ORIGINAL ARTICLE

Neuronal Gonadotrophin–Releasing Hormone (GnRH) and Astrocytic Gonadotrophin Inhibitory Hormone (GnIH) Immunoreactivity in the Adult Rat Hippocampus

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Gonadotrophin-releasing hormone (GnRH) and gonadotrophin inhibitory hormone (GnIH) are neuropeptides secreted by the hypothalamus that regulate reproduction. GnRH receptors are not only present in the anterior pituitary, but also are abundantly expressed in the hippocampus of rats, suggesting that GnRH regulates hippocampal function. GnIH inhibits pituitary gonadotrophin secretion and is also expressed in the hippocampus of a songbird; its role outside of the reproductive axis is not well established. In the present study, we employed immunohistochemistry to examine three forms of GnRH [mammalian GnRH-I (mGnRH-I), chicken GnRH-II (cGnRH-II) and lamprey GnRH-III (lGnRH-III)] and GnIH in the adult rat hippocampus. No mGnRH-I and cGnRH-II+ cell bodies were present in the hippocampus. Sparse mGnRH-I and cGnRH-II+ fibres were present within the CA1 and CA3 fields of the hippocampus, along the hippocampal fissure, and within the hilus of the dentate gyrus. No lGnRH-III was present in the rodent hippocampus. GnIH-immunoreactivity was present in the hippocampus in cell bodies that resembled astrocytes. Males had more GnIH+ cells in the hilus of the dentate gyrus than females. To confirm the GnIH+ cell body phenotype, we performed double-label immunofluorescence against GnIH, glial fibrillary acidic protein (GFAP) and NeuN. Immunofluorescence revealed that all GnIH+ cell bodies in the hippocampus also contained GFAP, a marker of astrocytes. Taken together, these data suggest that GnRH does not reach GnRH receptors in the rat hippocampus primarily via synaptic release. By contrast, GnIH might be synthesised locally in the rat hippocampus by astrocytes. These data shed light on the sites of action and possible functions of GnRH and GnIH outside of the hypothalamic-pituitary-gonadal axis.

Key words: astrocyte, oestradiol, LHRH, neurosteroid, progesterone, RFRP-3, sex difference, testosterone

Gonadotrophin-releasing hormone (GnRH) has long been known as the master hormone of reproduction and regulator of the hypothalamic-pituitary-gonadal (HPG) axis (1). In all vertebrates, the brain expresses multiple forms of GnRH (2). In mammals, the reproductive role of GnRH in the HPG axis is carried out by mammalian GnRH-I (mGnRH-I). mGnRH-I is produced in neurosecretory cells of

the medial preoptic area (mPOA) of the hypothalamus. These cells have terminals within the median eminence that release mGnRH-I into the hypophyseal portal system. mGnRH-I then travels to the anterior pituitary and binds to GnRH receptors (GnRHR), stimulating the release of the gonadotrophins, luteinising hormone (LH) and follicle-stimulating hormone (FSH) (3). LH and FSH travel to the

gonads, where they stimulate the production of sex steroids, such as oestradiol and testosterone, and promote maturation of gametes. A second form of GnRH, chicken GnRH-II (cGnRH-II), is expressed primarily in mesencephalic neurons and is highly conserved across vertebrates (2,4,5). The primary role of cGnRH-II is unclear, although it appears to chiefly regulate reproductive and feeding behaviours, rather than pituitary gonadotrophin release (6,7). Lamprey GnRH-III (lGnRH-III) is a more recently identified form of GnRH, first identified in teleost fish (8). lGnRH-III expression has been reported in the rat hypothalamus (9,10); however, lGnRH-III does not appear to be a robust stimulator of mammalian gonadotrophin release (11–13). Previous work from our group has demonstrated lGnRH-III expression in the songbird hippocampus (14), although this has never been examined in the rodent hippocampus.

Several lines of evidence suggest that GnRH modulates hippocampal function and plasticity. First, the hippocampus has the highest density of GnRHR within the brain; GnRHR in the rat hippocampus are concentrated in the stratum oriens and stratum radiatum of the CA1–4 fields (15). Rat hippocampal GnRHR mRNA expression is developmentally discrete from that of pituitary GnRHR, supporting a distinct role of hippocampal GnRHR from that of pituitary GnRHR (16). Second, GnRH administration up-regulates two markers of synaptic plasticity, synaptophysin and spinophilin, in rat hippocampal culture (16), increases dendritic spine density (17), and increases neural excitation via actions on ionotropic glutamate receptors (18,19). Third, GnRH administration up-regulates hippocampal oestradiol synthesis via actions on aromatase (17,20) and GnRHR colocalises with ERβ in CA1–4 (21). It is therefore possible that GnRH exerts neuromodulatory actions on the hippocampus through site-specific regulation of oestradiol, which has a well-established role in hippocampal neuroplasticity and neuroprotection (22). Taken together, these data strongly suggest that GnRH plays a neuromodulatory role in mammalian hippocampal function (23). However, previous immunohistochemistry studies have failed to provide robust evidence for endogenous GnRH+ cells or fibres in the mammalian hippocampus (4,24–27). This may be because previous studies did not examine multiple forms of GnRH in mammals, or it may be because GnRH is synthesised elsewhere in the brain and transported to the hippocampus via an alternate route; for example, via cerebrospinal fluid (CSF) (28).

Gonadotrophin inhibitory hormone (GnIH) is a neuropeptide recently identified in the quail brain (29). Subsequent to its discovery in quail, GnIH has been identified in a variety of vertebrates, including rats (30,31), although the roles of GnIH within the rodent brain are not yet well characterised. GnIH is produced by the hypothalamus (32,33); in avian models, it is released via the median eminence and acts on the anterior pituitary to suppress the synthesis and release of pituitary gonadotrophins (34–36). Whether GnIH negatively regulates the release of pituitary gonadotrophins in mammals remains unclear (31,37–40). In rats, GnIH+ cell bodies are present in the dorsomedial hypothalamus (31,37) and GnIH appears to directly inhibit GnRH secretion by neurosecretory cells of the mPOA (40–42). GnIH mRNA is expressed throughout the mammalian and avian brain, with robust expression in the hippocampus (43), and there is some evidence of GnIH+ fibres in the CA3 field of

the rat hippocampus (31). GnIH, similar to GnRH, may be involved in modulation of hippocampal plasticity, although there is a need to more fully characterise GnIH distribution in the rodent hippocampus.

The present study employs immunohistochemistry to characterise the distribution of mGnRH-I, cGnRH-II, lGnRH-III and GnIH in the adult rat hippocampus. We examined the distributions of GnRH and GnIH within (i) the hippocampus (CA1, CA3, hippocampal fissure and dentate gyrus); (ii) hippocampus-related areas (the fimbria hippocampus and the parenchyma surrounding the cerebral ventricles); and (iii) a circumventricular organ, the subfornical organ (SFO). We first examined sex and regional differences in hippocampal GnRH and GnIH-immunoreactivity (-IR) (Study 1). We subsequently performed double-label immunofluorescence with confocal microscopy to examine colocalisation of GnIH with NeuN and glial fibrillary acidic protein (GFAP), aiming to to confirm the neuronal or astrocytic phenotype of GnIH+ cells in the hippocampus.

Materials and methods

Animals

Adult (9–10 weeks old) male and female Long-Evans rats (Charles River, Saint-Constant, Quebec, Canada) were housed in two colony rooms (separated by sex) under a 12 : 12 h light/dark cycle with access to food and water available *ad lib*. The animals were given standard lab chow (RatDiet 5012; LabDiet, St Louis, MO, USA) and were housed in groups of four in clear cages with aspen chip bedding. Animals were handled daily for 1 week prior to experimental protocols.

One set of males (n = 8) and females (n = 16) was used for the 3,3′-diaminobenzidine (DAB) immunohistochemistry study (Study 1). There were twice as many females as males to account for potentially greater variability in female data as a result of the oestrous cycle (44). A second set of males (n = 5) and females (n = 5) was used for the double-label immunofluorescence study (Study 2). Animal housing, handling and tissue preparation were performed identically for both sets of animals.

All protocols were approved by the UBC Animal Care Committee (A11-0339) and conformed to the regulations established by the Canadian Council on Animal Care. All efforts were made to reduce animal suffering in the experimental protocols.

Tissue preparation

Animals were overdosed with chloral hydrate (140 mg/kg) via i.p. injection. Complete induction of anaesthesia required approximately 7.5 min. A blood sample was collected via cardiac puncture; blood was later centrifuged, and plasma was stored at −20 °C. Animals were perfused with 0.9% saline (60 ml) and freshly made 4% paraformaldehyde (120 ml). Brains were extracted and post-fixed in 4% paraformaldehyde at room temperature for 4 h. Brains were placed in 30% sucrose until they sank (at 4 °C for 72 h), flash frozen on powdered dry ice and stored at −80 °C. Coronal sections (40 μm) were cut with a cryostat. Tissue was collected into eight series in antifreeze solution and stored at −20 °C.

Study 1: GnRH and GnIH immunohistochemistry with DAB

The primary antibodies used were HU60, anti-chicken GnRH-II (anti-cGnRH-II), anti-lamprey GnRH-III (anti-lGnRH-III) and PAC123/124. HU60 is a rabbit

polyclonal antibody raised against mGnRH-I. HU60 recognises the mature forms of mGnRH-I and cGnRH-II, with little to no cross-reactivity with immature GnRH fragments or similar peptides (45). Anti-cGnRH-II is a rabbit polyclonal antibody with high specificity for cGnRH-II (46). Anti-cGnRH-II does not recognise mGnRH-I or mGnRH-I fragments (46). Anti-lGnRH-III is a rabbit polyclonal antibody raised against lGnRH-III (47). Anti-lGnRH-III does not recognise mGnRH-I but may cross-react with somatostatin (48). PAC123/124 is a rabbit polyclonal antibody raised against white-crowned sparrow GnIH (29). PAC123/124 has been validated in a variety of mammalian models, including rats (37,49).

For preadsorption controls, primary antibodies were incubated with a 10-fold Molar excess of immunising peptides on an orbital shaker at room temperature for 2 h prior to incubation with sections. Preadsorbing peptides used were: LH-RH human (catalogue number 20781; Anaspec, Fremont, CA, USA), LHRH II (catalogue number H-4278; Bachem, Torrance, CA, USA), LHRH lamprey III (catalogue number H-4258; Bachem).

Sections were removed from antifreeze, placed in acrylic mesh well plates and washed in Tris-buffered saline (TBS; 9×5 min). Sections were immersed for 30 min in 0.5% sodium borohydride. Endogenous peroxidase activity was quenched with a 30-min incubation in 0.5% H_2O_2 (in methanol for HU60; in TBS for anti-cGnRH-II, anti-lGnRH-III and PAC123/124). Tissue was incubated in 10% normal goat serum (NGS) for HU60, 5% NGS for anti-cGnRH-II and anti-lGnRH-III, and 10% NGS for PAC123/124 in TBS containing 0.2% TritonX-100 (TBS-T) for 2 h. Immediately afterwards, tissue was incubated overnight at 4 °C in primary antibody (HU60, dilution 1 : 1600; anti-cGnRH-II, dilution 1 : 6400; anti-lGnRH-III, dilution 1 : 6400; PAC123/124, dilution 1 : 5000) in 2% NGS in TBS-T. Optimal primary antibody concentrations were empirically determined. Tissue was washed in TBS (9×5 min) and incubated overnight at 4 °C in biotinylated goat anti-rabbit IgG secondary antibody (dilution 1 : 2000; Vector Laboratories, Burlingame, CA, USA). Tissue was again washed in TBS (9×5 min) and incubated for 1 h in avidin-biotin complex (ABC Elite kit; Vector Laboratories) in TBS-T. Immunoreactivity was visualised using DAB (Vector Laboratories) for 5 min for HU60, 2 min for anti-cGnRH-II, 4 min for anti-lGnRH-III and 3 min for PAC123/124. Unless otherwise indicated, all tissue was washed (5×5 min) with TBS between every step of the protocol.

Immediately following visualisation, tissue was rinsed thoroughly in TBS (4×5 min) and float-mounted in deionised H_2O onto gelatin-coated slides. Slides were left to air-dry overnight, dehydrated in serial ethanol solutions, cleared in xylene and coverslipped with Permount (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Plasma steroid levels

Steroids were extracted from plasma samples using solid phase extraction with C_{18} columns as described previously (50), with minor modifications (51,52). Columns (Bond Elut OH, 500 mg; Agilent Technologies Inc., Santa Clara, CA, USA) were primed with 3 ml of HPLC-grade methanol and equilibrated with 10 ml of deionised water. Plasma samples (30 µl for progesterone, 40 µl for testosterone and 70 µl for 17β-oestradiol) were diluted with 10 ml of deionised water and loaded onto columns. Samples were washed with 10 ml of 40% HPLC-grade methanol, and steroids were eluted with 5 ml of 90% HPLC-grade methanol. Eluates were then dried at 40 °C in a vacuum centrifuge (SPD111V Speedvac; Thermo Electron Corporation, Waltham, MA, USA). Samples were resuspended in assay buffer with ethanol ($\leq 1\%$) (50): 0.9% ethanol for progesterone, 1.0% ethanol for testosterone and 0.7% ethanol for 17β-oestradiol. Steroids were measured using specific and sensitive radioimmunoassays. Recovery was assessed by spiking plasma with known amounts of steroids and comparing these samples with unspiked samples from the same plasma pool. Values were corrected for recovery.

Progesterone was measured in duplicate using a commercial ^{125}I radioimmunoassay (#07120103; MP Biomedicals, Carlsbad, CA, USA) that we

modified to increase sensitivity (53). Then, 60 µl of tracer was added to 390 µl of sample, followed by 250 µl of primary antibody. Samples were incubated for 1 h at 37 °C. Then, 500 µl of precipitant (secondary antibody) was added, and samples were centrifuged at 1000 **g** for 20 min at 4 °C. Supernatants were decanted, and tubes were each counted for 1 min in a gamma counter. Testosterone and 17β-oestradiol were quantified in duplicate and singleton, respectively, using commercial radioimmunoassay kits (testosterone: #07189102; MP Biomedicals; 17β-oestradiol: DSL-4800; Beckman Coulter, Fullerton, CA, USA). These kits have been modified to increase sensitivity and have been validated for use with rodent plasma (52,54,55).

Microscopy and DAB quantification

Images were acquired with a light microscope (Eclipse 90i; Nikon, Tokyo, Japan). For HU60 (mGnRH-I and cGnRH-II), immunopositive staining was quantified in CA1, CA3, the hippocampal fissure, and the dentate gyrus in both the dorsal and ventral hippocampus. HU60-IR was further quantified in the fimbria hippocampus, along the parenchyma bordering the third ventricle, and in the SFO. For PAC123/124 (GnIH), immunopositive staining was quantified in the dentate gyrus only, in both the dorsal and ventral hippocampus. PAC123/124-IR was less abundant in other hippocampal regions, and was not quantified therein. Note that for anti-cGnRH-II and anti-lGnRH-III, hippocampal immunoreactivity was very sparse and thus not quantified.

NIS-ELEMENTS software (Nikon) was used for image capture on a Eclipse 90i microscope. Quantification of immunoreactivity was modelled in accordance with previously reported methods (14,56). Slides were coded using an online random number generator and the researcher (JKF) was blind to the sex of the subjects. Images were captured (using the 20 × objective) bilaterally from each brain region. Six background grey levels were measured from areas of the image that did not contain immunoreactivity. The average of the six background measurements was then calculated, and quantification threshold was set such that immunoreactivity 1.5 × darker than the average background grey level was counted for each image.

A region of interest (ROI) was applied to the image, from which the immunoreactive pixel area was counted. For HU60, in hippocampal areas, a ROI of 300 × 200 µm was overlain onto the image anywhere a fibre was present. The fibre location was classified by the researcher (CA1, CA3, fissure, dentate gyrus and fimbria; dorsal or ventral) and the pixel area of each fibre was used towards analysis. For the third ventricle, two ROIs of 100 × 400 µm each were overlain bilaterally onto the parenchyma surrounding the third ventricle. For SFO, a ROI of 500 × 200 µm was overlain on the center bottom area of each section of SFO. For PAC123/124, only immunoreactivity in the hilus of the dentate gyrus was quantified. For quantification of PAC123/124 in the dentate gyrus, a ROI of 400 × 400 µm was overlaid onto each image of the dentate gyrus between the most dorsal regions of the granule cell layer.

Once total pixel areas were collected for each subregion, the mean pixel area was determined by summing the total pixel area count for that subregion and dividing by the total number of ROIs measured. This procedure was conducted for each animal to gain a mean pixel area measurement for each ROI in each animal.

Statistical analysis

The effects of Sex (male versus female) and Region (dorsal versus ventral hippocampus) were analysed using two-way ANOVA in R, version 2.15 (R Foundation for Statistical Computing, Vienna, Austria) for hippocampal areas (CA1, CA3, hippocampal fissure and dentate gyrus for HU60; dentate gyrus for PAC123/124). Sex differences were analysed using Student's t-tests for the fimbria hippocampus, along the third ventricle, and SFO for HU60. Bivariate correlations between hippocampal immunoreactivity (for HU60 and

PAC123/124) and plasma steroid levels were performed using Pearson's r. $P \leq 0.05$ was considered statistically significant. Where appropriate, data were log-transformed before analysis.

Study 2: Phenotyping of GnIH+ cell bodies

Sections were removed from antifreeze and placed in acrylic mesh well plates with phosphate-buffered saline (PBS). Sections were rinsed in PBS (5×5 min) and immersed in 0.5% H_2O_2 in PBS for 30 min. Sections were again rinsed (5×5 min) in PBS and incubated in 5% normal donkey serum in PBS containing 0.1% Triton X-100 (PBS-T) for 2 h. Immediately afterwards, sections were incubated for 48 h at 4 °C in PBS-T containing primary antibodies: PAC123/124 (dilution 1 : 100 000) paired with one of two mouse monoclonal antibodies: anti-NeuN, a neuronal marker (dilution 1:500; MAB377; Millipore, Billerica, MA, USA) or anti-GFAP, an astrocyte marker (dilution 1 : 500; MAB360; Millipore). Tissue was thoroughly washed in PBS (5×5 min) and then incubated for 1 h in biotinylated donkey anti-rabbit (dilution 1 : 500; Jackson ImmunoResearch, West Grove, PA, USA) in PBS-T. Again, tissue was washed in PBS (5×5 min) followed by incubation in avidin-biotin-horseradish peroxidase complex (ABC Elite kit; Vector Laboratories) in PBS for 30 min. Sections were then incubated in a PBS solution containing 0.01% H_2O_2 and biotinylated tyramide (BT) for 10 min. BT was made as described previously (57). For fluorophore labelling, sections were incubated with streptavidin conjugated Alexa Fluor 488 (dilution 1 : 300; S32354; Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 647 donkey anti-mouse IgG (dilution 1 : 500; A31571; Invitrogen) in PBS-T for 3 h. Sections were washed in PBS (5×5 min) and float-mounted onto gelatin-coated slides and coverslipped with ProLong Gold Mountant with DAPI (P36931; Invitrogen).

For the preadsorption control, the anti-GnIH primary antibody was incubated with a 10-fold molar excess of immunising peptide on an orbital shaker for 1 h at room temperature and then overnight at 4 °C. The solution was then centrifuged, and the supernatant was diluted in PBS-T to reach the dilution of the PAC123/124 antibody at 1 : 100 000. The preadsorbing peptide used was white-crowned sparrow GnIH (58).

Confocal scanning laser microscopy

Z-section images were acquired with a confocal laser scanning microscope (TCS SP8; Leica Microsystems, Wetzlar, Germany), equipped with a multiline argon ion laser and a water-immersion $25 \times$ objective. Emitted fluorescence was detected at 488 nm in the green channel (for GnIH) or 647 nm in the far red channel (for NeuN or GFAP). A sequential scanning mode was used for all the images to avoid cross-talk from these two colour channels. High magnification images were collected (465×465 µm). Subcellular colocalisation was assessed by inspection of the resultant images at different Z-positions. Over 200 cells in the dentate gyrus and over 100 cells in the dorsomedial hypothalamus for each animal were inspected for the co-occurrence of GnIH with NeuN or GFAP in a single cell. The percentage of cells with colocalisation was calculated as a proportion of the total number of cells counted.

Results

Study 1

HU60 (GnRH1/2)-IR

As expected, GnRH1/2+ cell bodies and/or fibres were present within the mPOA of the hypothalamus and the median eminence (Fig. 1A,B). GnRH1/2+ fibres surrounded the cerebral aqueduct within the mes-

encephalic central grey (see Supporting information, Fig. S1). The presence of both hypothalamic and mesencephalic staining is consistent with HU60 recognising mGnRH-I and cGnRH-II. Preadsorption with mGnRH-I and cGnRH-II together completely abolished immunoreactivity throughout the brain (Fig. 1E), including all hippocampal staining. By contrast, preadsorption with only mGnRH-I reduced, but did not abolish, hypothalamic and hippocampal staining, and did not reduce mesencephalic staining. Preadsorption with only cGnRH-II reduced hypothalamic and hippocampal staining and completely abolished mesencephalic staining. Preadsorption with only lGnRH-III had no effect on observed staining.

GnRH1/2+ fibres, but not cell bodies, were observed throughout the dorsal and ventral hippocampus, including CA1–3, dentate gyrus and along the hippocampal fissure (Fig. 1C,D). Hippocampal GnRH1/2+ fibres were clearly and darkly labelled, and levels of background staining were very low. GnRH1/2+ fibres were not abundant in the hippocampus but were present throughout several hippocampal regions, in both males and females. GnRH1/2+ fibres, but not cell bodies, were also observed in hippocampal-associated white matter tracts: the indusium griseum and fimbria hippocampus (Fig. 2A). GnRH1/2+ fibres surrounded the third ventricle (Fig. 2B) and lateral ventricles. GnRH1/2-IR was also observed throughout circumventricular organs: the SFO (Fig. 2C) and organum vasculosum of the lamina terminalis. Fibre staining was also observed in the medial septum, bed nucleus of the stria terminalis and diagonal band of broca. GnRH1/2+ staining in the SFO, was especially abundant.

In CA3, there was no significant effect of Sex ($F_{1,23} = 1.91$, $P = 0.17$) on GnRH1/2-IR, although there was a significant effect of Region ($F_{1,23} = 11.72$, $P = 0.001$), with more GnRH1/2-IR in the ventral CA3 than the dorsal CA3 (Fig. 3). There was no significant Sex × Region interaction in CA3 ($F_{1,23} = 0.065$, $P = 0.80$). In CA1, dentate gyrus and hippocampal fissure, there were no significant effects of Sex (CA1: $F_{1,23} = 0.38$ $P = 0.54$; DG: $F_{1,23} = 0.007$, $P = 0.93$; Fissure: $F_{1,23} = 2.06$, $P = 0.16$) or Region (CA1: $F_{1,23} = 0.067$, $P = 0.80$; DG: $F_{1,23} = 1.67$, $P = 0.20$; Fissure: $F_{1,23} = 0.097$, $P = 0.76$) and no significant Sex × Region interactions (CA1: $F_{1,23} = 0.41$, $P = 0.52$; DG: $F_{1,23} = 0.12$, $P = 0.73$; Fissure: $F_{1,23} = 0.065$, $P = 0.80$). Levene's test for equality of variance revealed that females did not show greater variance in hippocampal GnRH staining than males ($F_{1,21} = 0.34$, $P = 0.59$).

There were no significant sex differences in GnRH1/2-IR in the fimbria hippocampus ($t_{21} = 1.12$, $P = 0.27$), along the third ventricle ($t_{21} = -0.63$, $P = 0.54$) or in the SFO ($t_{21} = 1.38$, $P = 0.18$) (Fig. 4).

Plasma steroid levels differed significantly between the sexes, with males having more plasma testosterone (males: 1.42 ± 0.38 ng/ml, females: 0.15 ± 0.02 ng/ml; $t_7 = -3.33$, $P = 0.01$) and females having more plasma progesterone (males: 1.77 ± 0.31 ng/ml, females: 11.57 ± 1.60 ng/ml, $t_{16} = 6.00$, $P < 0.001$). Plasma oestradiol was low in both sexes and not significantly different, although there was a trend in the expected direction (males: 3.85 ± 0.80 pg/ml; females: 5.68 ± 0.56 pg/ml; $t_{12} = 1.88$, $P = 0.08$). There were no significant correlations between plasma steroid levels and hippocampal GnRH1/2+ staining in either males or females (Table 1).
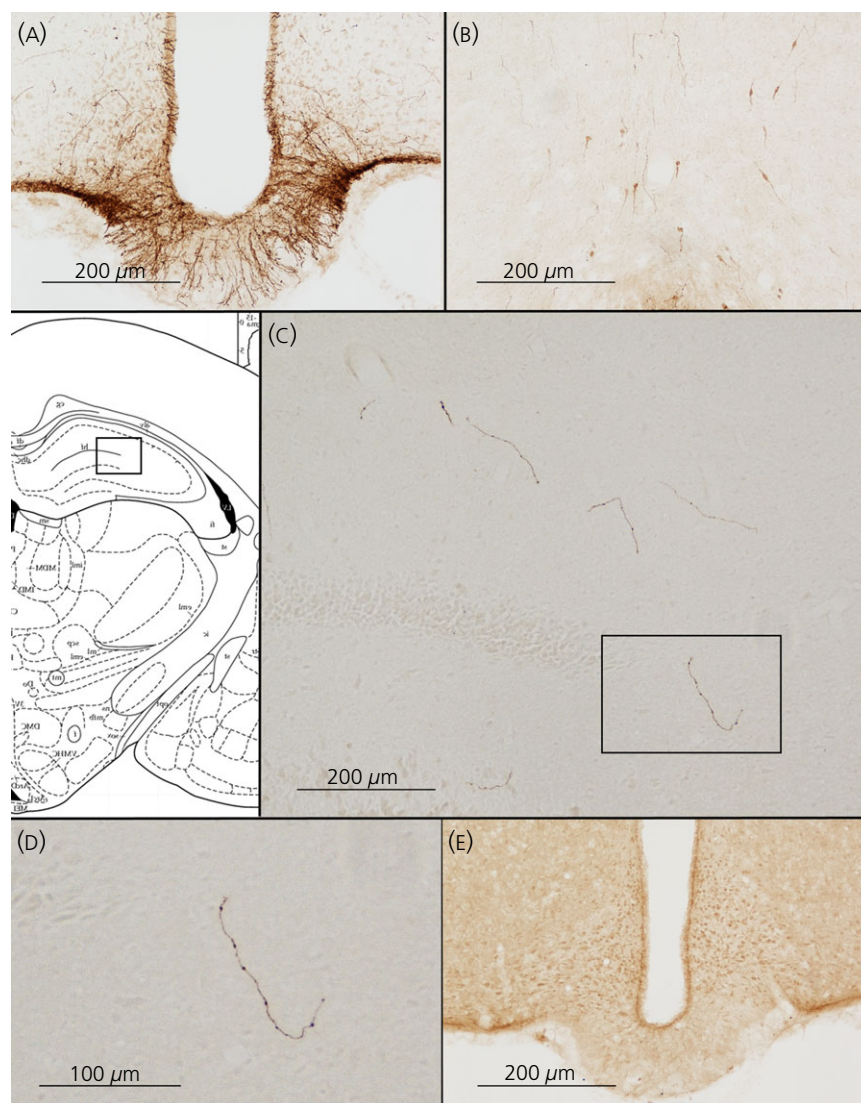
Fig. 1. HU60 [gonadotrophin-releasing hormone (GnRH)1/2] immunoreactivity in the rat brain. (A) Staining in the median eminence (positive control). (B) Staining in the medial preoptic area of the hypothalamus (positive control). (C) GnRH1/2+ beaded fibres in the dorsal hippocampus. Image is shown in the inset on the adjacent atlas image. (D) Higher magnification image of a hippocampal GnRH1/2+ fibre. Image is a higher magnification of the inset in (C). (E) Preadsorption with both mGnRH-I and cGnRH-II peptides abolished staining in the median eminence (negative control). (A–C, E) Scale bar = 200 μm. (D) Scale bar = 100 μm. Atlas source (97).
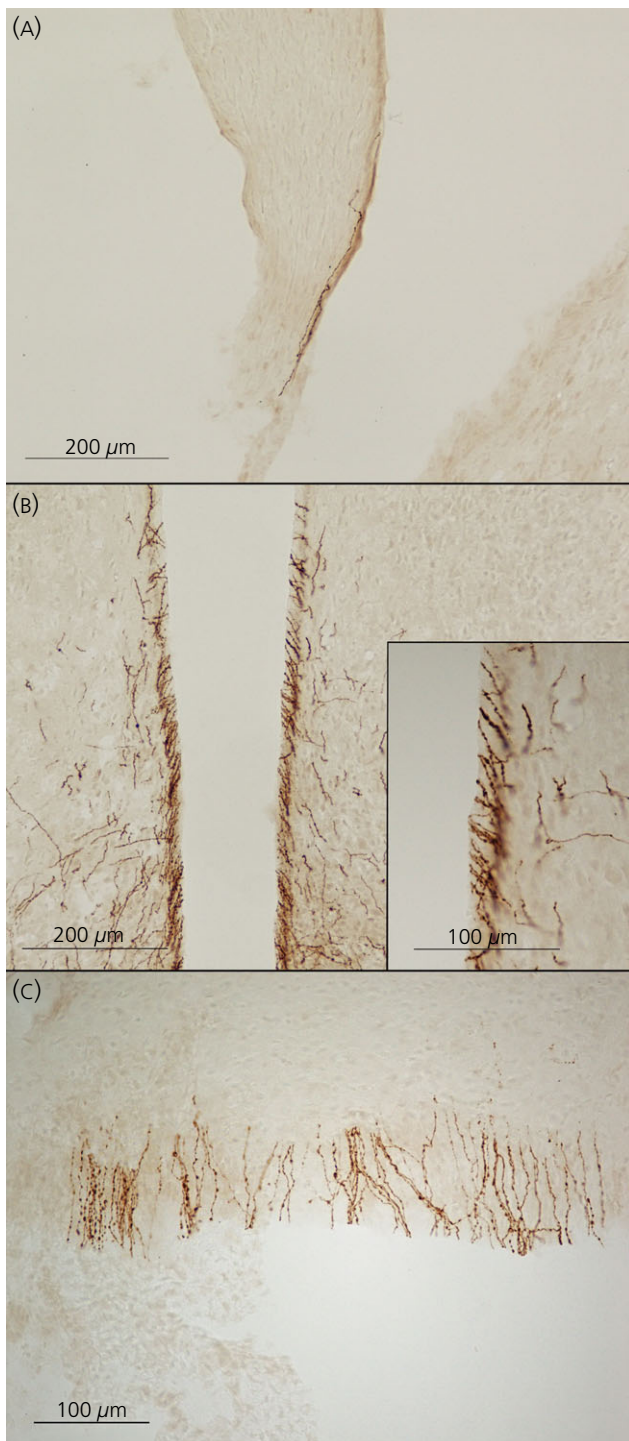
Anti-cGnRH-II-IR

As expected, cGnRH-II+ fibres were present in the hypothalamus, median eminence and mesencephalic central grey (Fig. 5A,B), consistent with this antibody recognising cGnRH-II. Preadsorption with only cGnRH-II completely abolished immunoreactivity throughout the brain, including all hippocampal staining (Fig. 5E). Preadsorption with only mGnRH-I or only lGnRH-III had no effect on staining.

cGnRH-II+ fibres, but not cell bodies, were observed in the dorsal and ventral hippocampus. Hippocampal cGnRH-II+ fibres were clearly and darkly labelled, and levels of background staining were very low (Fig. 5C,D). Overall, cGnRH-II+ fibres were less abundant than GnRH1/2+ fibres in the hippocampus but were nonetheless present in several hippocampal regions of both males and females.

In females, sparse cGnRH-II+ fibres were present in the ventral CA3, dorsal dentate gyrus, and dorsal and ventral hippocampal fissure. In males, the distribution of cGnRH-II+ fibres in males was similar, except that cGnRH-II+ fibres were present in dorsal (not ventral) CA3. cGnRH-II+ fibres were also present in the SFO, adjacent to the third and lateral ventricles, in the indusium griseum, and in the lateral septal nucleus. Note that cGnRH-II-IR in the hippocampus was too infrequent for quantification.
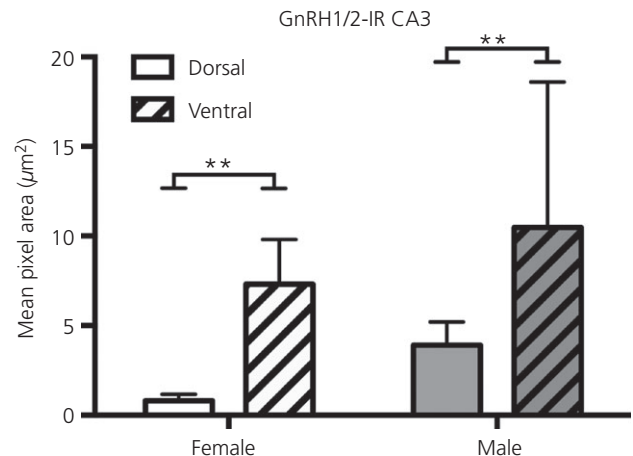
Anti-lGnRH-III-IR

lGnRH-III+ fibres were observed in the hypothalamus and median eminence (see Supporting information, Fig. S2); however, no lGnRH-III-IR was observed in the hippocampus. Preadsorptions

Fig. 3. Sex and regional differences in HU60 [gonadotrophin-releasing hormone (GnRH) 1/2] immunoreactivity in the rat hippocampus. There was a significant effect of region on GnRH1/2-IR in the CA3 field of the hippocampus, with significantly more GnRH1/2-IR in ventral CA3 than dorsal CA3. There was no effect of sex on GnRH1/2-IR the hippocampus. ** = $p < 0.01$.
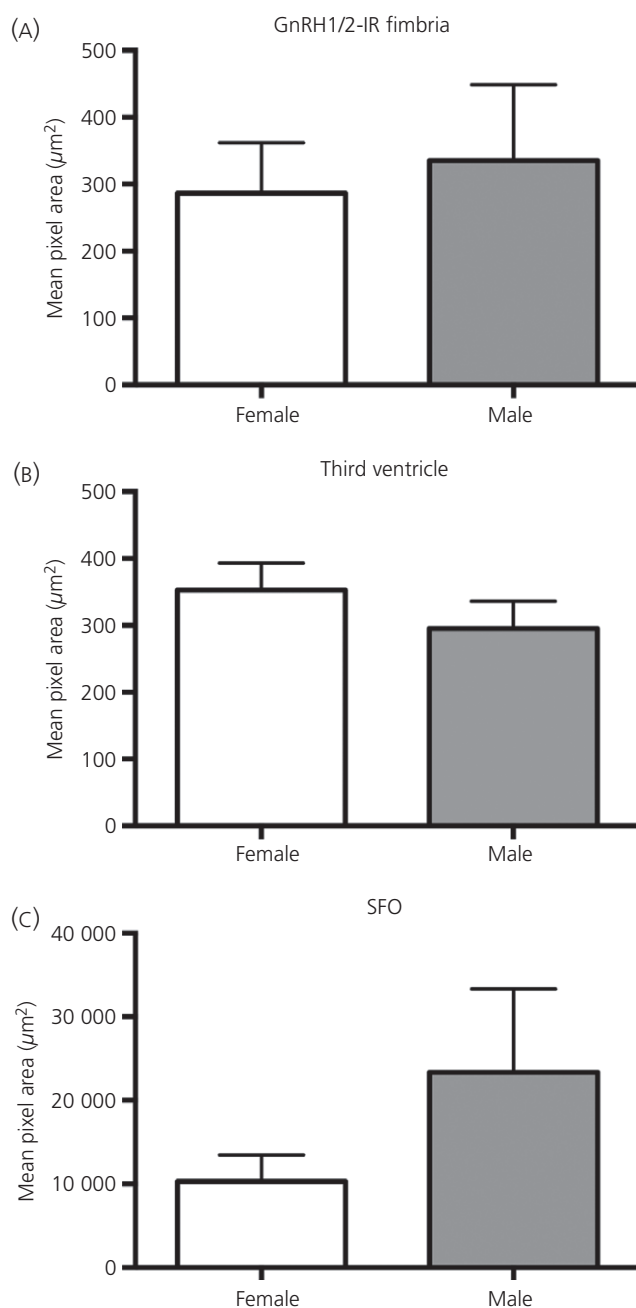
fibres were observed in the hypothalamus and median eminence; no lGnRH-III-IR was observed in the hippocampus.

PAC123/124 (GnIH)-IR

As expected, immunohistochemistry with PAC123/124 revealed immunopositive (GnIH+) cell bodies with neuronal morphology in the dorsomedial hypothalamus and GnIH+ fibres in the median eminence.

Interestingly, GnIH+ cell bodies were detected throughout the hippocampus (e.g. CA3) (Fig. 6A,B). The distribution was especially dense throughout the hilus of the dentate gyrus, for the entire rostral–caudal length of the hippocampus (Fig. 6C,D). Surprisingly, these hippocampal GnIH+ cells had an astrocytic morphology. In the dentate gyrus, there was a significant effect of Sex, with males having more GnIH-IR than females ($F_{1,21} = 4.77$, P = 0.04) (Fig. 7). There was no effect of Region on GnIH-IR ($F_{1,21} = 1.72$, P = 0.20) and no Sex × Region interaction ($F_{1,21} = 0.45$, P = 0.51) (Fig. 7).

Sparse GnIH+ fibre staining was not only present in the hippocampus, primarily in the dorsal CA1 and ventral CA3, but also in the ventral hippocampal fissure. For both sexes, GnIH+ fibres were observed most frequently in the ventral CA3. Females expressed GnIH+ fibres in the dorsal CA1 and dorsal hippocampal fissure. Males, in contrast to females, had GnIH+ fibre staining in the ventral CA1 and the ventral hippocampal fissure. GnIH+ fibres lined the third and lateral ventricles and the cerebral aqueduct. GnIH+ fibres were also present within hippocampal-associated white matter tracts: the fimbria hippocampus and SFO. Overall, GnIH+ fibres were far less abundant in the hippocampus than GnRH1/2+ and cGnRH-II+ fibres. GnIH+ fibres were also far less abundant than GnIH+ cell bodies. Levene's test for equality of variance revealed that females did not show increased variability in hippocampal GnIH staining than males ($F_{1,21} = 0.21$, P = 0.65). There were no significant correlations between plasma steroid levels and GnIH+ staining in either males or females (Table 2).

Fig. 2. HU60 [gonadotrophin-releasing hormone (GnRH)1/2] immunoreactive fibres in hippocampal-associated areas. GnRH1/2+ beaded fibres were observed (A) in the fimbria hippocampus, (B) surrounding the third ventricles and (C) in the subfornical organ, with fibres appearing on the ventral border adjacent to the third ventricle. (A, B) Scale bar = 200 μm. (C) Scale bar = 100 μm. * = p < 0.05.

against mGnRH-I and cGnRH-II peptides had no observed effect on lGnRH-III staining. Preadsorption with lGnRH-III peptide completely abolished observed staining throughout the rat brain. lGnRH-III+

(A)

GnRH1/2-IR fimbria

(B)

Third ventricle

(C)

SFO

Fig. 4. No sex differences in HU60 [gonadotrophin-releasing hormone (GnRH) 1/2] immunoreactivity in hippocampal-related areas. There were no significant sex differences in GnRH1/2-IR (A) in the fimbria hippocampus, (B) along the third ventricle or (c) in the subfornical organ (SFO).

Study 2

Phenotyping of GnIH+ cells with double-label immunofluorescence and confocal microscopy

GnIH and NeuN/GFAP double-label immunofluorescence staining was performed in the dorsomedial hypothalamus and the hippocampus. Over 100 cells in the dorsomedial hypothalamus were inspected per animal (n = 10; > 1000 cells total) and over 200 cells

Table 1. Correlations Between Plasma Steroids and Gonadotrophin-Releasing Hormone (GnRH)1/2+ Labelling in the Rat Hippocampus.

GnRH	Female		Male	
	R	P	R	P
Testosterone	−0.200	0.458	0.324	0.478
Progesterone	−0.217	0.418	0.479	0.277
Oestradiol	−0.271	0.310	0.095	0.840

in the hippocampus were inspected per animal (n = 10; > 2000 cells total).

As expected, 100% of GnIH+ cells in the dorsomedial hypothalamus were NeuN+, which is consistent with a neuronal phenotype (Fig. 8). Consistent with this, no GnIH+ cells in the dorsomedial hypothalamus were GFAP+ (Fig. 8).

By contrast, no GnIH+ cells in the hippocampus were NeuN+ and 100% of GnIH+ cells in the hippocampus were GFAP+ (Fig. 9), which is consistent with an astrocytic phenotype.

Preadsorption of the anti-GnIH primary antibody against the immunising peptide completely abolished hippocampal GnIH staining (see Supporting information, Fig. S3).

Discussion

In the present study, we report the presence of mGnRH-I+ fibres, cGnRH-II+ fibres and GnIH+ astrocytes in the rat hippocampus. Sparse mGnRH-I+ and cGnRH-II+ beaded fibres were present in CA1, CA3, along the hippocampal fissure and in the hilus of the dentate gyrus. The mGnRH-I+ and cGnRH-II+ fibres were also detected within hippocampus-related white matter tracts and surrounding the cerebral ventricles, with a particularly dense distribution around the third ventricle. No mGnRH-I+ or cGnRH-II+ cell bodies were detected within the hippocampus. lGnRH-III-IR was not detected in the hippocampus. GnIH-IR was detected in astrocytic cell bodies, as indicated by double-label immunofluorescence against GFAP. Infrequent GnIH fibre staining was detected in the hippocampus.

These data suggest that GnRH ligands may reach hippocampal GnRH receptors by mechanisms other than synaptic release. By contrast, GnIH is present in rat hippocampal astrocytes, suggesting that GnIH might be synthesised locally in the hippocampus. These findings provide evidence for regional differences in hippocampal GnRH expression in mammals, and the first evidence for GnIH expression in glial cells in the brain.

GnRH distribution in the rat hippocampus

HU60, a polyclonal antibody that recognises mGnRH-I and cGnRH-II, revealed sparse but unambiguous beaded fibre staining throughout the hippocampus in CA1, CA3, dentate gyrus and along the hippocampal fissure. GnRH1/2+ fibres were present throughout both the dorsal and ventral hippocampus, and were present at a higher density in the fimbria hippocampus. Additionally, GnRH1/2+ fibres were detected surrounding the third and lateral ventricles and in the SFO, consistent with previous studies (15,24,25,59). No
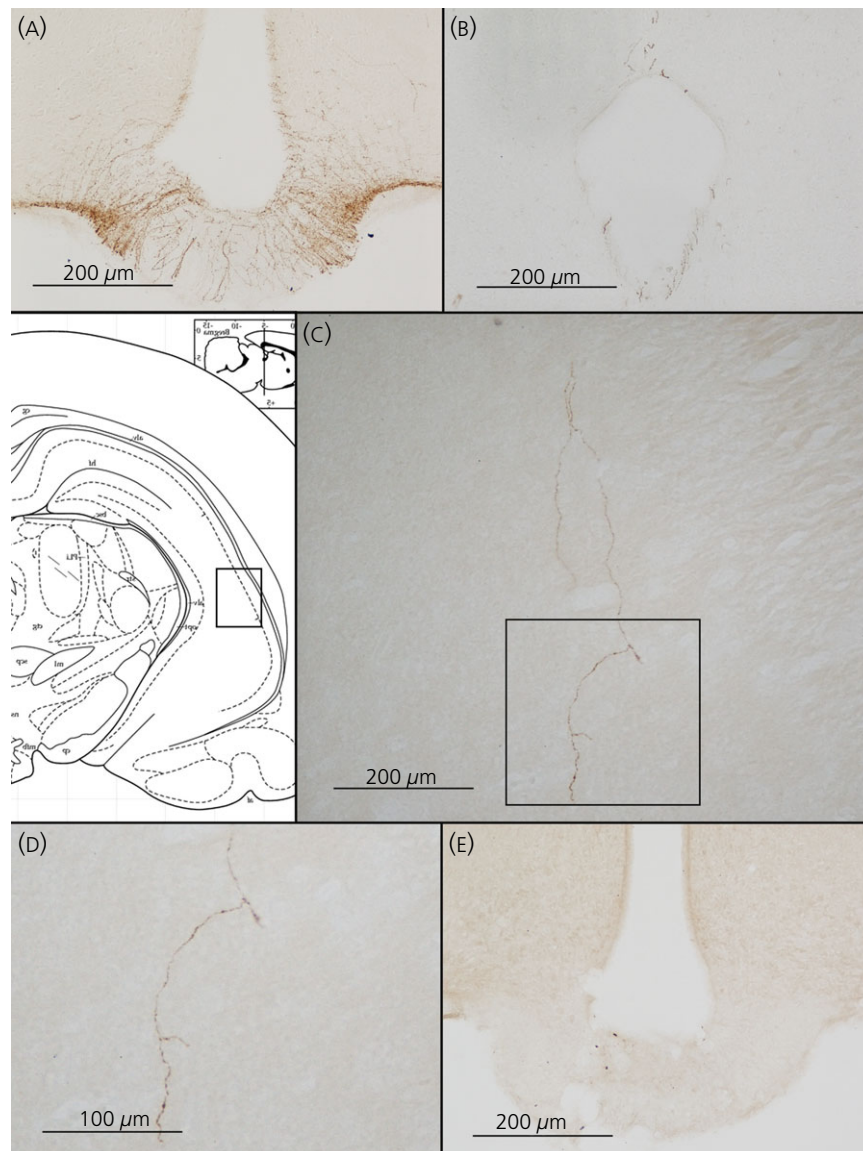
Fig. 5. Chicken gonadotrophin-releasing hormone (cGnRH)-II immunoreactivity in the rat brain. (A) Staining in the median eminence (positive control). (B) Staining in mesencephalic central grey (positive control). (C) cGnRH-II+ beaded fibres in the ventral hippocampus. Image is shown in the inset on the adjacent atlas image. (D) Higher magnification image of a hippocampal cGnRH-II+ fibre. Image is a higher magnification of the inset in (C). (E) Preadsorption with cGnRH-II peptide abolished staining in the median eminence (negative control). (A–C, E) Scale bar = 200 μm. (D) Scale bar = 100 μm. Atlas source (97).

GnRH1/2+ cell bodies were observed in the hippocampus or in hippocampal-related regions. Preadsorption with either mGnRH-I or cGnRH-II peptides did not completely abolish hippocampal staining, indicating the presence of both forms of GnRH in the rat hippocampus.

By contrast to our recent work in the songbird hippocampus (14), no sex differences were detected in rat GnRH1/2 hippocampal staining. We detected more GnRH1/2 staining in ventral rather than dorsal CA3. CA3 is a major input region of the hippocampal formation, receiving projections from the entorhinal cortex and integrating projections with the dentate gyrus (60). This finding is especially intriguing given the proposed importance of the ventral hippocampus with respect to regulating stress and affect rather than cognitive functions (61).

Using anti-cGnRH-II, a polyclonal antibody that specifically recognises cGnRH-II, we detected beaded cGnRH-II+ fibres throughout the dorsal and ventral hippocampus, similar to the staining with GnRH1/2. cGnRH-II+ fibres were less abundant than GnRH1/2+ fibres, consistent with the higher specificity of this antibody and previous studies investigating cGnRH-II (4,46,62). There has been very little previous work on cGnRH-II in the mammalian hippocampus; cGnRH-II peptides are present in the mammalian brain (4,6,62) and the presence of rare cGnRH-II+ fibres was reported in the CA1 field of the rat hippocampus (26).
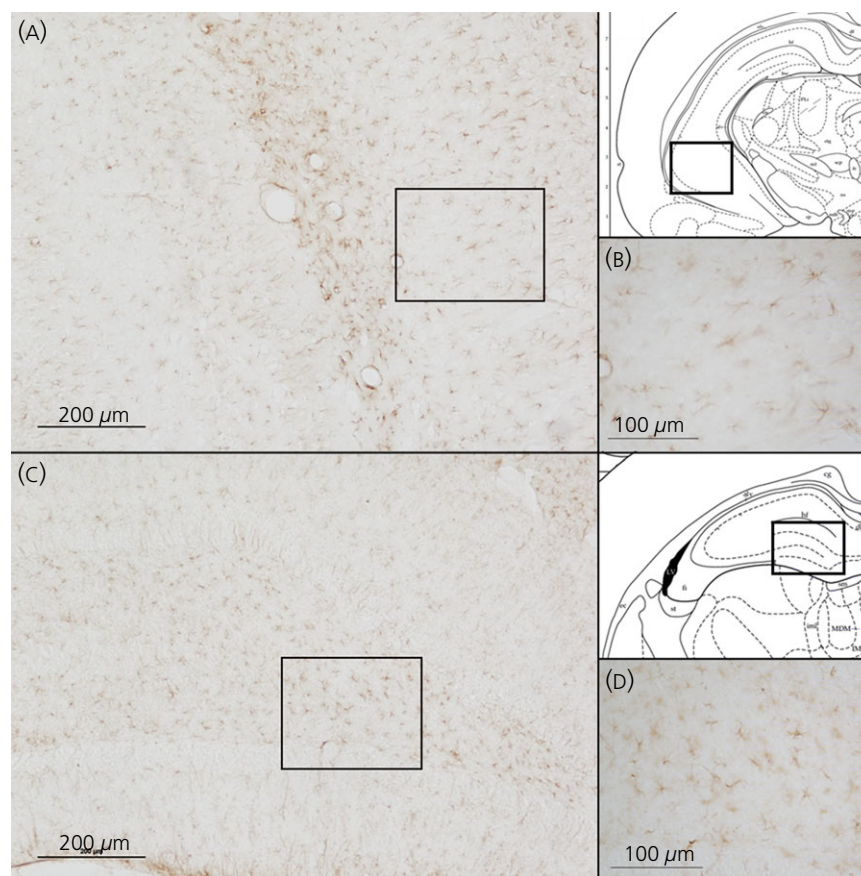
Fig. 6. PAC123/124 [gonadotrophin inhibitory hormone (GnIH)] immunoreactive cell bodies in the rat hippocampus. (A, B) Dense GnIH staining was observed in the ventral CA3. (C, D) GnIH staining in dorsal dentate gyrus. (B, D) Higher magnifications of the insets in (A) and (C), respectively. Note that the morphology of GnIH+ cells resembles that of astrocytes. (A, C) Scale bar = 200 μm. (B, D) Scale bar = 100 μm. Atlas source (95).
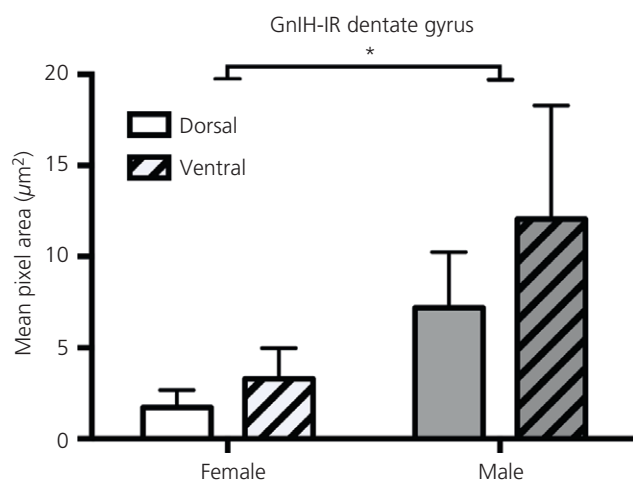
Fig. 7. Sex and regional differences in gonadotrophin inhibitory hormone (GnIH)-immunoreactivity (-IR) in the rat dentate gyrus. There was a significant effect of sex on GnIH-IR in the dentate gyrus, with males expressing more GnIH than females, * = p < 0.05.

Anti-lGnRH-III, a polyclonal antibody that recognises mature lGnRH-III, revealed some hypothalamic staining, consistent with previous studies (10,63), but no hippocampal staining. We conclude

Table 2. Correlations Between Plasma Steroids and Gonadotrophin Inhibitory Hormone (GnIH)+ Labelling in the Rat Hippocampus.

GnIH	Female		Male	
	R	P	R	P
Testosterone	−0.278	0.298	−0.518	0.234
Progesterone	0.259	0.332	−0.465	0.293
Oestradiol	0.233	0.382	−0.473	0.284

that lGnRH-III is not present in the rat hippocampus, in contrast to our recent work in the songbird hippocampus (14).

Our data suggest that mature forms of both mGnRH-I and cGnRH-II are present in sparse fibres in the adult rodent hippocampus; however, the lack of immunoreactive GnRH cell bodies suggests that mGnRH-I and cGnRH-II are not synthesised in the adult rat hippocampus.

GnIH distribution in the rat hippocampus

An unexpected finding of the present study was the abundance of GnIH+ cells in the hippocampus. The staining was present
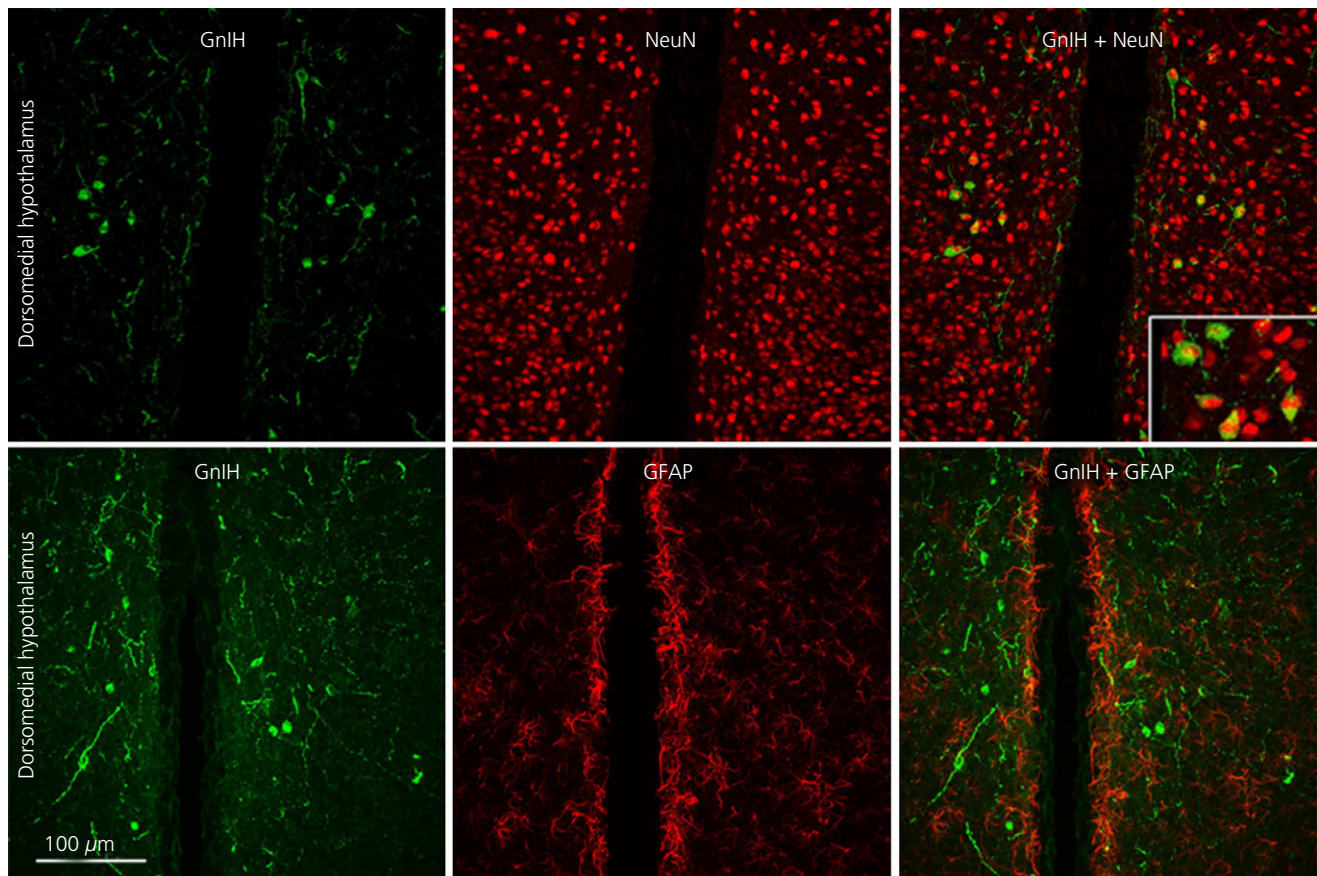
Fig. 8. Phenotyping of gonadotrophin inhibitory hormone (GnIH)+ cells in the dorsomedial hypothalamus via double-label immunofluorescence and confocal microscopy. Green, GnIH-immunoreactivity; red, NeuN or glial fibrillary acidic protein (GFAP) immunoreactivity. There is 100% co-localisation of GnIH with NeuN, and no co-localisation of GnIH with GFAP, in the dorsomedial hypothalamus. Scale bar = 100 μm.

throughout the dorsal–ventral extent of the hippocampus, although staining was densest within the hilus of the dentate gyrus. Surprisingly, these GnIH+ cells had a morphology resembling astrocytes. Double-label immunofluorescence revealed colocalisation of GnIH+ with GFAP in these cells, indicating that astrocytes in the rat hippocampus express GnIH protein. GnIH mRNA is present in the porcine hippocampus (43), suggesting that GnIH, unlike GnRH, is synthesised locally in the hippocampus. Additionally, males expressed more GnIH peptide in the dentate gyrus than females. The physiological relevance of this sex difference should be examined, especially with regard to known sex differences in hippocampal neurogenesis, plasticity and function (64). The present study contributes to a growing body of evidence for the presence of GnIH in the mammalian hippocampus (31,43,65).

## Hippocampal GnRH/GnIH-IR and circulating steroid levels

Hippocampal GnRH and GnIH may be influenced by circulating steroid levels, similar to hypothalamic GnRH and GnIH (44). GnRHR in the hippocampus increases after castration (66,67), although it is currently unknown how variations in peripheral steroid levels may impact hippocampal GnRH peptide. The present study did not observe any correlations between peripheral testosterone, oestradiol or progesterone levels and hippocampal GnRH or GnIH staining. We did not determine the oestrous phase in female animals to examine the impact of the oestrous cycle on hippocampal GnRH expression; however, we also did not observe increased variability in female hippocampal staining for either GnRH or GnIH relative to male staining, suggesting that circulating ovarian hormones do not increase the variability of hippocampal GnRH or GnIH expression. An absence of influence of circulating steroids on hippocampal GnRH levels may also account for the lack of sex difference in GnRH1/2 staining observed in the present study. The degree to which peripheral steroids regulate hippocampal GnRH or GnIH signalling has important implications for hippocampal function following a decline in circulating steroid levels (e.g. menopause in women, androgen deprivation therapy for treating prostate cancer in men) and should be examined further in future research.

Source of GnRH in the hippocampus

There is a clear discrepancy between the abundance of GnRHR expressed in the hippocampus (15,16,68,69) and the paucity of observed GnRH peptide expression. Rather than direct axonal projections, there may be an alternate route for GnRH with respect to
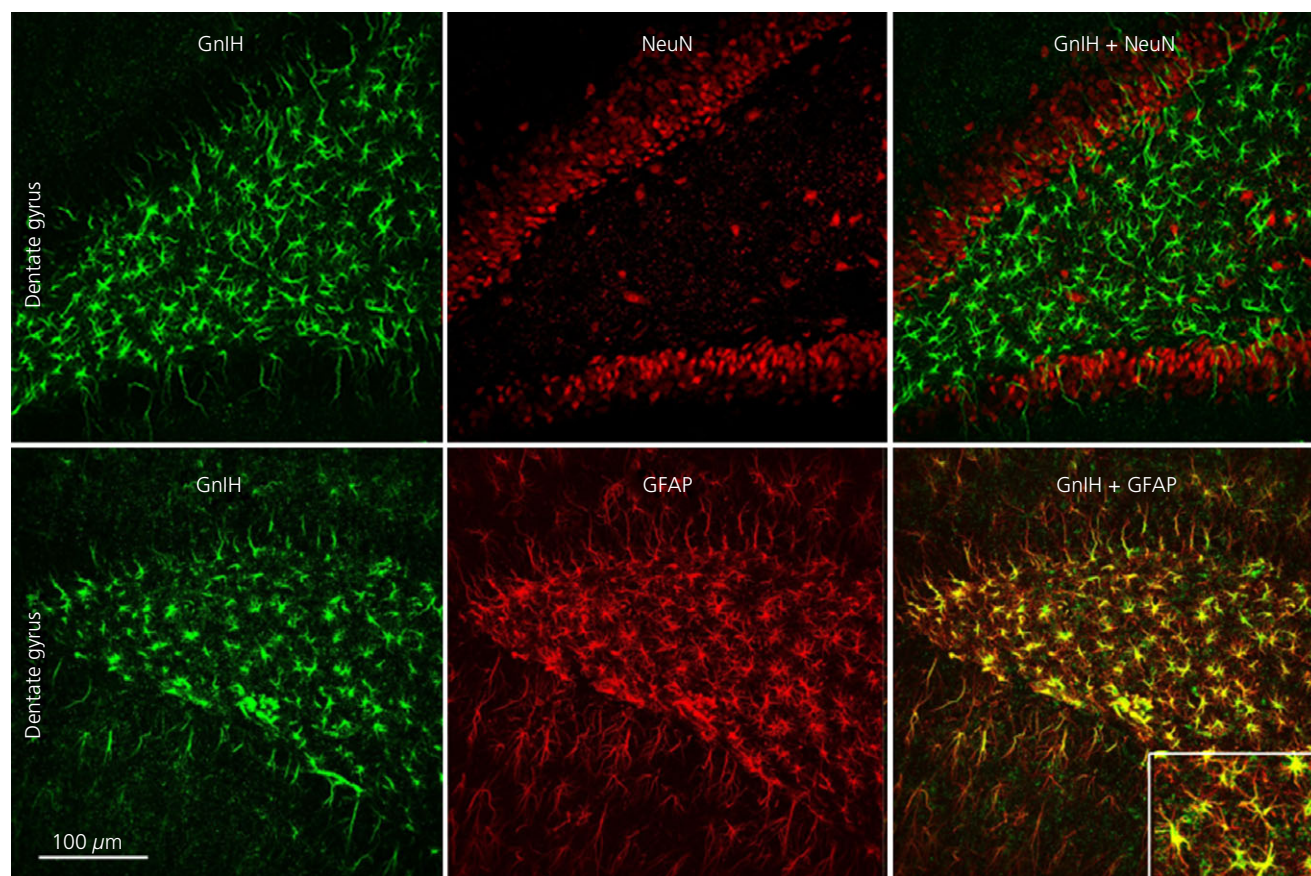
Fig. 9. Phenotyping of gonadotrophin inhibitory hormone (GnIH)+ cells in the dentate gyrus via double-label immunofluorescence and confocal microscopy. Green, GnIH-immunoreactivity; red, NeuN or glial fibrillary acidic protein (GFAP) immunoreactivity. There is no co-localisation of GnIH with NeuN, and 100% co-localisation of GnIH with GFAP, in the dentate gyrus. Scale bar = 100 μm.

reaching its receptors in the hippocampus, such as via volume transmission in the CSF. GnRH is present in the CSF at higher concentrations than in the hypophyseal portal blood (28), with high levels within the third ventricle CSF of mammals (70). Infusions of the GnRH receptor agonist ^{125}I-buserelin into the CSF of the lateral ventricles reveal tracer uptake in hippocampal CA1–3 (71), and injecting GnRH into the lateral ventricle increases synaptic spine density in the ipsilateral hippocampus (72). The dense GnRH+ fibre staining that we observed in the SFO suggests this region may be a site of GnRH uptake from the third ventricle, possibly with tanycyte cells mediating GnRH uptake from the CSF (73). Such a system would explain how GnRH could still be physiologically active within the hippocampus despite a low density of GnRH fibres. This discrepancy between abundant receptors and scarce fibre staining is not unique to GnRH amongst neuropeptides. For example, the amygdala is an important site of oxytocin action, and oxytocin receptors are densely expressed there. However, there is only sparse innervation of oxytocin-expressing fibres in the amygdala (74,75) but high levels of oxytocin in the CSF that are independently regulated from plasma oxytocin (76). Neuropeptides in the central nervous system therefore can act by nonsynaptic mechanisms of release and diffusion to distant target sites within the brain (77).

Another proposed pathway of hippocampal GnRH supply is via the medial septum to the hippocampus. GnRH could be transported along the septo-hippocampal pathway and enter the hippocampus via the fimbria. Hippocampus-specific GnRHR mRNA appears simultaneously with medial septal GnRH mRNA, at the time of establishment of septo-hippocampal connections (16), suggesting a possible pathway for GnRH transport to the hippocampus. Previous tracer studies on this as a source of GnRH to the hippocampus have been mixed, with some studies reporting no innervation (78) and others suggesting that there is (79). In the present study, mGnRH-I and cGnRH-II staining was found in both the septum and the diagonal band of broca, and GnRH+ fibres were detected throughout the fimbria hippocampus, perhaps lending support for a septo-hippocampal source of innervation to the hippocampus. However, given the high sensitivity of our protocols, which were optimised specifically for GnRH hippocampal straining, we would expect to see more abundant GnRH fibre staining, if GnRH were indeed reaching the hippocampus via synaptic transportation from the septum. Future tracing studies are required to determine possible sources of hippocampal GnRH from the SFO or septo-hippocampal projections; however, we consider that our data lend support to a volume transmission model of GnRH transportation to the hippocampus.

Possible functions of hippocampal GnRH and GnIH

There is a bourgeoning body of research indicating that steroids are produced locally within the central nervous system and that this local steroid production is highly regulated (80,81). One potential function of GnRH and GnIH within the hippocampus is to regulate local sex steroid production. Local oestradiol production has been demonstrated in the hippocampus and is up-regulated by GnRH administration *in vitro* (22,82–84). Various hippocampal subregions, including the CA1–3 fields and dentate gyrus, are known to express aromatase (85), and contain GnRH-IR in the present study. GnRH and GnIH are therefore likely candidates that act as regulators of local steroid production within the hippocampus, as they do in the HPG axis. GnRH is capable of rapidly modulating hippocampal dendritic spine density through actions on oestradiol synthesis (17), and astrocytes can also rapidly modulate hippocampal dendritic spine density (86). It is possible that hippocampal GnIH interacts with hippocampal GnRH to modulate synaptic connectivity in the hippocampus. The role of GnIH in endocrine regulation in hippocampal astrocytes is intriguing, given the demonstrated importance of astrocytes in modulating GnRH release in the hypothalamus (87). Astrocytes also act as modulators of glutamate and GABA signalling in the hippocampus (88); therefore, GnIH may have additional actions on neuronal excitability and plasticity in the hippocampus. Future research should explore the physiological role of hippocampal GnIH expression in astrocytes and its interactions with GnRH in regulating hippocampal oestradiol synthesis and neuroplasticity.

GnRH and GnIH may have important physiological roles in regulating the functions of oestradiol in the hippocampus, which may impact protective mechanisms against neural injury (89,90) or neurodegeneration (91–96). An understanding of the physiological roles of GnRH and GnIH in regulating hippocampal function could ultimately lead to valuable treatment avenues for brain injury and neurodegeneration.

Conclusions

The present study provides a systematic examination of mGnRH-I, cGnRH-II, lGnRH-III and GnIH in the rat hippocampus and related areas. In the hippocampus, GnRH+ cell bodies were not detected and GnRH+ fibres were present but sparse. GnRH+ fibres were more abundant in hippocampus-related areas, such as the fimbria hippocampus, medial septal nucleus, diagonal band of broca and subfornical organ. Taken together, these data are consistent with the hypothesis that GnRH reaches GnRHR in the hippocampus primarily via an alternate route, possibly through volume transmission via the CSF. With regard to GnIH, both GnIH+ cell bodies and GnIH+ fibres were present in the hippocampus, with high numbers of GnIH+ cells in the dentate gyrus. Interestingly, GnIH is expressed in hippocampal astrocytes, which may indicate GnIH involvement in local modulation of GnRH actions in the hippocampus. Taken together, these data shed light on the sites of action and possible functions of GnRH and GnIH outside of the HPG axis.

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Supporting Information

The following supplementary material is available:

Fig. S1. Photomicrograph of Gonadotrophin-releasing hormone (GnRH)1/2 immunoreactive fibres in the adult rat mesencephalic central grey, using the HU60 antibody.

Fig. S2. Photomicrograph lamprey gonadotrophin-releasing hormone (lGnRH)-III immunoreactive fibres in the median eminence. By contrast, no lGnRHIII staining was observed in the adult rat hippocampus.

Fig. S3. Preadsorption of anti-gonadotrophin inhibitory hormone antibody.