The gonadotropin-releasing hormone family of neuropeptides in the brain of human, bovine and rat: identification of a third isoform

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Abstract The mammalian gonadotropin-releasing hormone (GnRH-I), which regulates reproduction, was the first isoform of GnRH that was identified in mammals. Recently, we and others have demonstrated the existence of a second isoform of GnRH in the brain of mammals. The presence of a third isoform of GnRH, GnRH-III, in the brain of mammals is reported herein. GnRH-III, extracted from the brain of bovine and human, was purified by high performance liquid chromatography, using two distinct elution programs. In both, GnRH-III was eluted at the same positions as synthetic salmon GnRH, as demonstrated by radioimmunoassay. The luteinizing hormone-releasing activity of purified GnRH-III, using dispersed rat pituitary cells, was found to be similar to that of synthetic salmon GnRH. The total amount of GnRH-III, determined by radioimmunoassay, in the hypothalamus and midbrain of humans and calves is similar to that of GnRH-I. Immunohistochemical studies demonstrated GnRH-III-containing neurons in the hypothalamus and midbrain of human and GnRH-III fibers in the median eminence of rats. The distribution of GnRH-III in the brain suggests that in addition to a putative function as a neurohormone at the hypothalamic–pituitary axis, GnRH-III may have other functions. Our present results suggest that multiple isoforms of GnRH are present in the brain of mammals, and further studies are required in order to elucidate their biological functions.

Key words: Gonadotropin-releasing hormone/luteinizing hormone-releasing hormone isoform; Gonadotropin-releasing hormone, third isoform in mammalian brain; Salmon gonadotropin-releasing hormone-like peptide in mammals

1. Introduction

The gonadotropin-releasing hormone (GnRH-I, Table 1), originally isolated from the mammalian hypothalamus, plays a pivotal role as the physiologic regulator of reproduction [1,2]. This peptide is synthesized by hypothalamic neurosecretory cells and upon release it reaches the pituitary gland, by way of a specialized portal system, to induce the synthesis and secretion of the gonadotropic hormones luteinizing hormone (LH) and follicle-stimulating hormone. Today, a dozen isoforms of GnRH are known in vertebrates, which are conserved by 50–90% of their sequence as compared to GnRH-I [3]. In many of the lower vertebrate species, at least two different isoforms of GnRH are expressed in the brain [4]. One isoform, [His^5,Trp^7,Tyr^8]GnRH, named GnRH-II (Table 1), originally isolated from chicken brain [5], is expressed in almost all vertebrate classes. Until recently, only GnRH-I had been detected in the placental species of mammals [6]. GnRH-I was suggested to be not only the regulator of the secretion of the gonadotropic hormones, but also to play a role in a variety of other functions such as involvement in the regulation of sexual behavior in rats [7]. Lately, it was reported that a gene encoding GnRH-II is expressed in the human brain [8]. By using high performance liquid chromatography (HPLC), immunocytochemistry, radioimmunoassay (RIA), and molecular biology techniques, we have recently demonstrated the presence of GnRH-II in the brain of mouse, rat and human [9]. The presence of GnRH-II in non-human primates has also been demonstrated [10].

In several teleost fishes, three GnRH isoforms were identified in a single species [11–16]. We have therefore explored the possibility that multiple GnRH isoforms may be a common pattern in mammals and have looked for an additional isoform of GnRH in the mammalian brain. During our earlier studies using extracts of human and rat brains [9], we have observed weak immunoreactivity for GnRH-II in HPLC fractions corresponding to high acetonitrile concentrations that were used for column wash. Thus, we suspected that a third GnRH isoform is found in these extracts. This putative isoform is very hydrophobic, according to its HPLC elution profile, and should cross-react with our GnRH-II antibody. Based on these indications, we assumed that the immunoreactive peak may correspond to salmon GnRH (sGnRH; [Trp^7,Leu^8]GnRH-I, Table 1). sGnRH has a 1.5% cross-reactivity with our GnRH-II antibody (Table 2), and is the most hydrophobic GnRH isoform known in vertebrates, with the longest HPLC retention time [17,18]. Our present results indeed demonstrate a sGnRH-like peptide in the mammalian brain.

2. Materials and methods

2.1. Materials

Synthetic GnRH-I, GnRH-II and salmon GnRH were obtained from Peninsula Laboratories Inc. (San Carlos, CA, USA). Dogfish GnRH (dfGnRH) was synthesized and analyzed in our laboratory, using previously described procedures [19].

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Abbreviations: BSA, bovine serum albumin; GnRH, gonadotropin-releasing hormone; HPLC, high-performance liquid chromatography; LH, luteinizing hormone; RIA, radioimmunoassay; RP, reversed phase; TFA, trifluoroacetic acid

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2.2. Radioiodination and radioimmunoassay

Iodinations of synthetic GnRH-I, GnRH-II or sGnRH were carried out using the chloramine-T method. Free iodine was removed on a Sep-Pak C-18 cartridge and the ^{125}I-labeled peptides were separated from the unlabeled peptides by HPLC. GnRH concentrations in samples of brain extracts were determined by RIA as previously described [20].

2.3. Antibodies

The following antisera were used throughout this study: a polyclonal antibody against GnRH-I, prepared and characterized in our laboratory [20]. The specificity of this antibody was determined and is shown in Table 2. A polyclonal antibody against GnRH-II (aCII6) was kindly provided by Dr. K. Okuzawa (National Research Institute of Aquaculture, Watarai, Japan), and a polyclonal sGnRH antibody (sGnRH #2) was kindly donated by Dr. K. Aida (Fish Physiology Research Group, Department of Aquatic Bioscience, The University of Tokyo, Japan). The specificities of the two antibodies were previously defined [14,21,22] and are shown in Table 2. Similar results were obtained in specificity tests conducted in our laboratory, by performing dose–response studies of the interactions of relevant GnRH isoforms with these antibodies.

2.4. Tissue extraction

All animal experiments were carried out in compliance with the regulations of the Weizmann Institute of Science. Brains from 11-month-old male calves were obtained at a slaughterhouse and kept on dry ice for up to 2 h before homogenization. These brains were processed in two batches, of five and seven brains per batch. Specific regions of human brain were received during a routine autopsy at Rabin Medical Center from a 77-year-old man who died of pyonephrosis and from an 87-year-old man who died of bilateral bronchopneumonia. The bodies were kept for 38 h and 21 h, respectively, at 4°C until autopsy. The brain tissues from both human and cow were immersed in ice-cold 0.1 N HCl and homogenized by a Teflon-glass homogenizer. Following centrifugation of each homogenate (12 000×*g*, 30 min at 4°C), the supernatant was pumped onto Sep-Pak C-18 cartridges, eluted by methanol and evaporated by nitrogen. Following the methanol evaporation, all extracts were processed through reversed phase (RP) HPLC, performed on a Waters system composed of two Model 510 pumps, Model 680 automated gradient controller and Model 441 absorbance detector (Waters, Milford, MA, USA). The HPLC prepacked column which was employed was Lichrospher 100 RP-18, 250×4 mm (5 μm) (Merck, Darmstadt, Germany), and the flow rate was 1 ml/min. Eluent A, 0.1% trifluoroacetic acid (TFA) in water; eluent B, 75% CH_3CN in 0.1% TFA. The gradient program consisted of a linear gradient of eluent B 20–30% for 5 min, followed by isocratic elution of 30% eluent B for 30 min, followed by a linear gradient of eluent B 30–57% in 30 min, and finally column wash using 100% eluent B for 20 min. The fractions were evaporated to a volume of 0.2 ml and reconstituted with 0.1 M of phosphate buffer (PB, pH 7.4) containing 0.1% of bovine γ-globulin (Sigma, St. Louis, MO, USA). The HPLC fractions were assayed for GnRH-I, GnRH-II and sGnRH concentrations by RIA using the appropriate antisera; three different aliquots from every fraction were analyzed in each RIA system. The elution positions of the synthetic peptides were determined by HPLC injection (performed after all the extracts were eluted) of 100 ng of GnRH-I, GnRH-II, sGnRH and dogfish (df) GnRH, using the same conditions as used for the extracts. The elution positions were determined using the respective RIA systems. Between each of the HPLC separations of both extracts and standards, the column was thoroughly washed, and a blank run was monitored by RIA to ensure that the column was not contaminated. Recovery of peptides in the described extraction process was estimated by subjecting samples of iodinated GnRH-I, GnRH-II and sGnRH to identical extraction procedures. The reported amounts of all peptides were not corrected according to the estimated recovery (60–70%). Recovery was not improved by using a 80% methanol/20% 0.1 N HCl solution, instead of 0.1 N HCl solution, for tissue extraction.

2.5. Further purification

Fractions from HPLC separations of human and cow midbrains that were found to contain sGnRH-immunoreactive material (see above) were repurified in a second HPLC solvent system: eluent A, 0.1% TFA in water; eluent B, 75% isopropanol in 0.1% TFA. The column which was used was Lichrospher 100 RP-18, 125×4 mm (5 μm) (Merck, Darmstadt, Germany), and the flow rate was 0.8 ml/min. The gradient program consisted of a linear gradient of eluent B 0–20% for 5 min, followed by a gradient of 20–25% eluent B for 40 min, then 25% eluent B for 5 min, and finally the column was washed using 100% eluent B for 20 min. The fractions collected were evaporated and subjected to RIA for sGnRH, as detailed above. Synthetic GnRH markers were also injected in the same system, after all the extracts were eluted.

2.6. LH release from cultured, dispersed, pituitary cells

Cells from 21-day-old Wistar-derived female rats were dispersed as previously described [23] and incubated in 96-well plates (50 000 cells/well) at 37°C in M-199 medium containing 5% horse serum. After 48 h the cells were washed with M-199 medium containing 0.1% bovine serum albumin (BSA) and incubated for 4 h at 37°C with M-199/0.1% BSA (0.2 ml) containing the desired concentrations of the various peptides (three wells/experimental group). The incubation was terminated by removing the medium and diluting it in a ratio of 1:5 with 1% BSA in phosphate-buffered saline (PBS) solution. Three different aliquots from each sample were analyzed for LH concentration by RIA [19], using the kit kindly supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD) Rat Pituitary Program. Results are expressed in terms of the RP-3 reference preparation.

2.7. Immunocytochemical procedures

The localization of GnRH-III in the median eminence, hypothalamus and midbrain of rats and humans was carried out by application of a specific antibody, at a dilution of 1:10 000, to brain slices. This was followed by a biotinylated secondary antibody and of an avidin–biotin–horseradish peroxidase complex according to a procedure that we have previously described [9]. Human brain samples were obtained during routine autopsies, at Rabin Medical Center, from a 36-week-old male infant who died of bilateral polycystic kidneys and from an 81-year-old woman who died of pulmonary embolism. The bodies were kept for 8 h and 13 h, respectively, at 4°C until autopsy. The brain samples were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned (6 μm). The processing of the brain of mature female rats was carried out as described previously [9]. The sections were incubated for 15 min in 0.3% hydrogen peroxide to reduce endogenous peroxidase activity, and washed. The sections were then incubated for 2 h in a blocking medium containing 20% normal goat serum and 0.5% Triton X-100 to decrease non-specific staining. The sections were incubated overnight at 4°C with the antiserum (sGnRH#2), rinsed well and incubated for 4 h at room temperature with an avidin–biotin–peroxidase complex followed by nickel-intensified diaminobenzidine with hydrogen peroxide as a chromogen, as described earlier [9]. As controls for the specificity of staining we exposed sections to the antibody that was previously pre-absorbed with excess sGnRH or that was exposed to a solution from which the first antibody was omitted. In these control groups no immunoreactive staining was evident.

3. Results

Acid extracts of specific regions of cow and human brain were eluted through C-18 columns on HPLC, using an elution program that separates the three peptides of interest: GnRH-I, GnRH-II and sGnRH (Fig. 1, black arrows). The concentrations of these peptides in the eluate of the brain extracts were determined by RIA using antibodies specific to each GnRH isoform. Fig. 1 demonstrates that the elution profiles of the immunoreactive neuropeptides that were extracted from human hypothalamus, midbrain and pituitary stalk were identical to those of the three synthetic peptides. These results demonstrate for the first time the presence of a sGnRH-like peptide in human brain. The GnRH isoform with the highest homology to sGnRH among known GnRH isoforms, dfGnRH (Table 1), cross-reacts with the sGnRH antibody (Table 2). However, its retention time in the above HPLC

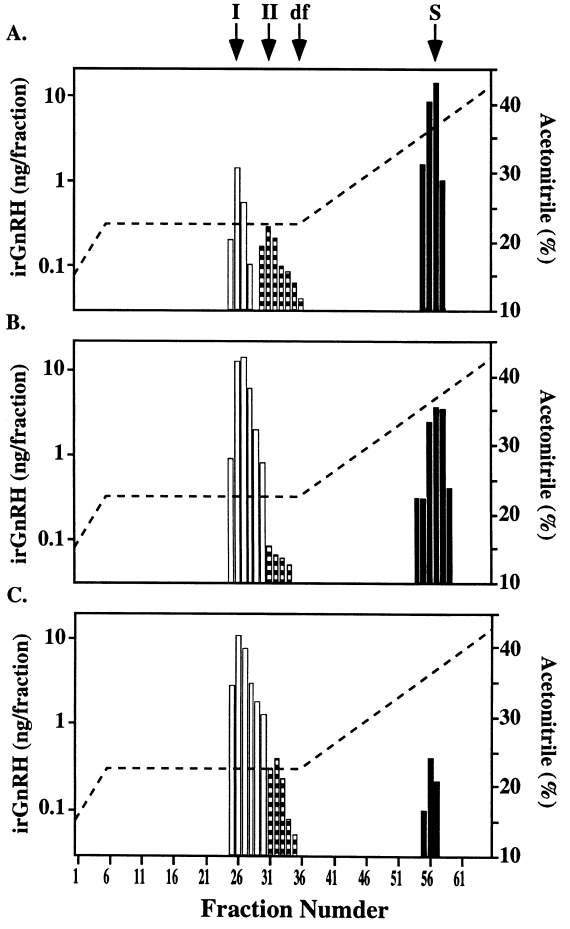

Fig. 1. RP-HPLC elution profile of GnRH-I, GnRH-II and GnRH-III extracted from human midbrain (A), hypothalamus (B), and pituitary stalk (C), using a water/acetonitrile solvent system with 0.1% TFA. The acetonitrile gradient is indicated by a broken line. Fractions (1 ml) of the eluate were collected, evaporated and reconstituted. All fractions were assayed by RIA for GnRH-I, GnRH-II and sGnRH immunoreactivity, using specific antibodies. The elution positions of synthetic GnRH-I (I), GnRH-II (II), salmon GnRH (S) and dogfish GnRH (df) are indicated by black arrows.

system is significantly different (Fig. 1). The sGnRH immunoreactive peak was further purified using a different HPLC system, with an almost isocratic gradient of isopropanol (Fig. 2). Again the extracted sGnRH-immunoreactive material co-eluted with the synthetic sGnRH in this system, further confirming the similarity of the extracted material to sGnRH.

The amounts of immunoreactive GnRH-I, GnRH-II and sGnRH-like peptides found in the various regions of the human brain (Fig. 1) have to be regarded only qualitatively, as they represent data that represent only one human brain sample. We also analyzed by HPLC and RIA an additional human sample of a combined extract from both hypothalamus and midbrain. This sample contained 66.1 ng of immunoreactive sGnRH (ir-sGnRH), as compared with a total of 32.2 ng in the respective regions of the human brain that is presented in Fig. 1.

In order to obtain qualitative data, we performed an identical extraction procedure using hypothalami and midbrains derived from 12 calves, processed in two batches. The hypothalami contained 1.1 ng ir-sGnRH and 8.1 ng ir-GnRH-I per calf, while the midbrains contained 13.7 ng ir-sGnRH and 0.8 ng ir-GnRH-I per calf. These results demonstrate that in calves ir-sGnRH is concentrated in the midbrain, while GnRH-I is more abundant in the hypothalamus.

We tested the LH-releasing activity of the ir-sGnRH using cultured rat pituitary cells. For this assay, we used GnRH-III extracted from calf midbrain, which was purified by the two HPLC systems that were described above. GnRH-III induced LH secretion in a dose-dependent manner (Fig. 3) that was similar in its potency to that of synthetic sGnRH, but much lower than the activity of GnRH-I. Fractions from a blank HPLC run, which correspond to the sGnRH-immunoreactive fractions, were collected as a control for this assay. LH release in this control group was similar to that of the second control

Table 1
Structure of relevant GnRH isoforms

	1 2 3 4 5 6 7 8 9 10
GnRH-I	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
GnRH-II	pGlu-His-Trp-Ser-<u>His</u>-Gly-<u>Trp</u>-<u>Tyr</u>-Pro-Gly-NH_2
sGnRH	pGlu-His-Trp-Ser-Tyr-Gly-<u>Trp</u>-<u>Leu</u>-Pro-Gly-NH_2
dfGnRH	pGlu-His-Trp-Ser-<u>His</u>-Gly-<u>Trp</u>-<u>Leu</u>-Pro-Gly-NH_2

Underlined residues differ from the sequence of GnRH-I.

Table 2
Specificity of antisera and RIA systems

Antiserum and titer	Percentage cross-reactivity			
	GnRH-I	GnRH-II	sGnRH	dfGnRH
YK-5 1:120 000	100	< 0.01	< 0.01	ND
aCII6 1:160 000	< 0.01	100	1.46	ND
sGnRH#2 1:240 000	< 0.01	1.58	100	80

The cross-reactivity of GnRH isoforms with antibodies aCII6 and sGnRH#2 have been reported [14,21,22] and were reevaluated in our assay systems.
ND, not determined.

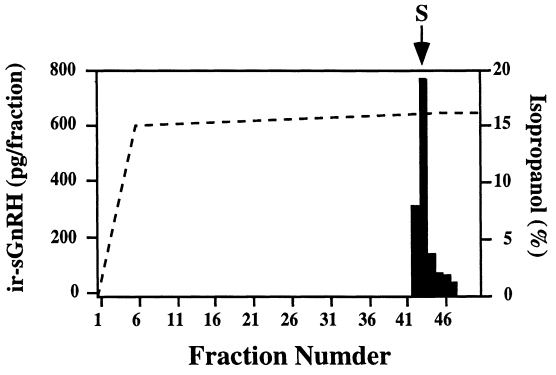

Fig. 2. Further purification of GnRH-III immunoreactive material from human midbrain by HPLC. An equivalent of 2.5 ng of ir-sGnRH, obtained as shown in Fig. 1, was eluted from an HPLC system using a water/isopropanol solvent system with 0.1% TFA. The isopropanol gradient is indicated by a broken line. Fractions (0.8 ml) of the eluate were collected, evaporated and reconstituted. All fractions were assayed for salmon GnRH-immunoreactive material, using RIA with specific antibodies for sGnRH. The elution position of synthetic salmon GnRH is indicated by a black arrow. GnRH-I and GnRH-II are eluted in this system in fractions 21 and 23, respectively.

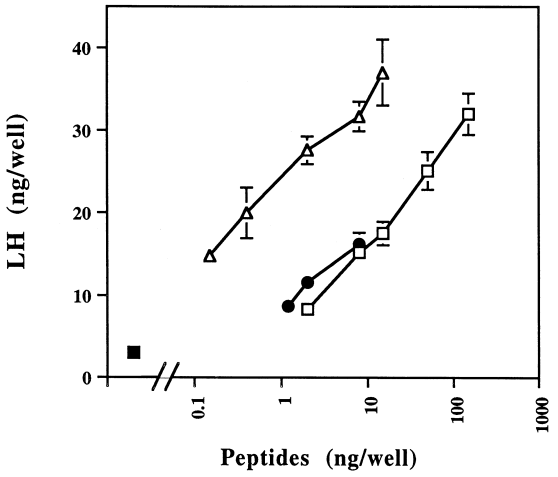

Fig. 3. Induction of LH release from dispersed rat pituitary cells by synthetic GnRH-I and salmon GnRH, as well as by GnRH-III extracted from calf brain (double-purified by HPLC). Cells were preincubated for 48 h after dispersion, washed and incubated for 4 h at 37°C with the examined peptides. Results are the mean ± S.E.M. of LH concentration (three wells/experimental group). The LH concentration in each well was determined by RIA, using three different dilutions of the samples. Two control groups for basal release of LH were utilized: one contained medium alone and to the other group, aliquots were added of fractions from a blank HPLC run, which corresponded to the sGnRH elution pattern. LH release in these control groups was similar (3.0 ± 0.7 versus 2.8 ± 0.7 ng/well, respectively). Closed square, basal LH release; open triangles, GnRH-I; open squares, sGnRH; closed circles, extracted GnRH-III.

group (M-199 containing 0.1% BSA) (2.8 ± 0.7 versus 3.0 ± 0.7 ng/well, respectively).

Immunohistochemical studies (Fig. 4), using the specific sGnRH antisera, demonstrated sGnRH-immunoreactive neurons in the hypothalamus and midbrain of both human and rat. As demonstrated in Fig. 4, GnRH-III immunoreactive granules (black arrows) are seen along nerve fibers in the rat median eminence (A). Immunoreactive GnRH-III neuronal cells (thick bold black arrows) and nerve fibers (black arrows) are found in the human midbrain, ventral to the cerebral aqueduct in the region of the oculomotor nucleus (B), as well as within the supraoptic area (C) and the preoptic area (D,E) of the human hypothalamus. Similar results were obtained in the rat hypothalamus and midbrain (data not shown).

4. Discussion

Using HPLC, RIA and immunocytochemistry, we have demonstrated the presence of a third GnRH isoform (GnRH-III) in the brain of cow, rat and human. This isoform coelutes with synthetic sGnRH in two distinct HPLC systems (Figs. 1 abd 2) and is recognized by antisera for salmon GnRH. sGnRH is the most hydrophobic isoform of the GnRH family and therefore its retention time on HPLC systems is significantly higher than that of the other known GnRH isoforms [17,18]. Thus, our results suggest that GnRH-III is either sGnRH itself, or a novel isoform that has identical elution patterns as those of sGnRH. Furthermore, GnRH-III is capable of inducing LH release, from cultured rat pituitary cells, in a dose response pattern that is similar to that of synthetic sGnRH (Fig. 3).

Salmon GnRH was previously identified in several fish species in combination with GnRH-II, and in some cases also together with a third GnRH isoform [11–15]. There is also evidence suggesting the existence of sGnRH, in combination with GnRH-I, in several amphibian species [24]. Lately, three GnRH isoforms were detected in a primitive mammal (*Hydrochaeris hydrochaeris*) by a combination of HPLC and RIA [25]. These isoforms were identified as GnRH-I, GnRH-II and sGnRH-like peptides, in agreement with the results presented herein for higher mammals.

The content of GnRH-III in the brain of humans and of calves is notable especially in the midbrain, where it is the dominant form of GnRH, as well as in the hypothalamus, where a considerable amount of GnRH-III is also found (Fig. 1). Thus, the total content of GnRH-III in the hypothalamus–midbrain unit of calves and humans is similar to that of GnRH-I. The fact that this isoform was not identified so far may be attributed to technical reasons. Being the most hydrophobic isoform in the GnRH family of peptides, GnRH-III is highly adsorbed onto glass or plastic at neutral pH.

Low concentrations of GnRH-III are found in the human pituitary stalk (Fig. 1C), as compared to the hypothalamus (Fig. 1B), whereas the total amounts of GnRH-I in the hypothalamus and in the pituitary stalk are similar. As the pituitary stalk is the site of storage of the hypothalamic hypophysiotropic neurohormones, these results further suggest that the anterior pituitary gland is not the main site of GnRH-III action. However, the demonstration of GnRH-III in neuronal fibers of the rat median eminence (Fig. 4A) suggests that GnRH-III is transported to the pituitary gland by way of the portal system and may interact with pituitary cells.

We found that the potency of GnRH-III in the induction of LH secretion from cultured rat pituitary cells is about 2.3% of that of GnRH-I (Fig. 3). It is therefore unlikely that the main physiological function of GnRH-III is in the regulation of gonadotropin secretion, as GnRH-I is known to be. Moreover, the localization of GnRH-III, mainly in the midbrain, also does not support a central role in the regulation of gonadotropin secretion. Thus, the role of GnRH-III in the hypothalamic–pituitary–gonadal axis may be conveyed by modulation of GnRH-I activity, or by the regulation of GnRH-I expression or release. In certain fishes, sGnRH was reported to induce release of prolactin [16] or growth hormone [26]. Nevertheless, preliminary results in our laboratory suggest that GnRH-III, in concentrations which induce LH release (Fig. 3), does not affect the release of these hormones from cultured rat pituitary cells. Today, it is evident that each of almost all neurotransmitters and neuropeptides has more than one receptor. Therefore, it is hard to conceive that the three GnRH isoforms that are 70–80% homologous to each other (Table 1) will share a common, single GnRH receptor. Indeed, the recent identification of two GnRH receptors in the goldfish [27] supports the assumption that multiple GnRH receptors are also present in mammals. The homology in the structure of the GnRH isoforms, however, may permit some cross-reactivity of GnRH-III and GnRH-II with the known mammalian GnRH receptor and thus result in the modulation of the activity of GnRH-I by these isoforms. Recently, it has been demonstrated that G protein-coupled receptor heterodimerization modulates receptor function. Thus, heterodimerization of two different opioid receptors (κ and δ) resulted in a new receptor that exhibited ligand binding and functional

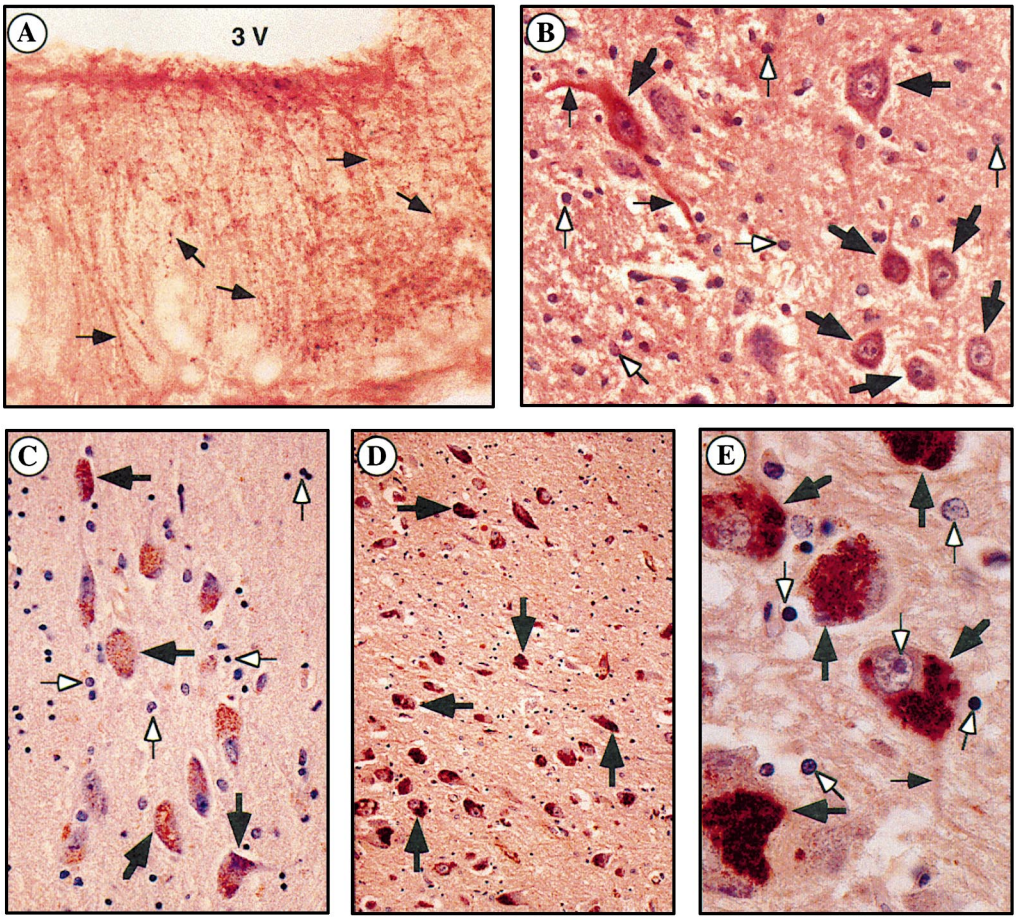

Fig. 4. Immunoreactive GnRH-III neuronal cells and fibers in coronal sections through the median eminence of a mature female rat (A), of the midbrain area from a 36-week-old infant (B), and of the hypothalamus from an 81-year-old women (C–E). In the rat median eminence, GnRH-III immunoreactive material (black arrows) is packed as dark granules lined along the neuronal fibers. In the human midbrain (B), GnRH-III immunoreactive neurons (thick bold arrows), and their processes (black arrows) are in the area of the oculomotor nucleus. Clusters of immunoreactive neurons are also found within the area of the supraoptic nucleus (C) and the preoptic area (D,E) of the human hypothalamus. Figures B–E were also stained by hematoxylin to demonstrate nuclei of cells (white arrows). 3V, third ventricle. A, ×240; B,C, ×64; D, ×32; E, ×160.

properties that were distinct from those of either receptor [28]. Similar results were obtained by demonstrating that heterodimerization is required for the formulation of functional $GABA_B$ receptors [29]. Indeed, it has already been demonstrated that dimerization of the GnRH receptor is essential for the induction of LH secretion [30,31]. The existence of several isoforms of GnRH that are capable of interaction with the known GnRH receptor, and the possible prevalence of additional, putative GnRH receptor(s) present multiple possibilities for the regulation of GnRH activity by heterodimerization.

GnRH-I was previously suggested to be involved in the induction of sexual behavior, since the administration of this peptide into the midbrain central gray of rats has been demonstrated to facilitate sexual behavior [7]. However, high doses of GnRH-I (μM range) were needed to elicit this effect. Also, discrepancies are evident between the reported biological potency of various GnRH-I analogs in the pituitary gland as compared to their potency in the brain. Therefore, the discovery of both GnRH-II and GnRH-III in the midbrain raises the possibility that either of these may serve as the physiological regulator of reproductive behavior.

The observation that three isoforms of GnRH are localized in identical brain regions of the mammalian brain (i.e. hypothalamus, midbrain, pituitary stalk) may suggest that these isoforms evolved from a common ancestor, by gene duplications and mutations. Later, these isoforms assumed distinct biological activities, although they may share some activities due to their structural similarities. The physiological significance of the existence of multiple isoforms of GnRH in the brain is not clear as yet, and unveiling their physiological functions is a challenge for future research. Nevertheless, our current conceptions on the neuroendocrine control of reproduction, as well as on the physiological functions of the different members of the GnRH family, have to be reevaluated.

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