# Second form of gonadotropin-releasing hormone in mouse: immunocytochemistry reveals hippocampal and periventricular distribution

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**Abstract Hypothalamic GnRH (GnRH-I) is known and named for its role in regulating reproductive function in vertebrates by controlling release of gonadotropins from the pituitary. However, another form of GnRH of unknown function (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly; GnRH-II) is expressed in the mesencephalon of all vertebrate classes except jawless fish. Here we show with immunocytochemical staining that the GnRH-II peptide is localized to the mouse midbrain as in other vertebrates, as well as in cells surrounding the ventricles and in cells adjacent to the hippocampus. Staining of adjacent sections using GnRH-I antibody revealed that the distribution of GnRH-I does not overlap with that of GnRH-II.**



*Key words:* GnRH; Ventricle; cGnRH-II; Brain

## 1. Introduction

Hypothalamic GnRH (GnRH-I) is the first in a cascade of hormones that regulates reproductive function in all vertebrates. Synthesized and secreted by neuroendocrine cells in the preoptic area of the hypothalamus, GnRH signals the anterior pituitary to release the gonadotropins that trigger sex hormone secretion and gametogenesis in the gonads. Although hypothalamic GnRH is the most studied, there are two other GnRH peptide forms that have been localized to different regions of the brain [1]. All known GnRH variants are comprised of ten amino acids: positions 1, 4, 9, and 10 are invariant, but positions 5 through 8 are highly variable [1]. Although GnRH-III (pGlu-His-Trp-Ser-Try-Gly-**Trp-Leu**-Pro-Gly; bold residues indicate differences from GnRH-I) has only been identified in fish [2], GnRH-II (pGlu-His-Trp-Ser-**His**-Gly-**Trp-Tyr**-Pro-Gly) has been found in all vertebrate classes except jawless fishes and has been localized to the mesencephalon in every species in which it has been studied [1]. GnRH-II was thought not to exist in placental mammals [3], but now its cDNA has been cloned and localized in several mammals including the tree shrew (*Tupaia glis*) [1], humans [4], and rhesus monkey [5].

Although the function of GnRH-II is unknown, its neuroanatomical distribution suggests that it has a physiological function distinct from that of GnRH-I. Immunocytochemical studies of GnRH forms in fish, amphibians, reptiles, birds, and mammals have consistently found that GnRH-II predominates in the posterior brain regions, while GnRH-I is usually limited to forebrain structures including the hypothalamus [1,6]. For example, using immunocytochemistry in the musk shrew (*Suncus murinus*), Rissman et al. [7] found no overlap in either the distribution of neurons containing GnRH-II and GnRH-I or in the projection sites of these neurons, primarily the median eminence and the medial habenula, respectively. Using both immunocytochemistry and in situ hybridization in the tree shrew, Kasten et al. [1] also found no overlap in the distribution of neurons containing the two forms of GnRH. In the rhesus macaque, immunocytochemical staining revealed a posterior system of neurons containing GnRH-II that projects throughout the brain to structures including the hindbrain, posterior pituitary, and spinal cord, and an anterior system of neurons containing GnRH-I which project from the hypothalamus to the pituitary or median eminence [8]. Urbanski et al. [5] used cRNA probes in the rhesus macaque to identify GnRH-II-expressing neurons in the midbrain, hippocampus, and several regions of the hypothalamus. However, the hypothalamic distribution of GnRH-II-expressing neurons did not overlap with that of GnRH-I-expressing neurons.

Neurons expressing these two forms of GnRH have strikingly different ontogenies, in addition to the differences in their distributions. GnRH-I-containing neurons originate in the olfactory placode and migrate to the preoptic area of the hypothalamus during development [3,9,10] whereas GnRH-II neurons originate within the mesencephalon [5].

Although GnRH-II has been isolated in every taxonomic class of jawed vertebrates, it was long thought not to exist in rodents [8]. Because GnRH-II peptide has been identified both in species which evolved more recently than rodents (such as primates) and in classes which predate rodents (e.g. fish, amphibians, reptiles, and birds), it seems highly unlikely that GnRH-II is not present in rodents, especially since a recent phylogenetic analysis [4] suggests that the multiple GnRH isoforms arose from a gene duplication event that preceded the appearance of vertebrates. In fact, it was recently shown that a peptide from mouse brain coelutes with GnRH-II using high pressure liquid chromatography and also has GnRH-II-like immunoreactivity [11]. Here we reexamine the presence of GnRH-II throughout the mouse brain using immunocytochemistry.

## 2. Materials and methods

Nine-month-old adult male mice (progeny of a B10.A×B10.BR F2 cross) were anesthetized with $CO_2$, perfused with 4.0% paraformaldehyde in PBS, and killed, all in accordance with guidelines of the Stanford University Administrative Panel on Laboratory Animal Care. The brains were postfixed for 5 h at room temperature and then overnight at 4°C, followed by embedding in agar, equilibration in 30% sucrose, and freezing in Tissue Tek O.C.T. medium (Sakura

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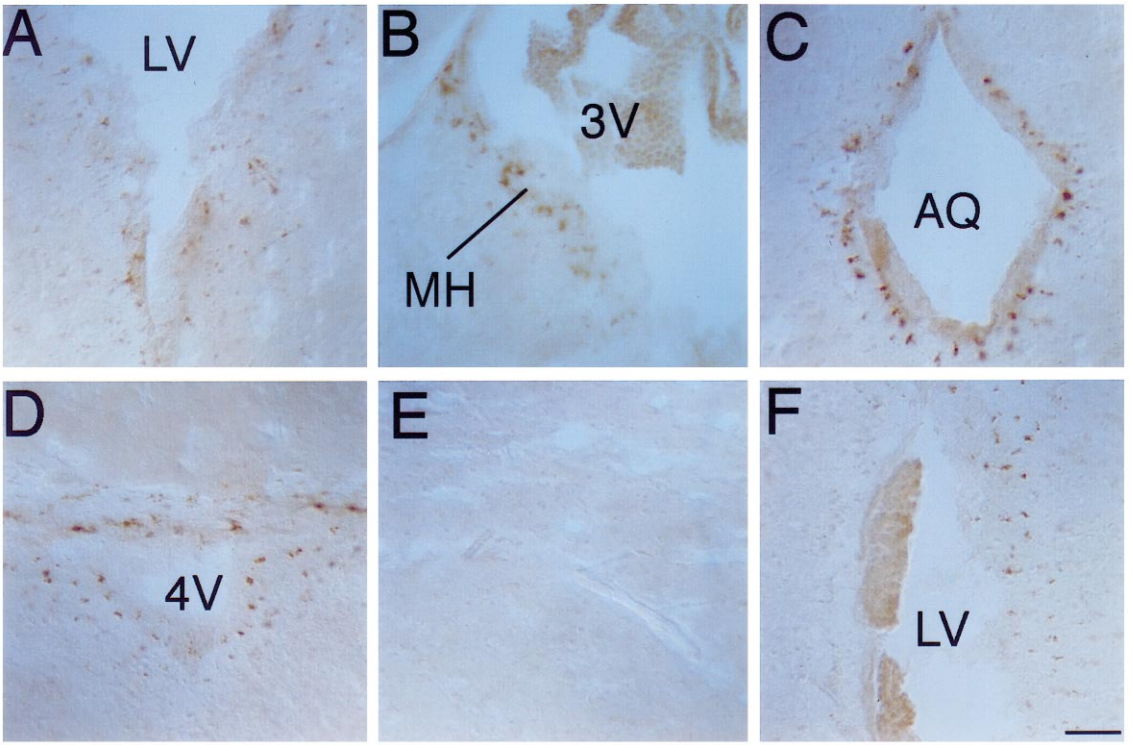


Fig. 1. Coronal sections of mouse brain showing GnRH-II immunoreactivity. LV, lateral ventricle; 3V, dorsal third ventricle; MH, medial habenula; AQ, aqueduct; 4V, fourth ventricle. Scale bar is 100 μm. A: Lateral ventricle region. B: Dorsal third ventricle region (medial habenula). C: Periaqueductal region. D: Fourth ventricle region. E: Medial septum-diagonal band. F: Tissue adjacent to hippocampus, near lateral ventricle.

Finetek, Torrence, CA). Each brain was cut coronally or sagittally into two sets of alternate 50 μm sections. The sections were rehydrated in PBS (four washes, 15 min each) and soaked in 3.0% hydrogen peroxide in PBS for 30 min to reduce endogenous peroxidase activity. The sections were again washed with PBS as above and blocked with 1.0% goat serum in PBT (0.2% BSA and 0.2% Triton X-100 in PBS) for 2 h to decrease non-specific background staining. Sections were incubated for 40 h at 4°C with GnRH-II polyclonal antiserum raised in rabbit (anti-cGnRH-II, Lot aCII6) [12,13] diluted 5000-fold in PBT containing 1.0% goat serum.

As controls for the specificity of the staining, in some cases alternate sections were either: (a) exposed to the primary antibody solution without primary antibody, (b) exposed to primary antibody preabsorbed with 100 μg GnRH-II peptide (American Peptide Company, Sunnyvale, CA) per ml of antibody solution, or (c) incubated with rabbit LHRH (GnRH-I) polyclonal antiserum (HU60) [14].

Sections were washed with PBT (four washes, 15 min each) and incubated for 10 h at 4°C with biotinylated goat anti-rabbit IgG secondary antibody diluted to 0.5% in PBT, then washed again with PBT and incubated with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 3 h at room temperature, and developed with 3,3′-diaminobenzidine (Sigma, St. Louis, MO) as chromogen. The sections were dehydrated in ethanol and cleared in xylene, mounted with Permount (Fisher Scientific, Fair Lawn, NJ), coverslipped, and examined using light microscopy.

## 3. Results

GnRH-II-immunopositive cells were located in areas surrounding each of the cerebral ventricles along most of their length: the lateral ventricles (Fig. 1A), third ventricle (dorsal third ventricle shown in Fig. 1B), midbrain aqueduct (Fig. 1C), and fourth ventricle (Fig. 1D). The cells surrounding the third ventricle (Fig. 1B) were located in the medial habenula. GnRH-II-immunopositive cells were also identified among neurons adjacent to the hippocampus (Fig. 1F). No GnRH-II immunoreactivity was observed in the medial septum-diagonal band (Fig. 1E) or hypothalamus, sites where we found extensive staining with GnRH-I (data not shown). Staining was eliminated by omission of primary antibody and preabsorption of the GnRH-II antiserum with GnRH-II. GnRH-II-immunoreactive cells were smaller in size ($\sim$10 μm in diameter) and more rounded in shape (Fig. 1, A–F) than the cells which were immunoreactive for GnRH-I, which were $\sim$20 μm in diameter and had a more fusiform shape (data not shown). All cells immunoreactive for GnRH-I or GnRH-II were stained in the cytoplasm but not in the nucleus. Immunoreactive processes of the GnRH-II-containing cells were very short and thin, and were confined to the same brain regions as GnRH-II-immunoreactive cell bodies. In contrast, GnRH-I-immunoreactive axons were long (often $>$250 μm), and could easily be traced to other regions of the brain.

In sections exposed to the GnRH-I antiserum, immunopositive cell bodies and processes were clearly identified in the medial septum-diagonal band and the hypothalamus. No cell bodies immunoreactive for GnRH-I were observed in the periventricular regions found to contain GnRH-II immunoreactivity. However, fibers immunoreactive for GnRH-I were identified in the areas surrounding the third ventricle, the aqueduct, and the fourth ventricle (not shown).

## 4. Discussion

The differential staining patterns produced by exposure to the GnRH-I and GnRH-II antisera respectively indicate that two distinct forms of GnRH exist in mouse and that these two

forms exhibit distinct patterns of immunoreactivity in the mouse brain. The morphology of the GnRH-II-immunopositive cells identified in this study is consistent with that of GnRH-II neurons previously identified in other species. In rhesus macaque monkey, GnRH-I-immunopositive cells are characteristically fusiform in shape with thick neurites and the cells containing GnRH-II are smaller and more oval-shaped, with fine, short processes [8]. Our results are consistent with these descriptions.

We found no evidence for the co-expression of GnRH-I and GnRH-II in cells within the mouse brain. However, Chen et al. [11] noted that a small number of cells in the mouse hypothalamus showed immunoreactivity for GnRH-I and GnRH-II. In the rhesus macaque, a recent study using the highly sensitive method of RNA-RNA in situ hybridization revealed no co-localization of GnRH-I and GnRH-II in the brain, including the hypothalamus [5], in accord with our results in the mouse using ICC. The limited evidence to date suggests that the question of GnRH-I and GnRH-II co-expression requires further investigation.

Although no cell bodies immunopositive for GnRH-I were identified in the regions surrounding the ventricles, fibers immunoreactive for GnRH-I were detected in these areas. This is consistent with previous reports of GnRH-I-immunopositive fibers in the ventricular and subarachnoid spaces of the rat brain. GnRH-I axons (but no cell bodies) have previously been identified near the lateral ventricles, third ventricle, and midbrain aqueduct of the rat brain, and some of these fibers were shown to reach the ventricular cavity [15]. The only GnRH-positive cell bodies we identified in periventricular regions of the mouse brain were immunopositive for GnRH-II.

The function(s) of GnRH-II remain a mystery, but its identification and localization suggest some possibilities. The spatial distribution is consistent with a neuromodulatory role, as further supported by earlier functional analyses [16,17]. GnRH has also been implicated in immune system activity based on several kinds of evidence: (a) the immunostimulatory effects of GnRH-I [18], (b) high GnRH-II expression, both in rhesus monkey spleen [5] and in human organs known to contain large number of mast cells (e.g. kidney, prostate, bone marrow) [4], (c) GnRH immunoreactivity in mast cells in the ring dove medial habenula [19,20], and (d) the presence of GnRH receptors in mouse thymus and spleen [18]. Likewise, our findings indicate the presence of GnRH-II in areas associated with mast cell function, because in the mouse, mast cells are located along the vasculature associated with the hippocampus and the lateral and third ventricles [21].

Although immunocytochemical, behavioral, and expression studies have provided important clues as to the distribution and possible sites of action of GnRH-II, the crucial steps in gaining a clearer picture of its physiological function will be the targeted deletion of the gene for GnRH-II in mouse and subsequent studies of mice lacking a functional GnRH-II gene.

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