A second isoform of gonadotropin-releasing hormone is present in the brain of human and rodents

Alon Chen[a], Dror Yahalom[a], Nurit Ben-Aroya[a], Ella Kaganovsky[b], Eli Okon[b], Yitzhak Koch[a,*]

[a] *Department of Neurobiology, Weizmann Institute of Science, Rehovot 76100, Israel*
[b] *Department of Pathology, Rabin Medical Center, Petah-Tikva 49100, Israel*

Received 22 July 1998; revised version received 17 August 1998

Abstract Gonadotropin-releasing hormone-I (GnRH-I), present in the mammalian hypothalamus, regulates reproduction. In this study we demonstrate, for the first time, that an additional isoform of GnRH, [His^5, Trp^7, Tyr^8] GnRH-I (GnRH-II) is present in the brain of the mouse, rat and human. Human and rat brain extracts contain two isoforms of GnRH, GnRH-I and GnRH-II, which exhibited identical chromatographic properties to the respective synthetic peptides, in high performance liquid chromatography. Using immunohistochemical techniques we have found that GnRH-II is present in neuronal cells that are localized mainly in the periaqueductal area as well as in the oculomotor and red nuclei of the midbrain. It is of interest to note that in the hypogonadal mouse, although the GnRH-I gene is deleted, GnRH-II is present. Substantial concentrations of GnRH-II are also present in the hypothalamus and stored in the human pituitary stalk or in the mouse median eminence. By using reverse transcription (RT)-PCR we have also found that while GnRH-II is not expressed in the cerebellum, it is expressed in all three structures of the brain stem: midbrain, pons and medulla oblongata.

Key words: GnRH/LHRH isoform; GnRH-II in human and rodents; Neuropeptide

1. Introduction

Gonadotropin-releasing hormone-I (GnRH-I), (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), 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 which regulate gonadal functions. Today, a dozen isoforms of GnRH are known in vertebrates, all are decapeptides that are conserved by 10–50% as compared to GnRH-I [3]. It can be stated, in general, that at least two different isoforms of GnRH are expressed in the brain of each of the lower vertebrate species [4]. One isoform, [His^5, Trp^7, Tyr^8] GnRH-I, named GnRH-II, originally isolated from chicken brain [5] is expressed in almost all vertebrate classes. GnRH-II, the most ancient and conserved form of GnRH has been found in cartilage and bony fish [6–9], amphibians [10–12], reptiles [13,14], birds [5,15], and metatherian species [16]. In the placental species of mammals, only GnRH-I has been detected [17], although recently GnRH-II was revealed in early evolved eutherian species such as the musk shrew and mole, of the insectivora order [16,18]. The form of GnRH which varies across vertebrate classes, is localized mainly in the hypothalamus and is responsible for the regulation of reproduction [19], whereas GnRH-II is localized mainly in extrahypothalamic brain areas, mostly in the midbrain [20,21]. Thus GnRH-II is an ancient isoform of GnRH and is structurally conserved for over 500 million years of evolution, suggesting that its neuronal functions are of utmost importance.

Recently it was reported that in the rhesus monkey immunoreactive GnRH-II neurons are scattered in the periaqueductal region of the midbrain, with a few positive cells in the basal hypothalamus [22]. However, GnRH-II positive cells could not be detected in the brain of several rodents [22]. It was also reported that a gene, encoding GnRH-II, is expressed in the human brain [23]. However, GnRH-II gene expression in the brain stem structures was not measured, nor the presence of the GnRH-II peptide in the human brain. By using high performance liquid chromatography (HPLC), immunocytochemistry, radioimmunoassay (RIA), and molecular biology techniques, we could demonstrate the presence of GnRH-II in the brain of the mouse, rat and human.

2. Materials and methods

2.1. Tissue processing for GnRH determination

All animal experiments were carried out in compliance with the regulations of the Weizmann Institute of Science. The brains of 50 female rats, 25 days old, were removed immediately after killing, immersed in ice-cold 0.1 N HCl and homogenized by a Teflon-glass homogenizer. Following centrifugation (12 000 × *g*, 30 min at 4°C) the supernatant was pumped onto columns of Sep-Pak C-18 cartridges, eluted by methanol and evaporated by nitrogen. Specific regions of a human brain were received during a routine autopsy at Rabin Medical Center from a 77-years-old man who died of pyonephrosis. The body was kept for 38 h at 4°C until autopsy. Samples from some of these regions were extracted for total RNA and the rest was homogenized in 0.1 N HCl and processed as described for the rat brain. The brain extracts were processed through reversed phase (RP) HPLC using C-18 columns, and eluted using the following conditions: eluent A, 0.1% trifluoroacetic acid in water; eluent B, 75% CH_3CN in 0.1% trifluoroacetic acid. The gradient program consisted of a linear gradient of eluent B 20–30% for 5 min at a flow rate of 1 ml/min, followed by isocratic elution of 30% eluent B for 35 min, and continued with 100% of eluent B for additional 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. All fractions were

*Corresponding author. Fax: +972 (8) 9344131.
E-mail: lhkoch@weizmann.weizmann.ac.il

Abbreviations: GnRH, gonadotropin-releasing hormone; HPLC, high performance liquid chromatography; PB, phosphate buffer; PBS, phosphate buffered saline; RIA, radioimmunoassay; RP, reversed phase; RT, reversed transcriptase

PII: S0014-5793(98)01064-3

assayed for GnRH-I and GnRH-II concentration by RIA using the appropriate antisera. The elution positions of the synthetic peptides were determined by application of 1 μg of GnRH-I and of GnRH-II (Phoenix Pharmaceuticals, Mountain View, CA; catalog no. 040-02 and 040-01, respectively; 99% purity by HPLC analysis). After thorough washing, a blank run was monitored by RIA to ensure that the column was not contaminated.

2.2. Radioiodination and radioimmunoassay

Iodination of synthetic GnRH-I or GnRH-II was carried out by using the chloramine-T method [24]. 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 [25].

2.3. Antibodies

The following antisera were used throughout this study: a polyclonal antibody against GnRH-I, prepared and characterized in our laboratory, was used for RIA [25]. GnRH-II did not displace any of the bound ^{125}I-GnRH-I even at a concentration that exceeded by 1000 times the GnRH-I concentration that is needed for displacing 50% of the tracer (20 ng vs. 20 pg). A monoclonal antibody against GnRH-I, kindly provided by Dr. H.F. Urbansky, was used at dilutions ranging from 1:4000 to 1:10 000 for the immunofluorescence studies; the specificity of this antibody (HU4H) was reported elsewhere [26]. A polyclonal antibody against GnRH-II (aCII6) was kindly provided by Dr. K. Okuzawa and its specificity was previously defined [27]. Additional specificity tests in our laboratory demonstrated that GnRH-I did not displace any of the bound ^{125}I-GnRH-II, even at a concentration that exceeded 2500 times the GnRH-II concentration that is needed to displace 50% of the tracer (20 ng vs. 8 pg). We have utilized dilutions ranging from 1:4000 to 1:10 000 of this antibody for the immunohistochemical studies.

2.4. Immunohistochemical procedure

The localization of GnRH-II in the midbrain of mouse, rat and human, was carried out by application of a specific antibody to the brain slices followed by a biotinylated secondary antibody, and of an avidine-biotin-horseradish peroxidase complex according to the procedures described by Eilam et al. [28]. Human brain samples were obtained during routine autopsies at Rabin Medical Center from a 36-weeks-old male infant who died of bilateral polycystic kidneys and from a 60-years-old man who died of pulmonary edema. The bodies were kept 8 h at 4°C until autopsy. The brain samples were fixed in 10% neutral buffered formalin, embedded in paraffin and cut into 6 μm thick sections. Ten-weeks-old female and male rats or mice were also used. The animals were deeply anesthetized with 10% chloral hydrate (2.5 ml/animal) and perfused through the left ventricle of the heart, with 0.1 M phosphate buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 M PBS and 4% sucrose. The brains were removed and immersed in a post-fixation solution (1% paraformaldehyde, 30% sucrose in 0.1 M PBS, pH 7.4) for at least one week. Frozen brains were cut by microtome, and sections (32 μm) were collected in a solution of 0.1 M PBS containing 0.1% sodium-azid, washed three times with PBS 0.1 M and incubated for 15 min in 0.3% hydrogen peroxide to reduce endogenous peroxidase activity. After an additional wash, the sections were incubated for 2 h in a blocking medium containing 20% normal goat serum and 0.5% Triton X-100. The sections were incubated over-night at 4°C with antisera to GnRH-II or GnRH-I, rinsed well and incubated with an avidin-biotin-peroxidase complex (ABC Vectastain, Vector Laboratories, Burlingame, CA) followed by nickel-intensified diaminobenzidine with hydrogen peroxide as the chromogen. In order to determine the specificity of the signals we have included several control groups in which the antibodies were preabsorbed with excess (100 μg) of GnRH-I or GnRH-II for 24 h. Additional control sections were incubated without the first antibody or with an antibody against vasopressin that is known to be expressed only in the paraventricular nucleus of the hypothalamus. Additional sections were stained by cresyl violet to confirm the neuroanatomical location of immunoreactivity.

2.5. Double fluorescence immunocytochemical analysis

Sections of the hpg mouse hypothalamus obtained from perfused brains of 3-months-old male were analyzed by double fluorescence

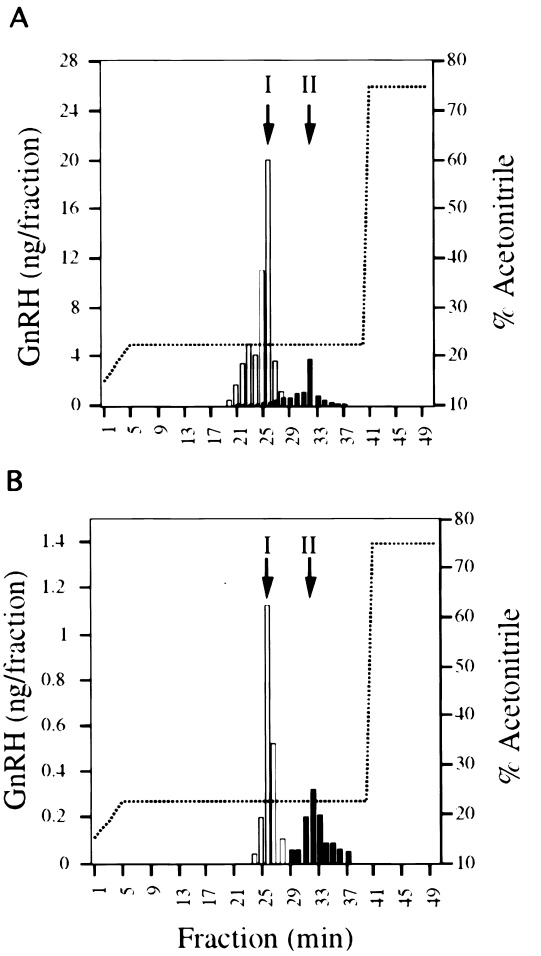

Fig. 1. RP HPLC elution profile of GnRH-I and GnRH-II extracted from rat brain (A) and from human midbrain (B). Fractions (1 ml) of the eluate were collected, evaporated and reconstituted. All fractions were assayed for GnRH-I and for GnRH-II by RIA, using specific antibodies for either GnRH-I (open columns) or GnRH-II (solid columns). The elution position of synthetic GnRH-I (I) or GnRH-II (II) is indicated by black arrows.

immunocytochemistry using fluorescence microscopy, according to the procedure described by Kim et al. [29]. The slices were washed (5 min×3) with PB and were permeabilized for 3 min with 0.5% Triton X-100, followed by three washes with PB. The slices were then incubated with 1% of bovine serum albumin (200 μl) in PB for 1 h at room temperature followed by the addition of the primary antibodies for 12–15 h at 4°C. The slices were then washed (5 min×3) with 0.1 M PB, and incubated for 2 h at room temperature with either fluorescein or rhodamine-conjugated secondary antibody as follows: goat anti-mouse conjugated to Cy2 (green fluorescence, Jackson, Immunoreaserch), goat anti-rabbit conjugated to rhodamin (red fluorescence, Jackson, Immunoreaserch), or both. Fluorescence was visualized by fluorescence microscopy using green and red filters for GnRH-I and GnRH-II, respectively.

2.6. Reversed transcription (RT)-PCR and Southern analysis

Total RNA was extracted by using Trizol RNA isolation reagent (Molecular Research Center, Cincinnati, OH) according to the protocol of the manufacturer. We have used RT-PCR [30] to amplify the levels of endogenous GnRH-II mRNA that may be present in the human midbrain, pons, medulla oblongata and cerebellum samples, using oligonucleotide primers that were described by White et al. [23]. The expression of the ribosomal protein S-14 [31], derived from the same tissue preparations, served as a control. Amplification was carried out for 31 cycles, the annealing temperature was decreased from

65 to 60°C and the final $MgCl_2$ concentration was 2.5 mM. The PCR products were transferred to a nylon membrane (Hybond-N+, Amersham) in 20×SSC solution for over-night and was baked in a vacuum oven at 80°C for 2 h. Pre-hybridization was performed in the presence of 6×SSC, 5×Denhart's solution, 5 mM EDTA and 0.2 mg/ml salmon sperm DNA for 3 h at 60°C. Over-night hybridization was performed at 64°C, in the presence of a ^{32}P labeled probe, specific to the GnRH-II that was described by White et al. [23] and exposed for 1 h using a phosphorimager (445SI, Molecular Dynamics).

3. Results

Acid extracts of whole rat brain or of specific regions of the human brain were eluted through RP C-18 columns on HPLC using an isocratic elution program, that separates synthetic GnRH-I from GnRH-II (Fig. 1, black arrows). The concentration of GnRH-I or GnRH-II in the eluate of the brain extracts was determined by RIA using specific antibodies to either GnRH-I or GnRH-II. Fig. 1 demonstrates that the elution profiles of the immunoreactive neuropeptides that were extracted from the brain were identical to those of the synthetic peptides. These results prove the existence of GnRH-II in the rat brain (Fig. 1A) and human midbrain (Fig. 1B). The total concentration of GnRH-II in the rat brain was found to be 15–20% of that of GnRH-I. The concentration of GnRH-II in the various regions of the human brain has to be regarded only qualitatively since they represent data obtained from a single brain of a 77-years-old person. Nevertheless, the highest amounts of GnRH-II were found in the midbrain region and in the pituitary stalk (1.2 ng, each), lower concentrations were found in the hypothalamus (0.36 ng), medulla oblongata (22 pg) and in the pons (18 pg). GnRH-II was undetectable in 1 g tissue samples taken from the frontal cortex or cerebellum. The presence of GnRH-II in the hypothalamus suggests that in addition to its possible role as a neurotransmitter in the brain, it may be also active as a neurohormone in the pituitary gland.

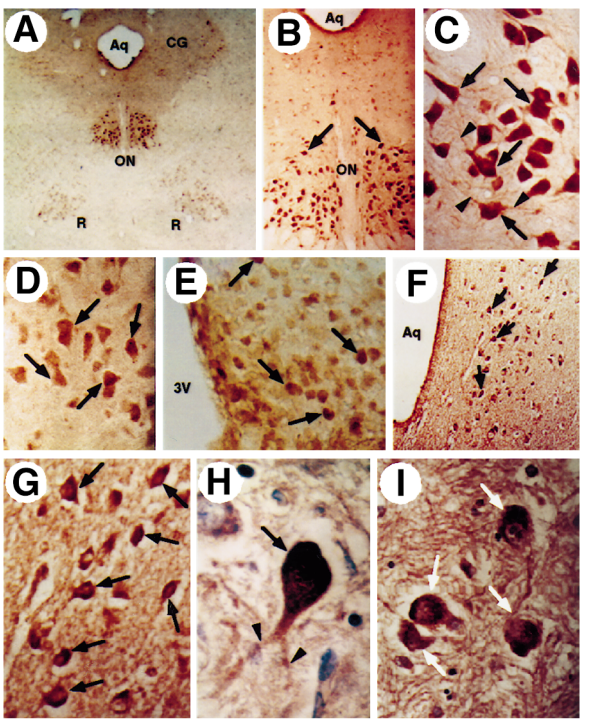

Fig. 2. Immunoreactive GnRH-II cells and fibers in coronal sections through the midbrain area of the mature female rat (A–C), of the hypogonadal mouse (D, E), of a 36-weeks-old infant (F–H), and of a 60-years-old man (I). In the rat, GnRH-II immunoreactive neurons (arrows) are visualized mainly in the oculomotor nucleus (ON) of the midbrain (A–C), and to a lesser extent in the red nucleus (R) (A). GnRH-II immunopositive neurons in the ON (D) and in the arcuate nucleus (E) of the hypogonadal mouse are seen. Dense accumulation of GnRH-II immunoreactive neurons are seen in the periaqueductal region of the human midbrain (F, G). The ON nucleus of the human is not as rich in GnRH-II immunopositive neuronal cells (H, I) as that of the rodents. Immunoreactive GnRH-II fibers and cell protuberants (arrowheads) are seen in many of the GnRH-II immunopositive neurons (C and H), Aq, aqueduct; CG, central grey; 3V, third ventricle A, ×3; B, F, ×12; E, ×24; C, D, G, ×48; H, ×72; H, ×120. H, I were stained also by hematoxyline to demonstrate nuclei of cells.

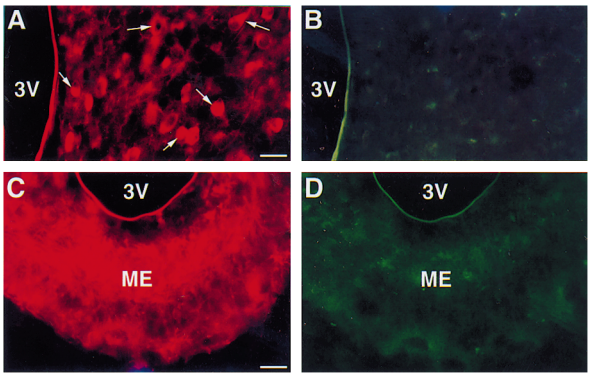

Fig. 3. Double fluorescence microscopy of the hypothalamic region of the hpg male mouse. GnRH-I monoclonal antibody was reacted with goat anti-mouse Cy2, whereas GnRH-II polyclonal antibody was reacted with goat anti-rabbit rhodamin. Immunoreactive cells (arrows), in the area of the arcuate nucleus of the hypothalamus, were observed only with the red filter (antibody against GnRH-II) (A) and not with the green filter (antibody against GnRH-I) (B). Massive immunoreactivy is seen in the median eminence that was reacted with an antiboby against GnRH-II (C), but not with an antibody against GnRH-I (D). ME, median eminence; 3V, third ventricle; scale bar, 10 μm.

The localization of GnRH-II in the midbrain of mouse, rat and human, was carried out by application of a specific antibody followed by biotinylated secondary antibody, and of an avidine-biotin-horseradish peroxidase complex. Fig. 2 demonstrates immunoreactive neurons in the midbrain region of the mouse, rat and human. Numerous immunoreactive GnRH-II neurons are found in the oculomotor nucleus of the rat midbrain (Fig. 2A–C) and to a lesser extent in the red nucleus (Fig. 2A). A similar distribution pattern of GnRH-II positive cells is also evident in the mouse (data not shown). It is well established that GnRH-I is not produced by the hypogonadal mouse due to a deletion in the GnRH-I gene [32,33]. We have therefore looked for immunopositive GnRH-II cells in the brain of the hypogonadal mouse. Indeed, we have found a similar distribution of GnRH-II immunoreactive neurons in the brain of the mutant mouse as compared to the normal mouse. GnRH-II neurons are concentrated in the oculomotor nucleus (Fig. 2D) and in the arcuate nucleus of the hypothalamus (Fig. 2E) of the hypogonadal mouse. Further studies on the hpg mouse brain were carried out using double fluorescence microscopy (Fig. 3). Slices of the hypothalamic region were reacted with a monoclonal antibody against GnRH-I and a polyclonal antibody against GnRH-II. GnRH-II immunoreactivity is clearly observed in the arcuate nucleus region

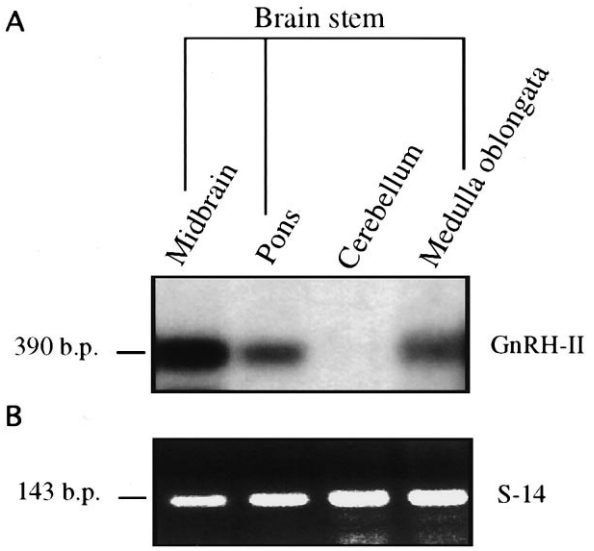

Fig. 4. A: Southern blot hybridization of amplified GnRH-II cDNA fragments. Amplified GnRH-II cDNA fragments from brain stem structures and cerebellum of human brain were hybridized to a human GnRH-II ^{32}P labeled oligonucleotide probe. Lanes: 1, midbrain; 2, pons; 3, cerebellum; 4, medulla oblongata. B: Parallel amplification of the ribosomal protein S-14 cDNA, derived from the same experimental groups as of A, after 1.5% agarose gel electrophoresis and ethidium bromide staining.

of the hypothalamus (Fig. 3A) and in the median eminence (Fig. 3C). As expected, these hypothalamic regions of the hpg mouse did not show any GnRH-I immunoreactivity (Fig. 3B and D). In the human, GnRH-II immunopositive neurons are seen in the periaqueductal region of the midbrain (Fig. 2F and G). In the rat and mouse, GnRH-II positive cells are also scattered in the periaqueductal region of the midbrain, but in the human they are much more abundant. On the other hand, the density of the GnRH-II immunopositive cells in the human oculomotor nucleus (Fig. 2H and I) is not as impressive as in the rodents. The specificity of the signals was tested by preabsorbing the GnRH-II antiserum with 100 μg of GnRH-II for 24 h, or by omitting the GnRH-II antiserum. In both cases no staining was evident whereas preabsorbtion with GnRH-I did not affect the staining. When we have replaced the GnRH-II antibody by a polyclonal antibody to vasopressin, staining was found only in the paraventricular nucleus of the rat hypothalamus.

RT-PCR and Southern hybridization have demonstrated (Fig. 4) that GnRH-II is expressed in the different regions of the human brain stem and especially in the midbrain. GnRH-II is not expressed in the cerebellum. The results of this experiment correlate well with our quantitative determinations of GnRH-II in these tissues.

4. Discussion

By using a variety of techniques, we have found an additional isoform of GnRH, GnRH-II, in the brain of the mouse, rat and human. GnRH-II was found to be present in neuronal cells that are localized mainly in the periaqueductal area as well as in the oculomotor and red nuclei of the midbrain. A similar distribution of GnRH-II was reported in amphibia, where this neuropeptide was restricted to the midbrain tegmentum [34], and in the musk shrew, one of the most primitive mammals. GnRH-II immunoreactive cells were found in the periaqueductal region, in the regions of the oculomotor and red nuclei as well as in the basal hypothalamus median eminence region of the musk shrew [20].

The strictly conserved structure of the GnRH-II peptide, from the primitive fish to the human, suggests that this neuropeptide possesses vital bioactivities. However, the biological functions of GnRH-II are practically still unknown. In the brain stem, GnRH-II may act as a neurotransmitter or as a neurotrophin and thus affect neuronal growth, differentiation and functions. In the bullfrog, application of exogenous GnRH-I has been reported to inhibit a specific voltage-dependent potassium current leading to depolarization of sympathetic neurons and to induction of a late, slow excitatory postsynaptic potential [35]. More recently it was found that GnRH-II is at least 1000 times more potent than GnRH-I in inducing this effect, suggesting it is the endogenous transmitter that mediates the excitatory postsynaptic potential [36,37]. Studies in mammals have indicated a role for GnRH in the process of sexual differentiation of the brain [38]. Other studies demonstrated the involvement of GnRH in the induction of sexual behavior. Thus, GnRH-I that was administered into the midbrain central grey has been demonstrated to facilitate sexual behavior [39]. However, the high doses of GnRH-I (mM range) that were needed to induce this phenomenon and the discovery of another isoform of GnRH in the midbrain raises the possibility that GnRH-II is the physiological regulator of mating behavior. This hypothesis is strengthened by the finding that the interaction of female musk shrews with males induces rapid changes in specific populations of GnRH immunoreactive cells [40]. A critical examination of the relevant literature projects discrepancies between the reported biological potency of various GnRH-I analogs in the pituitary gland as compared to their potency in the brain. Thus, analogs or fragments of GnRH-I that are unable to activate the pituitary gland could induce sexual behavior, whereas other GnRH analogs that are potent secretagogues of gonadotropins were unable to induce mating behavior [41,42]. These observations suggest that the activities of GnRH in the brain are not mediated by the known GnRH-I receptor, and imply that the behavioral effects of GnRH in the brain are mediated by an as yet unknown receptor.

The production of GnRH-II in the hypothalamus of mouse and human and its presence in the median eminence of the mouse and in the pituitary stalk of human, demonstrated herein, imply that in addition to its neurotransmitter roles, GnRH-II may possess neurohormonal functions at the pituitary gland. Previous studies, however, have demonstrated that its efficacy in inducing luteinizing hormone release is significantly lower than that of GnRH-I [43]. In view of the lower concentration of GnRH-II, as compared to GnRH-I, in the hypothalamus and considering its inferior capability to induce luteinizing hormone secretion, it seems that the role of GnRH-II in the hypothalamic pituitary gonadal axis may be limited to the modulation of GnRH-I activity. This assumption is supported by the fact that the presence of GnRH-II in the brain of the hypogonadal mouse (Fig. 2D and E, Fig. 3A and C) is not sufficient to cause normal gonadal development. Alternatively, GnRH-II may activate its intrinsic pituitary receptor and be responsible for the regulation of other pituitary functions.

Acknowledgements: The authors wish to thank Drs. K. Okuzawa, Japan, and H.F. Urbansky, USA, for supplying the antibodies for GnRH-II and GnRH-I, respectively; Dr. R. Eilam for helpful suggestions. This study was supported by the Grodetsky Center for Research of Higher Brain Functions of the Weizmann Institute of Science.

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