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Immunoreactive GnRH Type I Receptors in the Mouse and Sheep Brain

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Abstract

GnRH has been implicated in an array of functions outside the neuroendocrine reproductive axis. Previous investigations have reported extensive GnRH binding in numerous sites and this has been supported by *in situ* hybridization studies reporting GnRH receptor mRNA distribution. The present study on mice and sheep supports and extends these earlier investigations by revealing the distribution of cells immunoreactive for the GnRH receptor. In addition to sites previously shown to express GnRH receptors such as the hippocampus, amygdala and the arcuate nucleus, the improved resolution afforded by immunocytochemistry detected cells in the mitral cell lay of the olfactory bulb as well as the central grey of the mesencephalon. In addition, GnRH receptor immunoreactive neurons in the hippocampus and mesencephalon of the sheep were shown to colocalize with estrogen receptor β. Although GnRH may act at some of these sites to regulate reproductive processes, evidence is accumulating to support an extra-reproductive role for this hypothalamic decapeptide.

Keywords

neuromodulation; red nucleus; nucleus accumbens; central grey; preoptic area; arcuate nucleus; amygdala; bed nucleus of the stria terminalis; olfactory bulb

Introduction

Gonadotropin Releasing Hormone-I (GnRH) is a hypothalamic decapeptide that acts on gonadotropes in the anterior pituitary gland to release the gonadotropins, luteinizing hormone and follicle stimulating hormone. To affect this, GnRH binds to a specific G protein coupled receptor, the type I GnRH receptor (GnRHR) (1), which initiates several intracellular signaling events including a rise in intracellular calcium and activation of protein kinase C (2).

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For many years, GnRH has been implicated in a variety of extra-pituitary functions (3). Recent evidence also suggests that GnRH synthesis is not restricted to the hypothalamus (4,5). Moreover, GnRH binding and/or GnRHR mRNA have been detected in the testis (3), ovary (6), placenta (7), kidney (8), thymus and spleen (9), spinal cord (10) and several sites in the brain (11). Many reproductive tumors have also been found to express the GnRHR (12). Although the physiological function, if any, of GnRH at these GnRHR-expressing sites is largely unknown, there is strong evidence that GnRH plays a major role in sexual behavior (13-16). In this context, GnRH binding and/or GnRHR mRNA have been reported in areas of the brain associated with sexual behavior such as the hypothalamus and amygdala (17,18).

There is also evidence suggesting that GnRH may have non-reproductive functions and serve as a neuromodulator. GnRH depolarizes sympathetic ganglion neurons in the frog (19-21). In the rat, GnRH inhibits or stimulates hypothalamic (22,23), cerebellar (24) and preoptic area neurons (25,26). GnRH also alters thermoregulatory activity, when administered to septal neurons (27). Understanding the specific mechanisms by which GnRH affects these systems has been difficult to accomplish as the phenotype of the neurons and their corresponding efferent pathways remain unknown.

Using an antibody raised against the second extracellular loop common to the ovine and murine GnRHR, the present study describes the distribution of GnRHR-immunoreactive cells in the mouse brain. In addition, GnRHR-immunoreactive neurons were revealed in specific regions of the sheep brain. Visualization and mapping of the GnRHR in the mammalian brain provides further support for multifunctional physiological roles for GnRH.

Materials and Methods

Animals

Mice were housed under normal photoperiod and allowed food and water *ad libitum.* Ewes were sexually mature and housed in outdoor pens under natural photoperiod, fed hay daily and had access to water *ad libitum.* All procedures were conducted in accordance with University of Wyoming Animal Care and Use Committee.

Tissue Preparation

Male mice (n=5) were anesthetized with ketamine (Vedco Inc, St. Joseph, MO) and transcardially perfused with 100ml of 1% sodium nitrite in 0.9% NaCl, followed by 200ml of fixative (pH 7.4; 4% paraformaldehyde, 15% saturated picric acid in 0.1 M phosphate buffer solution (PBS)) and 100ml cryoprotectant (20% sucrose in 0.1M PBS). Sheep were killed through an overdose of sodium pentobarbitol before being decapitated and perfused through both carotid arteries with 1 liter of sodium nitrite solution, 3 liters of fixative, and 1 liter of cryoprotectant. After killing, brains were removed and stored in cryoprotectant overnight at 4°C. Mouse and sheep tissue were embedded in Tissue-Tech OCT compound (Miles Inc., Elkhart, NJ) and frozen by immersion in liquid nitrogen-cooled isopentane. Coronal sections (20μm) were mounted on silane-coated slides and stored at -80°C.

GnRH receptor antibody

The GnRH receptor antibody was raised in the rabbit against amino acids 193 to 212 (FSQCVTHCSFPQWWHQAFYN) of the ovine GnRH receptor. This is a highly conserved region of the second extracellular loop of the mammalian type I GnRHR and this sequence is 100% conserved in the sheep, cow, horse, mouse, rat and guinea pig and 95% conserved in the human, pig, dog and possum (BLAST search). It shows no overlap with any other receptor or peptide. The antibody was partially purified using protein G. We have previously validated the performance of this antibody on cell cultures (28). When used for immunohistochemical

examination of sections of mouse pituitary, our GnRHR antiserum discretely marked a subpopulation of cells within the anterior lobe. Colocalizing with an antibody against LHβ subunit indicated nearly complete colocalization of GnRHR and LHβ immunofluorescence (Fig. 1A). As gonadotropes are known to express GnRHR, this essential positive control provided important confirmatory evidence. A dilution series (1:10-1:10000) on pituitary sections was used to determine the optimal concentration for GnRHR visualization and the protein concentration was then established (11μg/ml). Excluding the primary or secondary antibody or using an inappropriate secondary antibody (goat anti-horse, goat anti-mouse) yielded no immunoreactivity. Absorbing the GnRHR antibody with the immunizing peptide (50μg/ml) completely blocked all immunoreactivity. We have been unable to visualize GnRHR immunoreactivity in brains that were not perfusion fixed or subjected to antigen retrieval (data not shown). Using an established (29) goat anti-luciferase polyclonal antibody (1:20; cr2029gap, Cortex Biochemical; San Leandro, CA), we performed a dual-labeling control study on transgenic mice containing the luciferase gene driven by 9100 base pairs of the ovine GnRH receptor promoter (30).

Additional controls

Western blots were performed on cell lines and mouse and bovine pituitary homogenates. Mouse and bovine pituitary glands were minced, rinsed free of blood and dissociated into single cells enzymatically (37°C; 90 min) using collagenase, hyaluronidase, and deoxyribonuclease. Dissociated cells were suspended in culture medium (DMEM supplemented with 10% FBS [Gemini Bio-Products, Inc.], 1% nonessential amino acids, 100IU/ml penicillin, and 100μg/ml streptomycin). Cells were plated in 100mm dishes and cultured for 2 days. αT3-1, LβT2, CHO cells and CHO cells expressing a GFP tagged GnRH receptor (31) were grown in 100mm dishes and maintained with high glucose DMEM containing 2mM glutamine, 100U penicillin/ml, 100μg streptomycin/ml and 1×nonessential amino acids (Mediatech, Herndon, VA), with 10% FBS (Gemini Bioproducts, Woodland, CA). All cells were grown in 5% CO_2 at 37°C in a humidified environment. Cells were washed in ice cold PBS and lysed in RIPA buffer containing 20mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP-40, 0.1% SDS, 0.5% deoxycholate and 0.2 mM PMSF. 6× sample buffer (300 mM Tris-HCl, pH 6.8, 60% glycerol, 30 mM DTT, 6% SDS) was added to yield a final concentration of 1×. Each lysate was heated (95°C; 5 min), subjected to SDS polyacrylamide gel electrophoresis (acrylamide:bis-acrylamide ratio of 29:1) and electro-blotted to nitrocellulose (Osmonics, Westborough, MA). The membrane was blocked in 5% non-fat dried milk in Tris buffered saline (TBS) and then incubated in the GnRH receptor antibody (1:2000; 2h). Blots were washed and incubated in HRP-conjugated anti-rabbit IgG (1:5000; 2 h; room temperature). Blots were then washed (6×10 min) with TBS and visualized by chemiluminescence using Pierce (Rockford, IL) SuperSignal reagents.

Immunocytochemistry

Sections were washed (3×3min) in 0.01M PBS and subjected to high temperature antigen retrieval as described (32). Slides were then washed, immersed in peroxidase-blocking solution (8 min; 1% hydrogen peroxide, 5% methanol in 0.01M PBS), washed, incubated with anti-GnRHR antibody (11μg/ml; 10% goat serum, 0.3% Triton in PBS; 48h), washed again, and immersed in biotinylated goat anti-rabbit IgG (1:200; 90 min; Jackson Immunoresearch, West Grove, PA). GnRHR immunoreactivity was visualized either chromogenically (n=3) with Vectastain ABC Elite kit (1:50; Vector, Burlingame, CA) and Ni-DAB, or fluorescently (n=5) with FITC-streptavidin (1:200; 90 min; Jackson Immunoresearch).

Hippocampal and mesencephalic sections from sheep were also subjected to dual-labeling for ERβ and the GnRHR. The specificity of the monoclonal ERβ antibody (EMR02; Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) has been reported (32). Sections were co-

incubated with the anti-ERβ (1:20) and anti-GnRHR (11μg/ml) antibodies for 48h and revealed using goat anti-rabbit IgG conjugated to FITC and goat anti-mouse IgG conjugated to Texas Red (both 1:200; 90 min; Jackson).

Neuronal regions were identified according to stereotaxic atlases of the mouse (33) and sheep (34) brains. Sections were stained and analyzed for immunoreactivity at 200μm intervals.

Western Blot

Brains from 2 additional mice were extirpated and areas encompassing certain regions (olfactory bulb, hippocampus, cerebral cortex in the region of the piriform cortex, Amygdala, hypothalamus) as well as lung tissue were homogenized in a mixture of 2.5% mercaptoethanol and 47.5% Laemmli's sample buffer (Bio-Rad, Hercules, CA) in de-ionized water. For pituitaries run in a separate Western, pituitaries from at least 3 mice were homogenized together. Samples were denatured for 10 minutes at 95°C and supernatants (75μg/lane) were run on a 4-15% Tris/HCl gel (Bio-Rad) in 10% Tris/glycine/SDS (Bio-Rad) in de-ionized H_2O. Gels were transferred onto a PVDF membrane (Millipore, Billerica, MA) in 12% methanol and 10% Tris/Glycine (Bio-Rad), in de-ionized H_2O. The membrane was blocked by 5% non-fat dry milk in 50mM Tris, 138mM NaCl, 2.7mM KCl and 0.05% Tween 20 (pH 8.0; Bio-Rad) and then incubated with the GnRHR antibody (0.5μg/ml). Immunoreactivity was revealed by incubation in HRP-conjugated goat anti-rabbit (1:200; 1h; room temp; Jackson Immunoresearch) followed by Pico chemiluminescent substrate (Pierce, Rockford, IL) and exposure to X-ray film (Kodak, Rochester, NY). As a control (data not shown) for the Western blot on pituitary and brain tissue from the mouse (n=2), a Western blot was performed and the 50kDa immunoreactive band detected. The blot was stripped and the primary antibody was absorbed in immunizing peptide (100μg/ml) prior to repeating the Western. No band was evident following preabsorption.

Results

In addition to control studies revealing the presence of GnRHR-labeling on gonadotropes (Fig. 1A). we also validated that the GnRH receptor could be detected in gonadotrope derived cells lines (αT3-1 and LβT2; Fig. 1EF) and in dissociated murine and bovine pituitaries (Fig. 1D-F). In stably expressing GnRH receptor-GFP-expressing CHO cells, a clear band was detected at approximately 75kDa (the additional weight expected due to the GFP) (Fig. 1E). All luciferase-immunoreactive cells in the brain were also immunoreactive for the GnRH receptor (Fig. 1B). Western blot analysis of brain tissue revealed a single band for all samples at approximately 50kDa (Fig. 1C). GnRHR was detected in tissue taken from the cerebral cortex, piriform cortex, hypothalamus, olfactory bulb, hippocampus and amygdala. No band was detected in lung tissue (negative control).

Immunohistochemical results were consistent with the Western blot data. Numerous regions in the mouse brain were found to contain GnRHR-immunoreactive neurons. The olfactory and limbic systems were noted to possess some of the most intensely labeled cells. GnRHR-immunoreactive neurons were detected throughout brain regions associated with the olfactory system. GnRHR-immunoreactive neurons were observed in the mitral cell layer of both the main (Fig 2A) and accessory olfactory bulb. Intense GnRHR labeling was present throughout the anterior olfactory nucleus, with no variation among the external, dorsal, medial, ventral, or lateral subdivisions of this nucleus. Strong staining was observed in the tenia tecta (Fig. 2I) and piriform cortex (Fig. 2F). In the piriform cortex, the majority of cell bodies within layer two were darkly stained, while scattered staining was observed within layer three. Immunoreactivity was detected throughout the olfactory tubercle, with intense staining in layer two and scattered labeling in layer three. Staining appeared to be restricted to the pyramidal cells. Numerous GnRHR expressing cells were observed within the entorhinal cortex.

Numerous limbic system regions were immunoreactive for the GnRHR, especially throughout CA1, CA2, CA3 and CA4 of the hippocampus (Fig. 2BC) as well as the dentate gyrus. Staining was almost exclusively localized to the pyramidal cell layer of the hippocampus. Only a few, scattered, GnRHR-immunoreactive cells were evident within the stratum oriens and radiatum. Staining was also seen within the indusium griseum (Fig. 2D), which is a dorsal subdivision of the hippocampus. A dense population of GnRHR-immunoreactive neurons was detected throughout the amygdala (Figs 1B, 2J).

A few strongly-labeled neurons were observed in the ventral, medial, and lateral divisions of the preoptic area and in the lateral septum. A few labeled cells were observed in the ventromedial hypothalamic nucleus but a dense population of GnRHR-expressing neurons was present throughout the arcuate nucleus (Fig. 2G) and ventromedial nucleus (Fig. 2L). Several other non-hypothalamic nuclei contained strong GnRHR immunoreactivity. These included the red nucleus, the periventricular nucleus of the thalamus, the bed nucleus of stria terminalis (BNST) and the nucleus accumbens.

GnRHR-immunoreactive neurons were observed within both the periaqueductal and pontine divisions of the central grey (Fig. 2K). Consistently strong immunoreactivity was observed throughout the cerebral cortex (Fig. 2EH). GnRHR-expressing cells were distributed throughout the cortex with the most immunoreactive neurons in layers two and three and proportionately fewer cells within deeper layers.

Only a few select regions of the sheep brain were investigated. As in the mouse, the hippocampus and dentate gyrus were extensively labeled (Fig. 3A-C). GnRHR-expressing neurons in the ovine hippocampus (Fig. 3C) and dentate gyrus (Fig. 3A) were clearly double-labeled for ERβ. GnRHR expressing cells in the ovine central grey were double labeled for ERβ (Fig. 3D). In the ewe, but not mouse, GnRHR immunolabeled cells were evident in the subfornical organ (Fig. 3F). Scattered GnRHR-immunoreactive neurons were evident in the POA but a dense population of GnRHR-expressing neurons was present in the ovine arcuate nucleus (Fig. 3G)

Discussion

GnRH, by virtue of its name, has been considered predominantly a reproductive hormone. Consequently, studies have focused almost exclusively on its ability to regulate pituitary gonadotrope activity. However, many studies have implicated GnRH as a regulator of sexual behavior. Other possible non-reproductive functions have been largely overlooked. It has been known for many years that GnRH affects neuronal activity in sites throughout the brain including areas associated with reproduction and areas associated with other functions. Moreover, the discovery that GnRH is released in large quantities into CSF (35-38) frees this decapeptide from the constraints of synaptic transmission enabling its sphere of influence to be more widespread. As some of the GnRHR-labeled neurons are also immunoreactive for ERβ, steroids are likely to affect GnRHR expression. Thus, it is possible that other regions within the brain may express GnRHR but were undetected in the present study. The present discussion will place these data in the context of putative reproductive roles. However, we acknowledge that many of the regions that we have identified may have either no physiological function or that their role is independent from the reproductive axis.

The presence of GnRHR within the hippocampus and dentate gyrus concurs with previous autoradiography, *in situ* hybridization and RT-PCR investigations in rodents (18,39-41) as well as a recent transgenic study in which human placental alkaline phosphatase was coupled to the GnRHR promoter (42). Immunoreactive GnRHR-expressing pyramidal neurons have been reported recently in the human hippocampus (43). Similarly, we found GnRHR-

immunoreactive neurons almost exclusively within the pyramidal cell layer, the dentate gyrus, and the indusium griseum, a dorsal extension of the hippocampus. There is evidence that the indusium griseum receives GnRH projections (44). Hippocampal pyramidal neurons in the rat take up I^{125}-Buserilin, a GnRHR agonist, when it is injected into the lateral ventricle (40), but endogenous GnRH is undetectable in the lateral ventricle (Caraty & Skinner, unpublished). GnRH alters the electrical properties of rat hippocampal pyramidal cells (45-47) and stimulates increased IP_3 production within these cells (48). Both these effects are modified by estrogen (45) in the rat and, as shown in sheep, these hippocampal and dentate gyrus GnRHR-expressing neurons co-express ERβ. Interestingly, as GnRH is likely to be elevated post-menopause (49), its action on these neurons may constitute an integral component of the neurodegenerative pathology that accompanies Alzheimer's disease (50).

Detection of GnRHR-immunoreactive neurons in the olfactory system (mitral cell layers of the olfactory and accessory olfactory bulbs, piriform cortex, tenia tecta and amygdala) concurs with and extends previous GnRHR mRNA and GnRH binding investigations in the rat (18, 51). GnRH has been detected in the hamster accessory olfactory bulb (52) and in the rat piriform cortex (51). The hamster tenia tecta contain a discrete population of GnRH-immunoreactive neurons, which are testosterone sensitive (4,53). GnRH has also been reported within the terminal nerve, which projects to several olfactory regions (54) and is a structure associated with reproductive behavior in hamsters (55,56). In some species (hamster: (57); mouse: (58); shrew: (59)), olfactory bulbectomy eliminates mating behavior. GnRH has been proposed to alter the detection of specific odors relevant to reproduction via a neuromodulatory effect (54). Such modulation may be the cause of variations in smell perception across the menstrual cycle (60,61). GnRHR-expressing neurons were distributed throughout the amygdala. Although some (40) have reported a limited distribution of GnRH binding sites in the rat amygdala, others have detected a high density of potential GnRH receptor expressing neurons in this region of the mouse (42) and rat (62). GnRH may access these receptors through neurons which project directly to the amygdala (63). Lesions of the amygdala decrease lordotic behavior in the rat (64) and prevent ovulation (63). Electrical stimulation of the rat (65) and cat (66) amygdala causes an increase in plasma gonadotropins and has been attributed to a direct connection between the amygdala and the preoptic area (67). As with the hippocampus, elevated levels of GnRH could act on the amygdala to affect the pathogenesis of Alzheimer's Disease.

GnRHR expression within the central grey concurs with previous GnRH binding data from the rat (40). Injections of GnRH into the rat central grey potentiated lordosis (68,69). GnRH immunoreactive fibers have been identified within the rat central grey (70) and significant amounts of GnRH have been extracted from midbrain preparations in this species (71). Additionally, the intimate association of the central grey with the 4^{th} ventricle and cerebral aqueduct allows potential access for CSF-borne GnRH. The presence of GnRHR-immunoreactive neurons within the ventromedial nucleus and arcuate nucleus agrees with previous work (11,18). GnRH has been shown to affect rat arcuate neuron activity (23). In the preoptic area, where scattered GnRHR-labeled cells are evident, GnRH has been shown to affect GnRH activity and/or release in the mouse (26) and rat (72,73). Indeed, some of the GnRHR-expressing neurons colocalize with GnRH in the developing ovine fetus (74).

GnRH binding has been reported previously within the cerebral cortex (40) and on dispersed cortical neurons in the rat (75). The characteristic GnRH binding next to the longitudinal fissure (40) was clearly evident in the present study. GnRH has been reported to depress the activity of cortical neurons (24,76). The GnRHR immunoreactivity observed in the cerebral cortex is widespread, suggesting that GnRH may act as common neuromodulatory peptide. The presence of GnRHR-immunoreactive neurons detected within the lateral septum concurs with previous work in the GnRHR-hPLAP transgenic mice (42). GnRH administered directly to septal

neurons affects thermoregulatory activity in the rat (27). In this context, it is noteworthy that dysregulation of the GnRH system has been suggested as a causative factor in hot flashes (77,78), but whether GnRH *per se* plays a central role in hot flash pathophysiology remains to be elucidated.

The presence of the GnRHR within the superior colliculus and red nucleus, which play a role in sensory detection and motor control (79), suggests that GnRH could modulate these functions. Previous studies have reported GnRH binding within the superior colliculus (11). While little evidence exists for endogenous GnRH within this area, GnRH has been detected in the tectum of the developing mouse (5). The GnRHR has not been detected previously in the red nucleus. The red nucleus has been implicated in movement, possesses cerebellar connections, and projects to the olivary nucleus (80,81). It is noteworthy that the red nucleus contains an abundance of dopamine neurons. GnRH inhibits the synthesis of dopamine (82), therefore GnRH may potentially act within the red nucleus to regulate dopamine production.

The presence of GnRHR-expressing neurons in the preoptic area and arcuate nucleus is in keeping with a recent GnRHR mRNA PCR investigation in the ewe (83) as well as physiological studies on this area. Specifically, GnRH has been shown to inhibit or stimulate hypothalamic (22,23) and preoptic area neurons (25,26). We (74) and others (26) have shown that at least some of the preoptic area GnRHR-expressing neurons also synthesize GnRH.

There are several areas where GnRHR immunoreactive neurons were detected in the present study that have not previously been identified as putative targets of this decapeptide. The observed GnRHR staining in the BNST is very close to the septal region where GnRH binding has been visualized in the rat (17) and a lack of acuity using autoradiography could account for the discrepancy. There is no evidence of GnRH-immunoreactive structures in the mammalian nucleus accumbens, but injection of GnRH into the rat nucleus accumbens impairs their ability to develop conditioned avoidance responses to aversive stimuli (84,85). Dopamine within the nucleus accumbens may be involved in avoidance conditioning as GnRH may inhibit dopamine synthesis (82).

The predicted molecular weight for the murine GnRHR is 37.7kDa. The larger approximately 50kDa band observed in the present study, corresponds to what we have previously reported on cell lines using this antibody (28), and is likely to represent a glycosylated form of the GnRHR (86). Others have demonstrated by autoradiography that the molecular weight of the GnRHR in the rat pituitary is approximately 50-60kDa (86-88) although lower molecular weight forms (40kDa) may also occur at lower expression (87). We cannot discount that these other weight forms of the GnRHR are expressed in neuronal cells. Either these different weight forms may be at a lower density than the 50kDa GnRHR form observed in the present study or the present antibody may be unable to recognize them. It is noteworthy that the functional molecular weight of the pituitary GnRHR is thought to be even larger (136kDa), which is suggestive of additional in situ associated components (89).

The present study is a fundamental first step in understanding the complex role of GnRH in mammalian physiology. Although GnRH is inextricably linked with reproduction, our study provides anatomical support for a much broader neuronal influence. Indeed, as Sharpe (90) noted nearly 3 decades ago, the hypophyseal portal system may have evolved as not only an elegant system to deliver hypothalamic peptides to the pituitary gland but also as a system to restrict the delivery of these peptides to this gland. If GnRH was released into the general circulation, our anatomical data suggest that it could have numerous other unintended effects.

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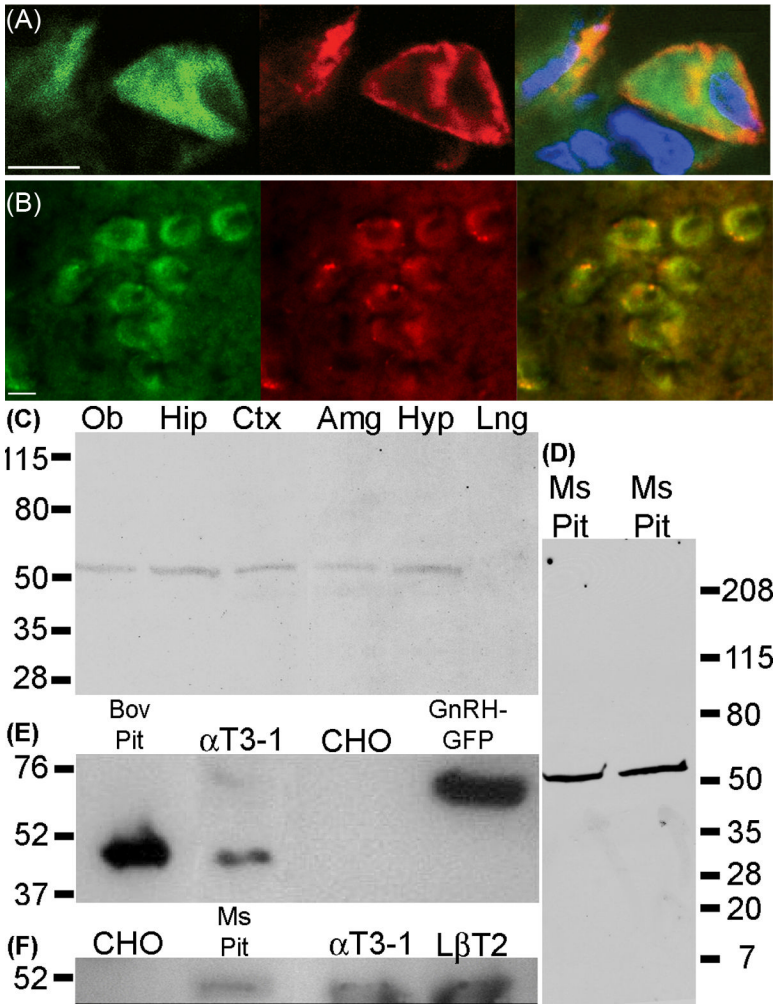

Figure 1.

Control studies supporting (**ABDEF**) the specificity of the GnRHR antibody used in these investigations and (**C**) that areas found to express GnRHR-immunoreactive neurons also presented an immunoreactive band in the Western blot. (**A**) Confocal image of GnRHR (green) and LHβ (red) immunoreactive cells in the mouse pituitary gland. The colocalization (yellow) is clearly evident in the right panel with DAPI-labeled (blue) nuclei. (**B**) GnRHR (green) expressing neurons in the amygdala that are also labeled for luciferase (red). The colocalization (yellow) is evident in the right panel. Scale bars 10μm. (**C**) Western blot on mouse tissue revealing a distinct immunoreactive band for the GnRHR at approximately 50kDa in olfactory bulb (Ob), hippocampus (Hip), cerebral cortex in the region of the piriform cortex (Ctx), Amygdala (Amg), hypothalamus (Hyp) but not in lung. (**D**) Western blot on pituitary homogenates from at least 3 mice per homogenate. (**E**) Western blot showing an approximate 50kDa immunoreactive band in bovine pituitary dispersed, αT3-1 and CHO cells. Importantly, no GnRH receptor immunoreactive band was evident in CHO cells unless they were transfected with the GnRH receptor. (**F**) Western blot showing a GnRH receptor immunoreactive band in dispersed mouse pituitary cells and in αT3-1 and LβT2 cells. Again, no labeling was evident in CHO cells.

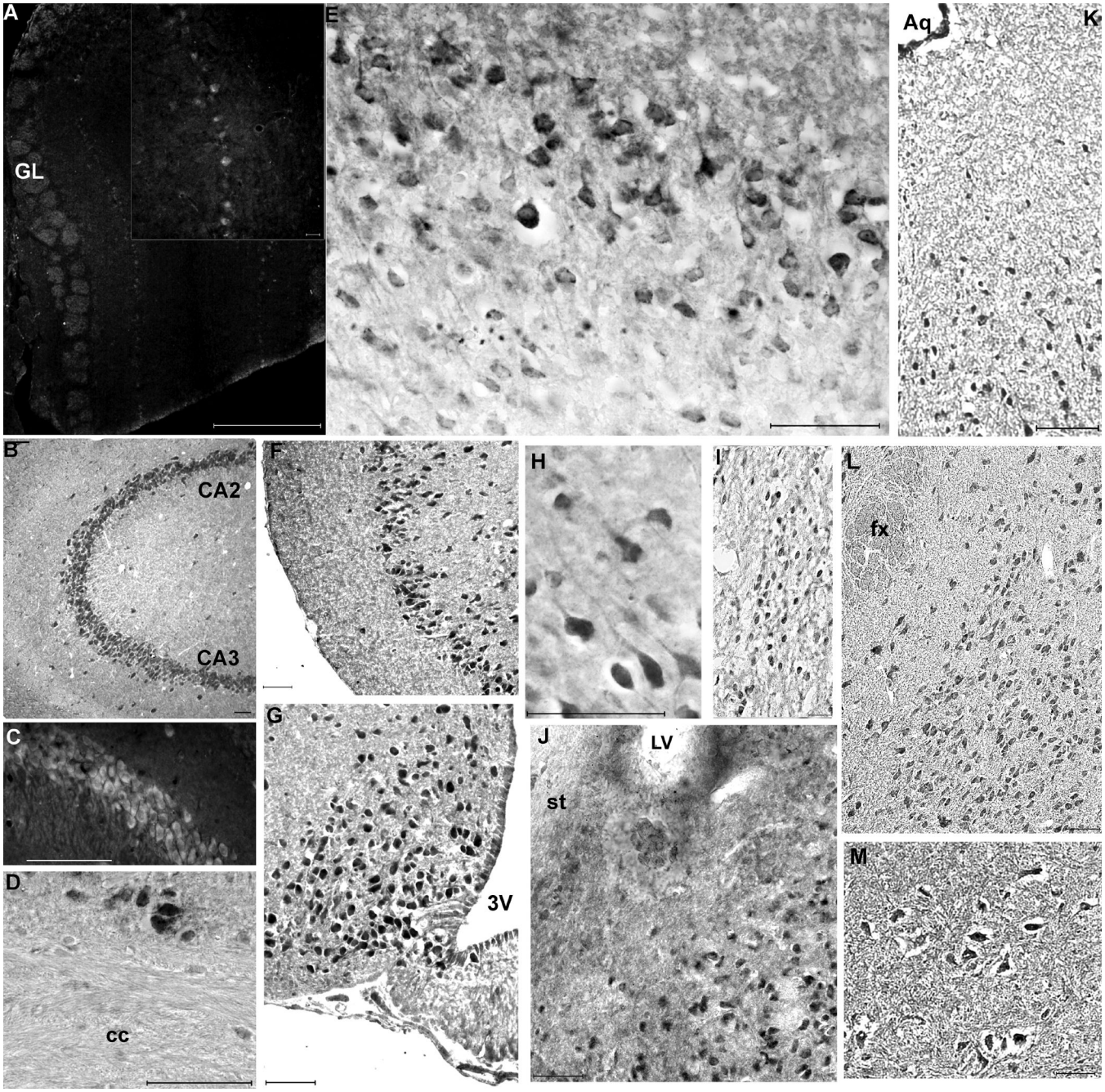

Figure 2.

GnRHR-immunoreactive cells were evident in numerous sites throughout the mouse brain. (**A**) Mitral cells in the olfactory bulb, (**B**,**C**) hippocampus, (**D**) indusium griseum, (**E**,**H**) cortex, (**F**) piriform cortex, (**G**) arcuate nucleus, (**I**) tenia tecta, (**J**) central amygdaloid nucleus, (**K**) periaqueductal gray, (**L**) ventromedial nucleus and (**M**) red nucleus. Scale bars 50μm

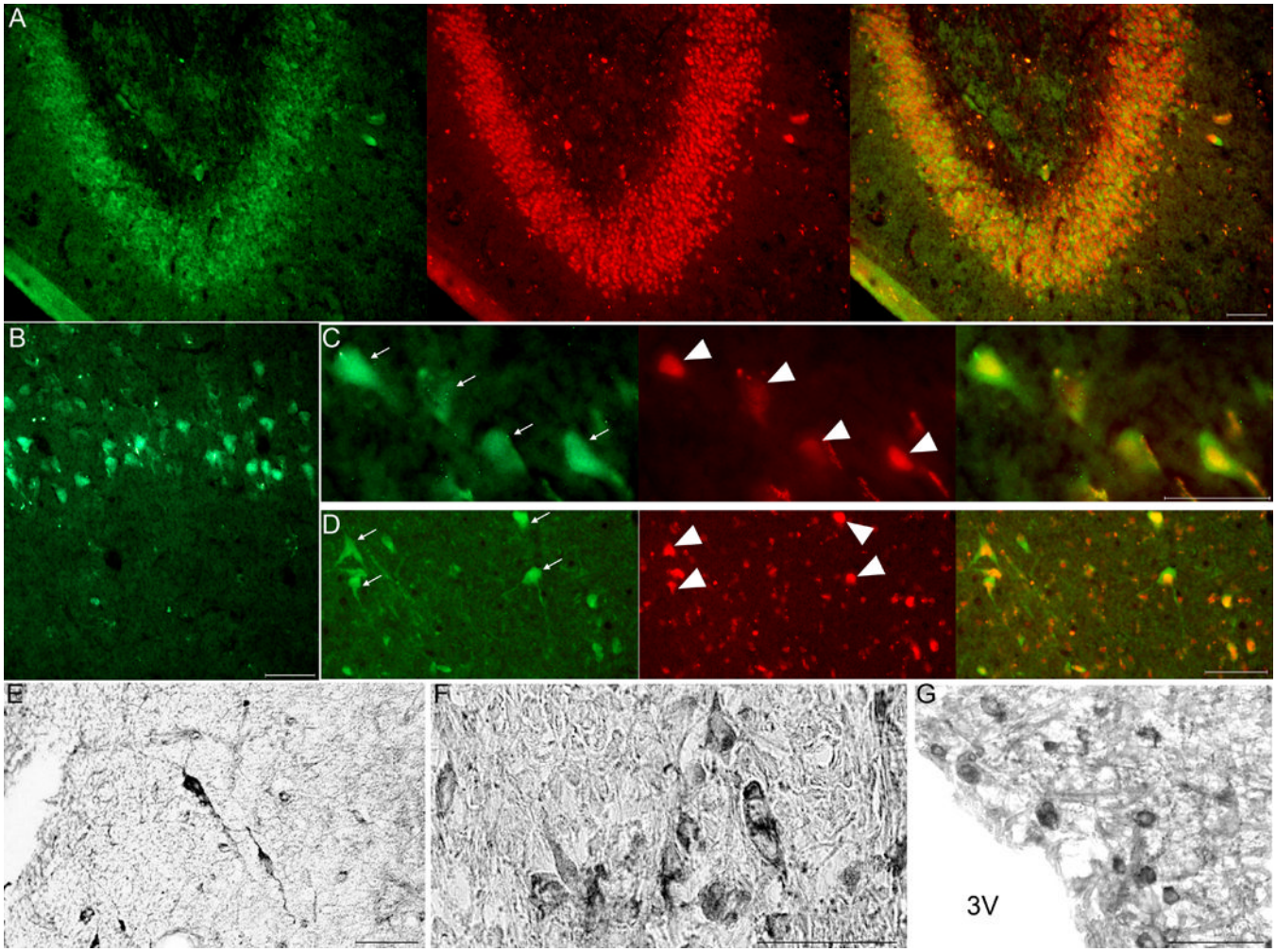

Figure 3.

GnRHR-immunoreactive (green/black) neurons in selective regions of the sheep brain. (**A**) dentate gyrus, (**B**, **C**) hippocampus, (**D**, **E**) mesencephalic central grey, (**F**) subfornical organ and (**G**) arcuate nucleus. GnRHR-expressing cells (green) also immunoreacted for ERβ (red nuclei) in the (**A**) dentate gyrus, (**C**) hippocampus and (**D**) central grey. GnRHR-immunoreactive neurons that also express ERβ-immunoreactive nuclei in **CD** are noted by white arrows and white arrowheads, respectively. Note in the central grey (**D**) that numerous cells, possibly glia, which are not immunoreactive for the GnRHR also express nuclear ERβ immunoreactivity. Dual-labeling (right hand side panels in **ACD**) is also evident in merged images, with GnRH-ERβ colocalization appearing yellow. Scale bars 50μm