly, these membrane ERs require G-protein coupled receptor (GPCR) signaling mechanisms for estrogen action (Terasawa et al., 2009).

Rapid action through membrane ERα and ERβ also requires a companion GPCR, metabotropic glutamate receptors (Boulware et al., 2005, 2007; Grove-Strawser et al., 2010). In primate GnRH neurons, a GPCR is also involved in rapid E_2 action, as pertussis toxin, a broad inhibitor of GPCR signaling, blocks the E_2-induced changes in $[Ca^{2+}]_i$ oscillations, their synchronization, and GnRH release (Noel et al., 2009).

GPR30, an orphan receptor coupled to Gαs proteins, binds E_2 (Revankar et al., 2005; Thomas et al., 2005). However, E_2 action through GPR30 does not require ERα or ERβ, as knockdown of GPR30 in ERα and ERβ negative cancer cells blocks E_2 action (Thomas et al., 2005). In cancer cell lines, E_2 action mediated by GPR30 mobilizes Gαs to stimulate adenylyl cyclase resulting in cyclic AMP synthesis and activates Gβγ signaling resulting in transactivation of epidermal growth factor receptor (Filardo et al., 2000, 2002). Importantly, unlike ER-X and STX-R, the sequence of GPR30 is known. Consequently, G1, a specific agonist for GPR30 (Bologa et al., 2006) and G15, a specific antagonist for GPR30 (Dennis et al., 2009) have been synthesized. Using these tools, we have studied the role of GPR30 in membrane-initiated E_2 action in primate GnRH neurons and found that GPR30 is, at least in part, responsible for rapid excitatory E_2 action. First, exposure of GnRH neurons to the GPR30 receptor agonist, G1, at 10 nM, but not 1 nM, induces an increase in $[Ca^{2+}]_i$ oscillations (Noel et al., 2009) and G1 at 100 nM stimulates GnRH release (Kenealy and Terasawa, unpublished observations). Second, siRNA knockdown of GPR30 completely blocks the E_2- and EDC-induced changes in $[Ca^{2+}]_i$ oscillations and their synchronization (Noel et al., 2009). Third, a high dose of ICI182,780 (1 μM) alone elicits changes in $[Ca^{2+}]_i$ oscillations similar to cancer cells, in which a high dose (1 μM) of ICI182,780 is an agonist for GPR30 (Filardo et al., 2000). Fourth, treatment with the GPR30 antagonist G15 blocks both the E_2-induced increase in $[Ca^{2+}]_i$ oscillations, their synchronization, and E_2-induced GnRH release, while G15 alone showed no significant effects (Kenealy and Terasawa, unpublished observations). Finally, GPR30 colocalizes in one-third of GnRH neurons in the monkey hypothalamus (Noel et al., 2009).

Because (1) a higher dose of G1 and EDC is required for the induction of changes in $[Ca^{2+}]_i$ oscillations and GnRH release, as compared to E_2 changes (1 nM) and (2) the effects of a higher dose of E_2 (10 nM) on changes in $[Ca^{2+}]_i$ oscillations is only partly blocked by GPR30 siRNA treatment (Kenealy et al., 2011b), we have speculated that additional membrane ERs may be involved in rapid E_2 action. STX-R was an enticing target to pursue, as STX (1) has been shown to mediate effects in hypothalamic cells in mutant mice lacking ERα, ERβ, and GPR30 receptors (Qiu et al., 2006, 2008), (2) modulates ion channels involved in membrane potential changes (Zhang et al., 2010), and (3) is potently inhibited by ICI182,780 (Qiu et al., 2003). Indeed, treatment of primate GnRH neurons with 10 nM STX induces an increase in $[Ca^{2+}]_i$ oscillations, their synchronization, and increases the percent of cells stimulated, similar to both E_2 (1 nM) and G1 (10 nM; Kenealy et al., 2011b). Interestingly, ICI182,780 blocks the STX-induced $[Ca^{2+}]_i$ oscillations and their synchronization. Furthermore, GPR30 knockdown does not influence the STX-induced changes in $[Ca^{2+}]_i$ oscillations and their synchronization. Finally, STX (10 and 100 nM) induces a dose dependent increase in GnRH release, although the effectiveness is smaller than that induced by E_2 at 1 nM (Kenealy et al., 2011b). Therefore, E_2 action in primate GnRH neurons is mediated through multiple receptor mechanisms (see **Figure 1**). This is not limited to primate GnRH neurons: in mouse GnRH neurons, in addition to E_2 action through ERβ (Temple et al., 2004) and STX–R (Zhang et al., 2010), E_2 through GPR30 modulates voltage gated calcium channels (Sun et al., 2010). Any interaction between STX-R and other ERs including GPR30 will become clear when the molecular nature of STX-R is identified.

PHYSIOLOGICAL SIGNIFICANCE OF RAPID ACTION IN GnRH NEURONS

Rapid E_2 action in hypothalamic neurons was first described nearly 40 years ago (Yagi, 1973; Kelly et al., 1976). During this period, a large number of reports on rapid steroid action in various types of neurons and in glia have been reported (Orchinik et al., 1991; Lagrange et al., 1995; Boulware et al., 2005, 2007; Fehrenbacher et al., 2009; Grove-Strawser et al., 2010; Kuo et al., 2010; Labombarda et al., 2010; Lebesgue et al., 2010). As discussed above, "rapid" timing of E_2 action is a membrane-initiated phenomenon, rather than "long term" E_2 action, which requires transcription after E_2 binds to cytoplasmic/nuclear ER. However, the physiological significance of rapid E_2 action in the hypothalamus, and specifically within the GnRH system, remains unclear.

It is unlikely that rapid, direct, excitatory action of E_2 plays a significant role in the classical positive and negative feedback mechanisms during the reproductive cycle. Rather, the enhancing and suppressing effects of E_2 are mediated by ERα. First, E_2 fails to induce LH surges in ERα knockout mice as well as in mutant mice lacking estrogen response element (ERE)-dependent ERα signaling (Glidewell-Kenney et al., 2007). By contrast, in ERβ knockout mice E_2 induces LH surges (Wintermantel et al., 2006), although these mice had ERβ splice variants (Krege et al., 1998), therefore, reexamination of the role of ERβ in positive feedback in mice with complete elimination of ERβ splice variants (Antal et al., 2008) is still needed. Similarly, cytochrome P450 aromatase knockout (ArKO) female mice, in which estradiol is absent, have acyclic estrus cycles characterized by elevated levels of LH, FSH, and testosterone (Fisher et al., 1998). Second, whereas convincing evidence for the presence of ERα in GnRH neurons has not been shown, the presence of ERα in interneurons that innervate GnRH neurons, such as neurons that synthesize kisspeptin, NPY, catecholamines, glutamate, and GABA have been consistently reported (Stumpf and Jennes, 1984; Leranth et al., 1992; Smith et al., 2005; Franceschini et al., 2006). Third, positive feedback effects of E_2 on LH surges require 16–48 h (depending on species) after its administration (Yamaji et al., 1971; Karsch and Foster, 1975) and negative feedback effects of E_2 require a minimum of 1–2 h lasting more than 12 h in rhesus monkeys (Chongthammakun and Terasawa, 1993; Mizuno and Terasawa, 2005). While it is possible that membrane ERα signaling may be in part responsible for negative feedback effects of E_2 on LH release, as E_2 can suppress LH levels in mice lacking ERE-dependent ERα signaling (Glidewell-Kenney et al., 2007), the genomic action of E_2 through ERα is indispensable for LH/GnRH surges. Taken together, ERα within interneurons, such as kisspeptin neurons that directly innervate GnRH neurons, play a role in E_2's feedback action.

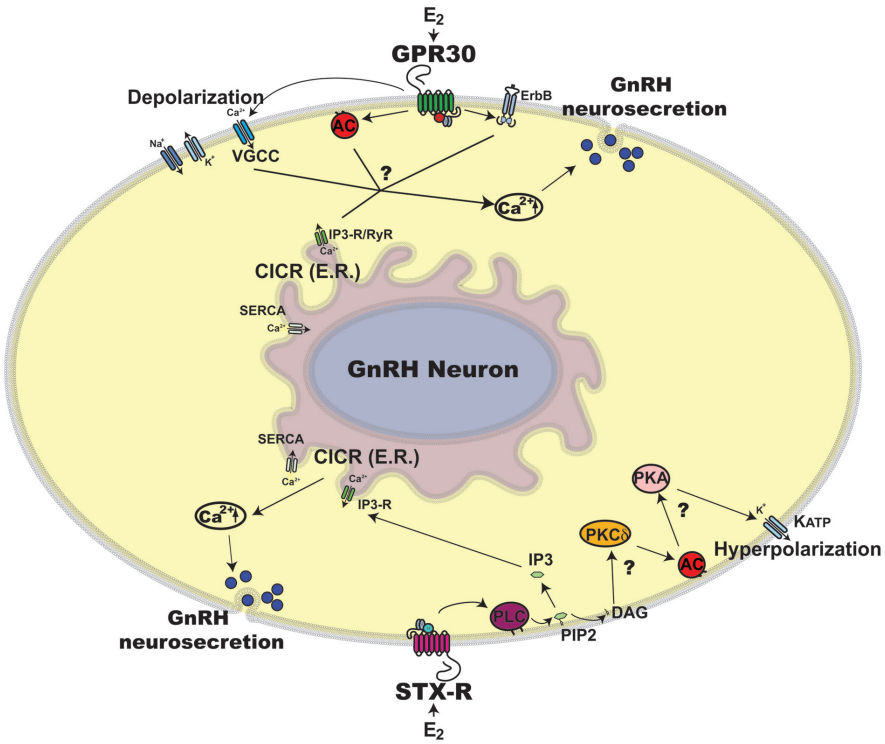

FIGURE 1 | Schematic illustration of rapid estradiol (E_2) action in primate GnRH neurons. Exposure of primate GnRH neurons to E_2 rapidly induces $[Ca^{2+}]_i$ oscillations and GnRH peptide release within 10 min (Noel et al., 2009; Kenealy et al., 2011b). Two possible mechanisms for the rapid E_2 action through GPR30 and STX-R are discussed in this article, although many details are yet to be clarified, which are noted by question marks in the scheme. First, E_2 binding to GPR30 may induce activation of two intracellular pathways: 1) E_2 activation through GPR30 depolarizes GnRH neuronal membrane via VGCCs (Sun et al., 2010), which allows $[Ca^{2+}]_e$ entry, resulting in CICR (Kenealy et al., 2011c) and 2) E_2 transactivates AC and/or ErbB pathways (Filardo et al., 2002), which also results in CICR. Second, E_2 binding to STX-R appears to cause 1) activation of CICR through a PLC and IP3-R mechanism leading to $[Ca^{2+}]_i$ increase (Kenealy et al., 2011b) and 2) activation of a PKCδ-AC-PKA mechanism resulting in hyperpolarization of the GnRH neuronal membrane through KATP channels (Zhang et al., 2010), which are essential for burst firing of GnRH neurons, hence neurosecretion.
Abbreviations: AC, adenylyl cyclase; Ca^{2+}, calcium; $[Ca^{2+}]_e$, extracellular Ca^{2+}; $[Ca^{2+}]_i$, intracellular Ca^{2+}; CICR, calcium induced calcium release; DAG, diacylglycerol; E_2, estradiol; ER, endoplasmic reticulum; ErbB, epidermal growth factor receptor; GnRH, gonadotropin-releasing hormone neuron; GPR30, G protein coupled estrogen receptor; IP3, inositol triphosphate; IP3-R, inositol triphosphate receptor; KATP, ATP sensitive potassium channel; PIP2, phosphatidylinositol biphosphate; PKA, protein kinase A; PKCδ, Protein kinase C delta; PLC, phospholipase C; RyR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; STX-R, membrane estrogen receptor sensitive to STX; VGCC, voltage gated calcium channel.

Based on the classical concept of positive and negative ovarian steroid feedback, doubt has been cast on the role of rapid excitatory E_2 action in the reproductive cycle (Herbison, 2009). It is still unknown whether E_2 directly alters secretory activity of GnRH neurons *in vivo*, as to date, *in vivo* effects of E_2 on GnRH release are tested only after systemic administration of E_2. Accumulating evidence suggests local effects of E_2 in various brain functions (Woolley, 2007). For example, a recent report in male song birds showing that forebrain E_2 concentrations rapidly (within 30 min) increase during social interaction with females (Remage-Healey et al., 2008) indicating the occurrence of rapid synthesis and release of E_2 in the brain (Saldanha et al., 2011). Moreover, another recent study by Konkle and McCarthy (2011) reports that E_2 levels of 20–30 pg/mg tissue are observed during the first 10 days after birth in the rat hypothalamus, regardless of the presence or absence of gonads. These observations raise the possibility that a relatively high amount of E_2 can be synthesized and released locally and rapidly in the hypothalamus under certain physiological conditions, although local concentrations of estradiol in the monkey brain are presently unknown. Therefore, we propose the hypothesis that local E_2 release in the hypothalamus may contribute to the activity of GnRH neurons.

As described above, our observations *in vitro* consistently show that E_2 is a potent frequency modulator of GnRH neurons (Terasawa et al., 2009). Thus, we can extend our hypothesis to suggest that local E_2 may modulate pulsatility of GnRH release in a subtle manner. Although the mechanism of pulsatile GnRH release is presently unclear, the concept that coordinated release of GnRH is due to synchronized activity among GnRH neurons is generally accepted. In fact, as a means to coordinate activity, GnRH neurons may communicate through dendro-dendritic interactions (Campbell et al., 2009). Because GnRH is released from the cell body and dendrites of GnRH neurons besides their neuroterminals (Fuenzalida et al., 2011), it is possible that rapid excitatory action of E_2 on GnRH release may contribute to the communication within dendro-dendritic bundles and consequently, modulate pulsatile GnRH release *in vivo*.

In rhesus monkeys and sheep, prenatal treatments with testosterone, which is aromatizable to E_2 in the brain, result in conditions similar to those of polycystic ovary syndrome (PCOS), one of the most common causes of infertility in women (Abbott et al., 1998; Padmanabhan et al., 2010). Importantly, this testosterone treatment in monkeys at a fetal age of E40-60 (early treatment), but not E100-115 (late treatment), results in LH hypersecretion, reduced hypothalamic sensitivity to negative steroid feedback, and follicular arrest with premature follicle differentiation (Dumesic et al., 2005), as seen in PCOS patients. Moreover, studies in humans show that PCOS women exhibit an accelerated frequency of LH pulses and presumably GnRH pulses (Marshall and Eagleson, 1999), indicating impairment of the GnRH pulse-generating mechanism. Importantly, the effective timing of early testosterone treatment (fetal age at E40-60) in rhesus monkeys (Dumesic et al., 2005) exactly corresponds to the period of GnRH neuronal maturation. After differentiation from progenitor cells at E32-34, GnRH neurons start migrating into the hypothalamus at E38 and settle down in the hypothalamus by E50 (Rønnekleiv and Resko, 1990; Quanbeck et al., 1997). In fact, this period appears critical for the maturation of GnRH neuronal function. Recently, we have shown that GnRH neurons gradually mature *in vitro* during the 3-week period, after GnRH neurons are dissected out from the nasal placode and started for culture at E36 (Kurian et al., 2010). It should be noted that although prenatal testosterone effects in monkeys may not solely be attributable to aromatized estrogens, it is also possible that rapid excitatory E_2 action described in this review may reflect developmental programming influenced by maternal steroidal environments during fetal development and elevated concentrations of cortisol and androgens (Schneider et al., 2002; Padmanabhan et al., 2010; Dunn et al., 2011).

CONCLUSION

E_2 action in the brain is not limited to the classical feedback control of the GnRH neuronal system. E_2 could be involved in excitatory and inhibitory neuronal function in the hypothalamus, as shown by Mermelstein and his colleagues in hippocampal neurons (Boulware et al., 2005, 2007). Moreover, E_2 action in GnRH neurons may be the consequence of events occurring *in utero*, such as developmental programming and sexual differentiation. Advances in technology and further detailed studies will ensure the importance of rapid action of estradiol in the hypothalamus.

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Estrogenic regulation of the GnRH neuron

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Reproductive function is regulated by the secretion of luteinizing hormone (LH) and follicle-stimulating hormone from the pituitary and the steroid hormones from the gonads. The dynamic changes in the levels of the reproductive hormones regulate secondary sex characteristics, gametogenesis, cellular function, and behavior. Hypothalamic GnRH neurons, with cell bodies located in the basal hypothalamus, represent the final common pathway for neuronally derived signals to the pituitary. As such, they serve as integrators of a dizzying array of signals including sensory inputs mediating information about circadian, seasonal, behavioral, pheromonal, and emotional cues. Additionally, information about peripheral physiological function may also be included in the integrative signal to the GnRH neuron. These signals may communicate information about metabolic status, disease, or infection. Gonadal steroid hormones arguably exert the most important effects on GnRH neuronal function. In both males and females, the gonadal steroid hormones exert negative feedback regulation on axis activity at both the level of the pituitary and the hypothalamus. These negative feedback loops regulate homeostasis of steroid hormone levels. In females, a cyclic reversal of estrogen feedback produces a positive feedback loop at both the hypothalamic and pituitary levels. Central positive feedback results in a dramatic increase in GnRH secretion (Moenter et al., 1992; Xia et al., 1992; Clarke, 1993; Sisk et al., 2001). This is coupled with an increase in pituitary sensitivity to GnRH (Savoy-Moore et al., 1980; Turzillo et al., 1995), which produces the massive surge in secretion of LH that triggers ovulation. While feedback regulation of the axis in males is in part mediated by estrogen receptors (ER), there is not a clear consensus as to the relative role of ER versus AR signaling in males (Lindzey et al., 1998; Wersinger et al., 1999). Therefore, this review will focus on estrogenic signaling in the female.

Keywords: GnRH, kisspeptin, GPR54, estrogen receptor, feedback, progesterone receptor, arcuate, AVPV

EVIDENCE FOR REGULATION OF GnRH GENE EXPRESSION BY ESTROGEN

Estrogen has a bimodal effect on the hypothalamus with both an inhibitory and stimulatory influence on GnRH secretion. The stimulatory effect of estrogen is seen at the end of the follicular phase where estrogen triggers the preovulatory GnRH surge (Sarkar et al., 1976; Karsch et al., 1997; Levine, 1997; Herbison, 1998; Simerly, 2002). The ability of estrogen to induce a surge declines with age in female rodents and women (Wise, 1982; Shaw et al., 2011) This appears to be due in large part to a decrease in estrogen induction of GnRH neurons, particularly in rodents (Downs and Wise, 2009), although a decline in pituitary responsiveness to GnRH contributing to attenuated luteinizing hormone (LH) surges has also been proposed (Shaw et al., 2009).

The inhibitory effect of estrogen on GnRH secretion and GnRH gene expression has been shown in *in vivo* studies in several mammalian species (Zoeller et al., 1988; Petersen et al., 1995). Studies in ewes indicate that estrogen inhibits GnRH pulse amplitude in the early follicular and luteal phase of the cycle (Caraty et al., 1989; Chongthammakun and Terasawa, 1993). *In vivo* and *in vitro* studies in the rat hypothalamus (Sarkar and Fink, 1980; Spratt and Herbison, 1997) indicate that estrogen inhibits GnRH mRNA expression and that this effect is localized to the rostral preoptic area of the hypothalamus. The inhibitory effect of estrogen seems to involve different anatomical sites in the hypothalamus than those associated with the stimulatory effect of estrogen on GnRH (Shander and Barraclough, 1980; Wiegand et al., 1980; Wray et al., 1989; Gibson et al., 1997; Caraty et al., 1998), which had indicated that the inhibitory and stimulatory effects may occur independently from one another. Negative feedback was localized to the arcuate and median eminence of the medial basal hypothalamus in these studies while positive feedback was mapped to the preoptic and suprachiasmatic nucleus. It is proposed that the biological substrate for these effects is kisspeptin, the hypothalamic protein previously found to be essential in pubertal onset (de Roux et al., 2003; Seminara et al., 2003). Estrogen has been shown to mediate a decrease in kisspeptin in the arcuate nucleus in contrast to increasing expression in the AVPV (Discussed below: Smith et al., 2006; Dungan et al., 2007).

In vitro evidence of negative estrogen regulation of rat GnRH gene expression includes transfection studies in both placental JEG-3 cells (Wierman et al., 1992) and in GT1-7 GnRH-expressing neuronal cells co-transfected with the estrogen receptor alpha (ER)α cDNA (Kepa et al., 1992). These *in vitro* studies indicated that estrogen decreases expression of the rat GnRH gene promoter. Studies of the-human GnRH promoter in transient transfection

experiments in JEG-3 cells co-transfected with ERα cDNA indicate estrogen-mediated regulation of the-human GnRH promoter (Radovick et al., 1991; Dong et al., 1996). Studies performed by Roy et al. (1999) demonstrated a decrease in GnRH mRNA levels in the GnRH-expressing neuronal cell line, GT1-7, treated with 17β-estradiol over a 48-h time course, starting as early as 12 h. Our laboratory has confirmed and extended these findings and observed a decrease in GnRH expression and secretion by estradiol in both the GN11 and GT1-7 GnRH-expressing cell lines and determined that these effects were primarily mediated by ERα in GT1-7 cells and by both ERα and ERβ in GN11 cells (Ng et al., 2009).

In addition, we have shown that estrogen down-regulates GnRH gene expression in a castration–estrogen replacement paradigm using a transgenic mouse model. This model was developed by targeting GnRH neurons with construct containing the GnRH gene promoter fused to a luciferase reporter gene (Radovick et al., 1994; Wolfe et al., 1995; Kim et al., 2007; −3446/+5-luc mice). These GnRH promoter elements were shown to specifically and reproducibly target hypothalamic GnRH neurons in transgenic mice. After treatment with estradiol, gonadectomized female mice exhibited an 80% reduction in hypothalamic luciferase expression. Although these *in vivo* studies do not prove that ER directly regulates GnRH expression in the hypothalamus, they do establish that GnRH gene expression is negatively regulated by estrogen at a transcriptional level.

THE ESTROGEN RECEPTOR

Estrogen is known to exert its effect through binding and activation of ERs. The ER is a member of the superfamily of nuclear receptors (Beato et al., 1995; Mangelsdorf et al., 1995), and is involved in transcriptional regulation of target genes. Two isoforms of ER (ERα and ERβ), with variable tissue distribution, have been described (White et al., 1987; Couse et al., 1997; Tremblay et al., 1997). ER isoforms share a common structural organization that includes six distinct functional domains, A to F, characteristic of members of the superfamily of nuclear receptors (Beato et al., 1995; Mangelsdorf et al., 1995). The A/B domain located at the N-terminal portion of the receptor contains a weak constitutive ligand-independent transcription activating function-l (AF-1); the C domain contains zinc finger-like motifs that are involved in binding to an estrogen response element (ERE); the D domain contains a hinge region that modulates DNA-binding; and the E/F domains contain the ligand-binding domain and a strong ligand-dependent activating function-2 (AF-2). Mouse ERα and ERβ share considerable homology in their DNA and ligand-binding domains (97 and 60% respectively), but display no sequence homology in their amino terminal domains (Tremblay et al., 1997). Mouse ERα has a slightly higher affinity for binding to a consensus ERE when compared to ERβ (Tremblay et al., 1997). Both ER isoforms, however, activate natural estrogen-responsive promoters to a similar extent in transient transfection studies.

THE CLASSICAL ER SIGNALING PATHWAY

The classic ER signaling pathway involves estrogen binding to ERs (Katzenellenbogen et al., 1993; Beato et al., 1995; Mangelsdorf et al., 1995) inducing a conformational change leading to receptor dimerization (Wang et al., 1995; Pettersson et al., 1997; Ogawa et al., 1998) and subsequent binding to an ERE located on promoter regions of target genes (Beato et al., 1995; Mangelsdorf et al., 1995). Binding of the ER complex to DNA activates gene transcription through its activating function domains, AF-1 and AF-2. AF-l and AF-2 can act independently, or synergize with one another to stimulate positive regulation of gene transcription (Tora et al., 1989; Berry et al., 1990). The ability of AF-1 and AF-2 to contribute to ER transcriptional activity seems to be cell- and promoter-specific (Tora et al., 1989; Tzukerman et al., 1994), and involves binding of AF-l and/or AF-2 to cofactors (Tzukerman et al., 1994; Shibata et al., 1997; Tremblay et al., 1999). Co-activators of ER include a family of related proteins known as the p160s: the SRC family, pCIP, and others (Onate et al., 1995; Horwitz et al., 1996; McKenna et al., 1999; Xu and O'Malley, 2002; Smith and O'Malley, 2004); and recruitment of p160 cofactors is sufficient for activation of the ER (Shang et al., 2000). p160s, in turn, interact with other co-activator proteins such as CREB-binding protein (CBP; Chakravarti et al., 1996), p300, and CBP-associated factor (P/CAF; Korzus et al., 1998). Other ER co-activators include RIP140 (Cavailles et al., 1995). Co-repressors of ER have also been described, including SHP (Seol et al., 1998; Johansson et al., 1999), NcoR (Lavinsky et al., 1998), and SMRT (Misiti et al., 1998).

THE RELATIVE ROLES OF ERα AND ERβ AND KISSPEPTIN IN THE CONTROL OF THE REPRODUCTIVE AXIS

The recent description of a patient with idiopathic hypogonadotropic hypogonadism with a mutation in a G-protein coupled receptor, referred to as GPR54, has added to our knowledge of signaling pathways involved in this complex system (de Roux et al., 2003; Seminara et al., 2003). GPR54 binds kisspeptin (kiss1) to regulate GnRH secretion (Kotani et al., 2001; Smith et al., 2005, 2006; d'Anglemont de Tassigny et al., 2008). Several studies have confirmed that GPR54 co-localizes with GnRH neurons (Irwig et al., 2004; Parhar et al., 2004; Han et al., 2005; Messager et al., 2005; Novaira et al., 2009). Central or systemic administration of kiss1 leads to GnRH and gonadotropin secretion in both prepubertal and adult animals (Gottsch et al., 2004; Matsui et al., 2004; Messager et al., 2005; Navarro et al., 2005; Shahab et al., 2005; Zhang et al., 2008). Confirming that kiss1 lies upstream of the GnRH neuron, mice who have a knock-out of GPR54, as well as humans that have mutations in GPR54, respond normally to GnRH.

In rats, ER isoform selective ligands have provided evidence that ERα is the predominant receptor isoform mediating negative feedback (Sanchez-Criado et al., 2006). However E2 is known to regulate gene transcription by binding to high affinity receptor dimers and by facilitating dimer formation between ERs (Katzenellenbogen et al., 1993). In fact, ERα and ERβ can form heterodimers in tissues where they are co-expressed (Pace et al., 1997; Powell et al., 2010) and the DBD was sufficient to allow for heterodimerization (Pace et al., 1997). This might explain the higher LH levels observed in ERα and ERβ double gene knock-out (KO) mice compared to ERα KO mice (Couse et al., 2003). The studies demonstrating the key role for ERα in negative feedback build on previous studies demonstrating an important role for kisspeptin for LH secretion during the entire estrous cycle despite dynamic changes in estrogen levels (Roa et al., 2006). Kiss1 neurons are activated during

the preovulatory period (Smith et al., 2006), and blockade of kiss1 secretion results in a lack of LH surge generation (Kinoshita et al., 2005). Two principal populations of kiss1 neurons are described in the hypothalamus; one in the arcuate nucleus and one in the AVPV which match well with the anatomical loci of estrogen positive and negative feedback discussed above (model is summarized in **Figure 1**). The arcuate population has been proposed to mediate estrogen negative feedback while the specific subset of neurons in the AVPV have been shown to be critical intermediates in transducing estradiol positive feedback required to induce ovulation (Popolow et al., 1981; Wiegand and Terasawa, 1982; Simerly and Swanson, 1987; Gu and Simerly, 1997). Within the AVPV of female mice, *Kiss1*-expressing neurons are activated by estrogen (Smith et al., 2005, 2006). Interestingly, perinatal treatment with the environmental estrogenic compound bisphenol A (BPA) produces males with female-like development of the kisspeptin neurons in the AVPV that correlates with the ability of BPA treated male mice to generate LH surges in response to high levels of estrogen (Bai et al., 2011). Kiss1 neurons have been shown to express ERα (and some ERβ), and kiss1 neurons in the AVPV secrete kiss1 in response to estrogen treatment. Since these neurons contact GnRH neurons, it has been proposed that kiss1 neurons in the AVPV are the locus of estrogen positive feedback leading to the gonadotropin surge. This complements studies from Wintermantel et al. (2006) that have shown neuronal ERα was required for generation of the LH surge in mice. The identity of these AVPV neurons is not entirely clear. While some have proposed that both ARC and AVPC kiss1 neurons may be GABA neurons (Petersen et al., 2003; Cravo et al., 2011) or glutaminergic neurons (Cravo et al., 2011) recent compelling evidence exists that a subset of both the ARC and AVPV kiss1 neurons also express galanin (Porteous et al., 2011; Kallo et al., 2012) and that a large number of ARC kiss1, and even some AVPV kiss1 neurons, co-express neurokinin B and dynorphin (Navarro et al., 2009). Whether kisspeptin is permissive or active in regulating GnRH neurons at the time of the LH surge is unknown (Dungan et al., 2007). Nonetheless, GnRH neuronal activity is increased by kiss1 and modulated by changes in estradiol levels (Pielecka-Fortuna et al., 2008).

EVIDENCE FOR ERs IN THE GnRH NEURON

Although the presence of ERs in the gonadotroph is well accepted and hence estrogen action on the gonadotroph is direct. The mechanism by which estrogen regulates GnRH neurons is not well understood. The more prevalent hypothesis is that the influence of estrogen on GnRH is not direct but is conveyed to GnRH neurons via presynaptic afferents from adjacent cells that express ER (now thought to be kiss1 neurons). This hypothesis is based on several *in vivo* double-labeling immunohistochemical studies examining the hypothalamus of many mammalian species where co-expression of GnRH and ER was not demonstrated (Shivers et al., 1983; Herbison and Theodosis, 1992; Watson et al., 1992; Herbison et al., 1993, 1995; Kalra, 1993; Lehman and Karsch, 1993; Navas et al., 1995; Sullivan et al., 1995; Lopez et al., 1996; Warembourg et al., 1998; Kelly et al., 2003; Garcia-Segura and McCarthy, 2004). Results from these studies indicate that, although ERs are highly expressed in the hypothalamus, few if any GnRH neurons express ER. Additionally then, the effects of estrogen on GnRH neurons would occur in an indirect manner by ERα and/or ERβ expressing neurons, or glial cells (Rage et al., 1997; Smith and Jennes, 2001; Prevot, 2002; Petersen et al., 2003). However, the original studies may have been hampered by an inability to detect a lower concentration of ERs in tissues, or by the castration paradigms that were used, which could alter ER expression levels.

An accumulating body of *in vitro* evidence currently indicates the presence of functional ERs in GnRH neurons. A number of these studies have used immortalized GnRH-expressing neuronal cell lines and have shown that they express ERα and/or ERβ (Shen et al., 1998; Butler et al., 1999; Roy et al., 1999; Hrabovszky et al., 2000; Kallo et al., 2001; Martinez-Morales et al., 2001) and respond to estrogen treatment by increasing galanin (Shen et al., 1998) and androgen receptor gene expression (Poletti et al., 1994). These studies show evidence for a direct estrogen effect on GnRH neurons, and suggest the presence of a functional ER signaling pathways in these neurons. ERβ may be the predominant ER in GnRH neurons (Hrabovszky et al., 2001; Kallo et al., 2001; Legan and Tsai, 2003; Petersen et al., 2003; Skinner and Dufourny, 2005); however, a separate immunohistochemistry study of female rats found ERα transcripts in GnRH neurons in the preoptic area (Butler et al., 1999). Prenatal GnRH neurons have been shown to express ERβ transcripts, however, the number of cells expressing ERβ decreased over time (Sharifi et al., 2002). These studies led to the most recent evidence in humans that ERβ colocalized with GnRH (Hrabovszky et al., 2007). With mounting evidence that

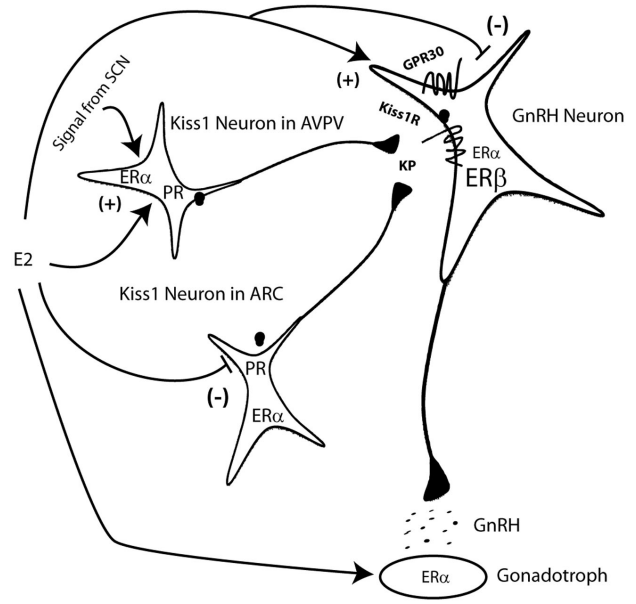

FIGURE 1 | Model of estrogenic regulation of the GnRH neuron. Estrogen (E2) from the ovary regulates the pituitary gonadotroph, the kiss1 neurons in the AVPV and the arcuate (ARC) nuclei and can also directly regulate the GnRH neuron. E2 regulation of kiss1 in both nuclei is regulated by ERα and progesterone receptor (PR). A daily signal from the SCN to the kiss1 neurons of the AVPV helps coordinate surge generation. Direct effects at the level of the GnRH neuron can be mediated by ERα, ERβ, or the membrane associated GPR30. Kiss1 (KP) regulates GnRH neurons by binding to and activating the kiss1 receptor (also known as GPR54).

GnRH neurons contain ERs, the functional significance remains to be determined as well as the intracellular signaling processes used. However, a direct, transcription-dependent mechanism on GnRH has been shown (Temple et al., 2004).

ESTROGEN RECEPTOR KNOCK-OUT PHENOTYPES

Studies from mice with generalized ERα disruption (ERαKO) indicate that ERα plays a major role in regulating the reproductive neuroendocrine–gonadal system (Lubahn et al., 1993; Couse et al., 1999; Schomberg et al., 1999; Dupont et al., 2000). ERα is widely expressed throughout the reproductive axis, including the hypothalamus, pituitary, gonads (both ovaries and testis), and uterus. Therefore, a generalized disruption of ERα would be predicted to affect the axis at various levels centrally and peripherally (Lubahn et al., 1993; Schomberg et al., 1999; Dupont et al., 2000), rendering the isolation of the effect of estrogen at any level impossible. Homozygous ERα KO mice display significant gonadal defects and impaired feedback regulation of the neuroendocrine axis. Homozygous ERα KO females were also infertile and were found to have hypoplastic uteri, large hemorrhagic cysts, and absent corpus luteum in their ovaries (Couse et al., 1995; Korach et al., 1996; Schomberg et al., 1999; Dupont et al., 2000).

In addition, studies in homozygous ERα KO females indicate that they exhibit 10-fold higher serum estrogen level than wild type controls. LH levels were also significantly increased in intact ERαKO females compared to those in WT females (Lindzey et al., 1998; Wersinger et al., 1999). Estrogen feedback regulation is thought to occur both at the level of the hypothalamus and at the level of the pituitary. Thus, elevated LH levels may indirectly reflect elevated GnRH levels due to loss of negative estrogen regulation at the level of the hypothalamus or may also reflect loss of negative estrogen regulation at the level of the pituitary. Similarly, LH and follicle-stimulating hormone (FSH) mRNA levels were higher in intact ERα KO compared to those in intact WT females, and were comparable to LH and FSH mRNA levels in ovariectomized, non-replaced WT females (Scully et al., 1997; Lindzey et al., 1998). These results suggest a role for estrogen in negative regulation of LH and FSH, but do not localize the effect to the level of the hypothalamus, and/or the pituitary.

Initial studies of ERβKO mice failed to identify a significant reproductive phenotype. However, it was noted later that ER knock-out mice produced by insertion of an ER allele containing a neomycin resistance gene could produce a chimeric protein that is partially estrogen-responsive (Couse et al., 1995; Antal et al., 2008; Chen et al., 2009). Complete ERβKO mice demonstrated subfertility (Krege et al., 1998; Dupont et al., 2000; Antal et al., 2008) though gonadotropin levels appear to be unchanged compared to control mice. These data suggest that ERβ may not be important for central E2 negative feedback of the axis. However, gonadotropin levels are less elevated in ERαKO versus the combined ERαβ KO mice, indicating that ERβ may have a negative feedback role in the axis (Couse et al., 2000). Interestingly, the role of ERβ may be to increase progesterone mediated effects at the level of the hypothalamus to produce the gonadotropin surge (Chappell and Levine, 2000).

Additional studies in ERαKO mice indicated a role for ERα in the positive regulation of LH secretion by the pituitary and inferred that negative regulation occurs at the hypothalamic level (Lindzey et al., 2006). Recently, ERα expression in neuronal cells was shown to be required for positive feedback of estradiol (Wintermantel et al., 2006). Furthermore, a pituitary-specific ERα KO mouse model, although being infertile, was shown to have normal levels of LH, suggesting that positive feedback regulation was impaired but negative regulation by estrogen was intact (Gieske et al., 2008). In contrast, we also generated a pituitary-specific KO of ERα and find elevated serum LH levels (Singh et al., 2009). It is not clear why these two models differ in regards to LH levels and may reflect differences in sampling or LH assay methodologies.

NON-CLASSICAL ER SIGNALING PATHWAYS

In the classical, or genomic pathway, ERs bind to an ERE on DNA to alter the transcription of genes (O'Malley and Tsai, 1992; Arbogast, 2008). However, ERα has been shown to signal through ERE-independent, non-classical, as well as non-genomic pathways (McEwen and Alves, 1999; Kousteni et al., 2001) through protein–protein interactions by tethering to other transcription factors such as AP-1 and NF-kB and the transcriptional complex (Stein and Yang, 1995; Ray et al., 1997; Kushner et al., 2000; Pedram et al., 2002). Furthermore, ERα has been shown to display antagonistic effects on ERα mediated transcription by interfering with recruitment of an AP-1 protein complex on DNA (Matthews et al., 2006). ER mediated, ligand-dependent signaling has been shown to involve cross-talk with growth factor mediated pathways through phosphorylation by MAPK activation (Kato et al., 1995; Couse and Korach, 1999; Feng et al., 2001; Masuhiro et al., 2005), and ERα has also been shown to induce rapid membrane-initiated signaling (Levin, 2005; Revankar et al., 2005; Pedram et al., 2006; Vasudevan and Pfaff, 2007).

Non-genomic effects of estradiol have been reported in several studies. For example, estradiol increased the phosphorylation of cAMP response element binding protein (CREB) in GnRH neurons (Abraham et al., 2003) as well as calcium oscillations (Abe et al., 2008) and potassium currents (DeFazio and Moenter, 2002; Farkas et al., 2007; Roepke et al., 2007; Zhang et al., 2007). Immunocytochemical localization was performed for ER isoforms in GT1-7 cells, localizing both receptors at the cell membrane to some degree (Navarro et al., 2003) and providing some evidence that the ERα isoform was responsible for norepinephrine responsiveness (Morales et al., 2007). Rapid signaling effects of estradiol have also been linked in hypothalamic neurons to activated protein kinase C pathways (Qiu et al., 2003). A membrane ER that is distinct from either ERα or ERβ has recently been identified. The GPR30 orphan receptor, a member of the G-protein coupled family of receptors, has been identified as a putative ER. GPR30 located in the endoplasmic reticulum, Golgi apparatus, and nuclear membranes have been shown to be activated by estradiol and other estrogen agonists (Filardo, 2002; Brailoiu et al., 2007; Prossnitz et al., 2008). GPR30 has been found in primate and mouse GnRH neurons (Noel et al., 2009; Sun et al., 2010) and is proposed to be located at the plasma membrane as membrane impermeable BSA conjugated E2 can exert rapid effects on GnRH function (Noel et al., 2009). Further complexity has recently been added with the report of a novel ER, acting as a G-protein coupled receptor in the hypothalamus (Qiu et al., 2008).

ROLE OF PROGESTERONE RECEPTORS (PR_A AND PR_B) IN THE POSITIVE FEEDBACK ACTIONS OF E_2

The preovulatory release of GnRH consists of a 2- to 4-h increase in the overall amount of GnRH secreted, occurring between 1600 and 2000 hours on the afternoon of proestrus (Levine and Ramirez, 1982). Classical neuroendocrine studies demonstrated that the CNS mechanisms that mediate the release of a GnRH surge require the integration of two obligatory signals – the preovulatory estrogen surge, and a daily neural signal that is synaptically conveyed from the circadian clock resident in the suprachiasmatic nucleus (Levine, 1997). The major action of estrogen is to "couple" the daily neural signal to the neuronal circuitries that mediate release of GnRH surges (Karsch and Foster, 1975; Legan and Karsch, 1975). In the absence of a sufficient elevation of estrogen, the daily signal is not communicated to neurons controlling the release of GnRH, and no surge takes place; with exposure of the hypothalamus to a sufficient estrogen stimulus, the pathways that convey the daily neural signal for the surge are rendered patent, and the GnRH is released into the hypophysial portal vasculature. At the same time, estrogen greatly enhances the responsiveness of the gonadotrophs to this burst of GnRH (Taga et al., 1982; Bauer-Dantoin et al., 1995; Shupnik, 1996). Both of these processes, along with the ability of GnRH to "self-prime" the estrogen-exposed pituitary to its own actions (Kamel et al., 1987) culminate in the release of a massive, but transient increase of LH on the afternoon of proestrus, which in turn triggers ovulation on the following morning of estrous.

How does estrogen couple the daily neuronal signal to the neurons responsible for the release of GnRH surges? One major locus of this integrative activity is the AVPV nucleus of the hypothalamus, where estrogen appears to activate ERα in neurons that receive afferents from the SCN and to project to GnRH neurons (possibly kiss1 neurons; Van der Beek et al., 1997; Tsukahara et al., 2008). We have examined the cellular actions of estrogen that may mediate these effects, focusing on the roles that PRs may play. Estrogen induces the expression of both isoforms of PR, PR_B, and the N-terminally truncated PR_A, in the AVPV, as well as other hypothalamic and preoptic nuclei. We have determined that induction of PRs is obligatory for the successful release of GnRH surges; thus, GnRH and LH surges are absent in ovarian intact and estrogen-treated PR gene knock-out (PRKO) mice (Chappell et al., 1997, 1999), and in rats treated with a progesterone receptor antagonist or intra-cerebroventricular PR antisense oligonucleotides (Chappell and Levine, 2000). We have proposed a model for the release of GnRH surges that includes (1) the induction of PRs by estrogen in AVPV neurons, (2) delivery of the daily neural signal from the SCN to AVPV neurons by neurotransmitter circuitries, and (3) the neurotransmitter-mediated activation of intracellular second messenger production that in turn transactivates the PRs in a ligand-independent manner; thereafter, (4) induces signals to kiss1 neurons that evoke the neurosecretion for the GnRH surge, which is (5) further amplified by ovarian progesterone release in response to the LH surge. The net result of all of these integrated physiological events is the release of a robust GnRH surge that is timed to trigger ovulation in concert with behavioral heat and maximal wakefulness, and uterine proliferation and differentiation thereby maximizing the chances for successful fertilization and implantation. The downstream targets of PR signaling that in turn initiate the GnRH surge process are not known, although we have provided evidence that one such target may be the neuropeptide Y (NPY) gene (Bauer-Dantoin et al., 1993). It is not clear how activated PRs may regulate NPY transcription, as there appear to be no classical palindromic PRE/GRE sites in the promoter of the preproNPY gene.

Estrogen's positive feedback actions also include a massive stimulation of pituitary responsiveness to GnRH stimulation. That E2-induced PR expression is obligatory for the manifestation of GnRH self-priming was demonstrated by the finding that this phenomenon is absent in PRKO animals (Chappell et al., 1999). The ability of progesterone to induce the GnRH self-priming response is rapid and depends upon mRNA and protein synthesis, but the cellular targets of PRs, whether activated by GnRH or progesterone, remain unclear.

Progesterone receptor in the ARC also likely contributes to negative feedback of the axis. Microimplants of the progesterone antagonist RU486 in the ARC, but not in the POA, blunt the negative feedback effects of progesterone in ewes (Goodman et al., 2011). The locus of action of the progesterone is likely the kisspeptin/dynorphin/neurokinin B expressing neurons in the arcuate (Goodman et al., 2004, 2011; Navarro et al., 2011).

CLASSICAL VERSUS NON-CLASSICAL PROGESTERONE RECEPTOR SIGNALING

Rapid effects of P have been documented in a variety of tissues, each potentially being mediated by one or more of several "non-classical" signaling mechanisms. Some of the rapid actions of P have been attributed to the ability of bound PR_A and PR_B to interact with the Src tyrosine kinase localized to the plasma membrane, which in turn prompts cellular responses via activation of the Src/Ras/Raf-1/MAPK signaling pathway (Faivre et al., 2008). At least three $PR_{A/B}$-independent pathways have also been identified that may mediate the effects of P in a variety of tissues and cell types. Progesterone is known to be rapidly metabolized in the brain to several neurosteroids, including allopregnanolone (3α-hydroxy-5α-pregnan-20-one; 3α5αTHP), which has been shown to modulate $GABA_A$ receptors in the brain (Majewska et al., 1986). Secondly, a progesterone membrane binding protein, progesterone receptor membrane component 1 (PGRMC1), has been suggested to mediate the ability of P to activate protein kinase G or other rapid signaling mechanisms in certain cells (Falkenstein et al., 1996).

The involvement of PRs in the positive feedback actions of E_2, nevertheless, is unequivocal, as both GnRH surges and GnRH self-priming mechanisms are inoperative in PRKO mice.

In summary, studies on estrogen effects on GnRH neuronal activity and secretion are wrought with complexity due to the various ER isoforms, direct versus indirect effects, classical versus non-classical estrogen signaling and the role of other hormones, such as testosterone (Eagleson et al., 2000; McGee et al., 2012) and progesterone, in modulating estrogen action on GnRH neurons. To overcome these difficulties, investigators have used a wide array of *in vitro* models, pharmacological tools, anatomical mapping, and transgenic and knock-out models. While these studies have helped clarify the role and mechanisms of estrogen action on GnRH neurons, many questions remain to be answered.

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Intracerebroventricular infusion of vasoactive intestinal peptide rescues the luteinizing hormone surge in middle-aged female rats

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Reproductive aging is characterized by delayed and attenuated luteinizing hormone (LH) surges apparent in middle-aged rats. The suprachiasmatic nucleus (SCN) contains the circadian clock that is responsible for the timing of diverse neuroendocrine rhythms. Electrophysiological studies suggest vasoactive intestinal peptide (VIP) originating from the SCN excites gonadotropin-releasing hormone (GnRH) neurons and affects daily patterns of GnRH–LH release. Age-related LH surge dysfunction correlates with reduced VIP mRNA expression in the SCN and fewer GnRH neurons with VIP contacts expressing c-fos, a marker of neuronal activation, on the day of the LH surge. To determine if age-related LH surge dysfunction reflects reduced VIP availability or altered VIP responsiveness under estradiol positive feedback conditions, we assessed the effect of intracerebroventricular (icv) VIP infusion on c-fos expression in GnRH neurons and on LH release in ovariohysterectomized, hormone-primed young and middle-aged rats. Icv infusion of VIP between 1300 and 1600 h significantly advanced the time of peak LH release, increased total and peak LH release, and increased the number of GnRH neurons expressing c-fos on the day of the LH surge in middle-aged rats. Surprisingly, icv infusion of VIP in young females significantly reduced the number of GnRH neurons expressing c-fos and delayed and reduced the LH surge. These observations suggest that a critical balance of VIP signaling is required to activate GnRH neurons for an appropriately timed and robust LH surge in young and middle-aged females. Age-related LH surge changes may, in part, result from decreased availability and reduced VIP-mediated neurotransmission under estradiol positive feedback conditions.

Keywords: vasoactive intestinal peptide, luteinizing hormone, gonadotropin-releasing hormone, aging

INTRODUCTION

An appropriately timed and robust preovulatory gonadotropin-releasing hormone (GnRH)–luteinizing hormone (LH) surge in rodents requires the convergence of synchronized neurochemical and hormonal events. Female reproductive aging is characterized by reduced responsiveness to estrogen positive feedback, resulting in a delayed and attenuated preovulatory LH surge (Cooper et al., 1980; Wise, 1982). Age-related changes in the magnitude of the LH surge reflect dysregulation of estradiol-regulated hypothalamic neurotransmission, which includes increased inhibitory input from GABA, reduced excitatory input from glutamate and kisspeptin, and reduced insulin-like growth factor-1 signaling (Neal-Perry et al., 2008, 2009; Lederman et al., 2010; Todd et al., 2010; Sun et al., 2011).

The suprachiasmatic nucleus (SCN) is hypothesized to integrate and synchronize all of the diverse neuroendocrine events required to activate GnRH neurons and to initiate an appropriately timed GnRH–LH surge (Brown-Grant and Raisman, 1977; Wiegand and Terasawa, 1982; Van der Beek et al., 1997; Colwell et al., 2003; de la Iglesia and Schwartz, 2006). Vasoactive intestinal peptide (VIP) neurons and vasopressin (AVP) positive cells located in the ventrolateral and dorsomedial SCN (Card et al., 1988) are hypothesized to be principal mediators of SCN modulation of the LH surge (van der Beek et al., 1993, 1994; Palm et al., 1999; Miller et al., 2006; Tsukahara, 2006). Moreover, available data suggest that a critical level of AVP and VIP signaling are required for appropriately timed LH pulse frequencies and induction of the LH surge under estradiol positive feedback conditions (Alexander et al., 1985; Harney et al., 1996; Krajnak et al., 1998; Palm et al., 1999; Colwell et al., 2003; Miller et al., 2006). Consistent with this hypothesis, icv infusion of AVP rescued LH release in transgenic mice with a mutation in the core circadian gene CLOCK and rescues the amplitude of the LH surge in rats that have AVP lesions in the SCN. Moreover VIP neurons located in the SCN project to GnRH neurons (Van der Beek et al., 1997), VIP activates GnRH neurons in a time- and estradiol-dependent fashion (van der Beek et al., 1994; Krajnak et al., 2001; Christian and Moenter, 2008), and VIP receptors are expressed on GnRH neurons and on astrocytes that are in close apposition to and ensheath GnRH neurons (Smith et al., 2000; Gerhold and Wise, 2006).

Additionally, exogenous infusion of VIP into the brain induces GnRH (Samson et al., 1981) and LH release in estradiol-primed females with SCN lesions (Palm et al., 1999). Conversely, intracerebroventricular (icv) infusion of VIP antiserum in intact females or VIP infusion in ovariectomized females blunts the LH surge and LH pulses, respectively (Alexander et al., 1985; Weick and Stobie, 1992, 1995).

Of interest, changes in the LH surge in old, irregularly cycling and persistent diestrus female rats correlate with reduced c-fos expression in SCN neurons, reduced VIP mRNA expression in the SCN, and reduced activation of GnRH neurons receiving VIP contacts (Krajnak et al., 1998, 2001). Additionally, the LH surge in young rats is delayed and attenuated by infusion of VIP antiserum into the third ventricle (van der Beek et al., 1999), infusion of VIP antisense oligonucleotides into the SCN (Harney et al., 1996), or thermal ablation of VIP neurons in the SCN (van der Beek et al., 1993). In contrast AVP mRNA expression in the SCN of reproductively aging females does not change (Krajnak et al., 1998). These data led us to hypothesize that the delayed and attenuated LH surge in regularly cycling, middle-aged rats results from reduced responsiveness to VIP, and/or reduced availability of VIP at the time of the surge. To test this hypothesis we (1) determined if hypothalamic VIP mRNA levels in regularly cycling middle-aged females are significantly less than in young females on the day of the LH surge; (2) determined if icv infusion of VIP rescues LH surge amplitude and/or restores the timing of the LH surge in regularly cycling middle-aged females; and (3) determined if icv infusion of VIP restores the activation of GnRH neurons, as determined by c-fos co-expression, in middle-aged females.

MATERIALS AND METHODS

ANIMALS AND HORMONE ADMINISTRATION

Young (2–3 months) and middle-aged (retired breeders, 9–11 months) female Sprague-Dawley rats (Taconic Farms, Germantown, NY, USA) were housed individually and maintained on a 14-h light, 10-h dark cycle (lights on at 0600) with free access to chow and water. Only rats with at least two regular 4–5 day estrous cycles were used. All procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Rats and were approved by the Institutional Animal Care and Use Committee at the Albert Einstein College of Medicine. All studies conditions were done in parallel. To induce LH surges, estradiol benzoate (EB) and progesterone (P; Steraloids Inc., Newport, RI, USA) were dissolved in peanut oil and administered subcutaneously in a volume of 0.1 ml. At 09 00 hours 7 days after ovariohysterectomy (OVX) and cannula placement, rats received the first of two daily injections of 2 μg of EB. At 09 00 hours 2 days after the first EB injection, rats were injected with 500 μg of P. This hormone regimen reliably produces LH surges in 75–80% of OVX female rats (Neal-Perry et al., 2005).

CANNULA PLACEMENT AND JUGULAR VEIN CATHETERIZATION

For OVX and stereotaxic surgery, rats were anesthetized with intramuscular ketamine/xylazine (80 and 4 mg/kg, respectively). After OVX, they were placed in a Kopf stereotaxic apparatus. A 22-gage icv guide cannula (Plastics One, Roanoke, VA, USA) was placed into the third ventricle (A/P +0.2 mm; M/L +0.0 mm; D/V −9.8 mm relative to Bregma) and plugged with a 26-gage dummy that extended 1 mm below the guide (Todd et al., 2010). Animals recovered for 6- to 7-days before further manipulations. Correct guide cannula placement was verified by tracking the path of the cannula in brain sections at the time of immunohistochemistry (IHC). Only those rats with correct placement of the icv cannulae were included in data analysis.

An indwelling catheter for serial blood sampling was placed in the jugular vein of anesthetized females 7 days after OVX and stereotaxic surgery as previously described (Neal-Perry et al., 2005). Catheters were kept patent by daily flushing with heparinized saline (50 IU). Blood collection (300 μl/sample) was initiated 2 days after the first EB injection starting at the time of the P injection and then every 2 h for 12 h. Blood was collected into tubes containing heparinized saline (10 IU), refrigerated overnight, and centrifuged at 10,000 × *g* for 20 min. Plasma was stored at −80°C until assayed for LH. An equal volume of warmed saline was infused after each blood collection to avoid hypovolemia.

ICV DRUG ADMINISTRATION

Normal saline was used to dissolve drugs and infused into all control rats. Two days after the first EB injection, the rats were connected to an automatic pump (Bioanalytical System Inc., West Lafayette, IN, USA) and attached to a tether allowing rats to move freely. Twenty micrograms of human VIP peptide (6 nmol; Bachem, CA, USA) or saline (vehicle/control) was infused into the third ventricle of young and middle-aged rats between 1300 and 1600 h at 2 nmol/15 μl/h, the time of the LH surge in young, reproductive-aged females (Neal-Perry et al., 2005).

LH RADIOIMMUNOASSAY

Plasma LH concentrations were measured in duplicate with rat double-antibody assays reagents provided by the Reproduction Ligand Assay and Analysis Core, General Clinical Research Center, University of Virginia (Charlottesville, VA, USA). The lower limit of the LH assay was 0.04 ng/ml and the intra- and inter-assay coefficients of variation were 3.9 and 5.7%, respectively. An LH surge was defined as greater than or equal to a 1.5-fold increase in serum LH levels from baseline for a minimum of two consecutive samples, and surge onset was considered to occur at the first of these samples (Todd et al., 2010). Baseline LH levels are defined by those at the time of P injection. Only those females who demonstrated an LH surge were included in the data analysis.

HYPOTHALAMIC DISSECTION, RNA PURIFICATION, REVERSE TRANSCRIPTION, AND REAL-TIME PCR FOR VIP

Independent groups OVX, young ($n = 5$–7), and middle-aged ($n = 5$–7) rats were primed with oil (control/vehicle) or EB and P as described above. Rats were killed 4 h after the P or last oil injection. The hypothalamus was dissected into anterior and posteriors halves (Neal-Perry et al., 2008), snap frozen on dry ice, and stored at −80°C until determination of VIP mRNA levels. DNA-free total RNA was purified using the RNeasy lipid minikit (Qiagen, Valencia, CA, USA) including a deoxyribonuclease step. Reverse transcription (RT) was performed using the high-capacity cDNA RT kit with ribonuclease inhibitor (Applied Biosystems, Foster

City, CA, USA) using 500 ng of RNA. Gene expression was measured by real-time PCR using TaqMan gene expression assays and master mix (Applied Biosystems) according to the manufacturer's instructions. 18S ribosomal RNA (18S rRNA, Fam probe, reference sequence Hs99999901_s1, context sequence X03205.1) was the endogenous control for VIP (Fam probe, reference sequence Rn01430567_m1; context sequence NM_053991.1). Real-time PCR was performed using an ABI PRISM 7900HT (Applied Biosystems) in simplex conditions using 50 ng of cDNA. Amplified transcripts for VIP were quantified using the comparative threshold cycle method and 18S rRNA as normalizer. The fold change in VIP expression was calculated as $2^{-\Delta\Delta CT}$ where CT = threshold cycle, ΔCT = CT (VIP) – CT (18S rRNA), $\Delta\Delta CT = \Delta CT$ (experimental) – ΔCT (reference). ΔCT (reference) was calculated using the mean of the ΔCT for the hypothalamus of OVX controls treated with oil.

IHC FOR GnRH AND c-fos

As per a modified protocol of Hoffman et al. independent groups of animals were perfused with 4% paraformaldehyde and 2.5% acrolein in phosphate buffer (pH 6.8) after VIP infusion between 1600 and 1730 h on the expected day of the LH surge (Hoffman and Le, 2004; Sun et al., 2011). Brains were post-fixed in 4% paraformaldehyde overnight at 4˚C, and then placed in 30% sucrose until they sank. This fixative method affords advantages that include improved antigen retrieval and improved signal to-noise ratios. Six sets of coronal sections (30 μm) starting at the level of the organum vasculosum of lamina terminalis (Bregma +0.48 mm) and continuing through the medial preoptic area (POA; Bregma −0.72 mm) were collected from each animal, with each set containing every sixth section. Sections were stored in cryoprotectant at −20˚C until processed for immunolabeling (Hoffman and Le, 2004).

As previously described (Sun et al., 2011) tissue sections were rinsed in potassium phosphate buffered saline (KPBS, 0.05 M, PH 7.4) to remove cryoprotectant, incubated in 1% sodium borohydrate to remove acrolein and then 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Sections were subsequently incubated in KPBS plus 0.04% Triton-X 100 (KPBS–Tx) and 1% bovine serum albumin (BSA) for 1 h at room temperature. Sections were incubated in goat anti-c-fos antibody (1:1000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in KPBS–Tx and 1% BSA for 48 h at 4˚C next and then incubated in biotinylated anti-goat immunoglobulin G (IgG, 1:600, Vector Laboratories, Burlingame, CA, USA) in KPBS–Tx for 1 h at room temperature. Finally sections were rinsed and incubated for 1 h in avidin biotin complex ('Elite' ABC kit, Vector Laboratories). After rinsing in KPBS and 0.175 M sodium acetate, the sections were stained in nickel sulfate (25 mg/ml) and diaminobenzidine–HCl (DAB, 0.2 mg/ml) in 0.175 M sodium acetate containing 30% H_2O_2 for 10 min followed by a final rinse in KPBS and sodium acetate. C-fos immunoreactive (ir) neurons were visualized as blue–black in the nuclei of neurons. For colocalization of c-fos in GnRH neurons, the sequence of reactions was repeated, substituting rabbit-anti GnRH antiserum as the primary antibody (1:5000, LR-5, a generous gift from Dr. R. Benoit, McGill University, Montreal, Canada) for 24 h at 4˚C. After rinsing, sections were incubated in biotinylated anti-rabbit IgG (Vector Laboratories) diluted (1:600) in KPBS–Tx for 1 h at room temperature, rinsed, and reacted with the avidin biotin complex as described above. A mixture of H_2O_2 and DAB–HCl in Tris (Sigma Aldrich Inc., St. Louis, MO, USA; 0.05 M, pH 7.2) was used as the chromogen to yield a brown staining in the cytoplasm. Sections were mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA). After drying overnight, the sections were dehydrated with ascending alcohol concentrations, cleared with xylenes, and coverslipped. A series of tissue sections was treated identically except without primary antibody to control for antibody specificity.

QUANTIFICATION GnRH AND c-fos-IMMUNOPOSITIVE CELLS

To quantify GnRH and c-fos-ir neurons, five sections of POA in the 1-in-6 series were viewed under a microscope (Zeiss Axioversion, Carl Zeiss, Thornwood, NY, USA; Sun et al., 2011). Sections corresponded to Plates 30–35 of the Paxinos and Watson atlas. Cells were considered c-fos-ir if they had blue/black nuclear staining with distinct nuclear boundaries. GnRH-ir cells were counted when the cell body was clearly identified and if they had brown cytoplasmic staining. GnRH neurons expressing c-fos were counted at 40× magnification; if cells had both brown cytoplasmic and blue/black nuclear staining, they were considered to be double labeled (**Figures 3A–H**). Cell counting was performed by two counters blinded to treatment (inter-rater variation < 10.5%) and average cell counts reported. Total GnRH and c-fos neurons as well as the percent of GnRH neurons expressing c-fos were calculated.

STATISTICAL ANALYSIS

Data are expressed as mean ± SEM. The area under the curve (AUC) for total LH release was calculated using Sigma Plot 10.0 (Systat Software, Inc., Chicago, IL, USA). A mixed design two-way ANOVA was used to determine the effect of time (repeated measure) and VIP (independent measure) on LH release. Two-way ANOVA (age × treatment) was used to determine differences in VIP mRNA and in total LH, GnRH, and c-fos cell numbers and percent of GnRH neurons expressing c-fos. $P < 0.05$ was considered statistically significant. Non-parametric testing was used when data were not normally distributed. Bonferroni or Newman–Keuls *post hoc* tests were performed as appropriate.

RESULTS

LH SURGES AND HYPOTHALAMIC VIP mRNA EXPRESSION IN YOUNG AND MIDDLE-AGED RATS

The EB and P-induced LH surge is delayed and attenuated in middle-aged rats compared to young rats (**Figures 1A,B**). Previous studies suggest that circadian-related changes in VIP cells of the SCN regulate the onset and peak of LH release in young rats (Colwell et al., 2003). Moreover, *in situ* hybridization suggests that old, irregularly cycling middle-aged, and persistent diestrus females have reduced VIP mRNA levels in the SCN and the POA on the day of the LH surge. To determine if hormone treatment affects hypothalamic VIP mRNA expression in regularly cycling, middle-aged females, we quantified VIP mRNA expression levels in the hypothalamus of a separate group of OVX control and EB + P-primed young and middle-aged females by real-time PCR. Neither age nor hormone treatment significantly affected

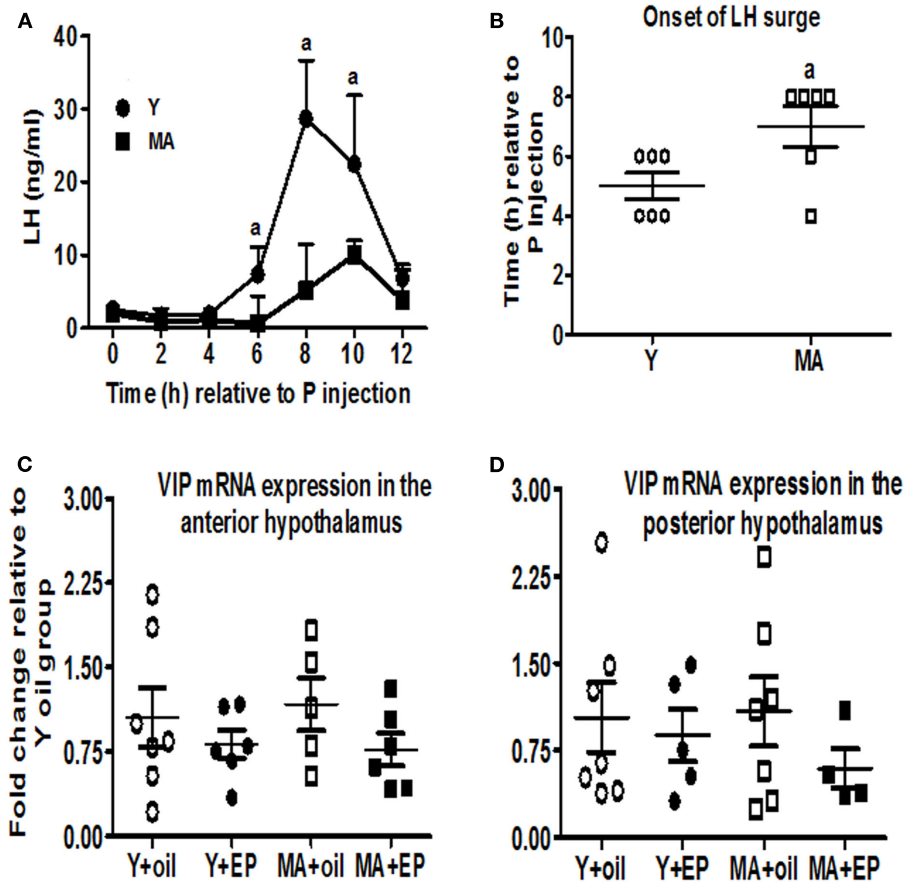

FIGURE 1 | Hypothalamic VIP mRNA expression is not reduced in regularly cycling, middle-aged females with delayed and attenuated LH surges. OVX young (Y; $n = 6$) and middle-aged (MA; $n = 6$) female rats received two daily doses of E2 benzoate (E: 2 μg) and one injection of progesterone (P: 500 μg) on the day of serum collection, or vehicle (oil). Serial blood sampling started at the time of P injection and continued every 2 h for 12 h. Time 0 represents the time of the P injection. Separate rats were used in RT-PCR experiments and were killed 52 h after the first injection of oil or E. Data are expressed as mean ± SEM. **(A)** LH surge in young (●) and middle-aged females (■) infused with icv saline. **(B)** Scatter plots of LH surge onset in young (○) and middle-aged females (□) infused with icv saline. **(C)** Scatter plots of VIP mRNA expression levels in the anterior hypothalamus of young (○, ●; $n = 6$–7) and middle-aged (□, ■; $n = 5$–6) rats primed with E + P (●, ■) or oil (○, □). **(D)** Scatter plots of VIP mRNA expression levels in the posterior hypothalamus of young (○, ●; $n = 5$–7) and middle-aged (□, ■; $n = 4$–7) rats primed with E + P (●, ■) or oil (○, □). [a] $P < 0.05$ vs. middle-aged.

total VIP mRNA levels in the anterior or posterior hypothalamus (**Figures 1C,D**).

ICV INFUSION OF VIP RESTORES LH SURGE AMPLITUDE AND ADVANCES PEAK RELEASE OF LH IN MIDDLE-AGED RATS

OVX, EB + P-primed rats were infused continuously with saline or VIP (6 nmol total dose) between 1300 and 1600 h on the day of the LH surge. There was a significant interaction between age and VIP treatment on total LH and peak LH release ($P < 0.01$). Middle-aged females infused with VIP exhibited a greater than twofold increase in total (**Figure 2C**; $P < 0.01$) and peak LH (**Figure 2E**; $P < 0.01$) release compared to middle-aged controls infused with saline. Moreover, VIP infusion rescued peak and total LH release in middle-aged females to levels that were equivalent to young control females (**Figures 2A–C,E**). VIP infusion did not advance the onset of the LH surge (**Figure 2D**); however, the time of peak LH release was significantly advanced in middle-aged females infused with VIP (**Figure 2F**; $P < 0.03$).

ICV INFUSION OF VIP ATTENUATES AND DELAYS THE LH SURGE IN YOUNG RATS

Young females infused with VIP had attenuated peak LH levels ($P < 0.05$) and released approximately 2/3 less total LH ($P < 0.01$) than young controls (**Figures 2A,E**). Peak LH release ($P < 0.05$) and the onset of the LH surge ($P < 0.05$) were significantly delayed in VIP-infused young rats (**Figures 2D,F**).

VIP INFUSION INCREASES THE PERCENT OF GnRH NEURONS EXPRESSING c-fos AND NUMBERS OF c-fos-ir NEURONS IN THE POA OF MIDDLE-AGED FEMALES

To determine if icv infusion of VIP increased the number of activated GnRH neurons, we quantified the percent of GnRH neurons expressing c-fos, a marker of neuronal activation, in hormone-primed middle-aged females infused with VIP. The number of GnRH neurons in middle-aged females infused with VIP or saline was not significantly different (**Figure 3J**). Similar to our previous study (Sun et al., 2011), approximately 20% of GnRH neurons

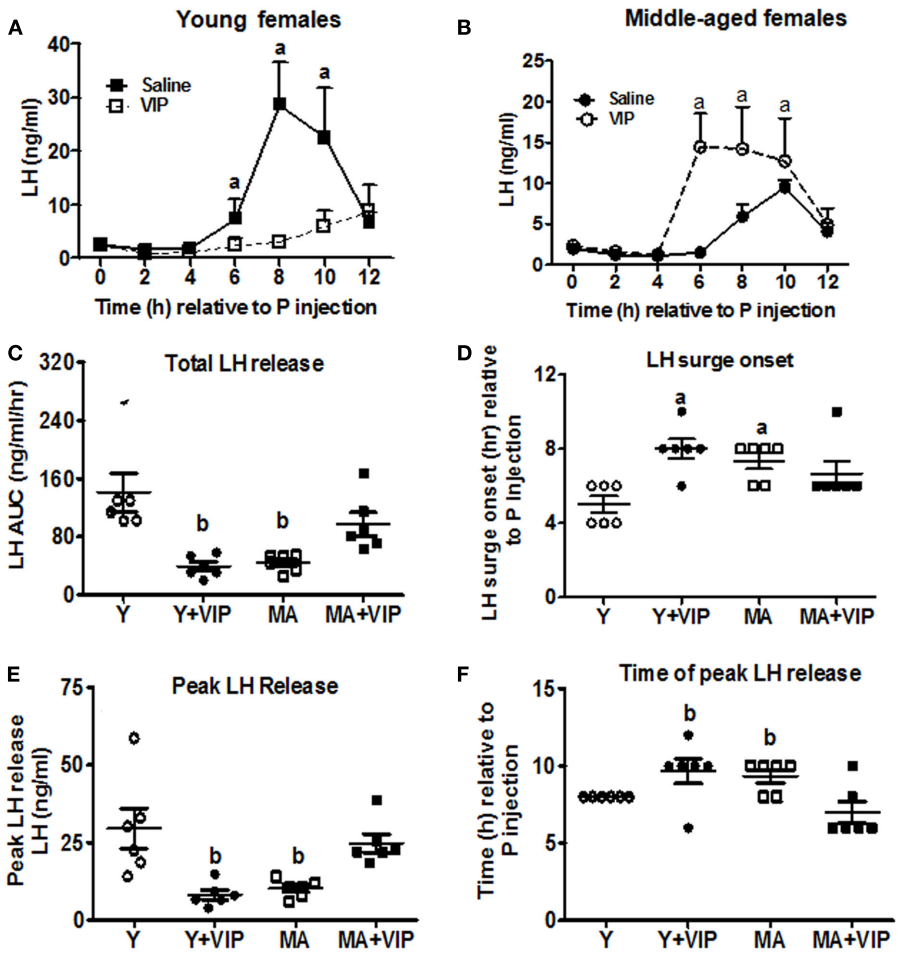

FIGURE 2 | Icv infusion of VIP restores the LH surge in middle-aged females but delays and attenuates the LH surge in young females. Data are expressed as mean ± SEM. OVX young (Y; $n = 6$) and middle-aged (MA; $n = 6$) rats were primed with two daily doses of E (2 μg) and one injection of P (500 μg) on the day of serum collection and infused with saline (control) or VIP (6 nmol) between 1300 and 1600 h. Serial blood sampling started at the time of P injection and continued every 2 h for 12 h. To compare the effect of VIP infusion on LH release in control females, we included the LH surge data for young and middle-aged, saline infused E + P-primed females shown in **Figure 1**. **(A)** LH surge in young females infused icv with saline (○) or VIP (●). **(B)** LH surge in middle-aged females infused icv with saline (□) or VIP (■). **(C)** Scatter plots of total LH release [area under the curve (AUC); ng/ml/h] in young (○, ●) and middle-aged (□, ■) females infused with icv saline (○, □) or VIP (●, ■). **(D)** Scatter plots of LH surge onset in young (○, ●) and middle-aged (□, ■) females infused with icv saline (○, □) or VIP (●, ■). **(E)** Scatter plots of peak LH release in young (○, ●) and middle-aged (□, ■) females infused icv with saline (○, □) or VIP (●, ■). **(F)** Scatter plot of time to peak LH release in young (○, ●) and middle-aged (□, ■) females infused with icv saline (○, □) or VIP (●, ■). [a] $P < 0.05$ vs. saline infused young rats. [b] $P < 0.05$ vs. saline infused young and VIP-infused middle-aged rats.

in the EB + P-primed middle-aged rats express c-fos (**Figure 3I**). Middle-aged rats primed with EB + P and infused with VIP had a threefold increase ($P < 0.01$) in the number of GnRH neurons expressing c-fos (**Figure 3I**). Additionally, icv infusion of VIP significantly increased the total number of c-fos-ir neurons in the POA of middle-aged rats by more than threefold ($P < 0.001$; **Figure 3K**).

VIP INFUSION DECREASES THE PERCENT OF GnRH NEURONS EXPRESSING c-fos AND THE NUMBERS OF GnRH-ir NEURONS IN YOUNG FEMALES

We also determined if VIP infusion modifies the percent of GnRH neurons activated in young females (**Figures 3A–H**). Consistent with our previous work (Sun et al., 2011), approximately 53% of GnRH neurons in young females express c-fos under hormonal conditions that typically induce a robust LH surge (Neal-Perry et al., 2005; **Figure 3I**). Compared to young control females, infusion of VIP significantly reduced the percent of GnRH neurons expressing c-fos by about 50% ($P < 0.05$; **Figure 3I**) to levels that were not significantly different than control middle-aged females (**Figure 3I**). Surprisingly, icv infusion of VIP significantly decreased the number of GnRH-ir neurons ($P < 0.05$; **Figure 3J**). Unlike middle-aged females, icv infusion of VIP in young females did not significantly affect c-fos expression in non-GnRH neurons (**Figure 3K**).

DISCUSSION

These data strongly suggest that a critical level of VIP signaling in the brain is important for an appropriately timed and

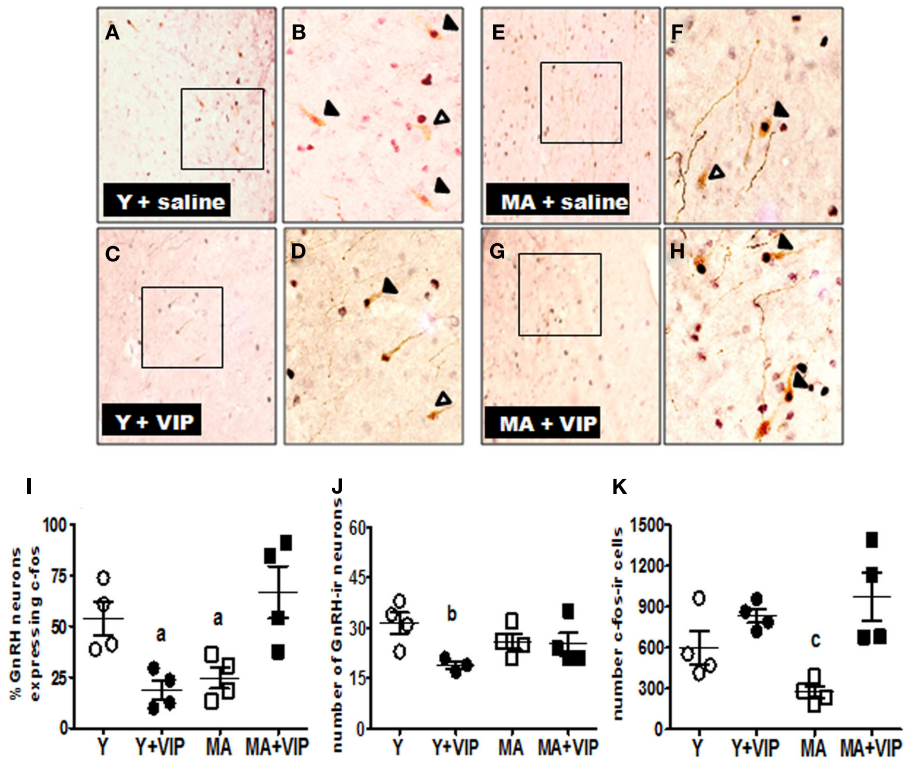

FIGURE 3 | Icv infusion of VIP increased numbers of GnRH/c-fos-ir and c-fos-ir neurons and decreased the number GnRH-ir and GnRH/c-fos-ir in the POA of middle-aged and young females, respectively. Middle-aged (MA; $n = 4$) and young (Y; $n = 4$) OVX rats primed with E (2 μg) and one injection of P (500 μg) on the day of serum collection were infused icv with saline (control) or VIP (6 nmol) between 1300 and 1600 h and then perfused between 1600 and 1730 h. Data are presented as mean ± SEM. Representative sections of double-label immunohistochemistry (20× and 40× magnification) showing GnRH neurons (brown cytoplasm) with c-fos-ir nuclei (black) in rats infused with saline **(A,B,E,F)** or VIP **(C,D,G,H)**. Open arrowhead indicate GnRH neurons. Closed arrowheads indicate GnRH neurons expressing c-fos. Scale bars, 10 μm. **(I)** Scatter plots of the percent of GnRH neurons expressing c-fos-ir in POA of young (○, ●) and middle-aged (□, ■) rats infused with saline (○, □) or VIP (●, ■). **(J)** Scatter plots of the total number of GnRH-ir neurons in the POA of young (○, ●) and middle-aged (□, ■) rats infused with saline (○, □) or VIP (●, ■). **(K)** Scatter plots of the total number of c-fos-ir cells in the POA of young (○, ●) and middle-ages (□, ■) rats infused with saline (○, □) or VIP (●, ■). [a] $P < 0.05$ vs. saline infused young and VIP-infused middle-aged rats. [b] $P < 0.05$ vs. saline infused young females. [c] $P < 0.05$ vs. saline infused young and young and middle-aged females infused with VIP.

robust LH surge in middle-aged rats. We hypothesized that age-related LH surge changes result from reduced release of central VIP rather than reduced synthesis of or responsiveness to the peptide. Consistent with our hypothesis, we found that the delayed and attenuated LH surge observed in regularly cycling middle-aged females is not associated with reduced hypothalamic VIP mRNA expression levels. Moreover, icv infusion of VIP in middle-aged, hormone-primed females rescued total and peak LH and the time of peak LH release. Icv infusion of VIP also increased the number of GnRH and non-GnRH neurons expressing c-fos to levels significantly greater than control middle-aged rats and equivalent to young controls. Icv infusion of VIP did not advance the onset of the LH surge in middle-aged females. Taken together, these findings are consistent with others suggesting that reduced VIP neurosignaling delays and attenuates the LH surge (Krajnak et al., 1998, 2001). They also strongly suggest that age-related changes in the LH surge reflect, in part, reduced release of VIP and not reduced responsiveness of middle-aged females to VIP under estradiol positive feedback conditions.

In contrast to its effects in middle-aged females, icv infusion of VIP does not advance the onset or increase the peak amplitude of the LH surge in young rats. Instead we demonstrate that increased brain VIP levels impose an opposite effect on young females. VIP infusion significantly reduced the percent of GnRH neurons expressing c-fos, delayed the onset of the LH surge and attenuated peak LH release in young females, thus creating a LH surge pattern reminiscent of one typically observed in middle-aged females. These data strongly suggest that, a critical level of VIP signaling is required in females to maintain appropriately timed and high amplitude preovulatory LH surges.

VIP mRNA EXPRESSION IN YOUNG AND MIDDLE-AGED HORMONE-PRIMED FEMALES

Neurons located in the SCN are hypothesized to regulate the circadian signal driving the daily LH surge in rodents (Brown-Grant and Raisman, 1977). GnRH neurons express VIP receptors (Smith et al., 2000), and VIP neurons in the SCN are hypothesized to project to 45% of GnRH neurons and to activate GnRH neurons in an estradiol- and time-dependent fashion (van der

Beek et al., 1993; Van der Beek et al., 1997; Christian and Moenter, 2008). Studies using *in situ* hybridization suggest that reduced expression of VIP mRNA in the SCN correlates with the reduced amplitude and delayed onset of LH surges in middle-aged rats (Harney et al., 1996; Krajnak et al., 1998). Consistent with these experiments the administration of VIP antiserum or VIP antisense oligonucleotide in young reproductive-aged females delays and attenuates the LH surge (Harney et al., 1996; van der Beek et al., 1999). We used quantitative RT-PCR to determine if age and/or hormone treatment significantly affected total VIP expression in anterior hypothalamus (location of the majority of GnRH neurons contacted by VIP terminals) or the posterior hypothalamus (location of the SCN and the majority of the VIP synthesizing neurons in this region). To our surprise we did not find a significant age-related difference in anterior or posterior hypothalamic VIP mRNA expression, thereby suggesting age-related changes in the LH surge do not correlate with reduced hypothalamic VIP mRNA levels in middle-aged females that still have regular estrous cycles. These findings differ from prior studies that report reduced VIP mRNA expression in the hypothalamus of irregularly cycling middle-aged and persistent diestrus old females (Krajnak et al., 1998). The main difference in our study and earlier studies is that we determined hypothalamic VIP mRNA levels in regularly cycling females instead of irregularly cycling (Krajnak et al., 1998) or persistent diestrus old females (Krajnak et al., 2001). Thus, it is possible that females with regular estrous cycles have not yet reached a stage when VIP mRNA synthesis declines. Alternatively, it is possible that changes in VIP mRNA expression are localized to the SCN and our technical approach does not detect fine local changes in SCN (Panda et al., 2002). Thus, it is possible that age-related differences in VIP expression in regularly cycling middle-aged females would be detected with microdissection of the SCN.

VIP PROMOTES A HIGH AMPLITUDE LH SURGE AND ACTIVATION OF GnRH NEURONS IN MIDDLE-AGED FEMALES

Age-related changes in the LH surge correlate with changes in the rhythmic expression of VIP mRNA and reduced VIP mRNA content in the SCN (Krajnak et al., 1998). Additionally, age-related LH surge dysfunction in irregularly cycling middle-aged and persistent diestrus old females is hypothesized to reflect reduced responsiveness to VIP, because reduced numbers of VIP-innervated GnRH neurons express c-fos on the day of the LH surge (Krajnak et al., 1998). Although we did not observe a significant reduction in hypothalamic VIP mRNA levels, it is still possible that age-related changes in the LH surge reflect reduced availability or release of VIP or altered responsiveness to VIP under estrogen positive feedback conditions (Krajnak et al., 2001). To address this question, we infused VIP into the third ventricle of middle-aged hormone-primed females and assessed onset and time of peak LH release, total and peak LH release, the percent of GnRH and non-GnRH neurons expressing c-fos in the POA. Icv infusion of VIP did not significantly advance the onset of the LH surge in middle-aged females. However, VIP significantly increased total and peak LH release and advanced the time of peak LH release in middle-aged females to levels seen in young controls. These *in vivo* experiments strongly support the hypothesis that reduced availability of VIP and not reduced responsiveness to VIP under estradiol positive feedback conditions contributes to the delayed and attenuated LH surge in regularly cycling, middle-aged females. Additionally, because we did not demonstrate an age-related reduction in hypothalamic VIP mRNA levels, reduced availability of VIP most likely reflects reduced VIP release rather than reduced peptide synthesis. This may also explain why significant numbers of GnRH neurons with VIP contacts in middle-aged females fail to express c-fos on the day of the LH surge (Krajnak et al., 2001). Interestingly, although VIP infusion advanced the time of peak LH release, it did not advance LH surge onset. These data suggest that timing of peak LH release and the onset time of the surge may be differentially regulated by VIP or that reduced VIP only partially explains age-related LH surge dysfunction.

Electrophysiological studies suggest that VIP stimulates GnRH neurons (Christian and Moenter, 2008). Consistent with electrophysiological studies, anatomical studies show as much as 60% of GnRH neurons with VIP contacts express c-fos on the day of the LH surge (Krajnak et al., 2001). Thus, we determined if VIP-induced LH release correlates with increased GnRH or non-GnRH neuronal activation in the POA on the day of the LH surge. Icv infusion of VIP increased the number of c-fos-expressing GnRH and non-GnRH neurons, thereby strongly suggesting that age-related LH surge dysfunction involves suboptimal VIP-dependent activation of hypothalamic neurons involved in the induction of the LH surge. Although our studies strongly suggest that VIP affects LH release in middle-aged rats by activating GnRH neurons, it is possible that the peptide increases LH by modulating GnRH release from axon terminals (Samson et al., 1981). Alternatively, because VIP also significantly increased c-fos expression in non-GnRH neurons, icv infusion of VIP may increase LH release in middle-aged rats by indirect activation of GnRH neurons.

The mechanisms resulting in reduced VIP neurotransmission in middle-aged females are unknown. We have demonstrated that female reproductive aging is characterized by reduced glutamatergic and increased GABAergic neurotransmission in the POA (Neal-Perry et al., 2005, 2008). VIP neurons located in the SCN receive glutamatergic afferents from the POA (Kiss et al., 2008). Thus it is possible that reduced availability of VIP peptide results from reduced excitatory afferent input to VIP neurons from glutamatergic neurons located in POA. Several studies have demonstrated that AVP neurosignaling is important for high amplitude LH surges in young reproductive-aged females (Palm et al., 1999; Miller et al., 2006). Our studies do not rule out the possibility that altered AVP neurosignaling also contributes to age-related LH surge dysfunction. Additional studies are required to test these hypotheses.

ADVERSE EFFECTS OF VIP ON ACTIVATION OF GnRH NEURON AND LH RELEASE

Several studies report that continuous VIP infusion in young reproductive-aged females reduces LH pulse frequency and blocks the LH surge (Alexander et al., 1985; Akema et al., 1988; Weick and Stobie, 1995). Consistent with other reports we found VIP infusion significantly decreased LH release in young rats (Weick and Stobie, 1992). VIP infusion also attenuated and delayed peak LH

release and the LH surge onset. Icv infusion of VIP also reduced the number of GnRH neurons co-expressing c-fos from 54 to 19%. To our surprise, VIP also significantly reduced numbers of GnRH-ir neurons. One interpretation of these data is that VIP reduced GnRH–LH release by reducing GnRH peptide expression and activation of GnRH neurons. The cellular mechanism(s) responsible for this surprising finding is (are) unclear. One other study reported a similar finding in seasonal breeding juncos in which the number of VIP-ir neurons inversely correlated with the number of GnRH-ir neurons; photosensitive (breeding) juncos have a low ratio of VIP-to-GnRH-ir neurons, and photorefractory (non-breeding) juncos have a high ratio of VIP-to-GnRH-ir neurons (Saldanha et al., 1994).

CONCLUSION

Previous studies in irregularly cycling middle-aged and persistent diestrus females suggest that delayed and attenuated LH surges might result from reduced hypothalamic VIP levels and responsiveness to VIP. This study strongly supports the hypothesis that age-related LH surge dysfunction in regularly cycling middle-aged females most likely reflects reduced VIP release and not abnormal responsiveness to VIP. Considerable evidence suggests age-related changes in the timing and magnitude of the LH surge in regularly cycling middle-aged females is associated with the failure of ovarian steroids to regulate a number of neurotransmitter systems involved in GnRH neuron activation. The cellular mechanism(s) that result in reduced VIP release may reflect age-related reduced responsiveness to estradiol of VIP containing neurons in the SCN. In contrast with findings in middle-aged rats, infusion of VIP in young females attenuates and delays the LH surge, and this is associated with reduced activation and reduced numbers of GnRH-ir neurons. These studies suggest that a critical level of VIP signaling is required for the maintenance of an appropriately timed and robust LH surge.

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Circadian control of neuroendocrine circuits regulating female reproductive function

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Female reproduction requires the precise temporal organization of interacting, estradiol-sensitive neural circuits that converge to optimally drive hypothalamo-pituitary–gonadal (HPG) axis functioning. In mammals, the master circadian pacemaker in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus coordinates reproductively relevant neuroendocrine events necessary to maximize reproductive success. Likewise, in species where periods of fertility are brief, circadian oversight of reproductive function ensures that estradiol-dependent increases in sexual motivation coincide with ovulation. Across species, including humans, disruptions to circadian timing (e.g., through rotating shift work, night shift work, poor sleep hygiene) lead to pronounced deficits in ovulation and fecundity. Despite the well-established roles for the circadian system in female reproductive functioning, the specific neural circuits and neurochemical mediators underlying these interactions are not fully understood. Most work to date has focused on the direct and indirect communication from the SCN to the gonadotropin-releasing hormone (GnRH) system in control of the preovulatory luteinizing hormone (LH) surge. However, the same clock genes underlying circadian rhythms at the cellular level in SCN cells are also common to target cell populations of the SCN, including the GnRH neuronal network. Exploring the means by which the master clock synergizes with subordinate clocks in GnRH cells and its upstream modulatory systems represents an exciting opportunity to further understand the role of endogenous timing systems in female reproduction. Herein we provide an overview of the state of knowledge regarding interactions between the circadian timing system and estradiol-sensitive neural circuits driving GnRH secretion and the preovulatory LH surge.

Keywords: suprachiasmatic, ovulation, kisspeptin, GnIH, RFRP-3, AVP, VIP

INTRODUCTION

The neural mechanisms regulating ovulation are under circadian control in many species, ensuring that the timing of greatest fertility coincides the period of maximal sexual motivation (Nequin et al., 1975; Sarkar et al., 1976). Superimposed upon this circadian control is a dependence of the reproductive cycle on estradiol to ensure proper maturation of the oocyte at the time of ovulation. The precise timing in the initiation of ovulation by a central, 24 h clock was first identified in a classic study by Everett and Sawyer (1950) who described the "neurogenic activation of the hypophysis" as a necessary component of ovulation. In this seminal paper, Everett and Sawyer reported that injections of barbiturate, within a 2-h time window, were capable of delaying ovulation in female rats by 24 h. Administration outside of this critical time window failed to prevent ovulation. Because the impact of the barbiturate injections was short-lived, whereas ovulation was delayed precisely 1 day, the authors postulated that a daily, neural signal triggers ovulation.

In mammals, a master pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus orchestrates circadian timing (Moore and Eichler, 1972; Stephan and Zucker, 1972). Circadian rhythms are endogenously generated (Lehman et al., 1987; Ralph et al., 1990) and synchronized to the external environment via direct neural projections from intrinsically photosensitive retinal ganglion cells to the circadian clock in the SCN (Berson et al., 2002; Hattar et al., 2002; Panda et al., 2002; Provencio et al., 2002; Ruby et al., 2002; Morin and Allen, 2006). The SCN communicates to neuroendocrine cells driving reproductive function through extensive direct and indirect neural projections (Boden and Kennaway, 2006; de la Iglesia and Schwartz, 2006; Kriegsfeld and Silver, 2006; Christian and Moenter, 2010). More recently, it has become evident that the gonadotropin-releasing hormone (GnRH) system does not respond passively to SCN signaling, but contains the same circadian clock "machinery" necessary to generate autonomous cellular oscillations, and these subordinate clocks likely mediate daily changes in sensitivity to SCN communication (Chappell et al., 2003; Gillespie et al., 2003; Resuehr et al., 2007; Zhao and Kriegsfeld, 2009; Tonsfeldt et al., 2011; Williams et al., 2011). Disruptions to SCN output signaling pathways or intrinsic activity of the cellular clockwork lead to gross deficits in female rodent ovulatory function and fecundity (Nunez and Stephan, 1977; Gray et al., 1978; Wiegand and Terasawa, 1982; Miller et al., 2004). The following sections will summarize the present understanding of interactions between the circadian system and estradiol responsive circuits required for mammalian female reproductive functioning and success.

CIRCADIAN CONTROL OF THE PREOVULATORY LH SURGE

Throughout the ovulatory cycle, concentrations of sex steroids and consequent stimulation of the developing ovarian follicle are controlled by a neuroendocrine cascade beginning with the secretion of hypothalamic GnRH into the hypophyseal portal system. In turn, GnRH acts on the anterior pituitary to stimulate the synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH then act on the gonads to regulate steroidogenesis and gametogenesis, respectively. The activity of the reproductive axis is controlled through the action of negative feedback effects of sex steroids, with this mechanism maintaining LH at low concentrations throughout most of the ovulatory cycle. In many spontaneously ovulating species, the preovulatory LH is initiated by the SCN on the day of proestrus, at a time closely preceding activity onset (Legan and Karsch, 1975; Mahoney et al., 2004; Chappell, 2005; Gibson et al., 2008). Paradoxically, high concentrations of estradiol are required for the SCN to trigger ovulation (i.e., positive feedback; Legan and Karsch, 1975; de la Iglesia and Schwartz, 2006; Kriegsfeld and Silver, 2006; Christian and Moenter, 2010). The site(s) of integration for positive and negative feedback effects of estradiol with the circadian timing system are complex and not fully understood. Moreover, it is unclear whether a single system participates in both negative and positive feedback, whether two independent systems differentially dominate throughout the cycle, or a combination of these two mechanisms of control determine the onset of the preovulatory GnRH surge.

To explore the role of the circadian system in female reproductive behavior, Syrian hamsters (*Mesocricetus auratus*) are frequently employed as a model system due to the exquisite precision in their circadian rhythms and reproductive behavior. When held in a light:dark (LD) cycle, for example, ovulation and the onset of behavioral receptivity occur precisely every 96 h in this species (Alleva et al., 1971). This rhythm in reproductive activity is endogenously generated and persists in constant conditions with a period four times their free-running circadian period. This fact was confirmed by investigating heat onset (i.e., propensity to display lordosis, the gonadotropin surge and vaginal cytology) in individual hamsters housed in constant darkness (DD) prior to and after administration of deuterium oxide (a treatment that result in a lengthening of the free-running period). As shifts in heat onset mirrored the free-running rhythm in locomotor activity, it became clear that the ovulatory cycle and locomotor rhythms are governed by a similar endogenous timing system (Fitzgerald and Zucker, 1976). Because estrous and activity onset were coupled temporally, it was suggested that the LH surge and locomotor activity are controlled by a single, endogenous oscillator, or a coupled, multioscillator system that regulates the rhythms of each process independently (Fitzgerald and Zucker, 1976). The former hypothesis postulated that the reproductive axis "tracks" four circadian cycles and ovulation occurs after the count is complete.

Converging lines of evidence over the next three decades established that both of these hypotheses are partially correct. We now know that the SCN provides a daily, stimulatory signal to the reproductive axis each day of the estrous cycle, closely preceding the active phase, in most spontaneously ovulating rodents (Legan and Karsch, 1975; Kriegsfeld and Silver, 2006), indicating that a single clock subserves both processes. However, this signal is only effective at stimulating the GnRH system to produce the LH surge in the presence of estradiol concentrations above a critical threshold. Prior to the day of proestrus, the developing ovarian follicles secrete insufficient estradiol to fulfill these criteria. The nature of the daily stimulatory signal from the SCN can be unmasked by implanting animals with estradiol capsules that result in proestrus concentrations of this hormone; in this case, daily LH surges occur (Legan and Karsch, 1975; Legan et al., 1975; Christian et al., 2005). Regarding the second hypothesis suggesting a multioscillator organization, although distinct clocks do not underlie locomotor rhythms and estrus, a hierarchical clock structure exists in which the SCN acts as the master pacemaker coordinating rhythmicity in subordinate oscillator systems of the reproductive axis, an arrangement discussed further below.

CIRCADIAN NEUROCHEMICAL COMMUNICATION

Whereas the same circadian pacemaker regulates locomotor and estrus onset, the communication modalities mediating each process likely differ. Transplants of fetal SCN tissue into bilaterally SCN-lesioned hamsters restores locomotor, but not endocrine, rhythms in the absence of neural outgrowth, suggesting that intact neural connections are required for endocrine rhythmicity whereas behavioral rhythms can be supported by a diffusible signal (Silver et al., 1990, 1996; Meyer-Bernstein et al., 1999). Confirmation of the importance of neural SCN communication in initiation of the GnRH/LH surge comes from studies using hamsters with "split" activity rhythms. When housed in constant light, some hamsters exhibit a splitting in behavior, with two daily activity bouts separated by 12 h, each reflecting an antiphase oscillation of the left and right sides of the bilateral SCN (de la Iglesia et al., 2000; Tavakoli-Nezhad and Schwartz, 2005; Yan et al., 2005). Under these circumstances, ovariectomized (OVX) hamsters treated with estradiol exhibited two LH surges in a 24-h period, each phase-locked to an individual activity bout (Swann and Turek, 1985).

The SCN sends pronounced, monosynaptic projections throughout the brain, including hypothalamic cell phenotypes driving reproductive function, principally ipsilaterally (DeVries et al., 1985; Watts and Swanson, 1987; van der Beek et al., 1997; Kriegsfeld et al., 2004). As a result, if a neural output signal from the SCN initiates the GnRH/LH surge, then one hemispheric set of GnRH neurons should be activated, ipsilateral to the activated SCN, with each locomotor activity bout. Conversely, if controlled by a diffusible signal, then the GnRH system should be activated concurrently on both sides of the brain, twice daily, 12 h apart. In a clever study by de la Iglesia et al. (2000) they found that each locomotor bout and LH surge is associated with unilateral activation of the GnRH system, ipsilateral to the activated SCN, confirming the importance of neural SCN communication to the GnRH system in ovulatory control. Both the GnRH system and several neurochemical systems upstream of the GnRH network receive SCN input and synergistically operate to precisely control the neuroendocrine events necessary to appropriately time ovulation and sexual motivation.

VASOACTIVE INTESTINAL POLYPEPTIDE

Neurons synthesizing vasoactive intestinal polypeptide (VIP) are located in the retinorecipient, ventrolateral SCN "core" (Ibata et al., 1989; Tanaka et al., 1993; Moore et al., 2002), and represent potential output signals conveying circadian information to target effector systems. VIPergic SCN neurons project monsynaptically to GnRH neurons (van der Beek et al., 1997; Horvath et al., 1998) that express the VIP receptor $VPAC_2$ (Smith et al., 2000). Several lines of evidence indicate an important role for this pathway in the timing of ovulation. First, GnRH neurons receiving VIPergic input preferentially express the neural activation marker, FOS, during the afternoon of the LH surge on the day of proestrus (van der Beek et al., 1994). Additionally, *in vivo* antisense antagonism of VIP production in the SCN abolishes GnRH/FOS activation in OVX + E2 primed female rats, suggesting the necessity of VIP output in triggering the afternoon GnRH surge (Harney et al., 1996; Gerhold et al., 2005). Furthermore, blocking the $VPAC_2$ receptor attenuates GnRH neuronal cell firing during the afternoon surge in female, estradiol-treated mice (Christian and Moenter, 2008). The expression of VIP afferents on GnRH neurons is sexually dimorphic, with female rats exhibiting higher VIPergic innervation (Horvath et al., 1998), suggestive of a specific role for VIP in estrous cycle regulation. From a developmental standpoint, the number of VIP-GnRH contacts increases between prepubertal and adult female rats (Kriegsfeld et al., 2002), and VIP-innervated GnRH neurons exhibit lower activation levels in middle-aged female rats, suggesting that this SCN peptide may be partially responsible for the initiation of reproductive senescence in female rodents (Krajnak et al., 2001). Together, these lines of evidence suggest that direct VIP projections from the SCN to the GnRH system positively drive the GnRH/LH surge.

Gonadotropin-releasing hormone neurons do not express estrogen receptor α (ERα), the estrogen receptor subtype mediating the positive feedback effects of estradiol (Herbison and Theodosis, 1992b; Dorling et al., 2003; Wintermantel et al., 2006), pointing to additional neurochemicals and neural loci at which stimulatory circadian and estrogenic signals converge. As described below, the positive feedback effects of estradiol necessary for surge generation occur at intermediate target nuclei that express abundant ERα. Several lines of evidence point to the anteroventral periventricular nucleus (AVPV) as an important site of circadian and estrogenic convergence necessary for initiating the GnRH/LH surge. The AVPV sends monosynaptic projections to GnRH cells, neurons in this region express FOS coincident with the LH surge, and lesions of the AVPV eliminate estrous cyclicity in both intact and ovariectomized, estradiol-treated rats (Wiegand et al., 1980; Wiegand and Terasawa, 1982; Ronnekleiv and Kelly, 1988; Gu and Simerly, 1997; Le et al., 1999). Moreover, the SCN sends pronounced monosynaptic projections to cells in the AVPV that express ERα (Herbison and Theodosis, 1992a,b; de la Iglesia et al., 1995; Watson et al., 1995; Shughrue et al., 1997). A multitude of neuropeptides and neurotransmitters are expressed within this relatively small nucleus, many of which express ERα, including glutamate, GABA, galanin, dynorphin, enkephalin, substance P, neurotensin, and kisspeptin and collectively represent potential sites for this convergence (reviewed in Herbison, 2008), with kisspeptin being the most well studied.

In addition to estrogen signaling in the AVPV, the SCN expresses estrogen receptors, providing the potential for direct actions on the master circadian pacemaker. In mice, a small proportion SCN cells express ERα, with a larger proportion expressing ERβ (Vida et al., 2008). In young female rats, ERβ mRNA exhibits a diurnal rhythm in the SCN that is dependent on estradiol concentrations, suggesting that the impact of estrogen on the SCN may be gated through time- and estradiol-dependent receptor turnover (Wilson et al., 2002; Shima et al., 2003). In human SCN, both ERα and ERβ are expressed (Kruijver and Swaab, 2002). In addition to direct actions on the SCN, estrogen may act indirectly to regulate circadian functioning; ERα-expressing cells in the preoptic area project to the SCN in female Syrian hamsters, providing an additional means of estrogen and circadian integration (de la Iglesia et al., 1999). The specific role of both direct and indirect estrogenic signaling to SCN in the preovulatory LH surge remains unspecified and represents an exciting opportunity for further investigation.

VASOPRESSIN

Vasopressinergic (AVPergic) cells in the dorsomedial SCN target ERα – expressing cells in the AVPV (Hoorneman and Buijs, 1982; DeVries et al., 1985; de la Iglesia et al., 1995; Watson et al., 1995), and AVP injections produce surge-like LH levels in SCN-lesioned, ovariectomized (OVX), estradiol-treated rats (Palm et al., 1999). Likewise, cells in this brain region express the vasopressin receptor, $V1_a$ (Ostrowski et al., 1994; Funabashi et al., 2000a). The convergence of circadian output signals and estradiol feedback within the AVPV has generated much interest in this nucleus as the integration site of these dual signals in the regulation of the GnRH surge. Indeed, anti-estrogens targeting the AVPV inhibit the LH surge in ovariectomized, estradiol-treated rats (Petersen and Barraclough, 1989).

Vasopressin gene transcription in the SCN is directly controlled by the molecular clockwork at the cellular level (Grace et al., 1999; Munoz et al., 2002) and is released in a circadian manner (Shinohara et al., 1994), with a peak coinciding with the onset of the LH surge (Schwartz et al., 1983; Kalsbeek et al., 1995). AVP, but not VIP, release is synchronous with GnRH secretion in co-cultures of medial preoptic area (mPOA) and SCN brains slices (Funabashi et al., 2000b), suggesting vasopressin may act as a circadian stimulator of GnRH. Furthermore, AVP injections directed at the mPOA produce surge-like LH levels in SCN-lesioned, ovariectomized, estradiol-treated rats (Palm et al., 1999). By contrast, central AVP receptor antagonists attenuate the LH surge in proestrus rats (Funabashi et al., 1999). Finally, the inability of *clock* mutant mice to generate an LH surge is associated with diminished *AVP* mRNA expression in the SCN, a phenotype that can be restored via central injections of AVP, further linking this peptide to the circadian control of ovulation (Miller et al., 2004). Importantly, central injections of AVP are only capable of inducing a surge-like pattern of GnRH secretion during a narrow time window in the afternoon (Palm et al., 2001b), suggesting additional temporal control at SCN target loci, a concept described further below.

RFAMIDE PEPTIDES: KISSPEPTIN AND GnIH

Despite the established role of the circadian clock and estradiol in the regulation of GnRH secretion and, ultimately, ovulation, the

specific targets of these requisite signals are less well understood. As described previously, the AVPV represents an important integration site for the positive feedback effects of estrogen and circadian signaling. Conversely, the neurochemical substrates and target loci at which circadian and estrogenic signals converge to maintain LH at low concentrations throughout the majority of the estrous cycle (i.e., negative feedback), and the mechanisms that suppress negative feedback at the time of the LH surge, remained unspecified. Converging lines of evidence from a number of reports point to two potent, reproductively relevant neuropeptides in the RFamide (Arg-Phe-NH_2) family, gonadotropin-inhibitory hormone (also known as RFamide-related peptide-3) and kisspeptin, in mediating circadian controlled estradiol negative and positive feedback, respectively (reviewed in Khan and Kauffman, 2011).

Kisspeptin

The discovery of kisspeptin has markedly impacted the field of reproductive biology since its initial isolation in human placenta and its recognition as the endogenous ligand for the orphan G-protein coupled receptor, GPR54 (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). Kisspeptin was initially characterized as a tumor metastasis suppressor sequence and originally named metastin (Lee et al., 1996). The Hershey, Pennsylvania group credited for this discovery shortened the name of the gene encoding the metastin peptide to *KiSS1*, reflecting its suppressor sequence "SS," while "Ki" was added to pay homage to an earlier discovery in the same town, the Hershey's Kiss (Lee et al., 1996). *KiSS1*-GPR54 signaling was discovered to play a critical role in reproductive physiology in 2003 when two groups reported that mutation of the *GPR54* receptor results in idiopathic hypophysiotropic hypogonadism in humans with an identical phenotype observed in mice with a targeted deletion in this receptor (de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003). Since these initial discoveries, numerous contributions by researchers working at varying levels of analysis indicate that kisspeptins are critical regulators of sexual differentiation and maturation as well as normal, adult reproductive functioning across mammalian species, including humans (de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003; Keen et al., 2008; Kauffman, 2009; Roseweir et al., 2009; Lehman et al., 2010a,b; Pineda et al., 2010a,b; Luque et al., 2011; Mayer and Boehm, 2011).

The *KiSS1* gene encodes a family of neuropeptides, kisspeptins, that act as pronounced stimulatory regulators of the GnRH system. The initial product is a 145 amino acid propeptide, kisspeptin-145, that is cleaved into the active 54 amino acid peptide, kisspeptin-54. Three shorter products, kisspeptin 10, 13, and 14 result from further cleavage of the 54 amino acid sequence; all are biologically active, bind with similar affinity to GPR54, and share an RFamide sequence on their C termini (Kotani et al., 2001; Muir et al., 2001). In rodents, *KiSS1* mRNA expressing cells are localized to the AVPV and arcuate (ARC) nuclei, with populations exhibiting a high percentage of ERα-expressing cells (Gottsch et al., 2004; Smith et al., 2005b; Clarkson and Herbison, 2006; Revel et al., 2006; Greives et al., 2007). The effects of estradiol on kisspeptin activity, however, varies by nucleus, with ovariectomy decreasing *KiSS1* mRNA in the AVPV and increasing *KiSS1* expression in the ARC, pointing to a role for kisspeptin in estradiol positive and negative feedback, respectively (Smith et al., 2005a,b). Analogous findings are seen in males, with castration increasing *KISS1* expression in the ARC and decreasing expression in the AVPV (Smith et al., 2005a,b; Kauffman, 2010). These effects are likely regulated both through direct actions of testosterone/dihydrotestosterone on androgen-responsive kisspeptin neurons and through aromatization of testosterone to estradiol, as kisspeptin neurons express both receptors and respond predictably to both sex steroids (Smith et al., 2005b). Exogenous kisspeptin administration potently induces LH release as well as upregulates FOS expression in GnRH neurons (Gottsch et al., 2004; Irwig et al., 2004; Matsui et al., 2004; Navarro et al., 2005a,b). In addition to direct actions of AVPV kisspeptin neurons on GnRH cells (Irwig et al., 2004; Han et al., 2005; Kinoshita et al., 2005; Clarkson and Herbison, 2006; Smith et al., 2008), there is some evidence that ARC kisspeptin cells may regulate the release of GnRH via direct actions on GnRH terminals (d'Anglemont de Tassigny et al., 2008). In one recent study, genetic ablation of kisspeptin cells or cells expressing GPR54 throughout development did not impact female pubertal development and the animals were fertile, although loss of GPR54-expressing cells resulted in blunted LH, reduced ovarian weights, and irregular estrous cycling (Mayer and Boehm, 2011). In adult animals, acute ablation of kisspeptin neurons markedly disrupted fertility and estrous cyclicity, whereas removal of ∼93% of GPR54-expressing GnRH cells resulted in more mild reductions in LH, fertility, and estrous cycling (Mayer and Boehm, 2011). Together, these findings suggest that kisspeptin signaling is required for adult female reproductive functioning and compensatory mechanisms can overcome the necessity for kisspeptin when this gene is inactivated throughout development. Given that as few as three GnRH neurons are sufficient to support activity of the hypothalamo-pituitary–gonadal (HPG) axis (Silverman et al., 1985; Gibson et al., 1988), it is unclear whether GPR54-expressing GnRH neurons can be dispensed within LH surge control.

As mentioned previously, the observation across rodent species that the SCN projects to the AVPV and this brain region is essential for production of the LH surge, combined with the knowledge that AVPV kisspeptin cells respond positively to estradiol, made these cells an attractive target of exploration in the initiation of GnRH/LH surge. *KiSS1* cells in the AVPV express FOS at the time of the LH surge in naturally cycling, ovariectomized, estradiol-treated rats (Smith et al., 2006; Adachi et al., 2007). This circadian pattern of expression persists in constant darkness (Robertson et al., 2009), suggesting endogenous circadian regulation of this cell population rather than reliance on external temporal cues. In mice, this daily pattern of *KiSS1* expression, and *KiSS1* cells expressing *c-fos*, is abolished by ovariectomy, and reinstated following steady-state estradiol replacement (Robertson et al., 2009), pointing to a permissive role for estradiol in the circadian control of *KiSS1* in this species.

These results point to either an endogenous, self-sustained rhythm in kisspeptin neurons, circadian control through upstream projections from the SCN, or a combination of both mechanisms of control. We examined both of these possibilities using Syrian hamsters. Consistent with findings in mice, FOS expression in kisspeptin immunoreactive (ir) cells expressed a daily rhythm in ovariectomized, estradiol-treated hamster, with peak

co-expression concomitant with the timing of the LH surge. In contrast to results observed in mice, ovariectomy results in a blunted rhythm of kisspeptin-FOS co-expression, but not its abolition (Williams et al., 2011). These latter findings point to potential species differences in the role estrogen plays in AVPV kisspeptin regulation and/or post-transcriptional modification of the *KiSS1* gene, resulting in differences in cells visualized with mRNA versus protein analyses. To determine whether the SCN projects to kisspeptin cells to mediate these observed rhythms, we examined projections from VIPergic and AVPergic SCN cells, given the role of these neuropeptides in positively driving the LH surge. We found that AVPergic SCN cells project directly to a majority of kisspeptin-ir cells, whereas VIPergic SCN cells did not (**Figure 1**). These results are consistent with previous findings in mice (with synapses confirmed at the electron microscopy level), with estrogen increasing the percent of GnRH cells with AVPergic terminal appositions (Vida et al., 2010).

Previous findings indicate that that administration of AVP can only induce the preovulatory LH surge within a narrow time window in rats (Palm et al., 2001b), suggesting a gated mechanism of control at SCN target loci. The gating of SCN information flow may be controlled within kisspeptin cells in the AVPV, at the level of GnRH neurons, or a combination of both mechanisms. To select among these possibilities, we examined whether: (1) kisspeptin cells within the AVPV respond in a time-dependent manner to AVP stimulation, and (2) if GnRH neurons display time-dependent sensitivity to kisspeptin signaling. If time-dependent sensitivity is controlled at the level of the AVPV, then one would expect kisspeptin cells to exhibit daily changes in sensitivity to AVP stimulation and contain an endogenous time-keeping mechanism. Alternatively, if the gating of control occurs within GnRH cells, then one would expect the GnRH system to display daily sensitivity in response to both AVP and kisspeptin administration. Our findings indicate that the kisspeptin system responds indiscriminately to AVP administration, regardless of time of day, whereas the GnRH system is only sensitive to kisspeptin stimulation at the time that the surge would normally occur (Williams et al., 2011). These results further support the notion that kisspeptin cells do not keep circadian time but, instead, their activity is driven by AVPergic SCN cells. However, these findings point to an important role for autonomous circadian oscillators in GnRH cells underlying time-dependent sensitivity to upstream signaling. Alternatively, it is possible that the master clock in the SCN communicates timing information to GnRH cells that do not maintain the capacity for endogenous rhythmicity. Given that SCN-derived VIPergic cells project monosynaptically to GnRH neurons, this cell phenotype represents an ideal candidate to communicate such timing information. Finally, a combination of both mechanisms may underlie such daily changes in GnRH cell sensitivity, with VIPergic SCN

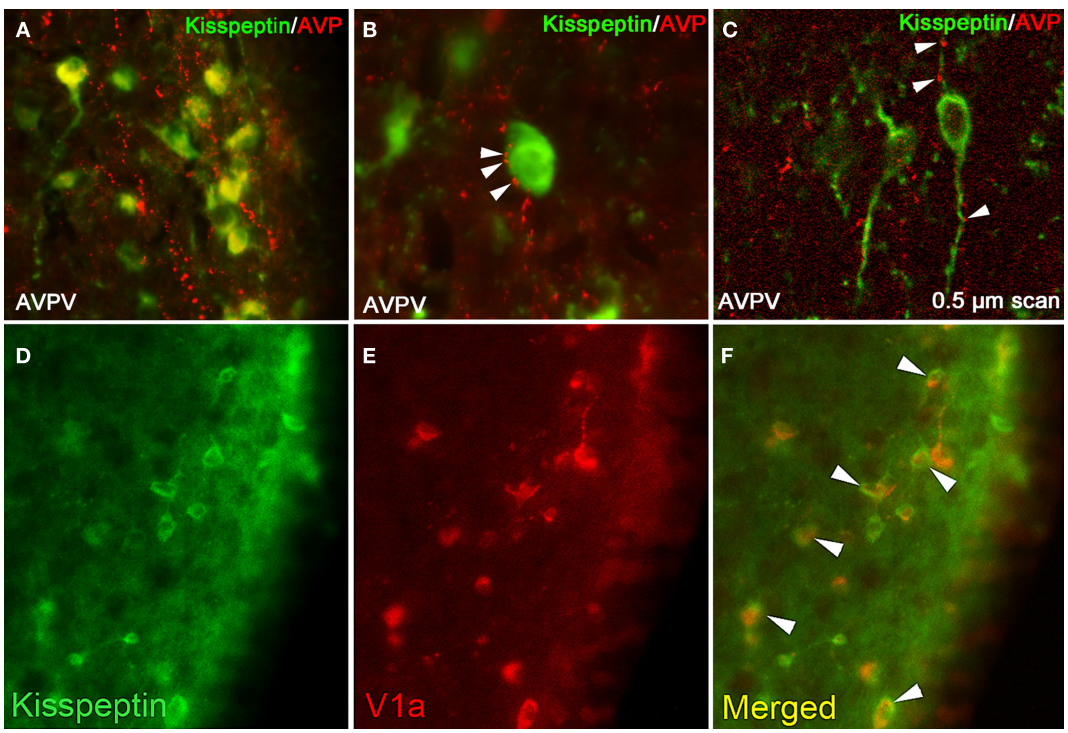

FIGURE 1 | Kisspeptin-ir cells in the hamster AVPV receive SCN-derived fiber contacts expressing AVP-ir. (A) Low-power photomicrographs of AVP-ir in the AVPV, in which kisspeptin cell bodies receive extensive AVP-ir fiber contacts. **(B)** High power photomicrograph showing several presumptive AVP-ir terminal boutons on a kisspeptin-ir cell body at the light level. **(C)** Confocal image (0.5 µm scan taken at ×400) confirming AVP-ir contacts upon kisspeptin-ir cell body and processes. In **(B,C)**, Arrows are indicative of close contacts. **(D–F)** Kisspeptin cells in the AVPV express the V1a receptor. **(D)** Low-power photomicrographs of kisspeptin-ir cells in the AVPV, **(E)** V1a-ir cells in the AVPV, and **(F)** the merged image showing overlap between kisspeptin-ir and V1a-ir. Modified from Williams et al. (2011).

communication synchronizing independent GnRH cellular oscillators to coordinate the timing in their responsiveness to upstream signaling.

At the cellular level, circadian rhythms are generated by 24-h autoregulatory transcriptional/translational feedback loops consisting of "clock" genes and their protein products (**Figure 2**; Reppert and Weaver, 2002; Maywood et al., 2007; Chen et al., 2009; Mohawk and Takahashi, 2011). In mammals, the feedback loop begins in the cell nucleus where CLOCK and BMAL1 proteins heterodimerize and drive the transcription of the Period (*Per1*, *Per2*, *and Per3*) and Cryptochrome (*Cry1* and *Cry2*) genes by binding to the E-box (CACGTG) domain on their gene promoters. Once translated, PER and CRY proteins build in the cytoplasm of the cell over the course of the day, and inevitably form hetero- and homodimers that feed back to the cell nucleus to inhibit CLOCK:BMAL1 mediated transcription. The timing of nuclear entry is balanced by regulatory kinases that phosphorylate the PER and CRY proteins, leading to their degradation (Lowrey et al., 2000; Wang et al., 2007; Mohawk and Takahashi, 2011). Two other promoter elements, DBP/E4BP4 binding elements (D boxes) and REV-ERBα/ROR binding elements (RREs; Ueda et al., 2005), also participate in cellular clock function. REV-ERBα, an orphan nuclear receptor, negatively regulates the activity of the CLOCK:BMAL1. The same mechanism controlling *Per* and *Cry* gene transcription also controls transcription of REV-ERBα. Similarly, the transcription factor DPB is positively regulated by the CLOCK:BMAL1 complex (Ripperger and Schibler, 2006) and acts as an important output mechanism, driving rhythmic transcription of other output genes via a PAR basic leucine zipper (PAR bZIP; Lavery et al., 1999).

Clock genes are expressed in the SCN, but also widely distributed throughout other brain regions and peripheral tissues. GnRH cells express the same clock genes that drive circadian rhythms at the cellular level in the SCN, both *in vitro* (Chappell et al., 2003; Zhao and Kriegsfeld, 2009) and *in vivo* (Olcese et al., 2003; Hickok and Tischkau, 2010; Matagne et al., 2012). Importantly, mice bearing a mutation in the essential circadian clock gene, *Clock*, display abnormal estrous cycles and abnormal LH surge induction in response AVP administration (Miller et al., 2004), suggesting an important role for clock genes (potentially in GnRH cells) in normal estrous cycling and ovulation. Consistent with our findings *in vivo*, immortalized GnRH neurons (i.e., GT1-7 cells) exhibit ∼24 h changes in sensitivity to kisspeptin and VIP signaling (Zhao and Kriegsfeld, 2009). These findings suggest that an endogenous timing mechanism in GnRH cells gates daily changes in responsiveness to upstream, stimulatory neurochemicals. More recently, Chappell and colleagues found that estrogen-treated GT1-7 cells exhibit a rhythm in *GPR54* expression that is abolished in the absence of estrogen (Tonsfeldt et al., 2011). These results are intriguing given the absence of ERα in this cell population, suggesting participation of ERβ in this rhythm of *GRP54* transcription. Whether GnRH neurons gate responsiveness to all upstream signals, only stimulatory input, or only those mediators regulating the LH surge remains an exciting opportunity for further inquiry. Likewise, given that all functional studies have been conducted *in vitro*, these findings should be interpreted cautiously as the immortalization process may lead to alterations in GnRH cellular functioning different from those *in vivo*.

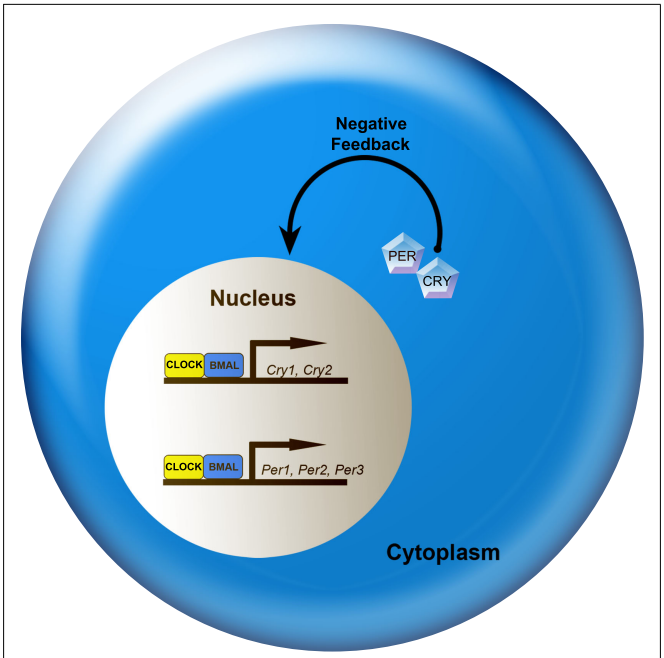

FIGURE 2 | A simplified model of the intracellular mechanisms responsible for mammalian circadian rhythm generation. The process begins when CLOCK and BMAL1 proteins dimerize to drive the transcription of the Per (Per1, Per2, and Per3) and Cry (Cry1 and Cry2) genes. In turn, Per and Cry are translocated to the cytoplasm and translated into their respective proteins. Throughout the day, PER and CRY proteins rise within the cell cytoplasm. When levels of PER and CRY reach a threshold, they form heterodimers, feed back to the cell nucleus and negatively regulate CLOCK:BMAL1 mediated transcription of their own genes. This feedback loop takes ∼24 h, thereby leading to an intracellular circadian rhythm. See text for additional details.

Gonadotropin-inhibitory hormone

Tsutsui et al. (2000) isolated a dodecapeptide, SIKPSAYLPLR-Famide, from Japanese quail brain. Neurons for this neuropeptide were concentrated in the paraventricular nucleus of the hypothalamus, with projections to the hypophyseal portal system, suggesting a role in anterior pituitary regulation. Using cultured quail pituitaries, Tsutsui and colleagues discovered a dose-dependent inhibition of gonadotropin release following administration of this novel peptide. Because this peptide directly inhibited gonadotropin release, they named it gonadotropin-inhibitory hormone, or GnIH (Tsutsui et al., 2000). Following this initial discovery, we examined the functional significance of GnIH in mammals, establishing this neuropeptide as robust inhibitor of the reproductive axis in Syrian hamsters, with identical expression patterns in mice and rats (Kriegsfeld et al., 2006). Since this initial characterization, it has become clear that GnIH acts as a pronounced negative regulator of HPG axis activity in all species investigated, including hamsters, mice, rats, cattle, sheep, non-human primates, and humans (reviewed in Bentley et al., 2010; Kriegsfeld et al., 2010; Smith and Clarke, 2010; Tsutsui et al., 2010).

In rats, hamsters and mice, GnIH neuronal cell bodies are tightly localized to the dorsomedial hypothalamus (DMH) and project directly to the POA, forming close appositions to GnRH cells (Kriegsfeld et al., 2006; Johnson et al., 2007), suggesting the potential for direct, neural regulation of the GnRH network. Administration of GnIH rapidly suppresses LH release in all mammalian species investigated to date (reviewed in Bentley et al., 2010; Kriegsfeld et al., 2010; Tsutsui et al., 2010). Direct application of GnIH to GnRH cells in brains slices from male and female mice decreased neural activity in a subset of cells (Ducret et al., 2009), supporting a suppressive role for this peptide via direct actions on GnRH neurons. Furthermore, electrophysiological recordings suggest a direct postsynaptic inhibition of GnRH cell firing may occur via GnIH-mediated hyperpolarization of potassium (K+) channels in vGluT2-GnRH neurons (Wu et al., 2009).

In addition to the direct action on GnRH, the actions of GnIH on pituitary gonadotropes remain more controversial. Suggestive evidence for this possibility comes from studies showing that the GnIH receptor (GPR147; Yin et al., 2005) is localized to rat and Syrian hamster pituitaries (Hinuma et al., 2000; Gibson et al., 2008), and GnIH-ir fibers have been reported to extend into the external layer of the median eminence (Gibson et al., 2008). Additionally, recent studies in which peripheral, but not central, injections of GnIH inhibits GnRH-elicited LH release, suggesting actions at the pituitary level (Murakami et al., 2008). In one study in rats, peripheral injections of the retrograde tracer, Fluorogold (FG), label sparse numbers of GnIH cells, suggesting that these cells do not contact the pituitary portal system (Rizwan et al., 2009). Despite showing few GnIH cells labeled with FG, intravenously administered GnIH rapidly (within 5 min) inhibited GnRH-induced LH release in the same study (Rizwan et al., 2009). These data indicate the potential for GnIH to act on pituitary gonadotropes or suggest that intravenously delivered GnIH crosses the blood-brain barrier to impact GnRH cell bodies or terminals. Whether or not endogenous GnIH acts on the pituitary in addition to GnRH cells in mammalian species requires further investigation to clarify whether discrepant findings represent interspecific differences or result from technical variation across studies.

Given the pronounced inhibitory actions of GnIH in the mammalian brain, we examined the possibility that this peptide participates in mediating the negative feedback effects of estradiol. Treatment of female rats with GnIH results in marked inhibition of GnRH neuronal activity at the time of the LH surge, providing support for this possibility (Anderson et al., 2009). In Syrian hamsters, we found that GnIH-ir cells express ERα and respond to acute estradiol treatment with increased FOS expression, suggesting activation by gonadal steroids (Kriegsfeld et al., 2006). Contrasting results were observed in one recent report, with treatment of mice with estrogen for 4 days leading to a decrease in GnIH mRNA expression (Molnar et al., 2011). The discrepancy in the impact of estrogen in mice and hamster may result from the timing at which the brains were collected for analysis – hamster brains were collected prior to the LH surge when negative feedback effects of estradiol are maximal, whereas when in the day mouse brains were sampled was not reported.

We next explored whether GnIH neurons might be a locus of integration for steroidal and circadian signals, providing a mechanism to coordinate the removal of estradiol negative feedback with SCN-mediated stimulation of the GnRH/LH surge (**Figure 3**). First, we examined the pattern of GnIH cellular activity, uncovering a daily pattern with trough activity at the time of the LH surge, suggesting the removal of negative feedback at this time (Gibson et al., 2008). Additionally, using anterograde tract tracing, we found that the SCN projects to a large proportion of GnIH cells, providing a mechanism for timing removal of negative drive on the GnRH system. Finally, by exploiting the "splitting" phenomenon seen in hamsters housed in LL described previously, we found that activation of the GnIH system is asymmetrical. Importantly, this asymmetry is opposite to that seen for the GnRH system, suggesting that the SCN concomitantly activates ipsilateral GnRH cells at the same time as removing the suppressive influence of GnIH on the same side of the brain (**Figure 3**; Gibson et al., 2008).

Recently, a meticulously comprehensive series of studies by Pineda et al. investigated the role of GnIH using a selective antagonist (RF9) of GnIH and a related peptide in the same family, neuropeptide FF (NPFF). Alterations in NPFF and interactions with its receptor do not alter gonadotrophin secretion (Pineda and Tena-Sempere, unpublished observations), suggesting that the effects of RF9 are likely mediated through blockade of the actions of RFRP-3 (Pineda et al., 2010b). The role of RF9 was investigated in male and female mice and rats. Injections of RF9 to cycling females led to a rapid and sustained, dose-dependent increase in LH both during estrus and diestrus, supporting a role for GnIH in maintaining low LH concentration through negative feedback. Analogous results were seen in males, with RF9 leading to a rapid and prolonged increase in LH and FSH. Together, these findings and those for kisspeptin, point to an important role for these opposing RFamide neuropeptides in the integration of positive and negative effects of estradiol with circadian signaling in the generation of the GnRH/LH surge.

It is likely that other neural loci upstream of the GnRH system are targets of the SCN involved in the timing of the LH surge. Dual-phenotype neurons expressing GABA/glutamate within the AVPV, for example, have been implicated in the control of GnRH activity and are regulated by estradiol feedback (Leranth et al., 1985; Jarry et al., 1995; Christian et al., 2009). GnRH neurons express AMPA, NMDA, $GABA_A$, and $GABA_B$ receptors (Gore et al., 1996; Spergel et al., 1999; Sullivan and Moenter, 2004; Sliwowska et al., 2006) and blockade of glutamatergic receptors prevents the LH surge in rats (Lopez et al., 1990; Brann and Mahesh, 1991). GnRH neurons respond to GABA and glutamate differentially across the day in female mice, with inhibitory responses in the morning during estrogen negative feedback and excitatory responses in the afternoon during estrogen positive feedback (Petersen et al., 2003; Christian and Moenter, 2007). Additionally, the number of AVPV-originating synaptic contacts onto GnRH neurons that express both GABA and glutamatergic vesicular transporters increases around the time of the LH surge (Ottem et al., 2004). Furthermore, these synaptic contacts are estradiol-sensitive, suggesting the coupling of circadian and estrogenic input mediates GABA/glutamate signaling to GnRH neurons (Ottem et al., 2004). Though no direct connections have been reported between the SCN and

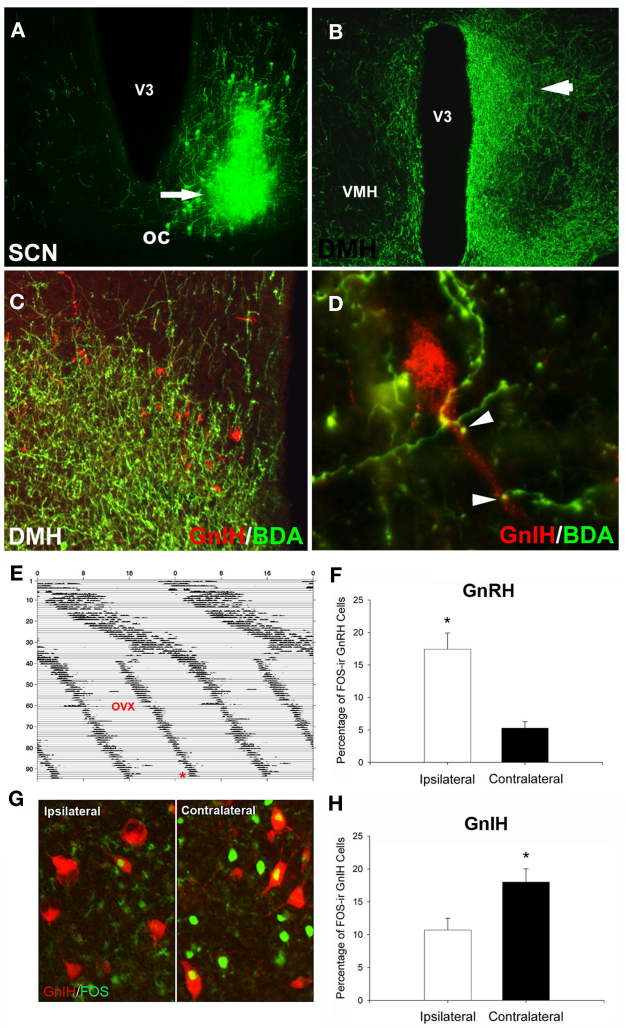

FIGURE 3 | Suprachiasmatic nucleus fibers project to GnIH-ir cells in the DMH. (A) Example injection site from an injection of biotinylated dextran amine (BDA) that filled the ventrolateral aspect of the SCN; **(B)** low-power photomicrograph indicating terminal fibers from the SCN project to the DMH, principally ipsilaterally. **(C,D)** Examples of SCN projections in close apposition to GnIH-ir cells in the DMH at the light level [**(C)**, low-power; **(D)**, high power]. **(E–H)** Lateralization of GnRH and GnIH activation in split hamsters. The pattern of SCN activation is lateralized in animals exhibiting two daily bouts of activity. GnIH cellular activity is lateralized and opposite to that of GnRH, indicating SCN-mediated removal of GnIH inhibition at the time of the LH surge. **(E)** Actogram of wheel-running activity in estradiol-implanted, ovariectomized (OVX) hamsters kept in constant light conditions (LL) from Day 1 onward (days indicated by *y* axis, hours by *x* axis). Split hamsters were killed (*) 1 h before the onset of one of the two activity bouts. **(G)** Photomicrograph of FOS activation in GnIH cells of split hamsters, showing ipsilaterally reduced GnIH activation when SCN and GnRH activation are high. Mean ± SEM) percentage of FOS-ir **(F)** GnRH and **(H)** GnIH cells in split hamsters. *Significantly different from the opposite hemisphere's activational state, $P < 0.05$. Modified from Gibson et al. (2008).

GABA/glutamate neurons within the AVPV, the expression of V1a receptors in GABA neurons within this nucleus (Kalamatianos et al., 2004) provides a potential SCN-mediated mechanism of control. It is possible that circadian input to this neuronal population comes indirectly through kisspeptin signaling; kisspeptin upregulates GABA transmission in the AVPV during estradiol negative (but not positive) feedback, suggesting local signaling within this nucleus (Pielecka-Fortuna and Moenter, 2010).

CIRCADIAN CONTROL OF PREGNANCY

In addition to the circadian control of ovulation, multiple lines of evidence indicate a critical role of the SCN in the regulation of prolactin (PRL) secretion and pregnancy maintenance. The release of prolactin, an adenohypophysis-derived hormone with myriad functions, most notably in lactation and pregnancy maintenance, is controlled by a balance of inhibitory and releasing factors. The circadian control of prolactin is abundantly clear, with a proestrus surge of PRL release occurring in the late afternoon. Additionally, ovariectomized rats treated with estradiol exhibit daily PRL surges around the time of the LH surge (Pan and Gala, 1985). Furthermore, as with the regulation of luteinizing hormone, ovariectomized, and estradiol-treated rats fail to exhibit a PRL surge following SCN lesions, suggesting a necessary role of the SCN in the release of PRL, either directly or indirectly (Pan and Gala, 1985).

Stimulation of the cervix during mating initiates twice daily prolactin surges, consisting of a diurnal, and nocturnal surge that maintains the corpora lutea and thus the secretion of progesterone, facilitating pregnancy maintenance. Evidence for the circadian regulation of the prolactin surge comes from studies similar to those linking the central clock to ovulation. The PRL surge is entrained to the light cycle and free-runs in constant conditions (Bethea and Neill, 1979; Yogev and Terkel, 1980), and SCN lesions prevent the mating-induced prolactin surges (Kawakami et al., 1980) underscoring the endogenous, circadian control of this phenomenon. Furthermore, pharmacological knockdown of the core SCN clock genes disrupts the PRL surge in rats (Poletini et al., 2007) suggesting a link between the molecular clock and the circadian drive of prolactin. Given the function of PRL in corpora lutea maintenance, disruptions in the circadian control of PRL may prevent the rise in corpora lutea-derived progesterone secretion and thus lead to an increased rate of abortion or fetal reabsorption (Milligan and Finn, 1997; Miller et al., 2004).

The SCN regulates secretory patterns of PRL regulators, including the primary PRL-inhibiting factor, dopamine (DA) and PRL-stimulating factor, oxytocin (Freeman et al., 2000). SCN-derived VIP is a likely regulatory factor in both cases, as VIPergic fibers project to dopaminergic neurons in the ARC and paraventricular nuclei (PVN; Teclemariam-Mesbah et al., 1997; Gerhold et al., 2001) and to oxytocin-secreting cells in the PVN (Teclemariam-Mesbah et al., 1997); areas considered to be critical for PRL regulation. DA cells in the ARC and cells in the PVN co-express the core circadian clock gene, *Per1*, suggesting a role for autonomous clocks operating in these cell types in mediating rhythms in PRL secretion (Kriegsfeld et al., 2003; Sellix et al., 2006). Furthermore, the rhythm of VIP in the paraventricular nucleus, where PRL-stimulating oxytocin neurons reside, is phase-locked to the rhythm of PRL, suggesting that this peptide may link the SCN to this regulatory system (Egli et al., 2004). Both DA and OXY neurons within the PVN express the VIP receptor, $VPAC_2$ (Gerhold et al., 2001; Egli et al., 2004), providing a mechanism of control between

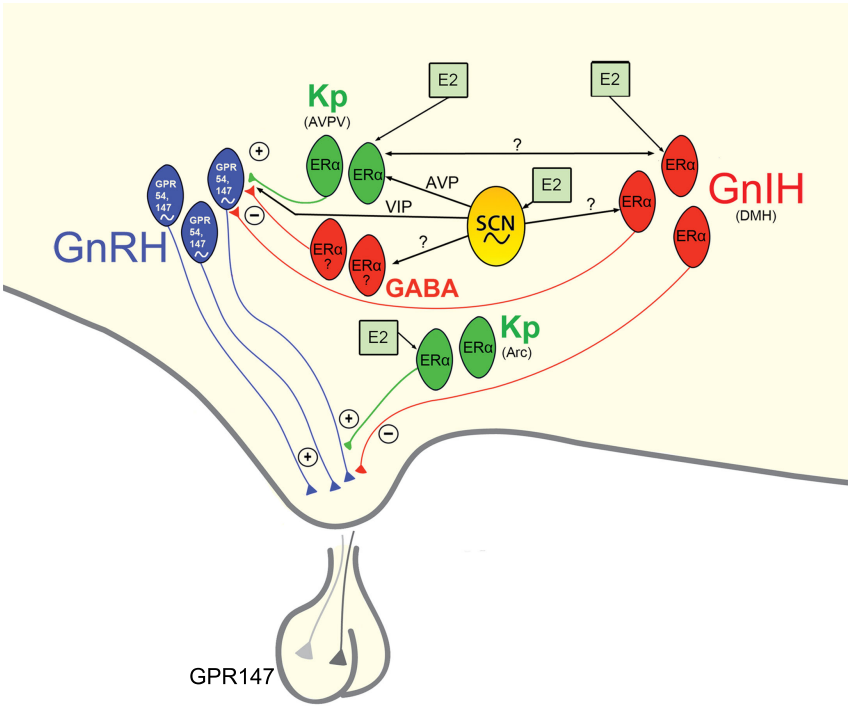

FIGURE 4 | Proposed model of circadian initiation of the preovulatory LH surge in spontaneously ovulating rodents by major positive and negative regulators of GnRH neuronal activity. Black lines depict monosynaptic projections from the SCN to GnRH neurons and to major positive (kisspeptin, Kp) and negative (GnIH, GABA) neurochemical mediators of the GnRH system that contain estrogen receptors. Kisspeptin cells in the AVPV are active at the time of the LH surge. Neurons containing ERα in the preoptic area and elsewhere are known to project to the SCN and to the vicinity of GnRH neurons and may play a role in mediating the circadian signal to GnRH neurons directly and/or indirectly. Whereas estrogen-responsive cells have not been definitively shown to project specifically to GnRH neurons, the emergence and sexual dimorphism of kisspeptin cells and fibers that project to GnRH cell bodies provide compelling evidence for the direct connection between these two neural phenotypes. Connections between the GnIH and GnRH systems indicate a putative role for GnIH in modulating the negative feedback effects of estrogen with SCN communication allowing for removal of negative feedback on the reproductive axis during the time of the LH surge. A similar role for SCN-GABA interactions is likely, although projections SCN projections and ERα expression specific to AVPV GABAergic cells have not been empirically examined. Kisspeptin cells in the ARC likely serve to modify GnRH output at the level of the terminal. See text for additional details.

the master clock and PRL regulatory neurons. Additionally, VIP antagonists directed at the SCN prevent the decrease in PVN DA, and eliminate the afternoon PRL surge in female rats (Harney et al., 1996). Finally, antisense oligonucleotide knockdown of clock genes within the SCN eliminates mating-induced PRL secretion (Poletini et al., 2007). Together, these results suggest a critical role for the circadian system in PRL regulation through VIPergic SCN communication to OXY and DA neurons, with possible additional contributions by autonomous clocks in these regulatory neuronal populations.

The circadian regulation of PRL secretion has important functional implications for female reproductive success. Female mice expressing a null mutation in the *Clock* gene exhibit disrupted ovulatory cyclicity, as well as a high incidence of pregnancy interruption (Miller et al., 2004). Spontaneous abortions and fetal reabsorption rates in these mice stem from insufficient PRL release during the initial stages of pregnancy, thus leading to blunted progesterone levels and insufficient support for the corpora lutea (Miller et al., 2004). The connections between the SCN and PRL release, and regulation of the *Prl* gene promoter by the core molecular clockwork via a non-canonical E-Box (Palm et al., 2001a; Poletini et al., 2010; Guillaumond et al., 2011), point to a direct link between the circadian system and pregnancy maintenance via the regulation of prolactin. These findings support the intriguing possibility that dysregulated PRL secretion may underlie the increased rate of reproductive health issues following prolonged circadian disruptions, including those observed in chronic shift workers (reviewed in Mahoney, 2010).

CONCLUSION AND CONSIDERATIONS

Given the pronounced impact of disruptions to circadian timing on female reproductive functioning and health, it is critical that a full understanding of the mechanisms underlying these processes be garnered. The SCN sits at the center of a hierarchy of temporal control, coordinating the activity of positive and negative upstream regulators of the HPG axis (**Figure 4**). In addition to receiving direct estrogenic feedback, the SCN also receive sex steroid feedback, indirectly, via estrogen-sensitive targets that communicate directly with the master pacemaker. Many reproductively relevant monosynaptic targets of the SCN express estrogen receptors, providing a further means of integration for circadian and estrogenic signaling necessary for optimal fecundity. Given the

ubiquity of autonomous clock function in the CNS and periphery, it is likely that many targets of the SCN contain independent clocks to temporally modulate their network environment and further ensure ideal timing of reproductive events. The means by which other well-established regulators of the HPG axis synergize with these circadian controlled circuits to optimize female reproductive health represents and exciting and vital opportunity for further enquiry.

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Differential roles of GnRH-I and GnRH-II neurons in the control of the primate reproductive axis

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In vertebrates, gonadotropin-releasing hormone (GnRH) represents the primary neuroendocrine link between the brain and the reproductive axis, and in some species up to three different forms of GnRH have been detected. Until recently, it had been assumed that humans and non-human primates only express one form (GnRH-I), but it is now clear they also express a second form (GnRH-II). GnRH-II, like GnRH-I, is highly effective at stimulating gonadotropin release, both *in vitro* and *in vivo*, but the neurons that produce GnRH-II are completely distinct from those producing GnRH-I. Moreover, GnRH-II and GnRH-I producing neurons respond very differently to estradiol; specifically, estradiol stimulates GnRH-II gene expression in the former and inhibit GnRH-I gene expression in the latter. Consequently, the negative feedback action of estradiol may be mediated exclusively by the subpopulation of GnRH neurons that express GnRH-I, while the positive feedback action may be mediated exclusively by the subpopulation that expresses GnRH-II. Taken together, these findings raise the possibility that two completely different GnRH neuronal systems participate in the control of primate reproductive physiology. The primary role of GnRH-I neurons is likely to be focused on the maintenance and modulation of tonic pulsatile LH release, whereas the primary role of GnRH-II neurons is likely to be focused on the generation of the preovulatory LH surge. This functional segregation of the primate neuroendocrine reproductive axis lends itself for novel targeted approaches to fertility control and for treatment of human reproductive disorders.

Keywords: estradiol, gonadotropin-releasing hormone, ovulation, rhesus macaque

THE NEUROENDOCRINE REPRODUCTIVE AXIS

In its essence, the mammalian neuroendocrine reproductive axis is composed of three main components, which act in a coordinated manner to control the onset of puberty and to subsequently maintain fertility (Silverman et al., 1994; Ojeda et al., 2006). The pulsatile secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the anterior pituitary gland to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In turn, these two gonadotropins then act on the gonads to stimulate maturation of gametes and to synthesize and secrete sex-steroid hormones. Important neuroendocrine feedback loops also exist. For example, although sex steroids contribute to the development and maintenance of fertility and play a role in other physiological and behavioral functions, they also feedback onto the hypothalamo-pituitary unit to modulate gonadotropin release. There is, however, a conundrum regarding the mechanism by which sex steroids do this. On the one hand, the ovarian steroid estradiol usually exerts a negative feedback action on GnRH and gonadotropin release, which is epitomized by the marked increases in circulating LH and FSH levels that occur at menopause or after ovariectomy (i.e., when estradiol levels are markedly attenuated). On the other hand, around the time of ovulation estradiol appears to exert a positive feedback action, causing the production of a gonadotropin surge, which serves as the ovulatory trigger. The traditional explanation for these two radically different effects of estradiol is that GnRH neurons respond differentially to low and high levels of this sex steroid – negatively when estradiol levels are low and positively when estradiol levels are high. Inherent in this argument, however, is the assumption that the GnRH neurons are relatively homogenous, despite their diffuse distribution pattern, and that individual GnRH neurons have the capacity to show both negative and positive feedback responses. Recent findings, especially from primate studies, suggest that this assumption is inaccurate. Not only have morphologically distinct GnRH neurons been observed in discrete subpopulations, in some species these subpopulations have been shown to express different molecular forms of GnRH. Importantly, different GnRH neuronal subpopulations respond differently to estradiol, suggesting that the negative and positive feedback actions are mediated by two distinctly different neuronal populations. This alternative view of the neuroendocrine reproductive axis lends itself to novel targeted approaches to fertility control and treatment of human reproductive disorders.

HETEROGENEITY IN THE PRIMATE GnRH NEURONAL SYSTEM

It has generally been assumed that GnRH neurons are all essentially similar in their responses to neurotransmitters. Indeed, this

is a fundamental assumption that underlies the extensive use of immortalized GnRH neurons for *in vitro* studies by many researchers, including members of this laboratory (e.g., Urbanski et al., 1996; Olcese et al., 2003; Garyfallou et al., 2006). However, this assumption is questionable because in humans GnRH neurons appear to show three distinct morphological types, based on cell size and GnRH cDNA probe labeling density (Rance et al., 1994; Krajewski et al., 2003): (1) small, heavily labeled, oval, or fusiform neurons, located primarily in the medial basal hypothalamus, ventral preoptic area, and periventricular zone; (2) small, oval, sparsely labeled neurons located in the septum, and dorsal preoptic region and scattered from the bed nucleus of the stria terminalis to the amygdala ("extended amygdala"); and (3) large round neurons (>500 μm two sectional profile area), intermediate in labeling density, scattered within the magnocellular basal forebrain complex, extended amygdala, ventral pallidum, and putamen. The pronounced differences in morphology, labeling density, and location of the three subtypes suggest that distinct functional subgroups of GnRH neurons exist in the human brain. Similar morphological subtypes have been observed in the brains of rhesus macaques (Urbanski et al., 1996), and it has been shown that the type 1 and type 3 neurons have characteristically distinct biochemical properties (e.g., they differ in their capacity to express glutamate and estrogen receptors). Currently, however, it is unclear whether the type 2 and 3 neuronal subtypes play any physiological role in the control of gonadotropin synthesis.

A second basic assumption, and the one that is most relevant to this hypothesis, is that pituitary gland activity is influenced by a single molecular form of GnRH. Although multiple molecular forms of GnRH have been identified in non-mammalian vertebrates (Sherwood et al., 1993; Fernald and White, 1999; Dubois et al., 2002; Roch et al., 2011), until recently only one form of this decapeptide was thought to exist in mammals. It now appears that at least a few eutherian mammals, including musk-shrews, tree-shrews, and humans express an additional form of GnRH (review: Herbison, 2006). This second form of GnRH is commonly referred to as "chicken GnRH-II," or simply GnRH-II, and it shows 70% similarity to mammalian GnRH (i.e., GnRH-I) at the amino acid level (**Figure 1**). The genomic and mRNA structures of GnRH-II resemble those of GnRH-I, although significant differences exist within the GnRH-associated peptide (GAP) regions of the respective genes (not shown); in addition, in humans the two molecules are encoded on different chromosomes (White et al., 1998). HPLC and immunocytochemical studies have shown that GnRH-II also exists in non-human primates (Lescheid et al., 1997), and this has been corroborated through the cloning of GnRH-II cDNA from the monkey brain (GenBank #228312; Urbanski et al., 1999). Importantly, GnRH-II gene expression has been demonstrated in the hypothalamus of monkeys, and shows a distribution pattern that is distinct from that of GnRH-I (**Figure 2**). GnRH-I expression has a diffuse expression pattern in the hypothalamus, whereas GnRH-II appears to be concentrated in specific nuclei such as the paraventricular, supraoptic, suprachiasmatic as well as the medial basal hypothalamus. Subsequent studies used double histochemical labeling to show that GnRH-I and GnRH-II are produced by two completely distinct populations of cells (Latimer et al., 2000).

INFLUENCE OF GnRH-II ON GONADOTROPIN RELEASE

Although several reviews of the mammalian GnRH system, have attempted to explain the physiological role of GnRH-II (Pawson et al., 2003; Terasawa, 2003; Cheng and Leung, 2005; Herbison, 2006; Kah et al., 2007), the physiological function of this most ancient and highly conserved form of GnRH in primates is still unclear. There is some evidence from the musk-shrew and marmoset that GnRH-II plays a role in coordinating reproductive behavior, although these behavioral effects are thought to be mediated by GnRH-II neurons located in the central regions of the midbrain rather than in the forebrain (Kauffman et al., 2005; Barnett et al., 2006). Rhesus macaques, however, show a high level of GnRH-II expression in the hypothalamus as well as in the midbrain (Urbanski et al., 1999; Latimer et al., 2001), which raises the interesting possibility that GnRH-II may contribute to the control of the primate reproductive neuroendocrine axis. Indeed, it has already been shown that GnRH-II is highly effective at stimulating LH and FSH release in rhesus monkeys *in vivo*, and from pituitary culture *in vitro* (Lescheid et al., 1997; Densmore and Urbanski, 2003; Kada et al., 2003). Furthermore, this activation can be blocked by Antide, a GnRH receptor-1 (GnRHR-1) specific antagonist (**Figure 3**). Taken together this suggests that both GnRH-I and GnRH-II act through the same receptor to

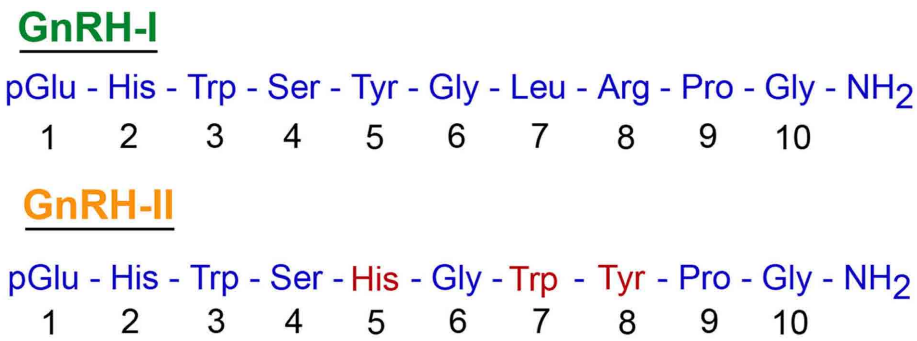

FIGURE 1 | A comparison between the amino acid sequences of GnRH-I and GnRH-II. Both decapeptides undergo similar post-translational modification, which includes conversion of Gln to pGlu at amino acid position 1 and amidation of the Gly at position 10. Note, 70% similarity in the amino acid sequence of the two decapeptides.

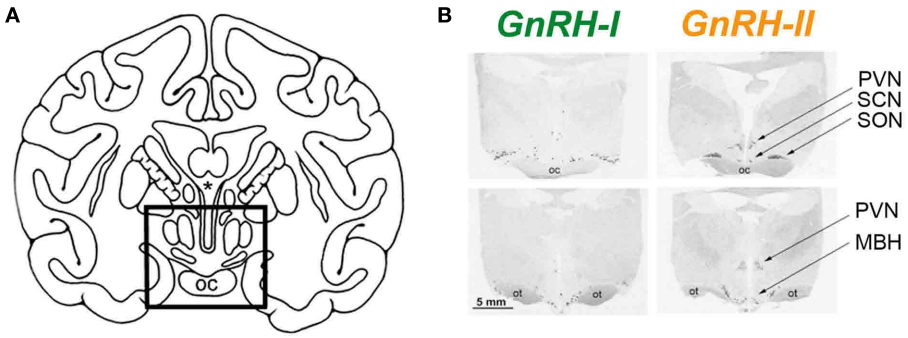

FIGURE 2 | Differential distribution of GnRH-I and GnRH-II mRNA in the rhesus macaque hypothalamus, as revealed by *in situ* hybridization histochemistry. (A) Line drawing showing hypothalamic region (in box). **(B)** Autoradiographs showing GnRH expression in the rostral (upper panels) and caudal (lower panels) hypothalamic sections. SON, supraoptic nucleus; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; MBH, medial basal hypothalamus; oc, optic chiasm; ot, optic tract. (Adapted from Urbanski et al., 1999, with permission from the Endocrine Society).

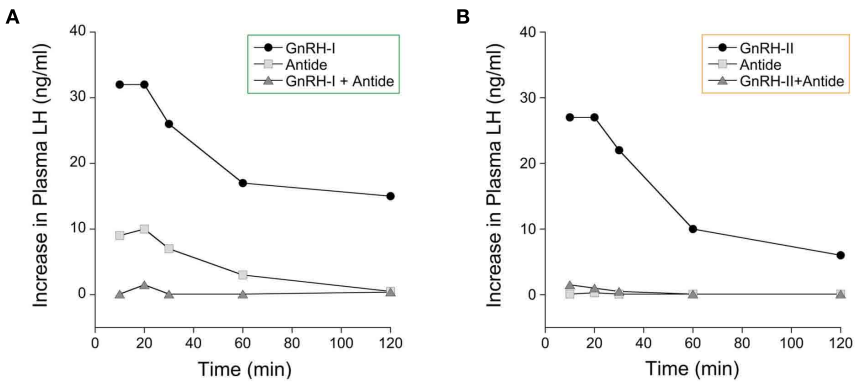

FIGURE 3 | Effect of GnRH on plasma LH levels in female rhesus macaques. GnRH-I or GnRH-II (both at 1 μg/kg body weight) were administered via an indwelling vascular catheter, and the effect of GnRH receptor-1 antagonist was determined by simultaneous administration of Antide (100 μg/kg body weight). The data demonstrate that **(A)** GnRH-I and **(B)** GnRH-II are both potent stimulators of LH release and that they both act through the same GnRH receptor (i.e., GnRH receptor-1). (Adapted from Densmore and Urbanski, 2003, with permission from the Endocrine Society).

stimulate gonadotropin release. Note that a second receptor for GnRH (GnRHR-2) has been cloned in monkeys and humans, but it probably does not have a specific role in controlling the reproductive neuroendocrine axis of primates; in monkeys the GnRHR-2 has a ubiquitous distribution pattern, while in humans there is a stop codon in the middle of its gene sequence which precludes its translation into a functional protein (Cheng and Leung, 2005; Herbison, 2006).

DIFFERENTIAL RESPONSES OF GnRH-I AND GnRH-II NEURONS TO ESTRADIOL

Although GnRH-I and GnRH-II can both stimulate gonadotropin release in the rhesus macaque (**Figure 3**), the neurons that produce them show marked differences in their responsiveness to estradiol. Firstly, the GnRH-II gene promoter contains estrogen response elements and the GnRH-II neurons express estradiol receptors (ERβ), whereas GnRH-I neurons do not (Sullivan et al., 1995; Densmore and Urbanski, 2004). Furthermore, semi-quantitative *in situ* hybridization histochemistry (Densmore and Urbanski, 2004) has shown that GnRH-II gene expression increases in the monkey medial basal hypothalamus after exposure to estradiol, whereas GnRH-I gene expression decreases (**Figure 4**). This observation is consistent with the central hypothesis that different GnRH neuronal subpopulations respond differentially to estradiol. Importantly, the result suggests that GnRH-II neurons are the primary mediators of positive estradiol feedback, whereas the GnRH-I neurons are the primary mediators of negative estradiol feedback. It is also consistent with the finding from other primate studies, showing that hypothalamic GnRH-I gene expression is elevated when estradiol levels are very low, such as after menopause or after ovariectomy (Rance and Uswandi, 1996; Abel et al., 1999). Additional support for the hypothesis comes from a microarray gene profiling study that examined hypothalamic GnRH-I and GnRH-II gene expression across the monkey menstrual cycle (Urbanski et al., 2009, 2010a). This study focused on three stages of the menstrual cycle that show three distinct sex-steroid profiles (**Figure 5**). During the early follicular (EF) phase, both estradiol and progesterone concentrations are low, whereas in the late follicular (LF) phase estradiol is highly elevated; during the mid-luteal (ML) phase, estradiol is moderately

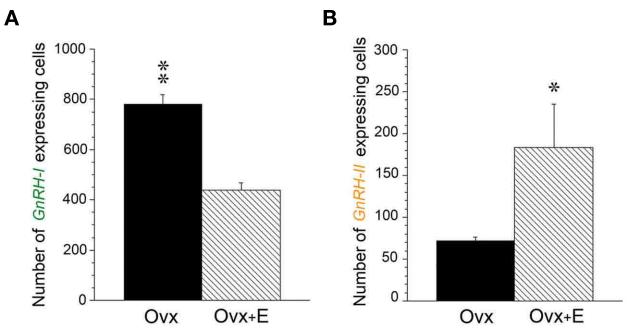

FIGURE 4 | Differential regulation of GnRH-I and GnRH-II gene expression by estradiol in ovariectomized rhesus macaques. (A) Histogram depicting number of cells expressing GnRH-I mRNA in the hypothalamus of ovariectomized (OVX) and ovariectomized/estradiol-treated (OVX + E) animals, as revealed by semi-quantitative *in situ* hybridization histochemistry. The number of detectable GnRH-I cells in the hypothalamus was markedly lower in the OVX + E animals than in the OVX animals. **(B)** Histogram depicting number of cells expressing GnRH-II mRNA in the MBH of OVX and OVX + E animals, as revealed by *in situ* hybridization. The number of detectable GnRH-II cells in the MBH was significantly greater in the OVX + E animals than in the OVX animals. The *in situ* hybridization was performed on a series of six coronal hypothalamic sections from each animal ($N = 3$/group); the sections were collected at ~200-μm intervals. $^{**}P < 0.01$, $^{*}P < 0.05$. (Adapted from Densmore and Urbanski, 2004, with permission from the Society for Endocrinology).

elevated and progesterone highly elevated (Downs and Urbanski, 2006). Despite differences in circulating sex-steroid concentrations during these three phases of the cycle, GnRH-I gene expression showed no significant change (**Figure 5A**); in marked contrast, GnRH-II gene expression showed a marked increase during the LF phase, in association with the elevated estradiol levels. The positive relationship between estradiol and GnRH-II gene expression, and the close temporal relationship between elevated GnRH-II gene expression and the preovulatory LH surge, suggests that the GnRH-II neurons play a dominant causal role in the preovulatory LH surge (**Figure 5B**). Further supportive evidence for this hypothesis comes from a recent study in which estradiol benzoate (EB; 42 μg/kg, s.c.) was administered to ovariectomized rhesus macaques (Urbanski et al., 2010b). In this well-established experimental model plasma estradiol levels reached a peak within 4 h of EB injection, and this was associated with an expected suppression of plasma LH levels, followed by a surge approximately 2 days later. Real-time-PCR showed that initial suppression of LH to be associated with a decrease in GnRH-I gene expression, which is consistent with estradiol exerting a negative influence on GnRH-I neurons. In marked contrast, GnRH-II gene expression increased following the estradiol peak, and reached a maximum just before the plasma LH surge, which is consistent with estradiol exerting a positive influence on GnRH-II neurons.

CENTRAL HYPOTHESIS

Based on these recent findings, mainly from rhesus macaques, it is hypothesized that GnRH-II neurons play a major role in the generation of the preovulatory LH surge in female primates,

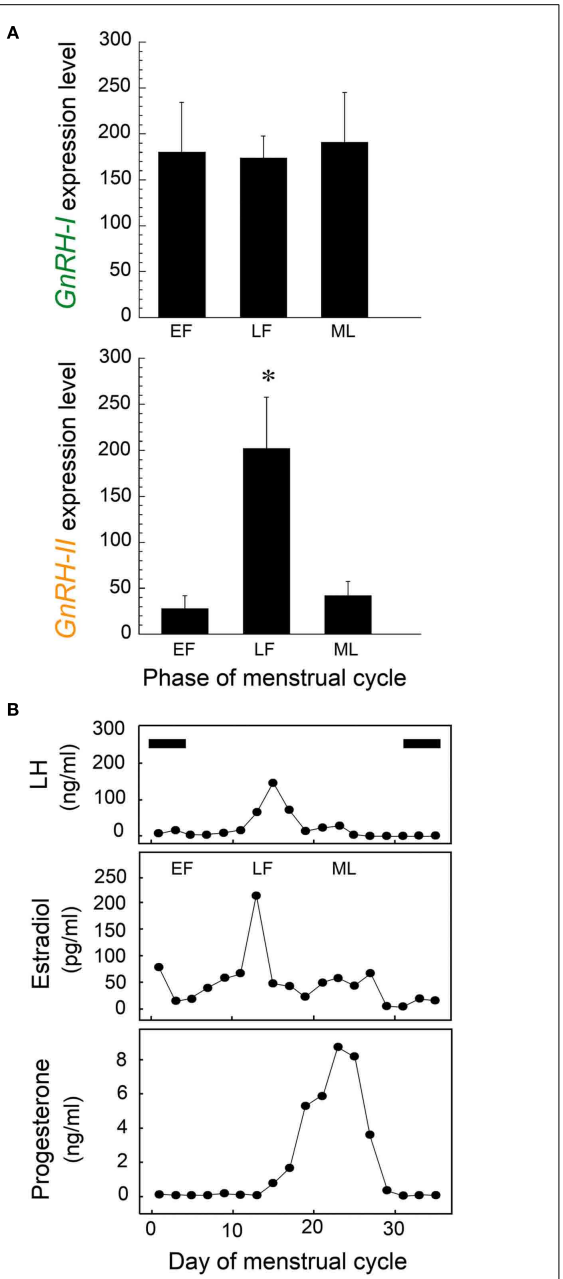

FIGURE 5 | (A) Mean expression of GnRH-I (upper panel) and GnRH-II (lower panel) mRNA levels in the rhesus monkey hypothalamus, as determined by Affymetrix GeneChip microarray analysis (HU133A plus 2.0; $N = 3$–4/group). The following three characteristic stages of the menstrual cycle were examined: EF, early follicular; LF, late follicular, ML, mid-luteal. The level of GnRH-I gene expression was similar across the menstrual cycle, whereas the level of GnRH-II gene expression showed a significant mid-cycle increase ($^{*}P < 0.05$). **(B)** Serum reproductive hormone profiles from a representative female rhesus macaque across a complete menstrual cycle. Note the temporal relationship between the mid-cycle preovulatory LH surge and the elevated serum estradiol levels of the LF phase. Periods of menstruation are indicated by black horizontal bars. Together the data suggest that estradiol-mediated activation of GnRH-II neurons may play a key role in triggering ovulation, whereas GnRH-I neurons are more likely to play a role in modulating tonic LH release and in follicular maturation. (Adapted from Urbanski et al., 2010a).

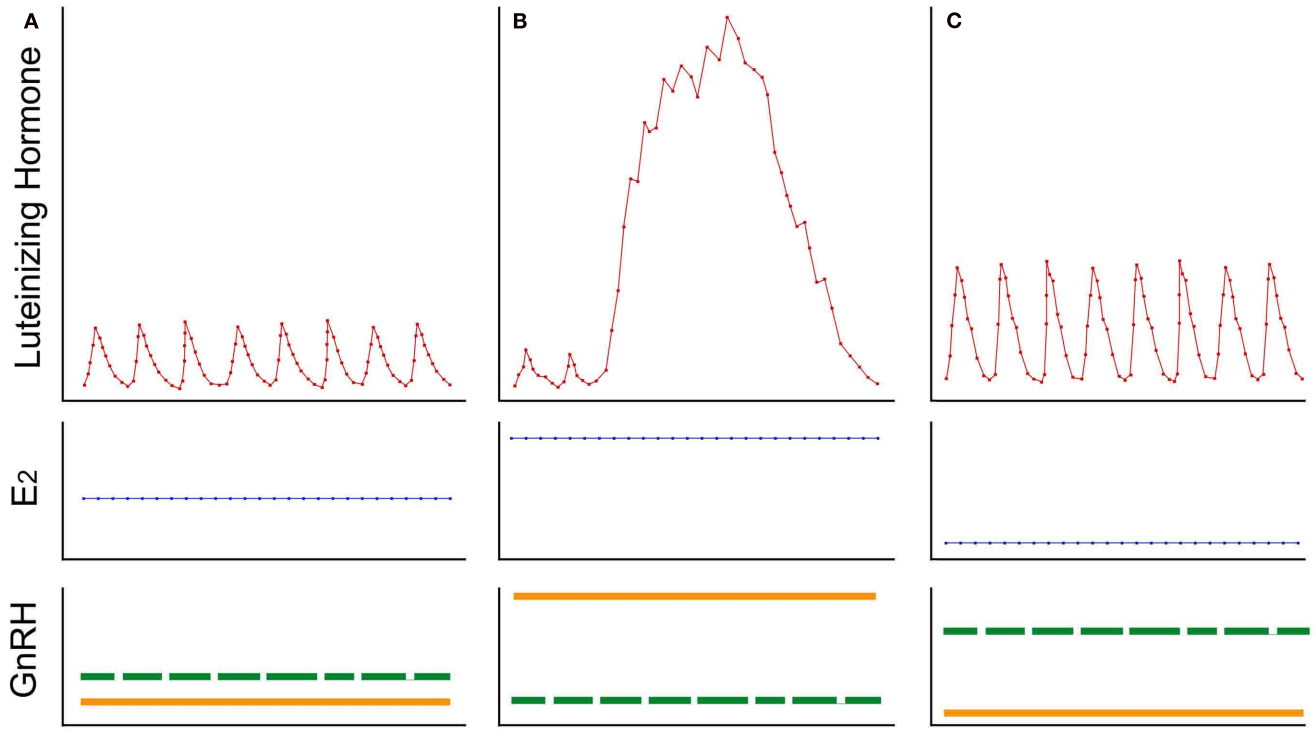

FIGURE 6 | A summary of the hypothesized mechanism by which estradiol (E_2) modulates luteinizing hormone (LH) release in female primates. Stylized representations of three distinct temporal patterns of LH release are depicted in the upper panels, while corresponding plasma E_2 levels and GnRH neuronal activity are represented in the middle and lower panels, respectively. **(A)** Low amplitude pulse of LH are typically observed during the follicular phase of the menstrual cycle; this is associated with low-to-medium plasma E_2 levels, which exert a moderate degree of negative feedback onto GnRH-I neurons (represented by the dashed green line) but cause little stimulation of GnRH-II neurons (represented by the solid orange line). **(B)** An LH surge typically occurs in the middle of the menstrual cycle; this is associated with high plasma E_2 levels, which inhibit GnRH-I neuronal activity but markedly stimulate synthesis and release of GnRH-II. **(C)** High amplitude pulses of LH are typically observed after ovariectomy and after menopause, as a result of the highly attenuated plasma E_2 levels; in the absence of significant E_2 negative feedback, GnRH-I neurons show increased activity whereas GnRH-II neurons lack positive feedback and so revert to a relatively quiescent state. Although it is commonly assumed that the same GnRH neurons can mediate both negative and positive E_2 feedback onto LH release, the data presented in this mini-review question this assumption. Instead, it is hypothesized that tonic and surge modes of LH release are orchestrated by two distinct GnRH neuronal populations, which in primates can be distinguished by their capacity to produce different molecular forms of GnRH (i.e., GnRH-I and GnRH-II, respectively) and to respond differentially to E_2.

whereas GnRH-I neurons mediate the negative feedback influence of estradiol on tonic gonadotropin release (**Figure 6**). It should be emphasized that this hypothesis does not invoke a special role for the GnRH-II molecule, other than suggesting that GnRH-II expression may be used to identify the estrogen-responsive neurons that are activated at the time of the LH surge. The hypothesis simply proposes that the menstrual cycle is orchestrated by the coordinated action of two separate subpopulations of GnRH neurons – one subpopulation responds to estradiol in a negative manner and is involved in stimulating the ovary during the follicular phase of the cycle, while the other subpopulation responds to estradiol in a positive manner and hyper-stimulates the pituitary gland to produce a mid-cycle LH surge.

In rodents it may be more difficult to distinguish between these two GnRH neuronal subpopulations because only one molecular form of GnRH (i.e., GnRH-I) has been equivocally shown to exist. Despite attempts by several laboratories, GnRH-II has still not been cloned in mice or rats, which is not surprising given that a BLAST search of the genome of these rodents reveals only one molecular form of GnRH (i.e., the traditional GnRH-I). Nevertheless, there is evidence from female rodents that activation of a specific subpopulation of GnRH-I neurons is associated with the preovulatory LH surge (Hiatt et al., 1992; Porkka-Heiskanen et al., 1994; Rubin and King, 1994). Therefore, it is plausible that the essence of the current hypothesis also applies to non-primate mammalian species.

For many years there was much debate as to whether a GnRH surge even existed in primates, because the primate pituitary gland appeared capable of producing an LH surge in response to tonic pulsatile GnRH stimulation (Knobil et al., 1980). Although there is now good evidence to suggest that an estradiol-induced GnRH surge does occur in primates, as in rodents, the key studies (Xia et al., 1992; Pau et al., 1993) were performed before the existence of GnRH-II was known in primates, and unfortunately relied on hormone immuno-assays that did not clearly distinguish between GnRH-I and the closely related GnRH-II molecule. Consequently, it is unclear if the assays measured GnRH-I exclusively or GnRH-I

plus GnRH-II. More recently, however, c-Fos immunohistochemistry has been used to examine activation of GnRH-I neurons around the time of the primate preovulatory surge. In marked contrast to rodents (Lee et al., 1990; Hoffman et al., 1993; Doan and Urbanski, 1994), however, there was no obvious increase in the number of c-Fos-expressing GnRH-I neurons at the time of the LH surge in primates (Witkin et al., 1994; Caston-Balderrama et al., 1998). This negative finding gives further credence to the hypothesis that GnRH-I neurons do not play a dominant role in stimulating the preovulatory LH surge in primates, and implies that this role is more likely to be mediated by GnRH-II neurons instead.

CONCLUDING REMARKS

Like rhesus macaques, humans express two molecular forms of GnRH (i.e., GnRH-I and GnRH-II). Given that GnRH-I and GnRH-II are produced by different neuronal populations, it is plausible that fertility in women is controlled by the coordinated action of two distinct GnRH neuronal subpopulations, rather than by a single homogenous population. If correct, this hypothesis has several implications, especially in primates where the distinct biochemical signatures of the two subpopulations lend themselves to targeted therapeutic interventions. For example, specific activation of the GnRH-II neurons could help to treat amenorrhea and improve fertility. Conversely, by specific silencing of the GnRH-II neurons it may be possible to selectively block ovulation while retaining normal tonic LH secretion, a strategy that could open up novel approaches to contraception without negatively impacting ovarian steroidogenesis.

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