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Journal
General and Comparative Endocrinology

ISSN
1095-6840

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Publication Date
1996

Peer reviewed

General and Comparative Endocrinology 104, 7–19 (1996)
Article No. 0135

Characterization of Two New preproGnRH mRNAs in the Tree Shrew: First Direct Evidence for Mesencephalic GnRH Gene Expression in a Placental Mammal

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Accepted May 6, 1996

Reproductive maturation and regulation is centrally orchestrated by gonadotropin-releasing hormone (GnRH). GnRH produced in the vertebrate hypothalamus acts on the pituitary to regulate gonadotropins. In nonplacental mammalian species, it has recently been shown that a second GnRH gene is expressed in mesencephalic cells. Here, we report the cDNA sequences and expression patterns for two distinct genes encoding the hypothalamic and mesencephalic GnRH forms in the brain of a placental mammal, the tree shrew (*Tupaia glis belangeri*). The novel mammalian GnRH form, designated here as [His^{5}Trp^{7}Tyr^8]GnRH (often called chicken GnRH II), is expressed in neurons of the mesencephalon and is the first nonhypothalamic form to be isolated from a mammal. Its peptide sequence is identical to the form previously reported in fish, amphibians, reptiles, and birds, revealing that it has remained unchanged for 500 million years. In contrast, the sequences of the hypothalamic GnRH decapeptides vary by as much as 50% across vertebrate species. The remarkable sequence conservation of mesencephalic GnRH suggests that it has been highly constrained throughout evolution, perhaps indicating an important, conserved nongonadotropic role. The discovery and localization of two mRNAs encoding distinct GnRH forms in an advanced mammal suggest that other mammals, including primates, may also have a second GnRH gene with expression localized in the midbrain.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. U63326 and U63327.

All vertebrates, from cyclostomes to primates, rely on gonadotropin-releasing hormone (GnRH) for the development and maintenance of reproductive function. GnRH synthesized within cells of the hypothalamus acts as a releaser of gonadotropins via secretion into the specialized circulatory system that serves the pituitary or through axons directly innervating the pituitary (Bushnik and Fernald, 1995). There, GnRH regulates the release of pituitary gonadotropins which, in turn, stimulate the release of steroid hormones from the gonads. During the 500 Myr of vertebrate evolution, the length of GnRH has remained at 10 amino acids, the amino (pGlu) and carboxy (Gly-amide) residues are unchanged, and 5 of the 10 residues are identical from lamprey to humans. The most stable region of the peptide, residues 1–3, is responsible for releasing gonadotropins, while the region with the most changes, residues 5–8, is thought to mediate receptor binding (Sherwood, 1987). Since the original isolation of the GnRH peptide (Amoss *et al.*, 1971; Matsuo *et al.*, 1971), cDNAs encoding hypothalamic forms have been cloned from mammals (Adelman *et al.*, 1986; Mason *et al.*, 1986) and, more recently, from

0016-6480/96 $18.00

nonmammalian species (Bond *et al.,* 1991; Klungland *et al.,* 1992; Suzuki *et al.,* 1992; Dunn *et al.,* 1993; Bogerd *et al.,* 1994; Hayes *et al.,* 1994; Okuzawa *et al.,* 1994a; Ashihara *et al.,* 1995; Gothilf *et al.,* 1995; Grober *et al.,* 1995; S. White *et al.,* 1995a).

In addition to the hypothalamic forms of GnRH, another form has been consistently detected. In many species, this form has been localized to mesencephalic perikarya (e.g., Mikami *et al.,* 1988; Amano *et al.,* 1991; Dellovade *et al.,* 1993; Muske and Moore, 1994; Yamamoto *et al.,* 1995) via immunohistochemical visualization with specific antibodies. In striking contrast to the variation in hypothalamic GnRH forms, the decapeptide expressed by mesencephalic cells is identical in all species in which the peptide sequence has been either determined (Miyamoto *et al.,* 1984; Lovejoy *et al.,* 1991a,b; Bogerd *et al.,* 1992; Ngamvongchon *et al.,* 1992) or deduced from isolated cDNAs (Bogerd *et al.,* 1994; White *et al.,* 1994). This is further supported by immunological and chromatographic evidence which demonstrates the presence of this form in every vertebrate class except jawless fishes (refer to reviews by King and Millar, 1991, 1992; Sherwood *et al.,* 1993b). It is perhaps surprising that despite it's being ubiquitous, the function of this form is still in question.

Nomenclature for the various forms of GnRH can be confusing. One convention uses names which refer to the organisms in which they were first isolated. For example, the form of GnRH which acts as the releasing peptide in mammals (Amoss *et al.,* 1971; Matsuo *et al.,* 1971) is often referred to as ''mammalian GnRH'' although it has since been found in amphibia and fish. Other nomenclatures have been used in which the multiple forms of GnRH within one species are arbitrarily labeled LHRH I (for the hypothalamic form) and LHRH II (for the extrahypothalamic form) as in Branton *et al.* (1986). Here we use a nomenclature that identifies the positions within the decapeptide at which the amino acids differ from the mammalian hypothalamic form [Arg^8]GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2). In this nomenclature, the mesencephalic form, previously called ''chicken GnRH II,'' is referred to as [His^{5}Trp^{7}Tyr^8]GnRH for it has the sequence (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-GlyNH_2).

Recently, cDNAs encoding [His^{5}Trp^{7}Tyr^8]GnRH have been sequenced from bony fish (Bogerd *et al.,* 1994; White *et al.,* 1994) and its mesencephalic localization has been confirmed using *in situ* hybridization (White *et al.,* 1994; Zandbergen *et al.,* 1995). Based on evidence from high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA) (King et al., 1989, 1990b, 1994a), some metatherian mammals (possum, *Trichosurus volpecula;* opossum, *Monodelphis domestica;* quoll, *Dasyurius viverrinus;* and bandicoot, *Isodon macrourus*) and two early evolved eutherian mammals (musk shrew, *Suncus murinus;* and mole, *Chysochloris asiatica*) are thought to express [His^{5}Trp^{7}Tyr^8]GnRH. More recently, immunohistochemistry has suggested that [His^{5}Trp^{7}Tyr^8]GnRH is localized within midbrain neurons of the musk shrew (Rissman *et al.,* 1995).

To discover whether there is a mammalian gene for this second, highly conserved GnRH form, we looked for GnRH-encoding mRNAs in another insectivore, a highly social placental mammal, the tree shrew (*Tupaia glis belangeri*). Tree shrews (*Scandentia*) seem a logical choice for this search since they closely resemble ancestral placental mammals (Romer, 1972) and are more recently evolved than rodents, but predate primates (Martin, 1990). Thus, due to their phylogenetic position, discovery of the gene in this taxa would strongly predict its presence in both rodents and primates.

Here we report the nucleotide sequences encoding two forms of GnRH in the tree shrew. A preliminary report of this result has already appeared (White *et al.,* 1995b). This discovery of more than one form of GnRH in a highly derived mammal supports the possibility that [His^{5}Trp^{7}Tyr^8]GnRH may also be present in primates.

MATERIALS AND METHODS

Animals

All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Tree shrews were anesthetized and then administered overdoses of pentobarbital. For use in nucleic acid analyses, brains were quickly dissected from the skulls. Animals used for immunohistochemistry were perfused with saline and then fixative (4% paraformaldehyde in 0.1 *M* phosphate buffer) through the right ventricle for 5 min prior to dissection.

Amplification and Subcloning of T. glis belangeri GnRH cDNAs

The brain of a male juvenile tree shrew was quickly frozen in ethanol on dry ice after removal. Poly(A)$^+$ mRNA was isolated and used as substrate for reverse transcriptase–polymerase chain reaction (RT–PCR) as previously described (White *et al.*, 1994) with the following modifications: cDNA transcription was primed with a bipartite 34-mer consisting of a 5′ *Hin*dIII restriction endonuclease (RE) site followed 3′ by $d(T)_{24}$. Reaction conditions, 5′ RACE strategy, subcloning, sequencing, and screening of clones were as previously described (White *et al.*, 1994).

Tissue Preparation and in Situ Hybridization

The brain of a female tree shrew was immersed in Tissue-Tek OCT compound (Miles, Inc.) and rapidly frozen on dry ice in ethanol. Coronal 40-μm sections were cut serially on a cryostat. Alternate sections were mounted on poly-L-lysine-coated slide pairs and dried at 40°. Sections were then fixed in 4% paraformaldehyde, rinsed in phosphate-buffered saline (PBS), ethanol dehydrated, and stored at −80°. Immediately prior to hybridization, tissue was rinsed with water, acetylated (2.5% acetic anhydride in 0.1 *M* triethanolamine buffer, pH 8), rinsed in PBS, and ethanol dehydrated. To control for cross-reactivity, alternate sections were hybridized to antisense probes made to either [Arg^8]GnRH or [His^{5}Trp^{7}Tyr^8]GnRH encoding mRNA. Riboprobes were generated and hybridized to tissue as previously described (White *et al.*, 1994) with minor modifications: cRNA antisense probes were transcribed from *Eco*RI (New England Biolabs)-linearized pBluescript II (SK$^+$) (Stratagene) plasmids containing the tree shrew preproGnRH cDNAs using T7 RNA polymerase (Epicentre Technologies) in the presence of digoxigenin–UTP. Probes were denatured in hybridization solution at 65° and then applied to each slide (120 μl per slide), without prehybridization, and incubated for 11 hr at 60°. Slides were washed in 4× standard sodium citrate buffer (SSC) at 60° and then treated with Ribonuclease A (5 μg/ml). Sections were washed in high-stringency washes (0.1 × SSC in 1 m*M* DTT at 60°). Immunological detection was achieved with anti-digoxygenin FAB fragments coupled to alkaline phosphatase, processed with NBT-BCIP substrate as described in the instructions for *in situ* hybridization from Boehringer-Mannheim's Genius 3 kit.

Northern Blot Analyses

RNA was isolated from brain tissue using Ultraspec II and RNA Tack resin (Biotecx) according to manufacturer's recommendations. From this total RNA fraction, poly(A)$^+$ mRNA was isolated using PolyATract mRNA isolation system III (Promega). Four micrograms of mRNA was loaded and electrophoretically separated on a 1.5% agarose, 7% formaldehyde gel alongside RNA molecular weight markers (Gibco-BRL). The mRNA was transferred by capillary to Nytran (Maximum Strength, Schleicher & Schuell) in alkaline transfer buffer and stained with 0.02% methylene blue in 0.3 *M* NaOAc to assess its quality. Labeled antisense probes for both the tree shrew [His^{5}Trp^{7}Tyr^8]-GnRH- and [Arg^8]GnRH-encoding messages were transcribed from vectors using T7 RNA polymerase incorporating [α-^{32}P]UTP to a specific activity of 3×10^8 cpm/μg. Probes were hybridized to blots at high stringency (50% formamide, 67°), washed (in 0.3 × SSC, 0.1% SDS, 0.25 *M* sodium pyrophosphate, 0.01 *M* EDTA, 70°), and imaged on X-ray film (Kodak) and Phosphorimager (Molecular Dynamics).

Immunohistochemical Analysis

Heads were removed from adult tree shrews and placed into fixative for at least an hour. Brains were removed from skull and dura and placed into 30% sucrose, 0.1 *M* phosphate buffer and stored at 4°. Brains were embedded in 30% sucrose, 1.5% agar and sunk in 30% sucrose, frozen at −20°, and sectioned coronally at 40 μm. Alternate sections were collected onto poly-L-lysine-coated slides, dried, and stored at −80° until use. Immunoreactive GnRH cells were labeled with antibodies to either synthetic [Arg^8]GnRH (No. 20075, INCStar Corp.) or to synthetic [His^{5}Trp^{7}Tyr^8]-GnRH (Millam *et al.*, 1989) and visualized with 3,3-diaminobenzidine (Davis and Fernald, 1990). Antibody cross-reactivity was tested with many forms of GnRH and GnRH analogues at doses producing 50% specific binding (Millam *et al.*, 1989). Cross-reactivities for anti-[His^{5}Trp^{7}Tyr^8]GnRH were >0.01% with most forms including [Arg^8]GnRH.

RESULTS

To identify GnRH cDNA sequences, we used RT–PCR following a strategy described by White *et al.* (1994). Complete coding sequences for two distinct GnRH mRNAs were isolated from tree shrew brain (Fig. 1A). The 470 bases of nucleotide sequence isolated from one transcript translate to an open reading frame of 92 amino acids including the decapeptide [Arg^8]GnRH, the typical mammalian hypothalamic form (Seeburg and Adelman, 1984; Adelman *et al.,* 1986). The other transcript isolated is composed of 415 nucleotides predicting a 114-amino-acid translation product containing [His^{5}Trp^{7}Tyr^8]GnRH, the sole GnRH peptide expressed by mesencephalic cells of nonmammalian vertebrates (Muske, 1993). The most 5′ AUG (located at nucleotides 69–71 in the [Arg^8]GnRH cDNA and at nucleotides 60–62 in the [His^{5}Trp^{7}Tyr^8]GnRH cDNA) initiates the open reading frame shown for each sequence in Fig. 1A. Sequence analysis of both transcripts predict preprohormone structures shared by all other members of the GnRH family: each encodes a signal sequence, the GnRH decapeptide, a conserved proteolytic site (Gly-Lys-Arg), and a GnRH-associated peptide (GAP). A second possible cleavage site at a dibasic residue pair within the GAP for [His^{5}Trp^{7}Tyr^8]-GnRH indicates the potential beginning of an additional associated peptide as in the cDNA encoding this form in a teleost fish (White *et al.,* 1994). Polyadenylation signals (located at nucleotides 449–454 in the [Arg^8]GnRH cDNA and at nucleotides 392–397 in the [His^{5}Trp^{7}Tyr^8]GnRH cDNA) precede poly(A) tails in each isolated sequence.

When comparing the amino acid sequences of each of the preprohormone components (Fig. 1B), it is clear that the decapeptide region has been conserved to a much greater degree than the GAP region. This is most likely due to differential selective pressure within the preprohormone itself. Decapeptides with identical amino acid sequences are seen in many classes (e.g., [Arg^8]GnRH is the hypothalamic form in amphibia, mammals, and many jawed fishes), and [His^{5}Trp^{7}Tyr^8]GnRH is seen in all classes other than agnathans. However, the GAP sequences are highly conserved only within each class.

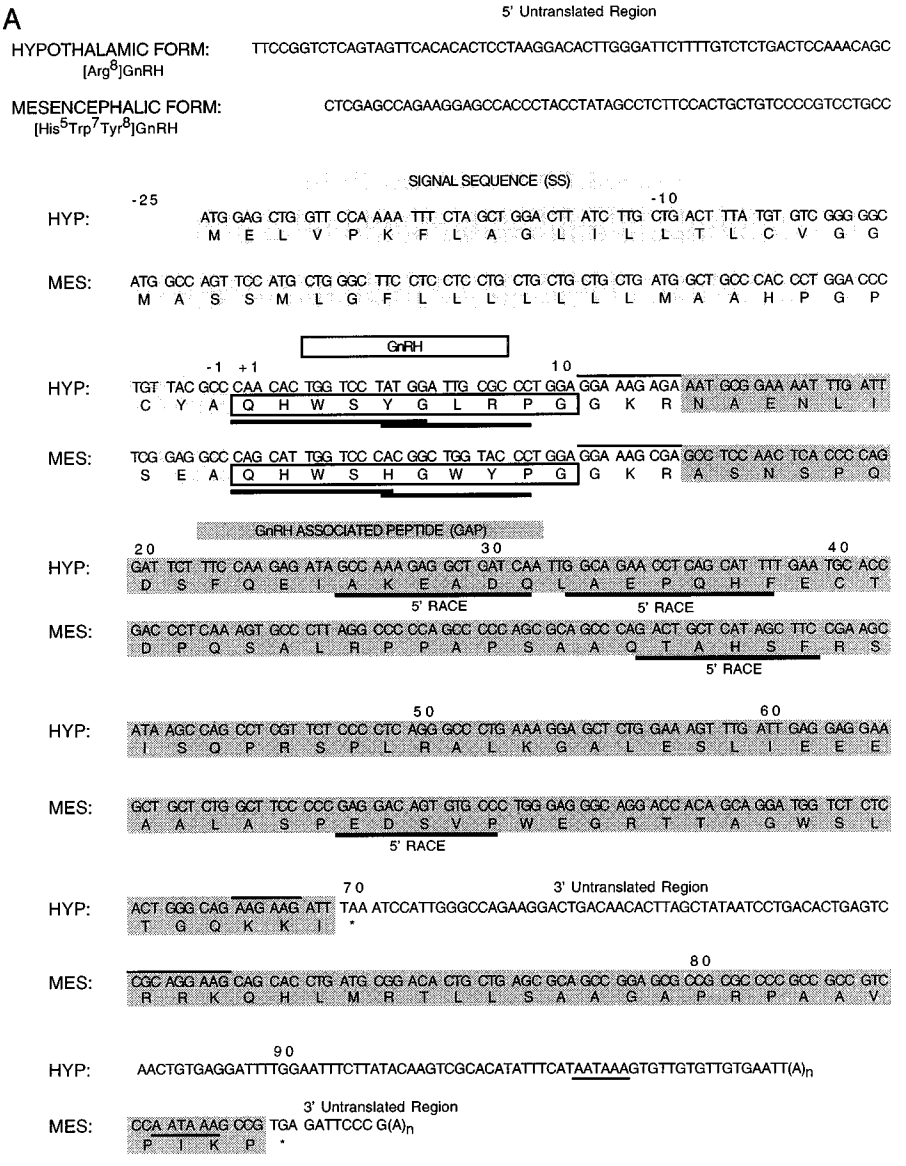
Interestingly, the messenger RNAs for these two preprohormones in the tree shrew are dramatically different in size. The [Arg^8]GnRH message is 0.62 kb, while the [His^{5}Trp^{7}Tyr^8]GnRH mRNA is 6.5 kb as measured using Northern analysis (data not shown). Since polyadenylation signals and poly(A) tails were found in each sequence indicating successful cloning of the 3′ end, the bulk of the mesencephalic [His^{5}Trp^{7}Tyr^8]-GnRH mRNA must consist of 5′ untranslated sequence (not isolated). This mRNA size difference does not exist between multiple reported forms in fish (Bogerd *et al.,* 1994; White *et al.,* 1994).

GnRH gene expression within the tree shrew brain was localized using both antibody staining and *in situ* hybridization. Results from the two methods were entirely consistent (see Fig. 2). [Arg^8]GnRH mRNA is expressed within a diffuse and continuous population of terminal nerve–septal–preoptic cells which extends along the ventromedial surface of the forebrain, from the olfactory bulb, increasing in abundance as they reach preoptic regions (Figs. 2B and 2C, left). This expression pattern is similar to that found in species where [Arg^8]GnRH acts as the releaser of gonadotropins (King and Millar, 1992; Hayes *et al.,* 1994). In contrast, [His^{5}Trp^{7}Tyr^8]GnRH mRNA is expressed in a distinct population of midbrain neurons (Figs. 2B and 2C, right) which form a dense cluster along the midline at the level of the red nucleus. This group extends as far rostral as the mammillary bodies and as far caudal as the cerebral peduncles, residing medial to the medial longitudinal fasciculi and ventral to the cerebral aqueduct and the third ventricle.

DISCUSSION

The data presented here show that two different GnRH genes are expressed in distinct neuronal populations in the brain of a placental mammal. Consistent with immunocytochemical studies in other species, the mRNA encoding the [Arg^8]GnRH form is expressed in cells within the septal–preoptic region while that encoding [His^{5}Trp^{7}Tyr^8]GnRH is found within the midbrain. The molecular architecture of both tree shrew transcripts is similar to that of all other GnRH preprohormones previously cloned: each contains a signal sequence followed by coding sequences for both GnRH and an associated peptide (GAP). The distinct localization of each form has been further confirmed

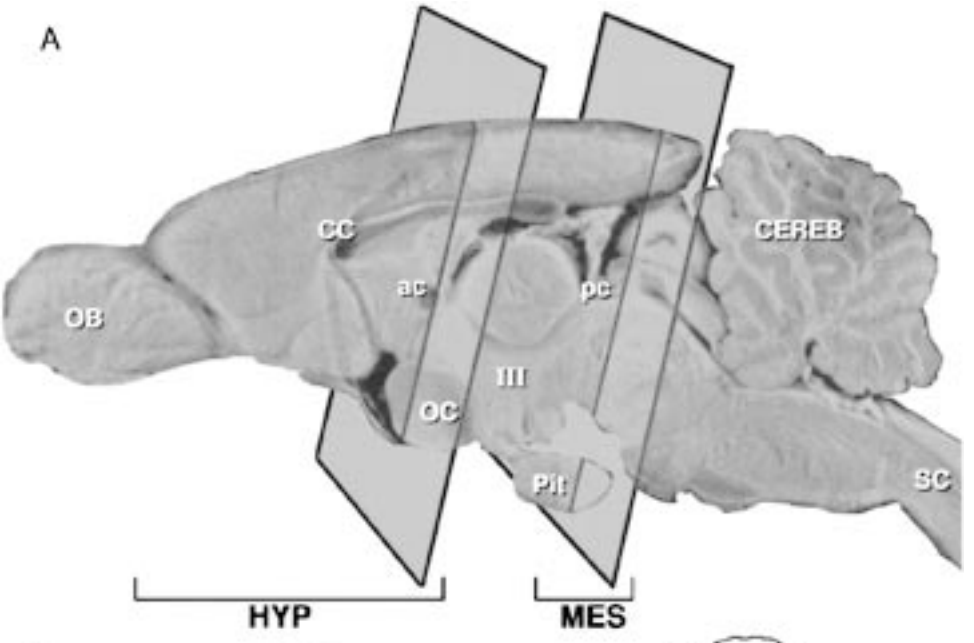
A



B

GnRH Forms Compared	SS		GnRH		GAP	
	% Iden	% Sim	% Iden	% Sim	% Iden	% Sim
Tree shrew hypothalamic vs. mesencephalic	22	48	70	80	16	36
Tree shrew hypothalamic vs. Fish hypothalamic	40	75	90	90	25	50
Tree shrew mesencephalic vs. Fish mesencephalic	39	65	100	100	10	34

FIG. 1. (A) Nucleotide and deduced amino acid sequences of the two forms of GnRH found in tree shrew brain are shown with functional domains of the preprohormone marked. Conserved proteolytic processing sites (overlined) follow each GnRH decapeptide (boxed). An additional potential site (overlined) appears within the GAP (dark shading) for [His^{5}Trp^{7}Tyr^8]GnRH as well as similar sites (also overlined) preceding the STOP codons in each cDNA as in most previously reported GAP sequences. Degenerate oligonucleotides made for nested PCR amplification of 3′ preprohormone sequences are underlined (bold). Regions complementary to oligonucleotides used to isolate the 5′ sequences in nested rapid amplification of cDNA ends (5′ RACE) are underlined (bold) and denoted as "5′ RACE" for each sequence. A polyadenylation signal in each sequence is underlined. (B) Homology among predicted amino acid sequences of GnRH preprohormones from different organisms shown as percentage of identity (% Iden) and percentage of similarity (% Sim) as calculated using Gap (Genetics Computer Group), software which employs the alignment algorithm described by Needleman *et al.* (1970). The signal sequence (SS), GnRH, and GAP are compared between the hypothalamic and mesencephalic forms in the tree shrew (top row), and between the hypothalamic forms (center row), and mesencephalic forms (bottom row) from the tree shrew and a teleost fish (*Haplochromis burtoni*) (White *et al.*, 1994).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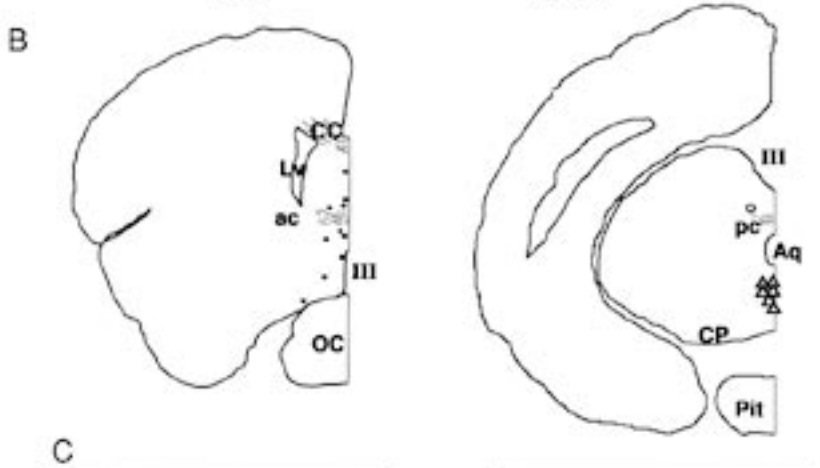
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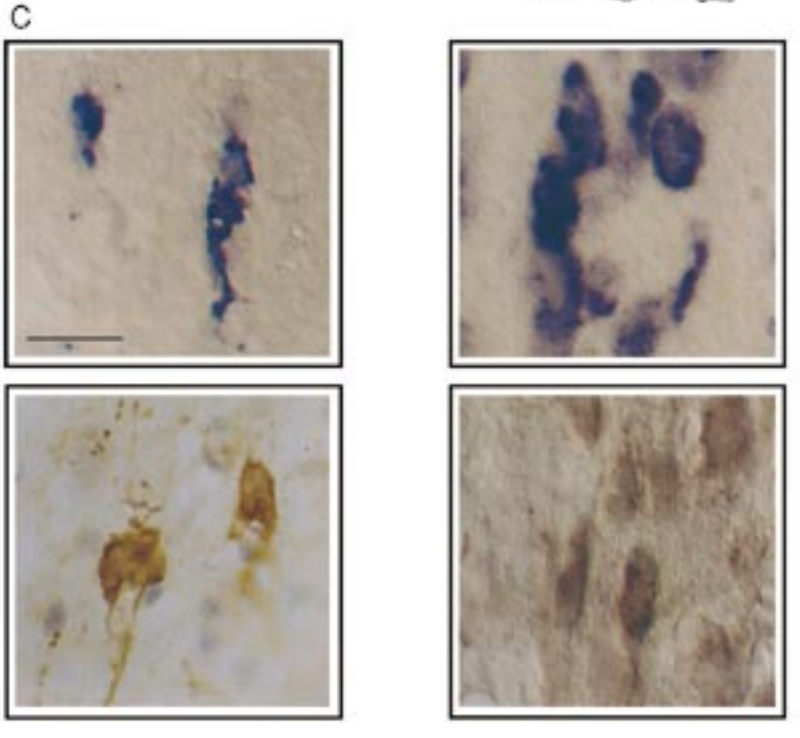
C

using antibodies directed against their GnRH peptide products.

Possible Roles of Multiple Forms of GnRH in One Organism

Why do multiple forms of GnRH exist? One obvious possibility is that each form plays a distinct role within the organism. While hypothalamic releasing forms are well-recognized as the central regulators of vertebrate reproductive physiology, a clear role, conserved across species, for the mesencephalic form has not yet been found. Many studies have focused on this form's possible function as an additional releasing hormone. In the goldfish, for example, [His^{5}Trp^{7}Tyr^8]GnRH is found in the pituitary (Rosenblum *et al.*, 1994) where it could stimulate release of gonadotropin and growth hormone (Murthy and Peter, 1994). Moreover, this decapeptide has remarkable gonadotropic biopotency across many species as shown by *in vivo* and *in vitro* pituitary receptor binding assays (for review see King and Millar, 1991). Despite this biopotency, the failure to find [His^{5}Trp^{7}Tyr^8]GnRH in the pituitary in most studies (e.g., Powell *et al.*, 1995) is consistent with hypotheses for extrahypothalamic functions.

An emerging premise is that [His^{5}Trp^{7}Tyr^8]GnRH also plays a role in reproductive behavior, albeit different from that of the hypothalamic form. This idea is based on three related experimental observations. First, in poecilid fish, spinal cord neurons thought to control sperm duct and oviduct contractility receive input from midbrain GnRH cells (Miller and Kriebel, 1986). Second, in newts, [His^{5}Trp^{7}Tyr^8]GnRH immunoreactivity shifts from midbrain cell bodies to terminal regions following the initiation of courtship (Muske *et al.*, 1995). Third, in the musk shrew, brain regions homologous to those regulating lordotic behavior in rats (Riskind and Moss, 1979) are richly innervated by [His^{5}Trp^{7}Tyr^8]GnRH-immunoreactive fibers (Rissman *et al.*, 1995).

GnRH also appears to have a neuromodulatory role in the peripheral nervous system. Jan *et al.* (1979 No. 302) first reported that an LHRH-like peptide mediates the late, slow excitatory postsynaptic potential (epsp) in the bullfrog sympathetic ganglia (reviewed in Branton *et al.*, 1986). Considered a likely candidate based upon its potency in eliciting the characteristic epsp (Jones, 1987), [His^{5}Trp^{7}Tyr^8]GnRH has since been identified immunologically and chromatographically (King and Millar, 1995) as the endogenous form of GnRH in these ganglia. Moreover, immunological evidence (Muske and Moore, 1990) suggests that it is part of a forebrain–spinal cord system of [His^{5}Trp^{7}Tyr^8]GnRH neurons. Possibly this peptide serves other functions at midbrain neuron terminals elsewhere in the CNS. If so, it appears that the ligand–receptor complex has placed stringent constraints on [His^{5}Trp^{7}Tyr^8]GnRH throughout evolution.

Other evidence has been interpreted to suggest that [His^{5}Trp^{7}Tyr^8]GnRH acts in the immune system. In ring doves, mast cells immunoreactive for GnRH appear within the habenula following courtship (Silver *et al.*, 1992). This staining persists despite preabsorption of the antibody with the [Arg^8]GnRH form, suggesting that these cells contain the [His^{5}Trp^{7}Tyr^8]GnRH form. Transient [His^{5}Trp^{7}Tyr^8]GnRH immunoreactivity has also been described in mast cells of musk shrew pups (Rissman *et al.*, 1995). In contrast, however, nucleotide probes indicate that rats and humans express the [Arg^8]GnRH form within lymphocytes (Wilson *et al.*, 1995). Perhaps the immune system, like the central nervous system, has utilized different forms of GnRH during the course of evolution. With multiple genetic probes for GnRH now available, it should be possible to test directly which form of GnRH is expressed in the immune cells of these animals.

FIG. 2. (A) Schematic sagittal view of the tree shrew (*T. glis belangeri*) brain, showing planes of section corresponding to the locations of the two populations of GnRH-containing neurons depicted below. Brackets indicate the rostrocaudal extent of each form. HYP refers to the location of the [Arg^8]GnRH hypothalamic form and MES refers to the location of the [His^{5}Trp^{7}Tyr^8]GnRH mesencephalic form. (B) Camera lucida drawings of representative coronal sections through the tree shrew brain showing the position of GnRH-containing neurons. The drawings illustrate sections through the hypothalamus (left) which contains [Arg^8]GnRH, the putative releasing form, and through the midbrain (right), which contains the mesencephalic [His^{5}Trp^{7}Tyr^8]GnRH form. ●, [Arg^8]GnRH cell locations; △, [His^{5}Trp^{7}Tyr^8]GnRH cell locations; III, third ventricle; ac, anterior commissure; Aq, cerebral aqueduct; CC, corpus callosum; CEREB, cerebellum; CP, cerebral peduncles; Lv, lateral ventricle; OB, olfactory bulb; OC, optic chiasm; pc, posterior commissure; Pit, pituitary; SC, spinal cord. (C) Photomicrographs of *in situ* hybridization (top) and immunocytochemical staining of the two different forms of GnRH to tree shrew brain sections. Left panels show [Arg^8]GnRH neurons while the right panels show [His^{5}Trp^{7}Tyr^8]GnRH neurons. Scale bar, 20 μm.

A

B

GnRH form	1	2	3	4	5	6	7	8	9	10
Releasing forms (●)										
[Arg^8]GnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	$GlyNH_2$
[Gln^8]GnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	$GlyNH_2$
[Ser^8]GnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	$GlyNH_2$
[Trp^{7}Leu^8]GnRH	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	$GlyNH_2$
[His^{5}Asn^8]GnRH	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	$GlyNH_2$
[His^{5}Trp^{7}Tyr^8]GnRH	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	$GlyNH_2$
[His^{5}Trp^{7}Leu^8]GnRH	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	$GlyNH_2$
[His^{5}Asp^{6}Trp^{7}Lys^8]GnRH	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	$GlyNH_2$
[Tyr^{3}Leu^{5}Glu^{6}Trp^{7}Lys^8]GnRH	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	$GlyNH_2$
Mesencephalic form (*)										
[His^{5}Trp^{7}Tyr^8]GnRH	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	$GlyNH_2$

GnRH form	Mammals SEQ	Mammals AB	Birds SEQ	Birds AB	Reptiles SEQ	Reptiles AB	Amphibians SEQ	Amphibians AB	Fishes SEQ	Fishes AB
Releasing forms (●)										
[Arg^8]GnRH	**7** 1-7	**11** 10-14					**1** 8	**8** 15-22	**1** 9	**13** 23-31
[Gln^8]GnRH			**1** 32,33	**5** 13,35-38	**1** 34	**5** 13,39-41				**1** 42
[Ser^8]GnRH									**2** 43-45	**1** 46
[Trp^{7}Leu^8]GnRH								**1** 17	**13** 45-53	**20** 13,28,30, 30,31,42, 46,54-64
[His^{5}Asn^8]GnRH									**2** 65-67	
[His^{5}Trp^{7}Tyr^8]GnRH									**1** 68	
[His^{5}Trp^{7}Leu^8]GnRH									**1** 69	**1** 30
[His^{5}Asp^{6}Trp^{7}Lys^8]GnRH									**1** 70	**1** 56
[Tyr^{3}Leu^{5}Glu^{6}Trp^{7}Lys^8]GnRH									**1** 71	**2** 28,56
Mesencephalic form (*)										
[His^{5}Trp^{7}Tyr^8]GnRH	**1** 6	**5** 10-12,72	**1** 73	**4** 35-38	**1** 34	**6** 13,39-41, 74		**9** 3,17,18, 20,21,75	**6** 44,65- 69,76	**23** 13,23-29, 46,55,58, 60,61,63, 64,77

FIG. 3. (A) *The relative locations* of the two principal systems of cells expressing GnRH are schematically shown in three representative vertebrate classes (Mikami *et al.*, 1988; Coe *et al.*, 1992; Bailhache *et al.*, 1994; White *et al.*, 1994; Rissman *et al.*, 1995; Yamamoto *et al.*, 1995). The forms produced in hypothalamic cells, responsible for the release of pituitary gonadotropins, are shown by the symbol (●), while the second form ([His^{5}Trp^{7}Tyr^8]GnRH) is shown by (*). The mesencencephalic localization of each form in the brain is conserved across classes. (B) (Top) The nine known forms of GnRH are shown above. Positions within the peptides which are unchanged are boxed while those which show conservative amino acid changes are shaded. The composition of the eighth residue varies the most. Note that nine different forms of GnRH serve as hypothalamic releasing forms whereas only one form of GnRH has been found in the midbrain. (Bottom) Distribution of the different forms found among species within each class. Numbers indicate how many species have been shown to express the indicated form of GnRH. Species in which the cDNA, the gene, or the peptide has been sequenced are listed under **SEQ.** Those species in which identification of the GnRH form depends upon the specificity of antibodies, as in studies using RIA with or without HPLC or in immunohistochemical studies, are listed under AB. Citations for each data point are given below that entry in the table. Citations from Table in B: 1. Adelman *et al.* (1986) 2. Jansen *et al.* (1995) 3. Matsuo *et al.* (1971) 4. Amoss *et al.* (1971) 5. Mason *et al.* (1986) 6. White *et al.* (1995b) 7. Ma *et al.* (1994) 8. Hayes *et al.* (1994) 9. Lescheid *et al.* (1995) 10. King *et al.* (1989) 11. King *et al.* (1994a) 12. King *et al.* (1990b) 13. King and Millar (1995) 14. Kelsall *et al.* (1990) 15. Cariello *et al.* (1989) 16. Conlon *et al.* (1993) 17. Iela *et al.* (1991) 18. Licht *et al.* (1994) 19. Muske *et al.* (1994) 20. Muske and Moore (1994) 21. Northcutt and Muske (1994) 22. Sherwood *et al.* (1986b) 23. Sherwood *et al.* (1991) 24. King *et al.* (1990a) 25. Joss *et al.* (1994) 26. King *et al.* (1995) 27. Parha and Iwata (1994) 28. Magliulo-Cepriano *et al.* (1994) 29. Lepretre *et al.* (1993) 30. D'Antonio *et al.* (1995) 31. Lovejoy *et al.* (1992b) 32. King and Millar (1982) 33. Dunn *et al.* (1993) 34. Lovejoy *et al.* (1991a) 35. Powell *et al.* (1987) 36. Mikami *et al.* (1988) 37. Millam *et al.* (1989) 38. Sherwood *et al.* (1988) 39. Sherwood and Whittier (1988) 40. Tsai and Licht (1993) 41. D'Aniello *et al.* (1994) 42. King and Millar (1985) 43b. White *et al.* (1995a) 44. Powell *et al.* (1994) 45.

Origin and Development of GnRH Cell Populations

Some insight about why and how different forms of GnRH arose has come from comparison of their developmental expression patterns. In mammals (Schwanzel-Fukuda and Pfaff, 1989) and other species (for review see Muske, 1993, and Hayes *et al.,* 1994), neurons destined to produce the releasing form of GnRH arise from the olfactory placode. These cells migrate centrally and take up positions along the ventral surface of the adult brain in a rostrocaudal continuum from the terminal nerve area to the hypothalamus. This placodal ancestry has suggested that, in addition to regulating gonadotropin release, GnRH in the terminal nerve neuronal population might coordinate sensory input at the time of reproductive activity. Functional segregation in this forebrain system is further suggested in fish whose GnRH-expressing neurons in the terminal nerve and hypothalamus express distinct GnRH genes (White *et al.,* 1995a). There has also been one report of transient expression of a form other than [Arg^8]GnRH in developing monkey forebrains (Quanbeck *et al.,* 1994).

There may also be species differences in the relative onset of expression for the mesencephalic and hypothalamic GnRH expression. During development, mesencephalic cells expressing [His^{5}Trp^{7}Tyr^8]GnRH appear to be of ventricular origin (Muske and Moore, 1990; R. White *et al.,* 1995) and in nonmammalian vertebrates, these cells express GnRH earlier than those derived from the placode. In the developing bullfrog, anti-[His^{5}Trp^{7}Tyr^8]GnRH immunoreactivity in the spinal cord appears early, with that in the midbrain (Muske and Moore, 1990), suggesting a common ontogeny which is separate from that of the later appearing hypothalamic GnRH neurons. However, in the musk shrew, anti-GnRH antibodies detect [Arg^8]GnRH prenatally while [His^{5}Trp^{7}Tyr^8]GnRH immunoreactivity is only apparent at Postnatal Day 2 (Gill *et al.,* 1995). It will be possible to discover the site and time of first expression for each form of GnRH with species-specific GnRH gene probes.

The Evolution of Multiple Forms of GnRH

The presumptive evolution of the GnRH peptide provides additional hints about the functions of its multiple forms. The GnRH decapeptide motif has an ancestry that predates vertebrates: Yeast α-mating factor bears homology to GnRH and exhibits releasing-factor properties when applied to mammalian pituitary tissue (Loumaye *et al.,* 1982). Further, immunological studies have detected GnRH-like molecules in several protochordate species (Georges and Dubois, 1980; Kelsall *et al.,* 1990). Evidence from one such protochordate lends evolutionary support to the idea that placodally derived GnRH neurons might have a sensory function. Specifically, in *Amphioxus,* a sensory structure known as Hatshek's pit is both open to environmental water and has GnRH immunoreactivity. Gorbman (1995) has proposed that this structure subserves chemoreception to environmental signals and cues the release of gonadotropin (Gorbman, 1995). Sequencing the gene encoding this GnRH-like protein could reveal whether it is the functional homologue to the vertebrate GnRH releasing form.

During vertebrate evolution, the conservation of mesencephalic [His^{5}Trp^{7}Tyr^8]GnRH, when contrasted with the variation among primary structures of releasing forms (Fig. 3B), suggests that [His^{5}Trp^{7}Tyr^8]GnRH has been subject to more stringent selective pressure. What functional requirements might account for this greater conservation? Perhaps [His^{5}Trp^{7}Tyr^8]GnRH acts in several contexts and hence is more stringently constrained during evolution by receptor–ligand interactions because its receptor demands greater fidelity of the peptide. For example, [His^{5}Trp^{7}Tyr^8]GnRH might be conserved because it integrates multiple inputs, acting at several sites in the nervous system as a central switch to control and coordinate the organism's reproductive behaviors.

In summary, work described here reveals the presence of genes for two GnRH peptides within a single mammalian species, revealing that the conservation of

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Gothilf *et al.* (1995) 46. Powell *et al.* (1995) 47. Bond *et al.* (1991) 48. Grober *et al.* (1995) 49. Ashihara *et al.* (1995) 50. Klungland *et al.* (1992) 51. Sherwood *et al.* (1983) 52. Suzuki *et al.* (1992) 53. Okuzawa *et al.* (1994) 54. Coe *et al.* (1992) 55. Kim *et al.* (1995) 56. Calvin *et al.* (1993) 57. Okuzawa *et al.* (1994) 58. Powell *et al.* (1986) 59. Sherwood *et al.* (1984) 60. Sherwood *et al.* (1993a) 61. Somoza *et al.* (1994) 62. Wu *et al.* (1986) 63. Yamamoto *et al.* (1995) 64. Yu *et al.* (1988) 65. Ngamvongchon *et al.* (1992) 66. Bogerd *et al.* (1992) 67. Bogerd *et al.* (1994) 68. Lovejoy *et al.* (1991b) 69. Lovejoy *et al.* (1992a) 70. Sower *et al.* (1993) 71. Sherwood *et al.* (1986a) 72. Rissman *et al.* (1995) 73. Miyamoto *et al.* (1984) 74. Powell *et al.* (1986) 75. King *et al.* (1994b) 76. White *et al.* (1994) 77. King *et al.* (1992).

[His^{5}Trp^{7}Tyr^8]GnRH extends to placental mammals. Whether and how [His^{5}Trp^{7}Tyr^8]GnRH is important in all vertebrates remains to be discovered. That it exists in a placental mammal, phylogenetically situated between rodents and primates, suggests that it may exist in all mammals. Development of GnRH gene probes within other select species should reveal how widespread this gene is and could extend our understanding of the functional, developmental and evolutionary roles of this molecule.

ACKNOWLEDGMENTS

We thank T. Adams, University of California, Davis, for providing specific antibodies for [His^{5}Trp^{7}Tyr^8]GnRH and D. Anderson, H. Fox, K. Hoke, R. Kopito, J. Kwan, K. Lindgren, R. Robison, R. White, and two anonymous reviewers for comments on the manuscript. H. Fox provided significant help in preparing the text. Supported in part by NIH HD 23799 and NS 34950 to R.D.F.

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