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Identification of a regulatory loop for the synthesis of neurosteroids: a steroidogenic acute regulatory protein–dependent mechanism involving hypothalamic–pituitary–gonadal axis receptors

Sivan Vadakkadath Meethal,* Tianbing Liu,* Hsien W. Chan,* Erika Ginsburg,† Andrea C. Wilson,* Danielle N. Gray,‡ Richard L. Bowen,§ Barbara K. Vonderhaar† and Craig S. Atwood*,¶,**

*Department of Medicine, University of Wisconsin and Geriatric Research, Education and Clinical Center, Veterans Administration Hospital, Madison, Wisconsin, USA

†Mammary Biology and Tumorigenesis Laboratory, National Cancer Institute, Bethesda, Maryland, USA

‡Department of Biology, Tuskegee University, Tuskegee, Alabama, USA

§OTB Research, Raleigh, North Carolina, USA

¶Institute of Pathology, Case Western Reserve University, Cleveland, Ohio, USA

**School of Exercise, Biomedical and Health Sciences, Edith Cowan University, Joondalup, Western Australia, Australia

Abstract

Brain sex steroids are derived from both peripheral (primarily gonadal) and local (neurosteroids) sources and are crucial for neurogenesis, neural differentiation and neural function. The mechanism(s) regulating the production of neurosteroids is not understood. To determine whether hypothalamic-pituitary-gonadal axis components previously detected in the extra-hypothalamic brain comprise a feedback loop to regulate neuro-sex steroid (NSS) production, we assessed dynamic changes in expression patterns of steroidogenic acute regulatory (StAR) protein, a key regulator of steroidogenesis, and key hypothalamic-pituitary-gonadal endocrine receptors, by modulating peripheral sex hormone levels in female mice. Ovariectomy (OVX; high serum gonadotropins, low serum sex steroids) had a differential effect on StAR protein levels in the extrahypothalamic brain; increasing the 30- and 32-kDa variants but decreasing the 37-kDa variant and is indicative of cholesterol transport into mitochondria for steroidogenesis. Treatment of OVX animals with E_2, P_4, or E_2 + P_4 for 3 days, which decreases OVX-induced increases in GnRH/gonadotropin production, reversed this pattern. Suppression of gonadotropin levels in OVX mice using the GnRH agonist leuprolide acetate inhibited the processing of the 37-kDa StAR protein into the 30-kDa StAR protein, confirming that the differential processing of brain StAR protein is regulated by gonadotropins. OVX dramatically suppressed extra-hypothalamic brain gonadotropin-releasing hormone 1 receptor expression, and was further suppressed in E_2- or P_4-treated OVX mice. Together, these data indicate the existence of endocrine and autocrine/paracrine feedback loops that regulate NSS synthesis. Further delineation of these feedback loops that regulate NSS production will aid in developing therapies to maintain brain sex steroid levels and cognition.

Keywords: Alzheimer's disease, estrogen receptor, feedback loop, gonadotropin-releasing hormone receptor I, neurosteroid, steroidogenic acute regulatory protein.

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Sex steroids are essential for normal brain function; they are neuroprotective, and promote neurogenesis, differentiation, neuronal survival and normal cognitive function (reviewed in Bates *et al.* 2005; Gleason *et al.* 2005; Simpkins *et al.* 2005; Vadakkadath Meethal and Atwood 2005). The abrupt loss of serum sex steroids with reproductive senescence not surprisingly correlates with an increased prevalence of cognitive disease in women (Jorm *et al.* 1987; McGonigal *et al.* 1993; Brookmeyer *et al.* 1998), while sex steroid replacement

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Address correspondence and reprint requests to Craig S. Atwood, University of Wisconsin-Madison School of Medicine and Public Health, Wm S. Middleton Memorial VA (GRECC 11G), 2500 Overlook Terrace, Madison, WI 53705, USA. E-mail: csa@medicine.wisc.edu

Abbreviations used:CEE, conjugated equine estrogens; ER, estrogen receptor; GnRHR, gonadotropin-releasing hormone 1 receptor; HPG, hypothalamic-pituitary-gonadal; LH, luteinizing hormone; MPA, medroxyprogesterone; NSS, neuro-sex steroid; OVX, ovariectomy; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; StAR, steroidogenic acute regulatory protein; TSPO, translocator protein.

therapy decreases the incidence (Henderson *et al.* 1994) and delays the onset of cognitive decline in women and men (reviewed in Gleason *et al.* 2005).

Brain sex steroids are derived from both circulating and local sources. Plasma sex steroids are derived predominantly from the gonads under the control of the hypothalamic-pituitary-gonadal (HPG) axis and cross the blood–brain barrier (BBB) (Pardridge and Mietus 1979a,b; Wilson *et al.* 2008). Locally produced neuro-sex steroids (NSS)* are synthesized within the nervous system *de novo* from cholesterol or steroidal precursors imported from peripheral sources (Baulieu 1997; Baulieu *et al.* 2001). Therefore, the concentration of sex steroids in the brain is a mixture of peripherally derived sex steroids, converted peripheral steroids, and NSS. NSS are synthesized in the central and peripheral nervous system primarily by neurons but also by astrocytes and oligodendrocytes (Jung-Testas *et al.* 1989, 1991; Guennoun *et al.* 1995; Robel and Baulieu 1995; Sanne and Krueger 1995; Furukawa *et al.* 1998; Kohchi *et al.* 1998; Ukena *et al.* 1998; Zwain and Yen 1999; Sinchak *et al.* 2003; Garcia-Ovejero *et al.* 2005; Rune and Frotscher 2005; Micevych *et al.* 2007). Despite the importance of brain sex steroids for normal cognitive function, the mechanisms regulating the production of NSS are poorly understood.

The transfer of cholesterol from the outer to the inner mitochondrial membrane is the rate-limiting step in hormone-induced steroid formation (Simpson and Waterman 1983; Jefcoate 2002). Cholesterol transport is mediated via a series of steps including the translocator protein (TSPO)-dependent import of steroidogenic acute regulatory protein (StAR) into mitochondria, and the association of TSPO with the outer/inner mitochondrial membrane contact sites (Rone *et al.* 2009). Upon gonadotropin stimulation, cytosolic 37-kDa StAR is transported to the outer mitochondrial membrane and processed into a 32-kDa and a mature 30-kDa protein found in the inner mitochondrial membrane (Stocco and Sodeman 1991; Hauet *et al.* 2005). Cholesterol transported into the mitochondria in this manner is then converted to pregnenolone by the action of cytochrome P450 cholesterol side chain cleavage enzyme located on the inner mitochondrial membrane (Hall 1985). Inhibition of StAR (or TSPO) expression rapidly inhibits MA-10 progesterone production and processing of the 37-kDa to the 30-kDa variant (Rone *et al.* 2009).

Recently we demonstrated that multiple variants of gonadotropin-releasing hormone 1 receptor (GnRHR) are expressed by pyramidal neurons in the human hippocampus, and in the entorhinal cortex and occipitotemporal gyrus (Wilson *et al.* 2006). Furthermore, GnRH was shown to signal via neuronal GnRHRs to induce luteinizing hormone (LH) expression, providing one explanation for the presence of cytoplasmic LH previously observed in human pyramidal neurons (Hostetter *et al.* 1981; Emanuele *et al.* 1983; Bowen *et al.* 2002). In a subsequent study, we demonstrated that LH signals via neuronal LH receptors (Lei *et al.* 1993; Liu *et al.* 2007) to modulate the expression of the cholesterol transport protein StAR, and increase NSS synthesis (Liu *et al.* 2007). In addition to these HPG axis components, all other components of the HPG axis feedback system including the sex steroid synthetic enzymes are present in the brain. In this regard, GnRH secreting neurons with efferent pathways leading into different regions of the brain including the hippocampus have been reported (Merchenthaler *et al.* 1984). The expression of kisspeptin, its receptor-G-protein-coupled receptor 54 (GPR54; Messager 2005; Messager *et al.* 2005; Smith 2008) and StAR (Furukawa *et al.* 1998; King *et al.* 2002; Sierra *et al.* 2003; Sierra 2004; Webber *et al.* 2006; Liu *et al.* 2007) also have been reported, as have enzymes of the steroidogenic pathways (Compagnone and Mellon 2000; Yu *et al.* 2002). These data suggest that both endocrine and/or autocrine/paracrine feedback loops may exist to modulate NSS production in the brain (Atwood *et al.* 2007).

Understanding whether feedback loops exist and how they might regulate brain sex steroid concentrations is complicated by the technical difficulties associated with determining the proportional contribution of locally produced and circulating sources of sex steroids to the total concentration of brain sex steroids. Further, identification of endocrine feedback axes requires the use of whole animals. To examine the potential for feedback loops, we have taken the *in vivo* approach of assessing the dynamic changes in expression patterns of StAR and key HPG endocrine receptors following the modulation of plasma sex steroid and gonadotropin levels. Peripheral sex steroids and gonadotropins were found to modulate brain GnRHR signaling and gonadotropin-induced, StAR-mediated uptake of mitochondrial cholesterol for NSS synthesis, thereby demonstrating the presence of endocrine feedback axes that regulate NSS synthesis. These results also support the presence of a brain NSS feedback axis.

Methods

Animals and tissue collection

Experiment 1

BALB/c mice were obtained from the Frederick Cancer Research and Development Center (Frederick, MD, USA). Animal experimentation was conducted under the guidelines described in the NIH Guide for the Care and Use of Laboratory Animals. Mice were kept with a ratio of 12 h light : 12 h darkness photoperiod and were allowed access to standard laboratory chow and water *ad libitum*.

Female mice (3 months of age) were either left intact, or ovariectomized (OVX) and treated with a cholesterol (control), 17β-estradiol (E_2), progesterone (P_4) or E_2 + P_4 pellet. OVX was

*Sex steroids synthesized in the brain from cholesterol or steroid intermediates transported into the brain. This term discriminates from corticoids synthesized in the brain, and other steroids synthesized by other organs.

performed as previously described (Wilson *et al.* 2008) and animals allowed to recover for 3 weeks to clear serum sex steroids. Sex steroids were administered in pellets (10 mg) with cholesterol as the carrier containing either E_2 (C : E_2, 2001 : 1), P (C : P_4, 2000 : 1000), E_2/P_4 (C : P_4 : E_2, 2002 : 1001 : 1) or cholesterol alone (C) and were implanted subcutaneously as previously described (Plaut *et al.* 1993). This treatment was previously shown to elevate circulating E_2 and P_4 levels at least five- to 10-fold during the first 3 days of treatment (Atwood *et al.* 2000).

Three days post-pellet insertion, animals were killed (sodium pentobarbital, 200 mg/kg, i.p.) and then transcardially perfused with phosphate-buffered saline (PBS, pH 7.4; Gibco, Rockville, MD, USA), the animals decapitated, brains removed and the cerebral hemispheres separated. One hemisphere was frozen, while the other was dropped fixed in methacarn. The frozen hemisphere was dissected to remove the hypothalamic region and then homogenized in 1 : 2 (w/v) media containing PBS, Triton X-100 1% (v/v), NP-40 0.2% (v/v) (Roche Diagnostics Corporation, Indianapolis, IN, USA), sodium dodecyl sulfate (SDS) 0.1% (w/v) and protease inhibitor [1 caplet/10 mL of PBS; Complete mini EDTA free caplets (cat. # 11836170001); Roche Diagnostics Corporation]. The tissue was homogenized on ice and then subjected to mild sonication (30 Hz for 15 s; 3 cycles with 30 s intervals under cold) and centrifuged at 750 *g* for 3 min. at 4°C and the supernatant used for immunoblot analyses.

Experiment 2

Female B6/SJL Tg2576 mice (Taconic, Germantown, NY, USA) housed in the Animal Resource Center at Case Western Reserve University (Cleveland, OH, USA) were provided *ad libitum* access to food and water and maintained on a 12 h light: 12 h dark photoperiod. All procedures performed on animals were reviewed and approved by the Animal Resource Center of Case Western Reserve University.

Mice at ~7.5 months of age were either bilaterally sham OVX or OVX and treated with or without leuprolide acetate for 9 months [until ~16.5 months of age (Wilson *et al.* 2008)]. OVX mice were left untreated or injected with leuprolide acetate, a well-known GnRH agonist (Lupron Depot; TAP Pharmaceuticals Inc., Lake Forest, IL, USA) which lowers LH/follicle-stimulating hormone (FSH) levels by desensitizing GnRHR signaling that subsequently leads to decreased LH/FSH secretion and a decrease in the serum concentration of sex steroids. Animals were injected intramuscularly with vehicle or leuprolide acetate (1.5 mg/kg) biweekly during the first month and monthly thereafter up to 9 months. Leuprolide acetate was mixed with a diluent in a pre-filled dual-chamber syringe containing sterile lyophilized microspheres to form a suspension. Leuprolide acetate is gradually released from microspheres over a 4-week period. Continuous treatment produces initial stimulation of the pituitary and an increase in blood gonadotropins, followed by a suppression of blood gonadotropins and sex steroids to castrate/post-menopausal levels within ~1 week. In females, both ovarian estrogen and androgen synthesis are inhibited. Using this paradigm, it is possible to assign changes in endpoints to changes in LH/FSH or sex steroids.

Mice were killed at 16 months of age with an injection of concentrated sodium barbital (50 μL each, 392 mg/mL, i.p.). Blood was collected at this time by heart puncture, the animals were then perfused with PBS (pH 7.4; Gibco), the animals decapitated and the brain removed from the skull. One brain hemisphere was frozen in liquid nitrogen and stored at −80°C prior to homogenization on ice in lysis buffer [20 mmol/L Tris–HCl, pH 7.6, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% SDS, and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA)]. The homogenate was then centrifuged for 10 min. at 10 000 *g* at 4°C and used for immunoblot analysis. Blood (100–400 μL) was collected into EDTA-coated tubes, which were centrifuged at 1000 *g* for 3 min. at 4°C, followed by centrifugation at 5000 *g* for 10 min. prior to the collection of plasma, which was stored at −80°C prior to analysis of gonadotropin levels (see Wilson *et al.* 2008 for details).

Immunoblot analyses

Following protein assay (Bicinchoninic Acid Protein assay kit; Pierce, Rockford, IL, USA) of samples, equal amounts of protein were loaded onto 10–20% tricine gels (Invitrogen, Carlsbad, CA, USA) for SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was fixed with glutaraldehyde (4%, v/v in tris-buffered saline with 0.1% Tween 20 for 30 min), blocked with milk (10%, w/v in tris-buffered saline with 0.1% Tween 20 for 2 h) and then probed with a rabbit polyclonal antibody against human StAR (1 : 1000; Dr Jerome Strauss, PA, USA), a goat polyclonal antibody against human β-actin (1 : 1000; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), a goat polyclonal antibody against GAPDH (1 : 1000; Santa Cruz Biotechnologies), a mouse monoclonal antibody against human GnRHR (1 : 900; a kind gift from Anjali Karande, Department of Biochemistry, Indian Institute of Science, Bangalore, India) and a rabbit polyclonal estrogen receptor (ERα; 1 : 500; Santa Cruz Biotechnologies) antibody overnight at 4°C. The blot was then incubated with the corresponding horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at 22°C, washed and developed with enhanced chemiluminescence reagent (Santa Cruz Biotechnology) as per the manufacturer's instructions. The chemiluminescent signal was captured on autoradiographs (Eastman Kodak, Rochester, NY, USA), which were scanned and the intensity of the autoradiograph signals (including a blank region) was determined using the NIH Image J Software (http://rsb.info.nih.gov/nih-image/). Control and treatment values were corrected for blank values, normalized to their respective β-actin band intensity and the results then expressed as mean ± SEM.

Statistical analyses

Statistical analysis was performed using one-way ANOVA followed by pair-wise comparisons with Fisher's protected least significant difference procedure to determine significant changes between treatment groups (Statview 5.0 & SuperAnova 3.0 programs; SAS Institute, Inc., Cary, NC, USA).

Results

Gonadal sex steroid production is controlled by feedback mechanisms within the HPG axis that tightly regulate cholesterol transport mediated by the StAR protein (reviewed in Miller 2007). Our recent studies in rat primary neurons and neuroblastoma cells indicate that LH induces NSS

production by increasing mitochondrial cholesterol transport by StAR protein and cytochrome P450 cholesterol side chain cleavage enzyme-mediated cleavage of cholesterol for NSS synthesis and secretion (Liu *et al.* 2007). This raised the question as to whether NSS production is regulated by autocrine/paracrine mechanisms within the brain or by a peripheral endocrine mechanism.

Hormonal regulation of brain StAR expression and processing

To determine if a similar feedback mechanism exists for the regulation of NSS synthesis in the brain, we treated 3-month-old ovariectomized female mice with and without E_2, P_4, or $E_2 + P_4$ for 3 days. Brain homogenates (excluding the hypothalamic region) were initially probed for StAR protein, an index of NSS synthesis (King *et al.* 2002). The 37-, intermediate 32- and mature 30-kDa forms of StAR were identified as per our previous analyses of StAR in human neuroblastoma cells, primary rat neurons (Liu *et al.* 2007) and human embryonic stem cells (Gallego *et al.* 2008). The brain expression of the 37-kDa StAR variant did not change markedly following OVX, indicating that decreased plasma sex steroids (and increased plasma gonadotropins) do not affect the brain expression of the 37-kDa StAR variant (Fig. 1). However, E_2 and/or P_4 treatments significantly increased the brain expression of the 37-kDa StAR variant (Fig. 1b). This increase in brain expression of the mature StAR variant appeared to be due to the suppression of the truncation of the 37-kDa StAR variant, as indicated by the slight attenuation in the expression of the 30-kDa and complete suppression of the 32-kDa variants with sex steroid treatment compared to OVX controls (Fig. 1b–d). As truncation of the 37-kDa variant to the mature 30-kDa variant of StAR is associated with cholesterol transport across the mitochondrial membrane (Stocco and Chen 1991; Stocco and Sodeman 1991; Yamazaki *et al.* 2006; Levine *et al.* 2007; Bose *et al.* 2008) these results suggest that peripheral sex steroids, or the down-regulation of gonadotropin production by exogenous sex steroids, regulates StAR-mediated uptake of cholesterol for NSS synthesis. Either way, these results indicate the presence of an endocrine feedback loop that regulates NSS synthesis.

To determine whether gonadotropins regulate StAR expression in the brain as implicated in the gonads (Pescador *et al.* 1997; Amsterdam *et al.* 2003; Tajima *et al.* 2003), or the processing of the protein, 7-month-old mice were either sham-operated (control), OVX (low plasma sex steroids, high gonadotropins) or OVX and treated with leuprolide acetate, a potent GnRH agonist (low plasma sex steroids and gonadotropins) for 9 months. Plasma collected at 16 months of age from these mice demonstrated that OVX significantly increases the concentration of plasma LH 8.4-fold (0.59 ± 0.08 ng/mL vs. 4.92 ± 0.65 ng/mL) and FSH 10.9-fold (4.8 ± 1.0 ng/mL vs. 52.6 ± 3.1 ng/mL) and that treatment of OVX mice with leuprolide acetate attenuated the OVX-induced increase in LH (0.70 ± 0.05 ng/mL) and FSH

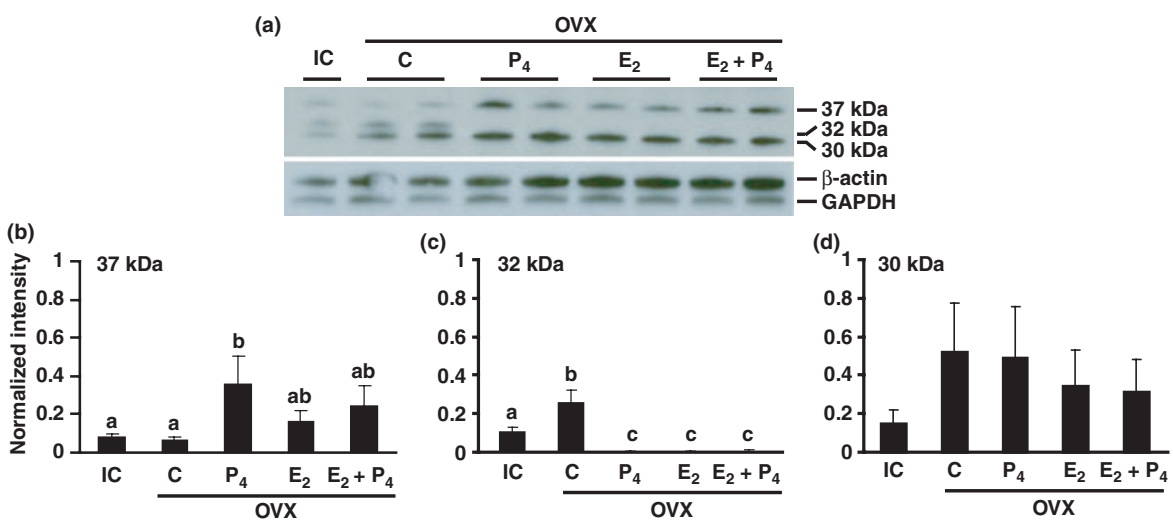

Fig. 1 Ovariectomy and treatment of mice with sex steroids modulates StAR variant expression in the extra-hypothalamic brain. Female mice (3 months of age) were either left intact (IC), or OVX and then s.c. administered with a pellet of cholesterol (control – C), estrogen (E_2), progesterone (P_4) or $E_2 + P_4$ for 3 days. (a) Brains were removed minus the hypothalamic region and immunoblots of homogenized brain tissue were probed with a rabbit polyclonal antibody against human StAR, a goat polyclonal antibody against human β-actin and a goat polyclonal antibody against GAPDH. Expression was quantified and normalized against β-actin for StAR 37-kDa (b), 32-kDa (c), and 30-kDa (d) variants. Results are presented as mean ± SEM; $n = 3$–4; significant differences between groups are indicated by *different* letters, $p < 0.05$, similar letters indicate no differences between groups. Quantitation against GAPDH gave similar results (data not shown).

(8.4 ± 1.0 ng/mL) as we have previously reported (Wilson *et al.* 2008). Age-related changes between 7 and 16 months of age in plasma LH (0.44 ± 0.11 ng/mL vs. 0.96 ± 0.07 ng/mL; 2.2-fold) and FSH (7.8 ± 2.2 ng/mL vs. 12.9 ± 2.2 ng/mL; 1.6-fold) were suggestive of partial ovarian function, as gonadotropin concentrations were significantly less than following OVX. Consistent with the results of Fig. 1, there was a trend towards an increase in the expression of the 30- and 32-kDa StAR variants following OVX in these older mice that correlated with the increase in plasma gonadotropins (Fig. 2), suggesting that the increase in plasma gonadotropins slightly increased cholesterol transport into the mitochondrion for NSS synthesis. However, OVX mice treated with leuprolide acetate displayed a marked and significant decrease in the brain expression of the 30-kDa StAR truncated variant and a non-significant trend towards a decrease in the expression of the 32-kDa variant. No change was observed in the expression of the 37-kDa StAR variant. These results suggest that suppressing plasma gonadotropin levels decreases cholesterol transport into the mitochondria for NSS synthesis. Together, these indirect means of manipulating plasma sex hormones suggest that brain StAR is differentially processed depending upon the concentration of plasma sex steroids and gonadotropins.

Hormonal regulation of brain GnRHR expression

As gonadotropin production is regulated by GnRH signaling in the pituitary (Kaiser *et al.* 1995), we examined how changes in plasma gonadotropins with OVX and sex steroid treatment affects brain GnRHR expression. GnRHR expression has previously been demonstrated in the rodent (Leblanc *et al.* 1988) and human (Wilson *et al.* 2006) brains and GnRH signaling induces neuronal LH expression (Wilson *et al.* 2006). Using the brain homogenates utilized in Fig. 1, we probed an immunoblot with the mouse monoclonal antibody against human GnRHR (Karande *et al.* 1995). There was a dramatic decrease in the brain expression of GnRHR variants, including the 64-kDa mature variant and 55- and 44-kDa truncated variants, following OVX (Fig. 3). We found similar expression pattern for the 64-kDa GnRHR variant using a anti-human GnRHR polyclonal antibody (data not shown). As there are large elevations in GnRH production by the hypothalamus post-reproduction in humans and monkeys (Hall *et al.* 2000; Gore *et al.* 2004), these results are likely explained by dramatic elevations in brain GnRH production following OVX. Levels of these GnRHR variants were further decreased upon administration to OVX mice of E_2, P_4, or E_2 + P_4 for 3 days as seen in pituitary cells (Cheon *et al.* 2001). In contrast, OVX and OVX plus sex steroid treatment induced the reverse expression pattern for the 30-kDa GnRHR variant that has previously been reported in the human brain (Wilson *et al.* 2006). These results suggest that peripheral sex steroids (and/or gonadotropins) can feedback to regulate brain GnRHR levels, as previously observed for hippocampal GnRHR (Badr *et al.* 1988; Jennes *et al.* 1995) and hypothalamic GnRH (Lindzey *et al.* 1998; Colledge 2008).

Hormonal regulation of brain ERα expression

Sex steroids down-regulate GnRHR expression in the extra-hypothalamic regions of the brain (Fig. 3), and it is known that sex steroids down-regulate GnRHR expression in the

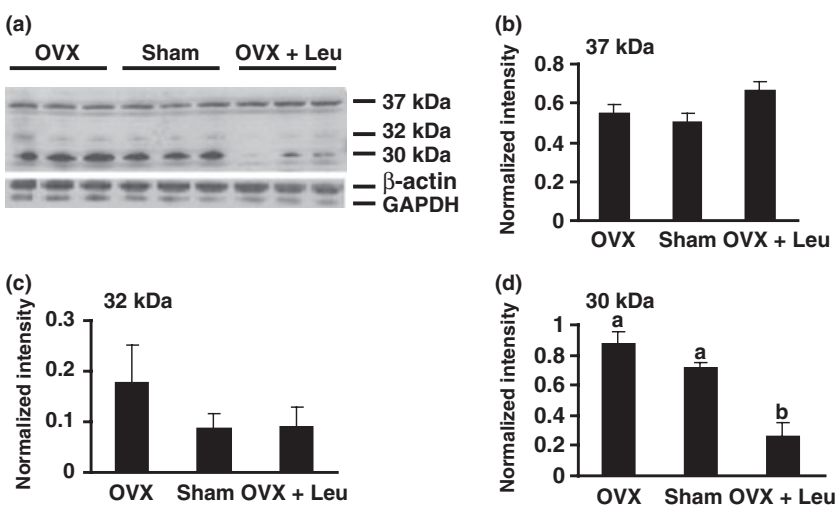

Fig. 2 Leuprolide acetate treatment of mice decreases StAR processing for the transport of mitochondrial cholesterol in the extra-hypothalamic brain. Female mice (~7.5 months of age) were either SHAM OVX or OVX and treated with or without leuprolide acetate for 9 months as described in the 'Methods'. (a) Brains were removed and immunoblots of homogenized brain tissue were probed using antibodies against human StAR, β-actin and GAPDH as described in Fig. 1. Expression was quantified and normalized against β-actin for StAR 37-kDa (b), 32-kDa (c) and 30-kDa (d) variants. The quantitation results are presented as mean ± SEM; $n = 3$; significant differences between groups are indicated by *different* letters; $p < 0.05$.

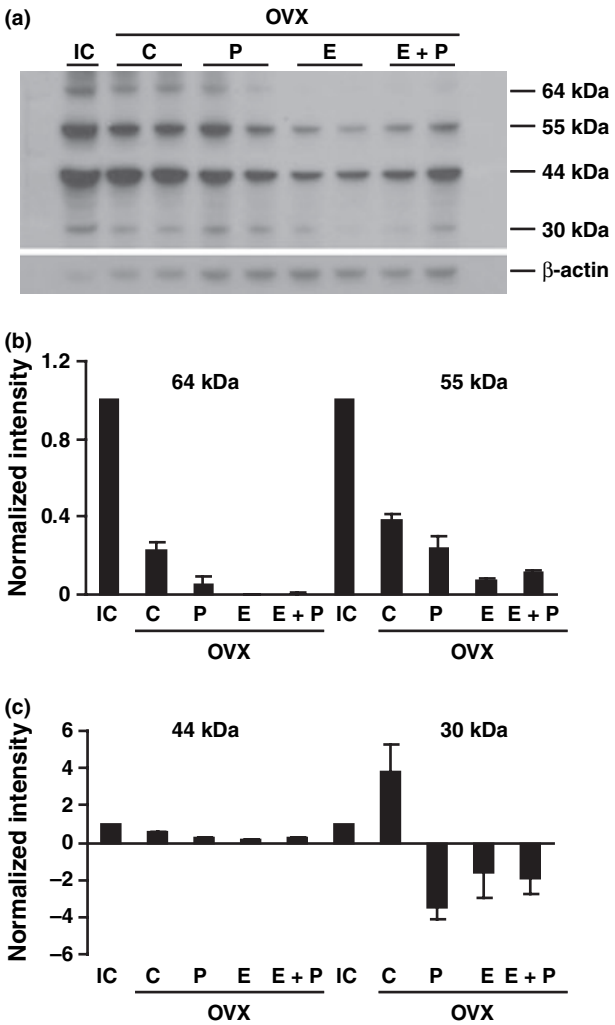

Fig. 3 Ovariectomy and treatment of mice with sex steroids modulates GnRHR expression in the extra-hypothalamic brain. (a) Immunoblots of brain homogenates from female mice described in Fig. 1 (intact control, OVX + C, E_2, P_4, or E_2 + P_4 for 3 days) were probed with a mouse monoclonal antibody against the N-terminus of human GnRHR, and antibodies against human β-actin and GAPDH. GnRHR expression was quantified and normalized against β-actin (43-kDa) for the (b) 64-kDa and 55-kDa, and (c) 44- and 30-kDa variants. Results are presented as mean ± SEM.

hypothalamus via signaling through sex steroid receptors. As ERα, but not ERβ, is indispensable to the negative-feedback effects of E_2 that maintain proper LH secretion from the pituitary (Couse *et al.* 2003), we examined whether peripheral changes in serum sex steroids altered ERα expression in extra-hypothalamic regions of the brain. OVX induced a non-significant decrease in the expression of ERα in the extra-hypothalamic brain (Fig. 4) and a significant decrease in the expression of ERα (37-kDa) in the brain with administration of E_2. Likewise, P_4 also significantly decreased the expression of extra-hypothalamic ERα

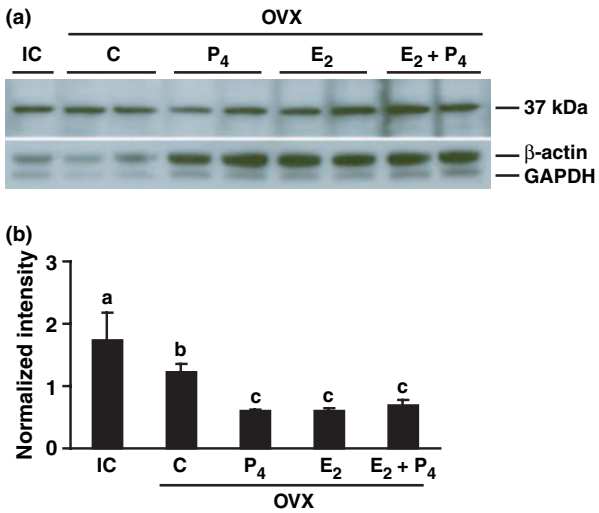

Fig. 4 Ovariectomy and treatment of mice with sex steroids modulates ERα expression in the extra-hypothalamic brain. (a) Immunoblots of brain homogenates from female mice described in Fig. 1 (intact control, OVX + C, E_2, P_4, or E_2 + P_4 for 3 days) were probed with a rabbit polyclonal antibody against human ERα, and antibodies against human β-actin and GAPDH. (b) ERα expression (37-kDa) was quantified and normalized against β-actin (43-kDa). Results are presented as mean ± SEM; significant differences between groups are indicated by *different* letters; $p < 0.05$.

(Fig. 4). These results indicate ligand-dependent regulation of the ERα as part of a negative feedback loop in the brain to regulate NSS synthesis based on the local/circulating sex steroid levels.

Quantitation of protein expression against GAPDH (data not shown) gave statistically similar results to protein expression quantitated against β-actin (Figs 1–4).

Discussion

This study demonstrates the existence of endocrine feedback pathways that regulate the local production of neurosteroids in the brain. These feedback pathways involve (i) the feedback of sex steroids produced by the gonads and (ii) the feedback of gonadotropins produced by the pituitary, on neurosteroid production in the brain. Our data indicate that E_2, P_4, or E_2 + P_4 potently suppresses the expression of brain ERα and GnRHR, and inhibits the processing of StAR for the transport of cholesterol into the mitochondrion (Figs 1–4), supporting the existence of a feedback loop that regulates GnRH and LH signaling for neurosteroid production (a Gonadal-Brain Neurosteroid Feedback Axis).

Our results indicate that gonadotropins regulate the processing of StAR protein in the brain for the transport of cholesterol into neuronal mitochondria (Fig. 2). OVX, which leads to decreased plasma sex steroids but increased plasma gonadotropins in both young (3-month) and older mice (16-month), increased the processing of StAR.

Conversely, suppression of gonadotropins with leuprolide acetate decreased the processing of StAR. The down-regulation in the processing of brain StAR following elevation of peripheral E_2 and P_4 (Fig. 1) is strongly supportive, as sex steroids negatively feedback on the hypothalamus/pituitary to suppress pituitary production of gonadotropins and their section into the circulation (Gharib *et al.* 1986; Papavasiliou *et al.* 1986; Caraty *et al.* 1989; Shupnik 1996; Wilson *et al.* 2008). These results are consistent with our previous studies demonstrating that leuprolide acetate suppresses the expression of the 30-kDa StAR variant in mouse brain, although in these intact animals the 37-kDa variant also was decreased (Liu *et al.* 2007). That LH regulates the expression and/or processing of StAR in the brain is supported by our *in vitro* studies showing LH increases the expression of StAR in human neuroblastoma cells and rat primary neurons (Liu *et al.* 2007). These findings parallel earlier studies in reproductive tissues demonstrating LH/human chorionic gonadotropin up-regulates StAR mRNA and/or protein expression in ovine luteal tissue (Juengel *et al.* 1995), human corpus luteum (Chung *et al.* 1998), mouse leydig tumor cells (Manna *et al.* 1999a,b), monkey granulosa cells (Chaffin *et al.* 2000) and porcine granulosa cells (Sekar *et al.* 2000a,b). Whether the GnRH agonist-induced suppression of StAR processing (Fig. 2) is a result of decreased peripheral gonadotropin transport into the brain, or of decreased brain gonadotropin expression, remains to be determined.

We also found that increasing peripheral E_2 and P_4 down-regulates ERα expression (Fig. 4), however previous workers have found a brain specific increase in ERα, but not ERβ, protein in the CA1 region of the hippocampus following E_2 treatment (Miller *et al.* 2005). E_2 elicits negative feedback inhibition of hypothalamic GnRH and pituitary LH release via ERα (Dorling *et al.* 2003), possibly as a result of E_2 modulation of GnRHR expression (Kaiser *et al.* 1993). In our study, E_2 and P_4 treatment of OVX animals induced a decrease in the expression of all GnRHR variants in the brain (Fig. 3) as previously reported for GnRHR mRNA and protein in the rat hippocampus (Badr *et al.* 1988; Jennes *et al.* 1995). It is not clear why OVX treatment also induced a decrease in brain GnRHR variants 64-, 55-, and 44-kDa, when one might expect a compensatory increase under conditions of low sex steroids. One possible explanation is that elevated GnRH levels post-OVX suppress GnRHR expression, as reported *in vitro* and *in vivo* following continuous GnRH exposure (Norwitz *et al.* 1999; Weiss *et al.* 2006). GnRHR expression may be sensitive to both sex steroid and GnRH concentration. GnRHR expression and signaling could therefore be modulated by endogenous NSS and GnRH synthesis as a result of high or low peripheral sex steroid or gonadotropin concentrations. *In vitro* data suggest the potential for GnRH signaling in the modulation of brain E_2 synthesis. Recently, Prange-Kiel and colleagues (Prange-Kiel *et al.* 2008) reported that E_2 synthesis, spine synapse density, and immunoreactivity of spinophilin, a reliable spine marker, are consistently up-regulated in a dose-dependent manner at low doses of GnRH but decrease at higher doses in hippocampal cultures.

The experiments conducted in this study examining StAR, GnRHR, and ERα expression in the extrahypothalamic hemisphere of a mouse brain clearly indicate that peripheral sex steroids and gonadotropins can modulate the expression and processing of StAR, GnRHR, and ERα. These results may not however be indicative of regional changes given our knowledge of the regional expression of proteins involved in NSS synthesis (see Appendix S1). Future studies using larger animals will determine how peripheral (and local) changes in sex hormones modulate regional NSS synthesis.

It is not clear whether endogenously produced NSS (Prange-Kiel *et al.* 2003; Hojo *et al.* 2004; Kretz *et al.* 2004; Liu *et al.* 2007) also can feedback to modulate brain production of NSS, however these data support the possibility of a brain axis that regulates NSS production. In support of this, we and others have previously shown that hippocampal and cortical neurons possess GnRHR (Wilson *et al.* 2006) and LH receptors (Lei *et al.* 1993; Liu *et al.* 2007) and that cultured neurons respond to GnRH and LH to induce LH expression (Wilson *et al.* 2006), StAR expression and NSS production (Liu *et al.* 2007), respectively. Moreover, other HPG axis components have been identified in the brain, including (i) kisspeptin and G-protein coupled receptor 54 (Messager 2005; Messager *et al.* 2005; Smith 2008), (ii) GnRH secreting neurons with efferent pathways into different regions of the brain including the hippocampus (Merchenthaler *et al.* 1984), (iii) LH (Bowen *et al.* 2002), (iv) StAR (Furukawa *et al.* 1998; King *et al.* 2002; Sierra *et al.* 2003; Sierra 2004; Webber *et al.* 2006; Liu *et al.* 2007), (v) enzymes of the steroidogenic pathways (Yu *et al.* 2002), and (vi) sex steroids (Compagnone and Mellon 2000; Baulieu *et al.* 2001). These data together indicate NSS synthesis could be regulated by autocrine/paracrine mechanisms as well as the endocrine mechanisms described above. This brain NSS feedback axis may be crucial for regulating the concentration of hippocampal NSS production required for normal neuron growth, development and function, including during the menstrual cycle.

As detailed above, brain sex steroid levels are influenced by circulating levels of sex hormones which vary depending on the reproductive state of the organism throughout life. The concentration of tissue and circulating sex hormones are known to change during development, puberty (Foster *et al.* 2000), the menstrual cycle, pregnancy (Shang *et al.* 2003), following castration, menopause, or andropause (Bowen and Atwood 2004) as well as with certain medical conditions such as polycystic ovary syndrome (Eldar-Geva *et al.* 2001). The post-reproductive changes in sex hormones are

particularly important because of their association with disease (Bowen and Atwood 2004). With menopause, the lack of negative feedback from the ovary (both E_2 and inhibin) is responsible for the unopposed elevation of GnRH release (Hall *et al.* 2000; Gore *et al.* 2004) and gonadotropin secretion following ovarian senescence leading to a three- to fourfold and a four- to 18-fold increase in the concentrations of serum LH and FSH, respectively (Chakravarti *et al.* 1976). Men also experience a greater than twofold, and threefold, increase in LH and FSH, respectively, as their reproductive function deteriorates (Neaves *et al.* 1984) during andropause. The influence of such increases in hypothalamic GnRH and pituitary gonadotropin production to the production of NSS is unclear. Hypothalamic GnRH production is most likely to enter other regions of the brain via efferent pathways; the short serum half-life of GnRH (~2–3 min.) (Redding *et al.* 1973; Fauconnier *et al.* 1978) would support autocrine release of GnRH within the brain, although it has been shown in rats that GnRH can cross the BBB (Dvorska *et al.* 1992). Likewise, gonadotropins synthesized by the pituitary enter the circulation and can cross the BBB (Toth *et al.* 2001), but it is possible they may also be transported retrogradely into the brain and bind neuronal LH/FSH receptors.

The above considerations are important for understanding how NSS concentrations are regulated. There are few studies that have assessed how peripheral sex steroid concentrations may influence the concentration of brain sex steroids and NSS production. In one study of mice that were OVX at 3 months of age, brain E_2 levels were found to remain at pre-OVX levels at 6 months despite the OVX-induced decrease in peripheral sex steroids (Yue *et al.* 2005). This maintenance of brain sex steroid levels in young mice despite low peripheral sex steroids is possibly because of an increased production of NSS as a result of OVX-induced increases in the production of circulating gonadotropins (that cross the BBB) (Bronson 1981; Naik *et al.* 1984) or neuronal gonadotropins (Wilson *et al.* 2006). However, in another study in aging men, brain testosterone concentrations have been shown to progressively decrease (Rosario *et al.* 2004) as serum LH levels increase with age. NSS concentrations also are decreased in elderly women with Alzheimer's disease versus age-matched controls even though serum E_2 levels are unchanged (Yue *et al.* 2005) and serum LH levels are elevated (Short *et al.* 2001) in such individuals. One possible explanation for the differences between these studies is neuronal reserve and/or the neuronal capacity to synthesize NSS at different ages. In the study by Li and colleagues (Yue *et al.* 2005), neurosteroid concentrations were measured in 6-month-old mice (i.e. relatively young animals), whereas in the human studies neurosteroid concentrations were measured in aged human brains, where both neuronal number and the capacity for NSS production may be decreased. An alternate explanation is that as the concentration of brain E_2 did not change in male subjects between 50 and 100 years of age (Rosario *et al.* 2004), brain testosterone may be derived largely from peripheral sources in comparison to E_2 and P_4. The interpretation of the above results is further complicated by the methodologies used to measure brain sex steroid concentrations and further studies are therefore warranted to determine whether peripheral changes in GnRH/gonadotropins impact NSS production differently throughout life. Our approach of examining changes in the expression pattern of brain StAR, GnRHR, and ER will be useful in future studies to examine the impact of peripheral changes in sex steroids, GnRH and gonadotropins on NSS production, and also in understanding the molecular mechanisms regulating the molecular cross-talk between peripheral sex hormones and NSS synthesis in balancing total brain sex steroid concentrations.

The contribution of NSS to total brain sex steroid content is unknown, however it is expected that following menopause and during andropause the contribution of NSS to total brain sex steroids will increase, especially as there are increases in hypothalamic GnRH and pituitary gonadotropin production that might be expected to promote NSS synthesis. Those individuals that can produce more NSS (and presumably have lower gonadotropin levels) would be expected to offset neurodegenerative changes longer than those whose innate NSS production is lower (and gonadotropin levels are higher), and this might be reflected in the concentrations of both NSS (and gonadotropins) in the brain and in the bloodstream. Certainly lower serum E_2 (Manly *et al.* 2000) and elevated serum LH (Bowen *et al.* 2000; Hogervorst *et al.* 2001; Rodrigues *et al.* 2008) are significantly associated with cognitive decline and Alzheimer's disease, i.e. a more dysregulated HPG axis correlates with neurological disease.

Hormone replacement therapy composed of E_2 and/or P_4 (the natural female sex steroids) might be expected to increase total brain sex steroid concentrations but decrease NSS synthesis as suggested by the experiments in this study (Figs 1 and 2). Administration of human sex steroids also would be predicted to decrease brain/pituitary gonadotropin/GnRH production. The requirement for normal adult concentrations of HPG axis hormones for normal brain health is strongly supported by the findings that physiological sex steroid replacement therapies enhance cognition in elderly post-menopausal women and decrease the incidence, and delay the onset, of cognitive decline among women following menopause (reviewed in Gleason *et al.* 2005). In elderly cognitively normal post-menopausal women, 13 studies indicate that *physiological* sex steroids (i.e. E_2) are important for maintaining or promoting cognitive health. In elderly demented subjects, all three studies where *physiological* estrogen (i.e. E_2) has been administered to individuals also have demonstrated improved cognitive function (Jacobs *et al.* 1998; Tan and Pu

2003; Gleason *et al.* 2005). Surprisingly, the effects of P_4 on cognition in normal or cognitively impaired post-menopausal women has not been studied despite its well documented neuroprotective functions in acute neurological diseases such as traumatic brain injury and stroke (Stein *et al.* 2008).

It is hard to predict how the axes that regulate NSS synthesis may respond to hormone replacement therapies utilizing conjugated equine estrogens (CEE) extracted from mare urine (e.g. Premarin). On the one hand, CEEs may antagonize further the already reduced E_2 signaling via ERs after menopause, and this is strongly supported by elevations (not decreases!) in the concentration of serum LH following high-dose CEE administration (Aono *et al.* 1977; Furuhashi *et al.* 1977). On the other hand, CEE-induced elevations in pituitary (and brain?) LH production might be expected to increase NSS synthesis, but whether this increased synthesis of NSS can reverse CEE antagonism of the ER is unclear and will be dependent upon the concentration of CEE used. Indeed, at lower doses, CEE decrease the concentration of serum gonadotropins (Dick *et al.* 1995; Blum *et al.* 1996; Uhler *et al.* 1998; Baracat *et al.* 1999; Yasui *et al.* 2001). This would presumably decrease NSS synthesis, and limit E_2 signaling (due both to the lower E_2 synthesis rate and the CEE antagonism of the ER). These conclusions are however complicated by the fact that CEEs increase serum [E_2]. Administration of 0.625 mg/day CEE increases serum [E_2] ~5-fold: ~4 pg/mL to 21 mg/mL; and [estrone] ~6-fold: ~22 pg/mL to 130 pg/mL (Yasui *et al.* 1999, 2001). The lower relative increase in serum [E_2] compared with [estrone] is likely insufficient to offset the ER antagonism by the CEE (the affinity of estrone sulfate for ER is 60% that of E_2)

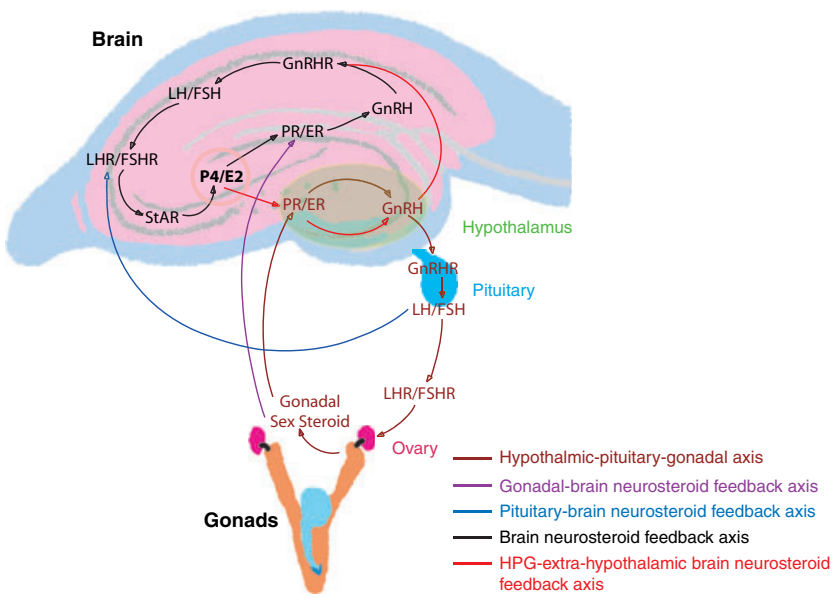

Fig. 5 A model of the endocrine with paracrine/autocrine regulatory feedback loops for the regulation of neurosteroid synthesis in the brain. Based on the dynamic expression changes in hormone receptors and StAR determined in this study and on the immunocytochemical localization of HPG axis components presented in the Appendix S1, we present a model of feedback pathways for regulating NSS production. The term 'brain' in each model refers to extrahypothalamic brain. *The Brain Neurosteroid Feedback Axis*: GnRH, synthesized by the hypothalamus (or extrahypothalamic brain) and transported via efferent pathways into the hippocampus signals via neuronal GnRHR to induce gonadotropin production which in turn signals via gonadotropin receptors on adjacent astrocytes, oligodendrocytes and neurons around the dentate gyrus to synthesize NSS. NSS feedback via ER present in the hippocampus to down-regulate GnRHR signaling. The level of signaling through autocrine/paracrine pathways will be dependent upon regional hormone receptor density (which will be dependent upon ligand regulated expression of these receptors). *The HPG-Brain Neurosteroid Feedback Axis*: Alternatively, NSS may feedback to the hypothalamus to modulate GPR54 signaling for hypothalamic GnRH production and brain (hippocampus) production of gonadotropins, or gonadotropin production by the pituitary. *The Gonadal-Brain Neurosteroid Feedback Axis*: Interconnected with the neurosteroid feedback axis, peripheral sex steroids can enter the brain and modulate GnRHR signaling and subsequently NSS synthesis (Figs 1 and 5b). *The Pituitary-Brain Neurosteroid Feedback Axis:* Also interconnected with the neurosteroid feedback axis, peripheral gonadotropins can enter the brain and modulate LH receptor (LHR) signaling and subsequently NSS synthesis. This cross-talk between the neurosteroid feedback axis and the HPG axis will impact NSS synthesis when peripheral concentrations of sex steroids and gonadotropins alter, such as during development, puberty, the menstrual cycle in women and during post-reproductive senescence. The scheme does not take into account the astroglial-neuron autocrine/paracrine unit that is likely the autocrine/paracrine feedback entity throughout the brain, but rather provides a global model of feedback axes that impact neurosteroid production. To simplify the figure we excluded kisspeptin/GPR54 signaling from the brain and the hypothalamus. The model is parsimonious with the bulk of the current data.

(Aono *et al.* 1977; Furuhashi *et al.* 1977). Irrespective, any therapy that decreases E_2 signaling or increases LH signaling is expected to promote neurodegeneration (reviewed in Atwood *et al.* 2005) and cannot be recommended for hormone replacement therapy in women. Indeed, recent, well-publicized studies have indicated that treatment with unphysiological sex steroids, such as CEE's extracted from urine, with or without the progesterone analog medroxyprogesterone (MPA), do not provide cognitive enhancement (reviewed in Turgeon *et al.* 2004; Gleason *et al.* 2005).

MPA is a synthetic progestin derived from 17α-hydroxyprogesterone that has clear differences in bioavailability, half-life, signaling pathways as well as possessing many non-progestagenic effects (Nilsen and Brinton 2003; Schindler *et al.* 2003; Wright *et al.* 2008). How MPA might alter neurosteroid production is unclear. MPA binds the androgen receptor, unlike P_4, and also activates glucocorticoid receptors at a much lower concentration than does P_4 (Schindler *et al.* 2003; Koubovec *et al.* 2005). MPA has clearly been shown to be ineffective as a neuroprotectant (Singh 2007). MPA fails to attenuate glutamate-induced increases in intracellular $[Ca^{2+}]$ and toxicity towards hippocampal neurons (Nilsen and Brinton 2002a,b; Nilsen *et al.* 2002). MPA, unlike P_4, blocks the beneficial effect of E_2 *in vitro* (Nilsen and Brinton 2002b). *In vivo*, P_4, when administered with E_2 protects against coronary vasopasm and myocardial ischemia, whereas MPA does not (Miyagawa *et al.* 1997; Rosano *et al.* 2000). Also unlike P_4, MPA does not provide protection from traumatic brain injury (Wright *et al.* 2008).

While our data indicate that the endocrine HPG axis can regulate neurosteroid production, whether the brain neurosteroid feedback axis regulates the reproductive HPG axis (the HPG-Brain Neurosteroid Feedback Axis) requires further investigation. In this respect, the production of neuroprogesterone by astrocytes (Zwain and Yen 1999; Sinchak *et al.* 2003; Micevych *et al.* 2007) located in the hypothalamus (Micevych *et al.* 2003) is thought to be required to trigger the LH surge (Micevych and Sinchak 2008). Thus, it is possible that brain NSS synthesis positively feedbacks on the HPG axis to regulate such processes as the pre-ovulatory surge of LH (e.g. Petersen *et al.* 2003; Herbison 2008).

Another implication of our results is that the change in expression of brain hormone receptors as a result of peripheral changes in sex steroids and gonadotropins may lead to altered signaling by those receptors involved in functions other than the regulation of NSS production. For example, the E_2 and P_4 induced decrease in GnRHR expression may modulate neuronal transmission, as GnRH1 and analogues can enhance synaptic transmission via ionotropic glutamate receptors (Lu *et al.* 1999). Similarly, P_4 induced suppression of ERα expression is consistent with previous findings in the rat uterus (Kraus and Katzenellenbogen 1993; Katzenellenbogen 2000), and indicate that cross-talk between these two sex steroids may play an important role in both the regulation of ERα signaling and progesterone receptor signaling in the brain.

Neurosteroids can modulate, either positively or negatively, neurotransmitter receptor-coupled ion channels such as $GABA_A$, NMDA, nicotinic acetylcholine, serotonin type 3, and can therefore directly affect synaptic activity and/or neuronal excitability. Given the importance of sex steroids to brain function (Compagnone and Mellon 2000), NSS production may be a mechanism to fine-tune the level of steroids in various regions of the brain.

Understanding the feedback loops in the brain that regulate NSS production has important implications for developing therapeutic strategies to maintain levels of brain sex steroids and cognition. Aside from the *Gonadal-Brain Neurosteroid Feedback Axis* discussed above, our findings that a GnRH agonist decreases StAR processing for the transport of cholesterol into the mitochondrion, supports the existence of a second feedback loop that regulates LH signaling for neurosteroid synthesis (*the Pituitary-Brain Neurosteroid Feedback Axis*). In addition, as these brain hormone receptors and StAR are regulated by peripheral sex steroids, it is likely they also respond to locally produced sex steroids, thereby suggesting the presence of a brain neurosteroid regulatory loop (*the Brain Neurosteroid Feedback Axis*) that is interconnected with the HPG axis to regulate NSS synthesis in the brain. Finally, the data also suggest that NSS could modulate HPG axis signaling (*the HPG-Extra-Hypothalamic Brain Neurosteroid Axis*). These feedback loops are presented schematically as a model in Fig. 5. Gonadotropin/sex steroid modulation of StAR processing also might be expected to alter corticosteroid production in the brain. Indeed, similar interconnected feedback loops might be expected for the hypothalamic-pituitary-adrenal axis, and for the regulation of steroid levels in other steroidogenic tissues of the body including the placenta.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Appendix S1 Description of studies relating to the localization of brain GnRH neurons and GnRH1, GnRH receptors, gonadotropins, gonadotropin receptors, StAR, sex steroids, sex steroid receptors, and kisspeptin and kisspeptin receptor.

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