Two molecular forms of gonadotropin-releasing hormone (GnRH-I and GnRH-II) are expressed by two separate populations of cells in the rhesus macaque hypothalamus

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Abstract

Gonadotropin-releasing hormone represents the primary neuroendocrine link between the brain and the reproductive axis, and at least two distinct molecular forms of this decapeptide (GnRH-I and GnRH-II) are known to be expressed in the forebrain of rhesus macaques (Macaca mulatta). Although the distribution pattern of the two corresponding mRNAs is largely dissimilar, their expression appears to show some overlap in specific regions of the hypothalamus; this raises the possibility that some cells express both molecular forms of GnRH. To resolve this issue, double-label histochemistry was performed on hypothalamic sections from six male rhesus macaques, using a monoclonal antibody to GnRH-I and a riboprobe to monkey GnRH-II mRNA. In total, more than 2000 GnRH neurons were examined but in no instance were GnRH-I peptide and GnRH-II mRNA found to be coexpressed. This finding emphasizes that GnRH-I and GnRH-II are synthesized by two distinct populations of hypothalamic neurons, and suggests that they may be regulated by different neuroendocrine pathways.

Keywords: Luteinizing hormone-releasing hormone; LHRH; In situ hybridization; Immunohistochemistry

1. Introduction

The neuropeptide, gonadotropin-releasing hormone (GnRH), plays a central role in stimulating the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland, so it represents the primary neuroendocrine interface between the brain and the reproductive axis. Although multiple forms of this decapeptide are known to exist in the brains of lower vertebrates [22], it has generally been assumed that eutherian mammals express only a single form (GnRH-I), commonly known as mammalian GnRH [15,17,18,26]. Recently, however, it has become evident that some mammals [3,5,7], including humans [2,30] and nonhuman primates [11,14,28], express a second form of GnRH (GnRH-II) which is commonly known as chicken-II GnRH [19]. From a phylogenetical perspective it is interesting that GnRH-II is highly conserved across the vertebrate classes. It appears to be identical in fish [6,8,20,21,25,31–33], amphibians [1,24,27], reptiles [23], birds [19], and mammals [3,5,7]. Nevertheless, the physiological function of GnRH-II remains unknown.

The peptide sequence of rhesus macaque GnRH-I (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) and the deduced peptide sequence of GnRH-II (pGlu-His-Trp-Ser-Ser-Gly-Trp-Tyr-Pro-Gly-NH₂) are 70% identical. On the other hand, the GnRH-associated peptide (GAP) regions of the respective precursor molecules are unique [16,28]. Furthermore, in humans GnRH-I and GnRH-II are encoded by two distinct genes [30]. Taken together, this suggests that the synthesis and release of GnRH-I and GnRH-II in primates may be controlled by different neuroendocrine pathways and that the two populations of GnRH-releasing neurons may play different physiological roles. Currently, however, it is unclear whether the same
neurons can synthesize both GnRH-I and GnRH-II. This possibility certainly exists in primates because GnRH-II is highly expressed in hypothalamic areas where GnRH-I has also been found, notably around the ventral hypothalamic tract and in the medial basal hypothalamus [11,28]. To resolve this issue, the present study used a combined immunohistochemical/molecular approach to examine the hypothalamic GnRH system of rhesus macaques, and to determine the extent to which GnRH-I neurons express mRNA encoding GnRH-II. Preliminary findings have already been published in abstract form [13].

2. Materials and methods

2.1. Animals

This study was approved by the Institutional Animal Care and Use Committee at the Oregon Regional Primate Research Center (ORPRC) and used six male rhesus macaques (Macaca mulatta), aged 0.6–15 years. They were cared for by the ORPRC in accordance with the NIH Guide for the Care and Use of Laboratory Animals and eventually were painlessly killed to provide a source of brain tissue both for this and other related studies.

2.2. Tissue preparation

The animals were deeply anesthetized using ketamine/pentobarbital according to procedures established by the Panel on Euthanasia of the American Veterinary Society. Their brains were fixed by perfusing 1 l of 0.9% saline through the ascending aorta at room temperature followed by 6.5 l of ice-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Hypothalami were blocked rostral to the optic chiasm and just rostral to the mammillary bodies. They were then immersed in fresh fixative for an additional 3 h (at 4°C) and cryoprotected, as previously described [4,9]. This involved their immersion in 0.02 M sodium phosphate buffer pH 7.4 containing glycerol (10% v/v) and dimethyl sulfoxide (DMSO; 2% v/v) for 24 h, followed by immersion in a more concentrated glycerol (20% v/v) phosphate/DMSO solution for an additional 72 h. The blocks were then rapidly frozen in...

Fig. 1. Regional distribution of GnRH mRNA in the rostral (upper panels) and caudal (lower panels) hypothalamus of male rhesus macaques, as revealed by in situ hybridization. Representative autoradiographs depicted in the left panels show a scattered pattern of GnRH-I mRNA expression, especially in ventral regions of the hypothalamus. Representative autoradiographs depicted in the right panels show a concentrated pattern of GnRH-II mRNA expression, especially in the supraoptic (SON), paraventricular (PVN), and suprachiasmatic nuclei (SCN), and also in the medial basal hypothalamus (MBH). oc = optic chiasm; ot = optic tract. Scale bar = 5 mm.
2-methyl butane (pre-cooled in an ethanol/dry-ice bath) and stored at −85°C. Subsequently, they were sectioned (25 μm) in the coronal plane using a freezing sliding microtome and then stored free-floating at −20°C in a cryoprotectant solution comprising 0.05 M sodium phosphate buffer (pH 7.3) with ethylene glycol (30% v/v) and glycerol (20% v/v).

2.3. Immunohistochemistry

Immunohistochemistry (IHC) was performed on a series of 12 hypothalamic sections from each animal, collected at approximately 200-μm intervals. All solutions made for this procedure used RNase-free, diethyl pyrocarbonate-treated water. Free-floating sections were washed three times, 5 min each, with Tris buffer A (0.05 M Tris, pH 7.6; containing 0.15 M sodium chloride and 1% Triton X-100, v/v). They were then incubated in 2% normal horse serum (Vector Laboratories, Burlingame, CA) in Tris buffer A for 20 min at room temperature and washed as before. Next, the sections were incubated overnight at 4°C with a previously characterized [29] GnRH-I monoclonal antibody (HU4H) at a 1:1000 dilution in Tris buffer A. They were then washed three times, 5 min each, incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories) at 1:1000 dilution in Tris buffer A for 1 h at room temperature, and again washed. To detect the signal, the sections were exposed to an avidin/biotin complex (Standard ABC kit; Vector Laboratories) for 1 h, washed three times in Tris buffer B (0.05 M Tris, pH 7.6; containing 0.15 M sodium chloride), 5 min each, and exposed to 3,3′-diaminobenzidine tetrachloride (1 mg/ml Tris buffer B; Sigma, St. Louis, MO) for 10 min, concluding with 3 more washes. The sections were then mounted on glass microscope slides (Fisherbrand Superfrost/Plus; Fisher, Auburn, WA), fan dried for 1 h, vacuum dried overnight, and stored at −85°C.

2.4. cRNA probe synthesis and in situ hybridization histochemistry

A 35S-labelled 430-nucleotide antisense riboprobe was transcribed using rhesus macaque GnRH-II precursor cDNA as the template. Because the GAP coding regions of the macaque GnRH-I and GnRH-II precursors are unique [16,28], this probe was found to specifically identify only those cells that express GnRH-II mRNA. The 35S-labelled riboprobe to rhesus macaque GnRH-I was transcribed from cDNA previously characterized by Ma et al. [16].

In situ hybridization (ISH) was performed, as previously described [4], on the series of hypothalamic sections that had initially been processed for IHC. First, the brain sections were brought to room temperature and post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 15 min, rinsed in phosphate buffer, and then were digested with Proteinase K (10 μg/ml) in Tris–EDTA buffer (pH 8.0; 100 mM Tris, 50 mM EDTA) for 30 min at 37°C. Next, they were acetylated, dehydrated with ascending concentrations of ethanol, and dried under vacuum for 2 h. They were then hybridized for 18 h at 65°C with 100 μl of 35S-labelled antisense riboprobe, diluted to 1 × 107 c.p.m./ml of hybridization buffer (50 mM dithiothreitol, 250 μg/ml tRNA, 50% formamide, 0.3 M sodium phosphate buffer).
chloride, 1 × Denhardt’s solution, 20 mM Tris (pH 8.0), 1 mM EDTA and 10% dextran sulfate). For the hybridization, glass coverslips were affixed to the slides by applying DPX mounting medium (BDH Laboratory Supplies, Poole, England) along the edges. They were subsequently removed after two 30-min soakings in 4 × saline–sodium citrate buffer (SSC; the 20 × stock solution comprised 175.3 g sodium chloride and 88.2 g sodium citrate per liter (pH 7.0)) containing 20 mM dithiothreitol (DTT). The sections were then incubated in Tris–EDTA buffer (pH 8.0; 10 mM Tris, 1 mM EDTA, 0.5 M sodium chloride) containing RNase A (10 μg/ml) for 30 min at 37°C, followed by two 30-min washes at room temperature with 2 × SSC containing 1 mM DTT. After a final 30-min wash at 70°C with 0.1 × SSC containing 1 mM DTT, they were dehydrated through ascending concentrations of ethanol, containing 0.3 M ammonium acetate, and air-dried for 30 min. To visualize the hybridization pattern, the sections were apposed to Hyperfilm β-max (Amersham Pharmacia Biotech, Piscataway, NJ) for 6 days (i.e., an exposure period that maintained the hybridization signal in the linear response range of the film).

For quantitation, the hypothalamic sections were again dehydrated using increasing concentrations of ethanol, defatted in xylenes for 1 h, and dipped in photographic emulsion (NTB-2, Eastman Kodak, Rochester, NY). They were exposed in a light-tight box for 12 days at 4°C, and then processed with Kodak developer (D-19) and fixer.

dehydrated with ethanol, cleared with xylenes, and finally coverslipped using DPX mounting medium. Cells were defined as having an obvious round or fusiform silver grain deposition pattern and a distinct central nuclear area. Both DAB-labelled (for IHC) and silver-grain-labelled cells (for ISH) were counted and the degree of colocalization determined.

3. Results

Representative autoradiographs depicting the general distribution pattern of GnRH-I and GnRH-II mRNAs in the hypothalamus are shown in Fig. 1. GnRH-I mRNA expression was found to be scattered widely throughout the hypothalamus, especially in the ventral regions (Fig. 1, left panels), whereas GnRH-II mRNA expression was found to be concentrated mainly in the supraoptic (SON) and paraventricular (PVN) nuclei, as well as in the medial basal hypothalamus (MBH) (Fig. 1, right panels). Despite the marked difference in the general distribution pattern of GnRH-I and GnRH-II mRNAs, some overlap was also evident, especially around the ventral hypothalamic tract and in the MBH.

To examine whether the GnRH-I neurons in these regions also express GnRH-II mRNA, some of the brain sections were first processed for IHC using a monoclonal antibody to GnRH-I and then for ISH using a riboprobe to

![Fig. 3. Representative photomicrographs of hypothalamic sections from male rhesus macaques, double-labelled using a procedure that combined immunohistochemistry (IHC) with in situ hybridization (ISH). The sections were initially processed for GnRH-I IHC and then for ISH, using a riboprobe to either GnRH-I mRNA (A,B) or to GnRH-II mRNA (C–F). In the bright-field photomicrographs (upper panels), GnRH-I-immunopositive cells are indicated by black arrows. In the corresponding dark-field photomicrographs (lower panels), cells expressing GnRH-I or GnRH-II mRNA are identified by regions of high silver grain density (indicated by white arrow heads). Note the colocalization of GnRH-I mRNA, but not GnRH-II mRNA in the GnRH-I immunopositive cells. Scale bars = 50 μm.]
monkey GnRH-II mRNA. The sections were then coated with photographic emulsion to allow microscopic examination for double labelling. Overall, three times as many GnRH-II as GnRH-I cells were detected (Table 1). More importantly, in no instance was GnRH-II mRNA found to be coexpressed with GnRH-I peptide (Fig. 2).

For control purposes, some of the GnRH-I-immunolabelled cells were processed for ISH using a riboprobe to monkey GnRH-I mRNA. As expected, numerous GnRH-belled cells were processed for ISH using a riboprobe to be coexpressed with GnRH-I peptide Fig. 2. Importantly, in no instance was GnRH-II mRNA found to be coexpressed with GnRH-II as GnRH-I cells were detected Table 1. More attention for double labelling. Overall, three times as many monkey GnRH-II mRNA. The sections were then coated with photographic emulsion to allow microscopic examination for double labelling. Overall, three times as many GnRH-II as GnRH-I cells were detected (Table 1). More importantly, in no instance was GnRH-II mRNA found to be coexpressed with GnRH-I peptide (Fig. 2).

For control purposes, some of the GnRH-I-immunolabelled cells were processed for ISH using a riboprobe to monkey GnRH-I mRNA. As expected, numerous GnRH-I-immunolabelled cells were found scattered around the ventral hypothalamic tract and in the MBH, and each one showed a high level of GnRH-I mRNA expression (Fig. 3A,B). This is in marked contrast to the results obtained when using the GnRH-II riboprobe; although several cells in the vicinity of the GnRH-I neurons showed a high level of hybridization to the GnRH-II riboprobe, the GnRH-I neurons themselves did not (Fig. 3C–F).

4. Discussion

The present results corroborate and extend previous ISH findings by showing a high level of GnRH-II mRNA expression in discrete regions of the primate hypothalamus [11,28]. These regions include three discrete hypothalamic areas: the supraoptic (SON), paraventricular (PVN), and suprachiasmatic (SCN) nuclei, as well as the medial basal hypothalamus (MBH). The significance of this finding is that the clustered pattern of GnRH-II mRNA is strikingly different from the more diffuse pattern of GnRH-I mRNA. Moreover, even in those regions where there appears to be some regional overlap between GnRH-I and GnRH-II mRNA expression, the individual cells express only one of the two molecular forms. Even though more than 2000 cells were examined, no coexpression was observed. Although the lack of GnRH-I/GnRH-II double-labelling may reflect a limitation of the techniques employed in this study, this possibility is highly unlikely. First, the combined IHC/ISH approach that was used in this study is well-established in our laboratory [10] and, moreover, was validated by demonstrating that GnRH-I-immunolabelled neurons also express GnRH-I mRNA. Second, GnRH-I-immunolabelled neurons were often observed within the same field of view as GnRH-II-positive cells, but in no instance was colocalization of GnRH-I peptide and GnRH-II mRNA evident. However, it remains to be elucidated whether or not the GnRH-II cells directly contact GnRH-I neurons, or other neuropeptidergic neurons that are abundant in the SON and PVN, or GnRH-II is colocalized with oxytocin and vasopressin.

Until recently, only a single molecular form of GnRH was thought to exist in the primate brain, so existing ideas about the neuroendocrine control of reproductive function in humans are still generally based on the involvement of only GnRH-I [15,17]. Therefore, the finding that in rhesus macaques GnRH-II mRNA is highly expressed in the hypothalamus and that GnRH-II can stimulate LH release in vivo [14] suggests that both molecular forms of GnRH may be involved in the physiological release of LH. Taken together, these findings suggest that the coordinated influence of GnRH-I and GnRH-II may be crucial to some aspects of reproductive function, such as ovulation or the onset of puberty. In support of the latter possibility, we have recently observed that the expression of GnRH-II mRNA is developmentally regulated in the hypothalamus of rhesus macaques [12], which suggests that GnRH-II may represent a primary trigger for the onset of puberty in primates.

Although the exact physiological role of GnRH-II remains to be elucidated, the present findings clearly show that its expression in the primate hypothalamus occurs in a population of cells that is completely distinct from that expressing GnRH-I. The marked difference in the distribution pattern of GnRH-I and GnRH-II expressing cells supports the view that these two neuropeptides are, to some extent, regulated differently and that they play different physiological roles. Moreover, because the reproductive axis of rhesus macaques closely resembles that of humans, the results question our basic assumptions about the etiology of human reproductive disorders and the involvement of only a single GnRH neuronal population.

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