Gonadotropin-Releasing Hormone Neurons Innervate Kisspeptin Neurons in the Female Mouse Brain

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Key Words

Rostral periventricular area of the third ventricle · Anteroventral periventricular nucleus · Arcuate nucleus · Asymmetric synapses · Reciprocal innervation · Oestrogen

Abstract

Kisspeptin (KP) neurons in the rostral periventricular area of the third ventricle (RP3V) and arcuate nucleus (Arc) are important elements in the neuronal circuitry regulating gonadotropin-releasing hormone (GnRH) secretion. KP and co-synthesised neuropeptides/neurotransmitters act directly on GnRH perikarya and processes. GnRH neurons not only form the final output pathway regulating the reproductive functions of the anterior pituitary gland, but also provide neuronal input to sites within the hypothalamus. The current double-label immunohistochemical studies investigated whether GnRH-immunoreactive (IR) projections to the RP3V and/or Arc establish morphological connections with KP-IR neurons at these sites. To optimise visualisation of KP immunoreactivity in, respectively, the RP3V and Arc, ovariectomised (OVX) oestrogen-treated and OVX oil-treated female mice were studied. Confocal laser microscopic analysis of im-

munofluorescent specimens revealed GnRH-IR axon varicosities in apposition to approximately 25% of the KP-IR neurons in the RP3V and 50% of the KP-IR neurons in the Arc. At the ultrastructural level, GnRH-IR neurons were seen to establish asymmetric synaptic contacts, which usually reflect excitatory neurotransmission, with KP-IR neurons in both the RP3V and Arc. Together with previous data, these findings indicate reciprocal connectivity between both of the KP cell populations and the GnRH neuronal system. The functional significance of the GnRH-IR input to the two separate KP cell populations requires electrophysiological investigation.

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Introduction

Gonadotropin-releasing hormone (GnRH)-producing neurons form the final common pathway for the hypothalamic neuronal circuitry that regulates gonadal functions. GnRH not only drives the anterior pituitary gland functions that govern ovarian steroidogenesis, follicular maturation and ovulation, but also acts as a neu-

romodulator within the brain [1, 2]. Accordingly, there is evidence that central administration of GnRH can inhibit the reproductive axis in sheep [3, 4] and rats [5, 6].

Previous immunohistochemical studies have identified putative sites for central actions of GnRH. Thus, GnRH-immunoreactive (IR) axon projections are detected in the preoptic area and anteroventral periventricular nucleus (AVPV) [1, 7, 8]; the latter plays an essential role in mediating the positive feedback effects of oestrogen that culminate in the surge release of GnRH [9]. GnRH-IR processes are also present in the mediobasal hypothalamus (MBH) [1, 7], including the arcuate nucleus (Arc), a site that is implicated in the pulsatile regulation of GnRH release [9] and in the negative feedback effect of oestrogen on GnRH secretion [10].

Oestrogen receptor (ER) α -expressing neurones mediate the positive [11] and negative [12] feedback effects of oestrogen onto the GnRH neurones. Given that the latter express ER β rather than ER α [13], the neurotransmitter phenotype and anatomical location of the ER α -expressing neurones that communicate oestrogen-induced signals to GnRH neurones have been the subject of intense investigation over the past three decades [14, 15]. The hypothalamic kisspeptin (KP) system appears to be involved in both the pulsatile [16] and surge [17–21] modes of GnRH release. It consists of two distinct cell populations located within the rostral periventricular area of the third ventricle (RP3V), including the AVPV and periventricular preoptic nucleus, and the Arc [22, 23]. Both of these KP cell populations express ER α [24, 25] and send projections that innervate GnRH-IR neurones [26, 27]; thus, they have the capacity to convey critically important oestrogen-dependent signals to GnRH neurones.

Given the evidence that KP neurones innervate GnRH neurones and that GnRH has actions within the hypothalamus that can influence the reproductive axis, we have used confocal and electron microscopic immunohistochemical approaches to test the hypothesis that GnRH neurones in female mice innervate KP-IR neurones in the RP3V and/or Arc.

Materials and Methods

Animals

Adult female CD1 mice (2–3 months old, 25–30 g b.w.; Charles River, Hungary) were housed under controlled lighting (12:12 h light-dark cycle; lights on at 07:00 h, Zeitgeber time (ZT)0) and temperature ($22 \pm 2^\circ\text{C}$), with access to food and water ad libitum. All studies were carried out with permission from the Animal Welfare Committee of the Institute of Experimental Medicine (No.

2285/003) and in accordance with legal requirements of the European Community (Decree 86/609/EEC). Surgery was performed on animals under deep anaesthesia induced by an intraperitoneally injected cocktail of ketamine (25 mg/kg b.w.), xylavet (5 mg/kg b.w.) and pipolphen (2.5 mg/kg b.w.) in saline.

Tissue Preparation for Confocal Microscopy

The animals were ovariectomised (OVX, day 0) and 7 days later (day 7) implanted subcutaneously with a capsule (ID 1.57 mm, OD 3.18 mm) containing either 17 β -oestradiol (0.625 μg in 20 μl sunflower oil; OVX+E₂) or vehicle (OVX+Oil) [28]. Three days after implantation (day 10), the mice were colchicine-treated intracerebroventricularly (40 μg in 4 μl 0.9% saline) and 24 h later (day 11) they were sacrificed at either ZT4–5 ($n = 5$ for OVX+E₂ and for OVX+Oil) or ZT11–12 ($n = 5$ for OVX+E₂; $n = 7$ for OVX+Oil), whether or not they had been treated with oestrogen; these times include, respectively, the negative and positive feedback phases of oestrogen's effects on LH release. The animals were perfused transcardially with phosphate-buffered saline (PBS; 0.1 M) containing 4% paraformaldehyde (PFA). The brains were post-fixed in 2% PFA/PBS solution for 24 h at 4°C , cryoprotected overnight in 25% sucrose and 25- μm thick coronal sections were cut on a freezing microtome. The sections were divided into three sequential pools and stored in antifreeze solution (30% ethylene glycol; 25% glycerol; 0.05 M phosphate buffer; pH 7.4) at -20°C until use. After the endogenous peroxidase activity had been quenched with 0.5% hydrogen peroxide (10 min), sections were permeabilised with 0.5% Triton X-100 (23,472-9, Sigma, 20 min). Finally, 2% normal horse serum was applied (20 min) to reduce non-specific antibody binding. Subsequent treatments and interim rinses in PBS (3×5 min) were carried out at room temperature, except for incubation in the primary antibody or fluorochrome conjugate, which took place at 4°C .

Tissue Preparation for Electron Microscopy

In order to investigate putative GnRH-KP appositions in the RP3V and Arc at the electron microscopic level, pre-embedding, double-label immunohistochemistry was carried out. For tissue preservation of oestrogen or oil-treated OVX animals ($n = 5$ each), which also received colchicine intracerebroventricularly, a mixture of 2% PFA and 4% acrolein was used. 30- μm thick coronal sections were cut with a vibratome and treated with 1% sodium borohydride (30 min), 0.5% H₂O₂ (15 min) and permeabilised with three freeze-thaw cycles, as described previously [29].

Double Immunofluorescence for GnRH and KP Immunoreactivity

Sections from the RP3V region of the OVX+E₂ animals and the Arc region of the OVX+Oil animals were incubated (72 h) in a cocktail of the guinea pig anti-GnRH (#1018, Hrabovszky, 1:10,000) [30] and rabbit anti-KP (#566, Caraty, 1:10,000) [25] primary antibodies. GnRH immunoreactivity was visualised with CY3-conjugated donkey anti-guinea pig IgG (#706-165-148, Jackson ImmunoResearch Laboratories, 1:1,000, 12 h). To visualise the KP-IR structures, the sections were incubated sequentially in biotinylated donkey anti-rabbit (#711-065-152, Jackson ImmunoResearch Laboratories, 1:1,000, 2 h), Vectastain ABC Elite solution (PK-6100, Vector Laboratories, 1:1,000, 1 h), biotinylated tyramide with H₂O₂ (1:1,000 and 0.006%, respectively; 0.5 h) and, finally, Alexa 488-streptavidin (S-11227, Molecular Probes, 1:500, 12 h).

Confocal Laser Analysis and 3-D Reconstruction of GnRH-IR Afferents to KP-IR Neurones in the RP3V and Arc

The double-labelled sections were analysed using a Radiance 2100 confocal microscope (Bio-Rad Laboratories, Hemel Hempstead, UK). Three sections were selected per brain from the RP3V (at bregma +0.38 to +0.26, +0.26 to +0.02 and +0.02 to -0.1 mm) and the Arc (at bregma -1.46 to -1.7, -1.94 to -2.06 and -2.3 to -2.54 mm) [31]. Multiple stacks of optical slices (512 × 512 pixels, z-steps 0.6 µm) were obtained by scanning all of the KP-IR neurones unilaterally in each of the selected coronal sections using a 60× oil immersion objective. The Alexa 488 and CY3 fluorochromes were detected with laser lines 488 and 543 nm, using dichroic/emission filters 560/500–540 nm and 650/560–610 nm, respectively. The separately recorded green and red channels were merged and displayed with the Laser Vox software (Bio-Rad) running on an IBM-compatible personal computer. The number of KP-IR neurones contacted by GnRH-IR processes and the incidence of GnRH-IR appositions on individual KP-IR neurones were quantified. Appositions were identified if no gap was visible between the juxtaposed profiles in at least one optical slice. Differences were assessed by ANOVA with a priori planned contrasts (IBM SPSS, Version 20) testing the hypotheses that the dependent variable differs (1) within each region across the time points and (2) between the regions at the same time point. Data are presented as mean ± SEM. Statistical significance was set at $p < 0.05$. The images acquired with the confocal laser microscope were further investigated using three-dimensional (3-D) analyses. The stack of optical slices was loaded into the visualisation software ImageSurfer 1.20 and the two channels containing images of consecutive optical slices were rendered in three dimensions with the Isosurface visualisation application. The isovalue was set individually for each image to minimise any noise, while maintaining the proper cellular boundaries. The isosurfaces generated from the two channels in the same optical volume were visualised to check for cell-to-cell contacts. This enabled verification of the findings from the two dimensional confocal image analyses.

Electron Microscopy to Investigate Synaptic Relations of GnRH-IR Varicosities and KP-IR Neurones

Sections from the RP3V and Arc of, respectively, OVX+E₂ and OVX+Oil mice were double labelled for GnRH and KP immunoreactivity. They were incubated (2 days) concurrently in the primary antibodies (guinea pig anti-GnRH, #1018, Hrabovszky, 1:600,000; rabbit anti-KP, #566, Caraty, 1:100,000). This was followed by visualisation of the GnRH-IR structures by incubating the sections sequentially in biotinylated donkey anti-guinea pig IgG (#706-065-148, Jackson ImmunoResearch Laboratories, 1:1,000, 1 day) and Vectastain ABC Elite solution (1:1,000, 1.5 h). The peroxidase reaction was carried out in the presence of H₂O₂ and nickel-diaminobenzidine (NiDAB), and post-intensified with silver-gold (SGI-NiDAB) [32]. After this reaction, the KP-IR structures were detected by incubating sections sequentially in biotinylated donkey anti-rabbit IgG (#711-065-152, Jackson ImmunoResearch Laboratories, 1:1,000, 1 day) and Vectastain ABC Elite solution (1:1,000; 1.5 h) and the peroxidase reaction was carried out in the presence of H₂O₂ and DAB alone. The double-labelled sections were then treated with 1% osmium tetroxide (1 h) and 2% uranyl acetate (in 70% ethanol; 40 min), dehydrated in an ascending series of ethanol and propylene oxide, and flat-embedded in

TAAB 812 medium epoxy resin between glass microscope slides pre-coated with a liquid release agent (#70880; Electron Microscopy Sciences, Fort Washington, Pa., USA). The resin was allowed to polymerise at 56°C for 2 days. The flat-embedded sections were initially investigated by light microscopy at 60× magnification; areas exhibiting appositions of GnRH-IR processes on the somatodendritic region of KP-IR neurones within the RP3V and Arc were selected for further processing. Semithin (1 µm) and ultrathin (50–60 nm) sections were cut with a Leica ultracut UCT ultramicrotome (Leica Microsystems, Vienna, Austria). The ultrathin sections were collected in ribbons onto Formvar-coated single-slot grids, contrasted with 2% lead citrate and examined with a Jeol-100C transmission electron microscope.

Immunohistochemical Controls

The specificities of the GnRH and KP primary antisera have been reported previously [25, 33]. Immunohistochemical controls included the use of increasing dilutions of the primary antisera, which resulted in a commensurate decrease and eventual disappearance of the immunostaining; omission of the primary antibodies or their preabsorption with corresponding peptide antigens resulted in complete loss of the immunostaining. The secondary antibodies employed here were designed for multiple labelling and pre-absorbed by the manufacturer with immunoglobulins from several species, including the one in which the other primary antibody had been raised.

Results

Confocal microscopic analysis revealed appositions between GnRH-IR processes and KP neurones. Electron microscopy identified synapses between GnRH-IR terminals and KP-IR neurones.

GnRH-IR Processes Form Appositions on KP-IR Perikarya and Dendrites in the RP3V and Arc

In keeping with the established distribution of GnRH in rats and mice, GnRH-IR cell bodies were found in the septo-preoptico-hypothalamic continuum and their processes were seen to course through and form varicosities in the RP3V (fig. 1a–c) and MBH (fig. 1d–f), two regions which contain KP-IR neurones. The expression of KP is differentially regulated by oestrogen in these two regions, being up-regulated in the RP3V and down-regulated in the Arc [24]; therefore, animal models with either high (OVX+E₂) or low (OVX+Oil) oestrogen levels were prepared to increase the detectability of KP in the respective regions. In addition, colchicine-treatment was employed to maximise peptide immunoreactivity in the somatodendritic region of KP neurones. Studies using double-label immunofluorescence identified KP-IR neurones in the RP3V within a plexus of GnRH-IR processes; some of these processes run close to the ependy-

Fig. 1. Representative coronal images of the mouse brain showing immunohistochemical double labelling for GnRH (red) and KP (green) at three rostro-caudal levels of the RP3V (**a–c**) and of the MBH (**d–f**). KP-IR cell bodies in the AVPV, periventricular (Pe) and Arc nuclei are intermingled within a plexus of varicose GnRH processes. Some of these processes run close to the ependymal layer of the third ventricle (3V) in the AVPV and PE (**a–c**), others arch away from the 3V (**d, e**) or form a dense ventral bundle coursing towards the median eminence (**d–f**). Scale bars: 50 μ m (**a–f**).

Table 1. Percentage ($\pm$ SEM) of KP-IR neurones found with apposed GnRH-IR axon varicosities and the mean ($\pm$ SEM) number of such appositions detected on those KP-IR neurones in the RP3V and Arc

	RP3V		Arc	
	at ZT4–5 (n = 5)	at ZT11–12 (n = 7)	at ZT4–5 (n = 5)	at ZT11–12 (n = 5)
Percentage of KP-IR neurones with GnRH-IR appositions	23.79 $\pm$ 6.93	28.19 $\pm$ 4.41	45.99 $\pm$ 3.02*	55.13 $\pm$ 4.62*
Number of GnRH-IR appositions per innervated KP neurones	2.57 $\pm$ 0.47	2.97 $\pm$ 0.26	3.31 $\pm$ 0.31	3.57 $\pm$ 0.31

The animals were sacrificed at ZT4–5 or at ZT11–12. Neither parameter in the RP3V or Arc shows a significant difference between animals sacrificed at the two ZTs. * Significantly greater ($p < 0.003$) percentage of KP-IR neurones with GnRH-IR appositions in the Arc than in the RP3V at the corresponding time.

mal layer of the third ventricle near the most medial KP-IR neurones (fig. 1a–c, 3a). In the Arc, KP-IR neurones were found in the vicinity of GnRH-IR processes, which were either arching away from the third ventricle (fig. 1d, e) or forming a dense ventral bundle coursing towards the median eminence (fig. 1d–f). Confocal microscopic analysis and 3-D reconstruction of the labelled cellular profiles identified GnRH-IR axon varicosities juxtaposed to KP-IR perikarya or dendrites in both the RP3V

(fig. 2a, b) and Arc (fig. 2c, d). In the animal models used for quantifying the innervation in the RP3V (colchicine-treated OVX+E₂ mice) and in the Arc (colchicine-treated OVX+Oil mice), GnRH-IR axon varicosities were seen to contact approximately 25% of the RP3V KP-IR neurones [23.79 $\pm$ 6.93% at ZT4–5 (n = 5) and 28.19 $\pm$ 4.41% at ZT11–12 (n = 7)] and approximately 50% of the Arc KP-IR neurones [45.99 $\pm$ 3.02% at ZT4–5 (n = 5) and 55.13 $\pm$ 4.62% at ZT11–12 (n = 5)]; the percentage

Fig. 2. High-power photomicrographs (**a**, **c** = optical slices; **b**, **d** = projected image of reconstructed Z stacks) of sections immunohistochemically double labelled for GnRH (red) and KP (green). GnRH-IR varicose processes (red) are in apposition to KP-IR cell bodies (arrowheads) or processes (arrows) in the AVPV (**a**, **b**) and Arc (**c**, **d**). Scale bars: 20 µm (**a**, **c**), 10 µm (**b**, **d**).

was unaffected by the ZT (table 1). At each of the investigated times there was a significantly greater ($p < 0.003$) percentage of KP-IR neurones with GnRH-IR appositions in the Arc than in the RP3V. At both sites, the mean number of GnRH-IR appositions on their target KP-IR neurones was approximately 3; this number was unaffected by the ZT (table 1).

GnRH-IR Axon Terminals Establish Asymmetric Synapses on KP-IR Neurones in the RP3V and Arc

The immunofluorescent identification of appositions between GnRH-IR varicosities and KP-IR neurones in the RP3V and ARC prompted an initial investigation for classical synapses at the ultrastructural level. Pre-embedding immunohistochemical double labelling (fig. 3a), employing two distinct electron dense markers, enabled the electron microscopic identification of pre- and post-synaptic elements. This made it possible to select contact sites in flat-embedded sections. Analysis of the selected areas in serial ultrathin sections identified 13 cases of either axo-somatic (fig. 3b1, b2, c) or axo-dendritic (fig. 3d1–d6) synapses between GnRH-IR terminals and KP-IR neurones. The GnRH-IR presynaptic profiles contained abundant small round clear vesicles and a few dense core granules that were heavily labelled with silver grains. Each of the 13 synapses detected in the RP3V ($n = 4$) and ARC ($n = 9$) was found to be asymmetric.

Discussion

Previous evidence indicating that GnRH-IR processes establish synaptic connections within the hypothalamus has prompted expansion of the classic concept of GnRH as merely a hypophysiotrophic factor. Identified targets for such synapses include neurones exhibiting immunoreactivity for ERα in the preoptic area [34] and GnRH neurones themselves [35]. The present findings add KP-IR neurones to this list; the immunofluorescent and electron microscopic evidence shows them to be targeted by GnRH-IR processes in both the RP3V and the Arc. Such evidence does not address the question of whether GnRH or a co-transmitter is the active synaptic agent. Nevertheless, there is functional evidence that GnRH per se can regulate the activity of various hypothalamic neuronal systems [36–40]. Its actions appear to include the ultrashort feedback regulation of GnRH secretion, as indicated by the single-cell RT-PCR discovery of GnRH-R expression in GnRH neurones [41–43]. In the context of the present findings, it is noteworthy that previous studies involving ligand binding, immunohistochemistry, in situ hybridisation histochemistry or transgenic animals [44–48] have identified GnRH-R-expressing neurones in the rodent RP3V and Arc, sites which contain very few GnRH neurones in those animals; a neurotransmitter phenotype for these GnRH-R-positive cells remains to be determined.

Fig. 3. Double-label immunohistochemical detection of KP-IR neurones and GnRH-IR processes visualised with, respectively, the DAB brown reaction product (medium electron dense in the electron microscope) and the silver-gold intensified nickel-DAB black reaction product (highly electron dense in the electron microscope). Example of appositions between GnRH-IR varicosities and KP-IR neurones in the AVPV, identified under bright field conditions (arrowheads in **a** and its **inset**). At the electron microscopic

level, the detected synapses were asymmetric. Arrowheads indicate axo-somatic synapses in the RP3V in **b1**, **b2** (showing the same synapse in adjacent ultrathin sections) and **c**. A representative axo-dendritic synapse is shown in the Arc in **d1–d6** (showing the same synapse in adjacent ultrathin sections). Nu = Nucleus; nl = non-labelled; 3V = third ventricle. Scale bars: 20 µm (**a**), 10 µm (**inset**), 500 nm (**b1**, **d1–d6**), 250 nm (**b2**, **c**).

In rodents, RP3V KP neurones provide a direct input to GnRH neurones [26, 27, 49]; this is of critical importance in generating the GnRH surge during oestrogen's positive feedback phase [17–21]. The present study provides evidence for connections in the opposite direction. These may provide an indirect route for GnRH to regulate its own release, thereby supplementing the ultrashort feedback onto GnRH neurones.

The present study also provides evidence for GnRH-IR synaptic input to KP-IR neurones in the Arc. This population of KP neurones has been implicated in the generation of GnRH pulses and in the negative feedback effects of gonadal steroids on the reproductive neuroendocrine axis [50, 51]. The hypothesis that the Arc KP neurones actively participate in that negative feedback has been challenged by recent findings; they show Arc KP-GFP cells to be almost completely silent in the absence of oestrogen, the proportion of the silent neurones being significantly higher in OVX mice than in those that are ovari-intact [52]. It should be noted that these effects were observed in partially deafferented neurones in coronal

slices; further research will be needed to establish the significance of the inputs to Arc KP neurones that are lost in that preparation. Such considerations indicate the importance of elucidating the respective functions of the projections from GnRH neurones to Arc KP neurones, as shown in the present study, and the previously reported projections in the opposite direction [27].

Given that galanin is a peptide co-transmitter within GnRH neurones [53], it may have a functional role in the synaptic communications identified here. The expression of galanin in rodent GnRH neurones is sexually differentiated [54–56]. In females, it has been implicated in the regulation of both the surge [57] and pulsatile [58, 59] release of LH. The effect of the co-expressed galanin on GnRH secretion may derive from an autocrine action on the GnRH/galanin neurones, since both GalR1 and GalR2 mRNAs have been detected in mouse GnRH neurones [41]. A further role for the co-expressed galanin may involve regulation of non-GnRH RP3V and/or Arc neurones that contribute to the control of GnRH release. This possibility is supported by the presence of galanin recep-

tor expression in the RP3V (GalR1 and GalR3) and Arc (GalR1) [60–62] and by the capacity of galanin to activate as yet unidentified neurones at both of those sites [63]. Consequently, it will be important to determine whether KP neurones in the RP3V and/or Arc express GnRH-R and/or GalR1/3. Considering that galanin is also expressed in subsets of RP3V and Arc KP neurones [27, 64], the possibility of its bidirectional involvement in this circuitry merits attention.

The present study identified exclusively asymmetric synapses between GnRH-IR processes and KP-IR neurones in the RP3V and Arc. Although exceptions have been reported, this asymmetric morphology usually reflects excitatory neurotransmission [65]. The presence of the glutamatergic marker, VGLUT2, in most GnRH neurones in rats and mice [66, 67] is consistent with putative excitatory glutamatergic transmission from GnRH neurones at the synapses in question. Furthermore, the abundant small round clear vesicles that were seen in the pre-synaptic profiles may indicate the presence of glutamate [68]. In contrast, studies on rats and monkeys have found the predominant synaptic morphology between GnRH-IR terminals and GnRH-IR neurones to be symmetric [35, 69, 70]. Whether such relations reflect the previously cited capacity of GnRH to inhibit the reproductive axis [3–6] remains to be determined. Further research will be required to establish whether these two classes of GnRH-IR synapses derive from (1) functionally different subpopulations of GnRH neurones or (2) GnRH neurones that are capable of establishing functionally different synapses on phenotypically distinct target neurones.

It should be recognised that the current study involved colchicine treatment. The extent to which this intervention and the presence or absence of oestrogen may have affected the pre- and post-synaptic sites in the two KP neuronal populations requires detailed investigation. In the present study, GnRH-IR axon varicosities contacted approximately 25% of the RP3V KP neurones in the oestrogen-treated animals, but they reached approximately

50% of the Arc KP neurones in the absence of oestrogen. Previous studies have shown that oestrogen increases not only the incidence of vasopressin-IR afferents to RP3V KP neurones [29], but also the local expression of V1a vasopressin receptors [71]. Presence of this steroid has also been shown to determine the effects of a stressor such as fasting not only on LH release, but also on RP3V KP expression [72]. Consequently, the precise effects of oestrogen on the dynamics of the GnRH/KP interactions reported here require further research.

In summary, by demonstrating appositions and asymmetric synapses between GnRH-IR processes and KP-IR neurones, the data presented here provide the first morphological evidence for GnRH-IR innervation of KP neurones in both the RP3V and Arc. The results add a new perspective to the previously reported projections from these KP neuronal populations to GnRH neurones [26, 27]. The projections in the opposite direction may supplement the ultrashort feedback onto GnRH neurones with indirect autoregulatory mechanisms established through the RP3V and Arc KP neurones. Thus, these GnRH-IR terminals may provide substrates for the final common pathway neurones to influence signals from the oestrogen-responsive KP neuronal populations that contribute to the surge and/or pulsatile release of GnRH. The functional significance of the GnRH-IR input to KP neurones and the existence of the regulatory loop we propose based on our new neuroanatomical data will need to be addressed in electrophysiological experiments.

Acknowledgements

Support contributed by grants from the National Science Foundation of Hungary (OTKA K101326, OTKA K83710, OTKA K100722). The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007–2013) under Grant Agreement 245009. The authors wish to thank Barna László for his excellent technical assistance.

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