GLUTAMATERGIC AND GABA-ERGIC INNERVATION OF HUMAN GONADOTROPIN-RELEASING HORMONE-I NEURONS

Abbreviated title: Glutamate and GABA in inputs to GnRH neurons

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Abstract

Amino acid neurotransmitters in synaptic afferents to hypothalamic gonadotropin-releasing hormone-I (GnRH) neurons are critically involved in the neuroendocrine control of reproduction. While in rodents the major amino acid neurotransmitter in these afferents is GABA, glutamatergic axons also innervate GnRH neurons directly. Our aim with the present study was to address the relative contribution of GABAergic and glutamatergic axons to the afferent control of human GnRH neurons. Formalin-fixed hypothalamic samples were obtained from adult male individuals (n=8) at autopsies and their coronal sections processed for dual-label immunohistochemical studies. GABAergic axons were labeled with vesicular inhibitory amino acid transporter (VIAAT) antibodies, whereas glutamatergic axons were detected with antisera against the major vesicular glutamate transporter isoforms, VGLUT1 and VGLUT2. The relative incidences of GABAergic and glutamatergic axonal appositions to GnRH-immunoreactive neurons were compared quantitatively in two regions, the infundibular and paraventricular nuclei. Results showed that GABAergic axons established the most frequently encountered type of axo-somatic apposition. Glutamatergic contacts occurred in significantly lower numbers, with similar contributions by their VGLUT1 and VGLUT2 subclasses. The innervation pattern was different on GnRH dendrites where the combined incidence of glutamatergic (VGLUT1+VGLUT2) contacts slightly exceeded that of the GABAergic appositions. We conclude that GABA represents the major amino acid neurotransmitter in axo-somatic afferents to human GnRH neurons, whereas glutamatergic inputs occur somewhat more frequently than GABAergic inputs on GnRH dendrites. Unlike in rats, the GnRH system of the human receives innervation from the VGLUT1, in addition to the VGLUT2, subclass of glutamatergic neurons.

Introduction

Projections of type I gonadotropin-releasing hormone (GnRH) synthesizing neurons to the pericapillary space of the hypophysial portal blood vasculature represent the final common output way of the hypothalamus in the neuroendocrine control of reproduction (1). The neurosecretory activity of GnRH neurons is regulated by a variety of neurotransmitters/neuromodulators (2), which include the dominant inhibitory and excitatory amino acid neurotransmitters of the hypothalamus, γ-aminobutyric acid (GABA) and L-glutamate, respectively (3, 4).

Evidence mostly from studies of laboratory rodents indicates that GABA exerts multiple central effects on the reproductive axis and represents the principal neurotransmitter in the synaptic control of GnRH neuronal functions (2). GnRH neurons receive an abundant synaptic input from GABAergic neurons (5) and express functional receptors for both ionotropic $GABA_A$ (6-8) and metabotropic $GABA_B$ (9) receptors. All GnRH neurons in mice exhibit $GABA_A$ receptor mediated postsynaptic currents (7, 8). The putative importance of GABA in the afferent control of human GnRH neurons requires clarification.

In addition to GABA, the major excitatory amino acid neurotransmitter L-glutamate is also critically involved in the hypothalamic control of the reproductive axis (2), via regulating the onset of puberty (10) and the pulse (11) and surge (12) modes of GnRH secretion. In laboratory rodents, at least some of the glutamatergic actions are exerted directly on GnRH neurons which express ionotropic receptors for glutamate (6, 13, 14) and exhibit spontaneous excitatory postsynaptic currents that are mostly mediated by AMPA receptors (15, 16). Prior to exocytotic release, glutamate is accumulated into synaptic vesicles by one of the three distinct subtypes of vesicular glutamate transporters (VGLUT1-3), out of which VGLUT2 represents the dominant isoform in the rodent hypothalamus. In rats, glutamatergic fibers expressing VGLUT2 account for most of the glutamatergic innervation of hypothalamic neuroendocrine cells (17, 18). Specifically, glutamatergic neurons of the VGLUT2, but not the VGLUT1, phenotype innervate GnRH cells, with terminals preferentially targeting the dendritic compartment (17). The relative abundances of the

VGLUT1 and VGLUT2 isoforms in the human hypothalamus and their contribution to a putative glutamatergic input to GnRH neurons have not been addressed yet.

In the present study we used dual-label immunohistochemistry to visualize GABAergic afferents and glutamatergic afferents to GnRH neurons of the human hypothalamus. GABAergic terminals were detected with a primary antiserum directed against the vesicular inhibitory amino acid transporter (VIAAT)(19), whereas two distinct subclasses of glutamatergic terminals were detected with VGLUT1 and VGLUT2 antisera, respectively. A quantitative light microscopic analysis was carried out separately in the infundibular (Inf) and paraventricular nuclei (Pa), to determine the relative abundances of GABAergic and glutamatergic neuronal contacts onto GnRH-immunoreactive (IR) cell bodies and dendrites as well as the relative incidences of VGLUT1-IR *vs*. VGLUT2-IR glutamatergic contacts.

Materials and methods

Human subjects

Human hypothalamic samples from eight male individuals (between 30 and 70 years of age) were collected from autopsies at the Forensic Medicine Department of the University of Debrecen with permission from the Regional Committee of Science and Research Ethics of the University of Debrecen (DEOEC RKEB/IKEB: 3183-2010). Selection criteria included sudden causes of death, lack of history of neurological and endocrine disorders. *Post mortem* delay was kept below 36h.

Section preparation

Following dissection, the hypothalamic tissue blocks were rinsed briefly with running tap water and then, immersion-fixed with 4% formaldehyde in 0.1M phosphate buffer saline (PBS; pH 7.4) for 7 days at 4°C. Following fixation, the blocks were trimmed further to include the optic chiasma rostrally, the mammillary bodies caudally and the anterior commissure dorsally (20). Bilateral sagittal cuts were made 2cm lateral from the midline. The blocks were finally bisected and then, infiltrated with 20% sucrose for 5 days at 4°C. The right hemihypothalami were placed in a freezing mold, surrounded with Jung tissue

freezing medium (Leica Microsystems, Nussloch Gmbh, Germany; diluted 1:1 with 0.9% sodium chloride solution), snap-frozen on powdered dry ice, and sectioned coronally at 30µm with a Leica SM 2000R freezing microtome (Leica Microsystems).

Pretreatments

The tissues were permeabilized and endogenous peroxidase activity reduced using a mixture of 0.2% Triton X-100 and 0.5% H_2O_2 in PBS for 30 min. Antigen epitopes were unmasked by antigen retrieval using a 0.1M citrate buffer (pH 6.0) treatment at 80 °C for 30 min.

Immunohistochemical detection of VIAAT-, VGLUT1- or VGLUT2-IR fibers

To detect GABAergic terminals, every 60^{th} section from each block was incubated in polyclonal antisera against VIAAT for 48 h at 4°C. Another two series of sections were used similarly to visualize VGLUT1 and VGLUT2 immunoreactivities, respectively. As described previously (21, 22), the affinity-purified primary antibodies were raised in goats against GST-fusion constructs, which included mouseVIAAT (aa 31-112), mouseVGLUT1 (aa 531-560) and mouseVGLUT2 (aa 559-582) sequences. The antibodies were diluted at 1:2000 in normal horse serum (NHS) and reacted sequentially with biotin-SP-antigoat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:500) and the ABC Elite reagent (Vector Laboratories, Burlingame, CA, USA; 1:1000) for 60 min each. The peroxidase signal was visualized with nickel-intensified diaminobenzidine chromogen and then, post-intensified with silver-gold (23).

Specificity control experiments either used primary antibodies that were preabsorbed with 1µg/ml of the relevant immunization antigen or immunohistochemical procedures from which the primary antibody step was omitted. Positive control experiments used a different set of primary antibodies against GST-fusion constructs of the three vesicular transporters: guinea pig anti-mouseVIAAT (N82; aa 31-112; 1:2000), rabbit anti-mouseVGLUT1 (C30; aa 531-560; 1:2000) and mouse anti-humanVGLUT2 (aa 542-582; #228; 1:8000). As described for the goat primary antisera, these control antibodies were reacted with appropriate biotinylated secondary antibodies (Jackson ImmunoResearch) and then, with the ABC reagent.

Finally, the peroxidase reaction was developed using the silver-gold intensified nickel-diaminobenzidine chromogen.

Detection of GnRH neurons

Following the visualization of amino acidergic fibers with the black silver-gold-intensified nickel-diaminobenzidine chromogen, GnRH immunoreactivity was detected. First, the sections were incubated overnight with a guinea pig primary antiserum against the mammalian form of GnRH (GnRH-I; #1018; 1:5000), followed by biotin-SP-antiguinea pig IgG (Jackson ImmunoResearch Laboratories; 1:500) and the ABC Elite reagent (1:1000). The peroxidase signal was developed with the brown diaminobenzidine chromogen. For characterization and specificity testing of this guinea pig GnRH antiserum, see (24).

Section mounting and coverslipping

The dual-immunolabeled sections were mounted on microscope slides from Elvanol, air-dried, dehydrated with 95% (5 min), followed by 100% (2X5 min) ethanol, cleared with xylene (2X5 min) and coverslipped with DPX mounting medium (Fluka Chemie; Buchs, Switzerland). The microscopic images were scanned with an AxioCam MRc 5 digital camera mounted on a Zeiss AxioImager M1 microscope using the AxioVision 4.6 software (Carl Zeiss, Göttingen, Germany).

Quantitative analysis of axo-somatic and axo-dendritic neuronal contacts

One-to-three sections per double-labeling experiment were selected from each human subject to determine the number of axo-somatic and axo-dendritic contacts on GnRH neurons. To take into account the dendrites and exclude the axons of GnRH neurons, the analysis was restricted to GnRH-IR fibers that exhibited a non-varicose appearance.

The regional density of GABAergic and glutamatergic fibers was highly variable which could cause region-specific differences in the innervation pattern of GnRH neurons. Therefore, the quantitative analysis of contacts was carried out separately in two regions where sufficient numbers of GnRH neurons could be analyzed, the Inf and the Pa. The sections were coded and randomized from the three double-labeling experiments. Counting was carried out using a 63X oil-immersion objective. A contact was defined using

stringent criteria that were applied consistently, i.e. the axon and the GnRH-IR profile had to be in the same focus plane without any visible intervening gap (24-27). For each subject and region, the mean number of contacts per GnRH soma and the mean number of contacts per 10µm GnRH dendrite were determined. Counts obtained from the Inf and the Pa were expressed as the mean of 5-8 individuals, for each of the three types of labeling. The relative abundances of VIAAT/GnRH, VGLUT1/GnRH and VGLUT2/GnRH contacts as well as putative region-dependent variations in the incidences of the different types of input were compared statistically by one-way ANOVA, followed by Newman-Keuls post hoc test.

Double-labeling fluorescent immunohistochemistry

To demonstrate neuronal appositions in confocal images, a set of sections was treated with a mixture of 0.5% H_2O_2 and 0.2% Triton X-100 for 30 min. To reduce tissue autofluorescence caused by neuronal lipofuscin deposits, the sections were pretreated with Sudan black (24). For immunofluorescent labeling, the sections were incubated in the goat anti-VIAAT, anti-VGLUT1 or anti-VGLUT2 antisera (diluted at 1:2000 with 2% NHS in PBS) for 48h at 4°C, then, in biotin-SP-antigoat IgG (Jackson ImmunoResearch Laboratories; 1:500) for 60 min and in ABC Elite reagent (Vector Laboratories; 1:1000) for 60 min. Then, biotinylated tyramide was deposited on peroxidase-containing sites according to the manufacturer's instructions (TSA kit; NEN Life Science Products, Boston, MA). Biotin-tyramide deposits were finally reacted with Cy3-conjugated-streptavidin (Jackson ImmunoResearch; 1:1000) for 60 min. Subsequently, immunoreactivity for GnRH was detected using the guinea pig GnRH antiserum (1:5000; 48h) which was reacted with FITC-conjugated donkey anti-guinea pig IgG (Jackson ImmunoResearch; 1:250; 2h). Photographic illustrations were prepared with a Radiance 2100 confocal microscope (Bio-Rad Laboratories, Hemel Hempstead, UK) using laser excitation lines 488 nm for FITC and 543 nm for Cy3 and dichroic/emission filters 560 nm/500–530 nm for FITC and 560–610 nm for Cy3. To eliminate emission cross-talk, single optical slices were collected in "lambda strobing" mode in a way that only one excitation laser and the corresponding emission detector were active during a line scan. The digital images were processed with the Adobe Photoshop CS software (Adobe Systems, San José, CA, USA) at a

resolution of 300 dpi. Brightness and contrast were adjusted when needed. Neuronal appositions were illustrated on single 0.7µm optical slices.

Results

The immunohistochemical detection of VIAAT, VGLUT1 and VGLUT2 revealed differentially patterned signals which reached varying intensity levels within distinct hypothalamic nuclei (Fig. 1). The punctate appearance of the immunoreactive fibers was characteristic of the subcellular distribution of small clear vesicles within amino acidergic axon terminals. Examples for the varying regional densities of the three types of signal in the infundibular (Inf), ventromedial (VMH), supraoptic (SO) and paraventricular (Pa) nuclei are illustrated in Figure 1. Control experiments showed the absence of labeling if primary antibodies were either omitted from the immunohistochemical procedure or substituted with working dilutions that also contained 1µg/ml of the immunization antigens (Figs. 2D-F). In addition, very similar labeling patterns could be obtained with the use of a different set of primary antisera as positive controls (compare lower to upper panels in Fig. 2), in further support of specificity.

The immunohistochemical detection of GnRH revealed a few cell bodies and dendrites per section that were scattered over large areas in the human hypothalamus. To eliminate area-dependent variations, the comparative analysis of the different types of amino acidergic inputs to GnRH neurons was carried out region-specifically in two distinct hypothalamic nuclei, the Inf and the Pa. The most dense input to GnRH neurons of the Inf was by VIAAT-IR axons. These fibers formed numerous contacts onto the cell bodies and dendrites of GnRH-IR neurons (Fig. 3A). Glutamatergic axons of both the VGLUT1 (Figs. 3C, D) and VGLUT2 (Figs. 3E, F) phenotypes were also juxtaposed to GnRH neurons. Overall, both the VGLUT1-IR and the VGLUT2-IR innervation appeared less heavy, compared with the VIAAT-IR input. Confocal microscopic analysis of dual-immunofluorescent specimens confirmed that GnRH neurons receive VIAAT-

IR and glutamatergic afferent inputs, without visible gaps between the juxtaposed neuronal profiles (Figs. 3B, G).

The quantitative analysis of neuronal contacts onto GnRH cell bodies of the Inf revealed that the mean incidence of axo-somatic contacts (contacts/perikaryon) was 72.4% lower in case of VGLUT1 and 59.8% lower in case of VGLUT2 than the incidence of VIAAT-IR contacts. These differences were statistically significant by one-way ANOVA, followed by Newman-Keuls test (VGLUT1 *vs.* VIAAT: P=0.003; VGLUT2 *vs.* VIAAT: P=0.009; Fig. 4A). The mean incidence of VGLUT2-IR contacts was somewhat higher compared with the mean incidence of VGLUT1-IR appositions, but statistical difference was not detected (P=0.35).

The most frequently encountered phenotype of axo-dendritic appositions in the Inf (No of contacts/10µm GnRH dendrite length) was also established by VIAAT-IR fibers. Although VGLUT1-IR contacts were less frequent by 57.8% and VGLUT2-IR contacts by 33.5% than the VIAAT-IR appositions, their combined incidence on GnRH dendrites exceeded that of the VIAAT-IR axo-dendritic inputs by 8.6% (Fig. 4B). No obvious age-dependence could be revealed in the number of axo-somatic or axo-dendritic contacts with regression analysis.

The relative abundances of different inputs to GnRH neurons of the Pa (Figs. 5A and B) showed identical tendencies to those described for the Inf (Figs. 4A and B). The following statistically significant differences were identified: axo-somatic VGLUT1 *vs.* VIAAT: P=0.0006; axo-somatic VGLUT2 *vs.* VIAAT: P=0.002; axo-dendritic VGLUT1 *vs.* VIAAT: P=0.04.

Comparison of the incidences of axo-somatic and axo-dendritic inputs in the two regions has not revealed any significant regional difference between the innervation patterns of GnRH neurons in the Inf and the Pa.

Discussion

GABAergic regulation of human GnRH neurons

As reviewed recently (28), a large body of evidence mainly obtained from rodents indicates that GABA influences many aspects of GnRH neuronal functions. In its direct actions on GnRH neurons, the dominant effects appear to be mediated by postsynaptic $GABA_A$ receptors which are ligand-gated ion channels composed of five subunits (29). Functional $GABA_A$ receptors have also been detected in GnRH neurons (6, 7). The modulation of $GABA_A$ receptor mediated synaptic transmission to GnRH neurons has been implicated in metabolic (8), sex steroid (30) and circadian (31) signaling to GnRH neurons. A long lasting debate reviewed recently (28) now appears to end with the consensus view that the dominant effect of $GABA_A$ receptor mediated neurotransmission to GnRH neurons is excitatory in mice and rats (7, 32) which is explained by the sustained high intracellular chloride concentration of adult GnRH neurons. In view that in rodents, GnRH neuron activity is increased by both GABA and glutamate, retrograde endocannabinoid signaling may represent an important regulatory mechanism under physiological and pathological conditions whereby GnRH neurons in mice regulate their excitatory GABAergic inputs (33). In addition, GABA can also reduce the excitability of GnRH neurons via metabotropic $GABA_B$ receptors which activate an inwardly rectifying K^+ current (34). It will require clarification to what extent the above electrophysiological observations allow us to conclude about the GABAergic mechanisms of action upon the primate GnRH neuronal system.

It is likely that VGAT-IR afferents innervating human GnRH neurons arise from multiple sources. In the absence of literature about the amino acid phenotype of human hypothalamic nuclei, it is difficult to speculate aboute these resources. The scattered distribution of human GnRH neurons (35) also raises the possibility that these sources are not the same at the different hypothalamic sites. In rodents, a considerable degree of segregation exists between hypothalamic GABAergic and glutamatergic cell groups (expressing glutamic acid decarboxylase and VGLUT2 mRNAs, respectively), as indicated by results of comparative *in situ* hybridization experiments (20). Many GABAergic systems afferent to GnRH neurons may exhibit

an additional peptidergic neurotransmitter/neuromodulator phenotype. Accordingly, peptidergic neurons co-synthesizing neuropeptide Y with agouti-related protein establish symmetrical synapses with murine GnRH neurons which is indicative of GABAergic neurotransmission (25). The abundant innervation of human GnRH neurons by neuropeptide Y-IR fibers (35) may be partly analoguous to this afferent system arising from the rodent arcuate nucleus. In mice, positive estrogen feedback is exerted in the anteroventral periventricular nucleus and neurons in this region partly use GABAergic mechanism for communication with GnRH neurons (36). A subset of GABAergic neurons at this site express kisspeptin mRNA (37) and a subset of kisspeptin-IR synapses on GnRH neurons exhibit symmetric morphology (38), suggesting use of combined GABAergic and peptidergic mechanisms in their communication with the GnRH system. Kisspeptin-immunoreactive neurons also innervate abundantly human GnRH neurons (24, 39), but their amino acid neurotransmitter phenotype is not known. A particularly interesting cell group in the anteroventral periventricular nucleus of the female rat contains glutamatergic as well as GABAergic markers. These GABA/glutamate dual-phenotype cells innervate GnRH neurons and exhibit sexual dimorphism and plastic chemotype changes at the time of the LH surge (40). Finally, we have to note that although VIAAT is a well-established marker for GABAergic cells (19), it also participates in vesicular packaging of glycine. We can not entirely rule out the possibility that some VIAAT-IR fibers we detected in the human hypothalamus are not GABAergic, but rather, ascend to the hypothalamus from a glycinergic cell group of the brainstem (41). However, the existence of a significant glycinergic input to GnRH neurons is unlikely given that, at least in mice, GABA and glutamate together account for the vast majority of fast synaptic currents recorded from GnRH neurons (15, 42).

Glutamatergic innervation of GnRH neurons by VGLUT1 and VGLUT2-immunoreactive axons

There is compelling evidence that the excitatory amino acid neurotransmitter L-glutamate plays a crucial role in the central regulation of reproduction via acting on the GnRH neurosecretory system. Accordingly, intravenous N-methyl-D,L-aspartate infusion can induce precocious puberty in immature rats

(10) and ionotropic glutamate receptor activation has been implicated in both the pulse (11) and the surge (12) modes of GnRH neurosecretion. Glutamate release into the preoptic area is increased during the LH surge (43, 44) and this increase is attenuated during reproductive aging (45). Conversely, inhibition of either the NMDA or the AMPA glutamate receptors is capable of blocking the LH surge (46, 47). Previous immunohistochemical evidence from laboratory rodents indicates that glutamate can regulate GnRH neurons at the level of GnRH cell bodies and dendrites in the preoptic area which receive VGLUT2-IR synapses (17, 18) and exhibit immunoreactivity for ionotropic glutamate receptors (14). In addition to acting postsynaptically, functional evidence indicates that glutamate can inhibit GABA release onto GnRH neurons via Group II and III metabotropic glutamate heteroreceptors that are present on GABAergic synaptic afferents (42).

The current immunohistochemical study provides evidence that GnRH neurons in the human hypothalamus, similarly to rat GnRH neurons (17), receive direct VGLUT2-IR axo-somatic and axo-dendritic inputs. Unlike rat GnRH neurons, GnRH neurons of the human Inf and Pa were also contacted by VGLUT1-IR afferents in our study. The mean incidence of these VGLUT1-IR contacts was only slightly lower compared with that of the VGLUT2-IR juxtapositions. The sources of glutamatergic inputs to GnRH cells are presently unclear. They can be of both hypothalamic and extrahypothalamic origins. The hypothalamus of the rat only contains glutamatergic neurons of the VGLUT2 phenotype (18). Provided that this is also the case in the human, VGLUT2-IR contacts on GnRH neurons can originate from both hypothalamic and extrahypothalamic excitatory neurons, whereas VGLUT1-IR contacts are more likely to arise exclusively from extrahypothalamic sources.

Beyond the large body of evidence to support the role of glutamate in rodent reproduction, there is abundant literature to also indicate that glutamatergic mechanisms are involved in primate puberty onset (48) and GnRH secretion (49, 50). To our knowledge, our present study is the first to use the vesicular glutamate transporters as highly specific glutamatergic markers to analyze direct glutamate/GnRH

interactions in primate hypothalami. Early immunohistochemical work on monkeys with antibodies against glutamate provided evidence for immunoreactive glutamate in axon terminals that establish asymmetrical synapses with GnRH-IR neurons (51). The use of specific antisera against VGLUT1 and VGLUT2 provided us a tool to also distinguish between the two major subclasses of glutamatergic afferents to human GnRH neurons. Our analysis provided light microscopic evidence for VGLUT1-IR and VGLUT2-IR inputs to human GnRH cell bodies, in addition to GnRH dendrites. While the existence of this axo-somatic excitatory input is in accordance with the electron microscopic observation of VGLUT2-IR synapses on GnRH-IR cell bodies in rats (17), it is somewhat in conflict with previous immuno-electronmicroscopic results from Goldsmith and colleagues who found that excitatory inputs only target the dendritic compartment of GnRH neurons in monkeys (51). The different conclusion of these studies may result from potential species differences and/or the use of different immunohistochemical approaches and marker antigens.

In addition to acting on GnRH neurons via afferent regulatory pathways, glutamate may also influence GnRH secretion via autocrine/paracrine mechanisms, as suggested by the presence of VGLUT2 mRNA and immunoreactivity in GnRH neurons of the rat (52). In this rodent species, the endogenous glutamate which is likely released by GnRH neurons into the median eminence may act on the GnRH terminals which exhibit immunoreactivity for the KA2 and NR1 ionotropic glutamate receptor subunits (53), are apposed to glutamatergic axons (18, 53) and respond to glutamate and ionotropic glutamate receptor agonists with a Ca^{2+}-dependent release of GnRH (53). As we reviewed recently (54), glutamate target cells may also include glutamate receptor expressing tanycytes and endothelial cells in the median eminence.

While the primary goal of the present study was to analyze the amino acid neurotransmitters in neuronal afferents to the human GnRH neuronal system, the confocal analysis of dual-immunofluorescent specimens also allowed us to address the presence of VGLUT2 immunoreactivity in GnRH neurosecretory axon terminals targeting the postinfundibular eminence (55). In this study we have found no evidence for

any VGLUT2 signal in GnRH-IR neurosecretory axons. This somewhat unexpected negative finding may suggest a species difference and raises the possibility that GnRH neurons in the human, unlike in the rat (52), do not express the glutamatergic marker VGLUT2. Alternatively, the colocalization of the two signals could have failed because of technical reasons. VGLUT2 expression might be of too low levels in human GnRH neurons to be detected with the immunofluorescent detection method. Electron microscopic studies provided evidence that VGLUT2 is localized to small-clear vesicles in the rat median eminence (56). It might be technically difficult to find GnRH-IR axon segments that co-contain small clear vesicles with VGLUT2 and large dense-core granules with GnRH. It is interesting to note that so far we have not been able to detect VGLUT2 immunoreactivity in GnRH-IR axon terminals of the mouse median eminence either (unpublished observation), despite recent evidence for the VGLUT2 phenotype of mouse GnRH neurons from the VGLUT2-GFP transgenic mouse model (57).

Relative incidences of GABAergic and glutamatergic appositions to human GnRH cells

GnRH neurons in the human hypothalamus are distributed over a large area (35). To obtain an estimate about the relative importance of GABAergic and glutamatergic inputs to GnRH cells, we carried out a quantitative analysis of neuronal contacts at high-power. To eliminate regional variations, we have carried out the analysis of inputs separately in two hypothalamic nuclei, the Inf and the Pa. Although our results indicate that in these two regions the relative incidences of the three types of amino acidergic inputs are highly similar, the possibility exists that the innervation of GnRH neurons is different elsewhere in the human hypothalamus.

Both in the Inf and the Pa, the VIAAT-IR axo-somatic appositions outnumbered the glutamatergic (VGLUT1-IR+VGLUT2-IR) axo-somatic appositions. This GABAergic dominance is in accordance with the electrophysiological observations on mice that $GABA_A$ receptor mediated postsynaptic currents (PSCs)

are present in all GnRH neurons (8), whereas glutamatergic excitatory PSCs are less abundant and only detectable in 20-35% of the GnRH cell bodies (15).

The VIAAT-IR GABAergic appositions also represented the most frequently encountered type of axo-dendritic contact. However, the combined incidence of VGLUT1-IR+VGLUT2-IR inputs on the dendritic compartment somewhat exceeded that of the VIAAT-IR inputs (by 8.6% in the Inf and by 26.3% in the Pa). This glutamatergic dominance on GnRH dendrites is in accordance with the general tendency of glutamatergic inputs to target dendrites and also with the specific observation on rats that VGLUT2-IR axons preferentially innervate the dendritic compartment of GnRH neurons (17). Although excitatory PSCs generated by these dendritic inputs might be undetectable in GnRH cell bodies using whole-cell patch-clamp electrophysiology (15), their physiological importance may still be crucial considering that most of the action potentials, at least in mice, appear to originate from the dendritic compartment of GnRH neurons (58).

Technical considerations

Some of the technical limitations of the quantitative analysis we used in the present study should be mentioned. First, recent three-dimensional reconstruction of biocytin-filled mouse GnRH neurons has provided evidence that the dendrites of GnRH neurons are much longer and their arborization richer than previously assumed from their immunohistochemical image (59). Therefore, it is important to emphasize that we had to restrict the quantitative analysis of inputs to the GnRH-IR dendritic segments that are relatively thick and close to GnRH cell bodies.

Second, the approach of using the high-power light microscopic analysis of neuronal contacts, even with a shallow depth of field, has a somewhat limited capability to determine the absolute number of glutamatergic and GABAergic afferent inputs to GnRH neurons. Some appositions on top and below the GnRH neurons might remain undetected, causing false negatives. On the other hand, many light

microscopic contacts might be devoid of synaptic specializations at the electron microscopic level, which would cause false positive counts in the quantification. Even with these limitations, we argue that such quantitative studies are capable of providing an estimate of the relative ratios of VIAAT-IR, VGLUT1-IR and VGLUT2-IR inputs if the analysis relies on the use of randomized samples and consistent judgements by an experienced investigator who is blind to the applied immunohistochemical procedures. Clear trends and statistically significant differences in our quantitative results, as well as earlier quantitative studies using successfully a similar approach (24-27), confirm the feasibility and value of such analyses.

In summary, in this study we show that GABAergic axons expressing VIAAT immunoreactivity and glutamatergic axons of both the VGLUT1 and VGLUT2 phenotypes abundantly innervate both the somatic and dendritic compartments of human GnRH neurons. We report the dominance of GABAergic over glutamatergic inputs to GnRH-IR somata in the Inf as well as the Pa. This finding is in accordance with published observations on mouse GnRH neurons about the dominance of GABAergic over glutamatergic miniature postsynaptic currents. As opposed to the somatic compartment of GnRH neurons, the dendrites received somewhat more glutamatergic (VGLUT1+VGLUT2) than GABAergic inputs. This excitatory afferentation may have an important contribution to the generation of action potentials which, at least in mice (58), tend to originate from the dendritic compartment of GnRH neurons.

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Legends

Figure 1. Identification of GABAergic and glutamatergic fibers in different hypothalamic nuclei. Most hypothalamic sites receive GABAergic innervation (immunoreactive to VIAAT; **A, D, G, J**) as well as glutamatergic innervation of both the VGLUT1 (**B**, **E**, **H**, **K**) and VGLUT2 (**C**, **F**, **I**, **L**) phenotypes. Note that the fine punctate appearance of the immunohistochemical signals (silver-gold intensified Ni-DAB chromogen) is in accordance with the accumulation of the vesicular neurotransmitter transporters in small synaptic vesicles within amino acidergic axon terminals. The differential distribution of the three types of fibers in distinct anatomical regions is illustrated from the hypothalamic infundibular (Inf; **A-C**), ventromedial (VMH; **D-F**), supraoptic (SO; **G-I**) and paraventricular (Pa; **J-L**) nuclei. Scale bar=50μm.

Figure 2. Results of specificity testing for the goat VIAAT, VGLUT1 and VGLUT2 antisera

In preabsorption experiments, 1:2000 working dilutions of the goat VIAAT, VGLUT1 and VGLUT2 antisera were preincubated overnight with 1µg/ml of the fusion proteins used to generate the antisera. Test sections with (**D-F**) and without (**A-C**) preabsorption were processed in parallel. Note the complete abolishment of immunohistochemical labeling using the preabsorbed primary antibodies (**D-F**) in representative test sections of the infundibular (Inf) and ventromedial (VMH) hypothalamic nuclei. Additional test experiments used three different polyclonal antibodies on neighboring sections as positive controls. The punctate immunolabeling obtained with the guinea pig anti-mouseVIAAT (N82; **G**), rabbit anti-mouseVGLUT1 (C30; **H**) and mouse anti-humanVGLUT2 (#228; **I**) antibodies are highly reminiscent to those obtained with the goat polyclonal antibodies (**A-C**). Scale bar=100µm.

Figure 3. Demonstration of GABAergic and glutamatergic inputs to GnRH-IR neurons of the infundibular nucleus.

Arrows in dual-immunohistochemical (**A, C-F**) and dual-immunofluorescent (**B, G**) images illustrate the axo-somatic and axo-dendritic contacts of VIAAT-IR GABAergic (**A, B**) and the VGLUT1-IR (**C, D**) and VGLUT2-IR (**E-G**) subclassess of glutamatergic axons to GnRH neurons of the infundibular nucleus (Inf). Note that the most dense innervation to GnRH perikarya is provided by GABAergic fibers, whereas glutamatergic fibers of both the VGLUT1 and VGLUT2 phenotypes also contribute substantially. Note that the dendrites of GnRH-IR neurons in lower part of panel D can be readily distinguished form GnRH-IR axons (upper part of panel D), the latter exhibiting numerous varicosities (arrowheads) interconnected by thin intervaricose axon segments. The dendrites of GnRH neurons receive GABAergic and glutamatergic (combined VGLUT1 and VGLUT2) inputs in similar numbers. For quantitative analysis of the three types of input in the infundibular and paraventricular nuclei, see Figures 4 and 5, respectively. A confocal image of the infundibular stalk (InfS) from dual-immunofluorescent specimens (**H**) illustrates the

segregation between GnRH-IR (green puncta) and VGLUT2-IR (red puncta) fibers around the putative portal blood vessels (BV). Note the conspicuous accumulation of VGLUT2-IR fibers around the superficial network of portal capillaries (55). Unlike observed previously in the median eminence of rats (52), GnRH-IR fibers appear to be devoid of any VGLUT2 labeling in the 0.7μm optical slice (**I**). Scale bar=20μm in **A-G**, **J** and 300μm in **H**.

Figure 4. Relative incidences of GABAergic (VIAAT) and glutamatergic (of the VGLUT1 and VGLUT2 phenotypes) neuronal appositions to the cell bodies and dendrites of GnRH neurons in the infundibular nucleus.

High-power light microscopic analysis of dual-immunolabeled sections was used to determine the relative incidences of GABAergic and glutamatergic contacts onto the somata (**A**) and dendrites (**B**) of GnRH-IR neurons in the infundibular nucleus (Inf). The counts were obtained from 1-3 sections per subject and expressed as the mean of the 5-8 individuals ± SEM. Quantitative analysis of axo-somatic contacts established that the main input to the cell bodies of GnRH neurons is provided by VIAAT-IR GABAergic axons (**A**). Glutamatergic axons of both the VGLUT1 and VGLUT2 phenotypes also innervate GnRH-IR perikarya, although the mean incidences of these contacts are significantly lower and only reach 27.6% and 40.2%, respectively, of the incidence of VIAAT-IR contacts (**P<0.01 by ANOVA, followed by Newman-Keuls). The relative incidence of the three types of contacts on GnRH-IR dendrites (expressed as the mean number of contacts/10μm dendrite ± SEM of 5-8 individuals; **B**) exhibits a similar trend, but glutamatergic inputs here have higher relative contributions (VGLUT1-IR inputs representing 42.2% and VGLUT2-IR inputs representing 66.5% of the VIAAT-IR contacts). The combined contribution of VGLUT1-IR and VGLUT2-IR inputs exceeds that of VIAAT-IR inputs by 8.6%.

Figure 5. Relative incidences of GABAergic and glutamatergic appositions to the cell bodies and dendrites of GnRH neurons in the paraventricular nucleus.

High-power light microscopic analysis of neuronal contacts was also carried out in the paraventricular nucleus (Pa) to see if the innervation pattern is different in this region. The relative incidences of GABAergic and glutamatergic appositions to the somata (**A**) and dendrites (**B**) of GnRH-IR neurons show similar tendencies to those observed in the infundibular nucleus (Figure 4). Quantitative analysis of axo-somatic contacts established that the main input to the cell bodies of GnRH neurons is provided by VIAAT-IR GABAergic axons (**A**). Glutamatergic axons of both the VGLUT1 and VGLUT2 phenotypes also innervate GnRH-IR perikarya, although the mean incidence of their afferent contacts are significantly lower (29.8% and 46.6%, respectively, of the incidence of VIAAT-IR contacts; *$P<0.05$ and **$P<0.01$ by ANOVA, followed by Newman-Keuls. The incidence of the three types of contacts on GnRH-IR dendrites (expressed as the mean number of contacts/10µm dendrite ± SEM of 5-8 individuals; **B**) changes similarly, but glutamatergic inputs here have higher relative contributions (VGLUT1 input: 57.0% of VIAAT-IR contacts; VGLUT2 input: 69.3% of VIAAT-IR contacts). The combined contribution of VGLUT1-IR and VGLUT2-IR inputs exceeds that of VIAAT-IR inputs by 26.3%.

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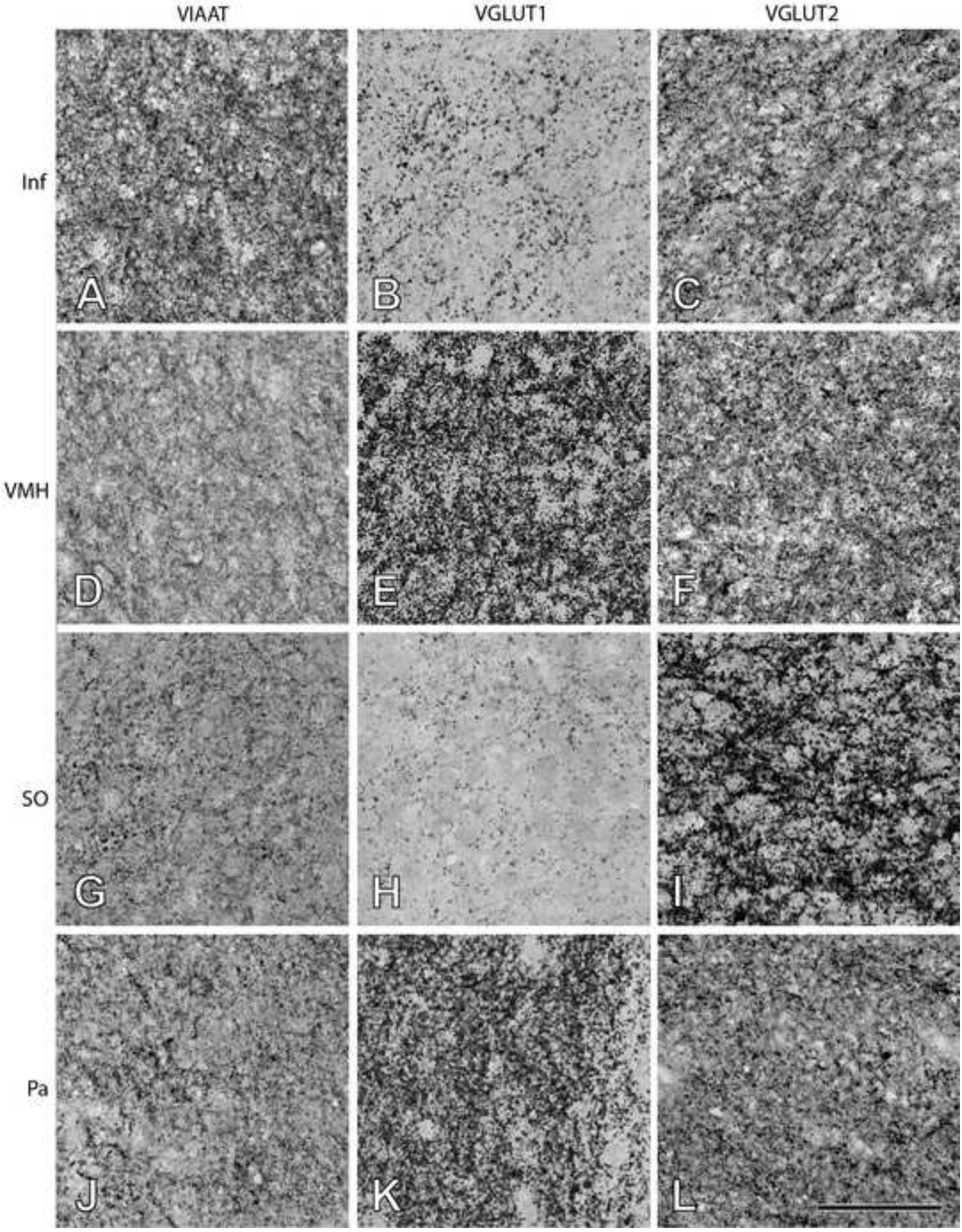

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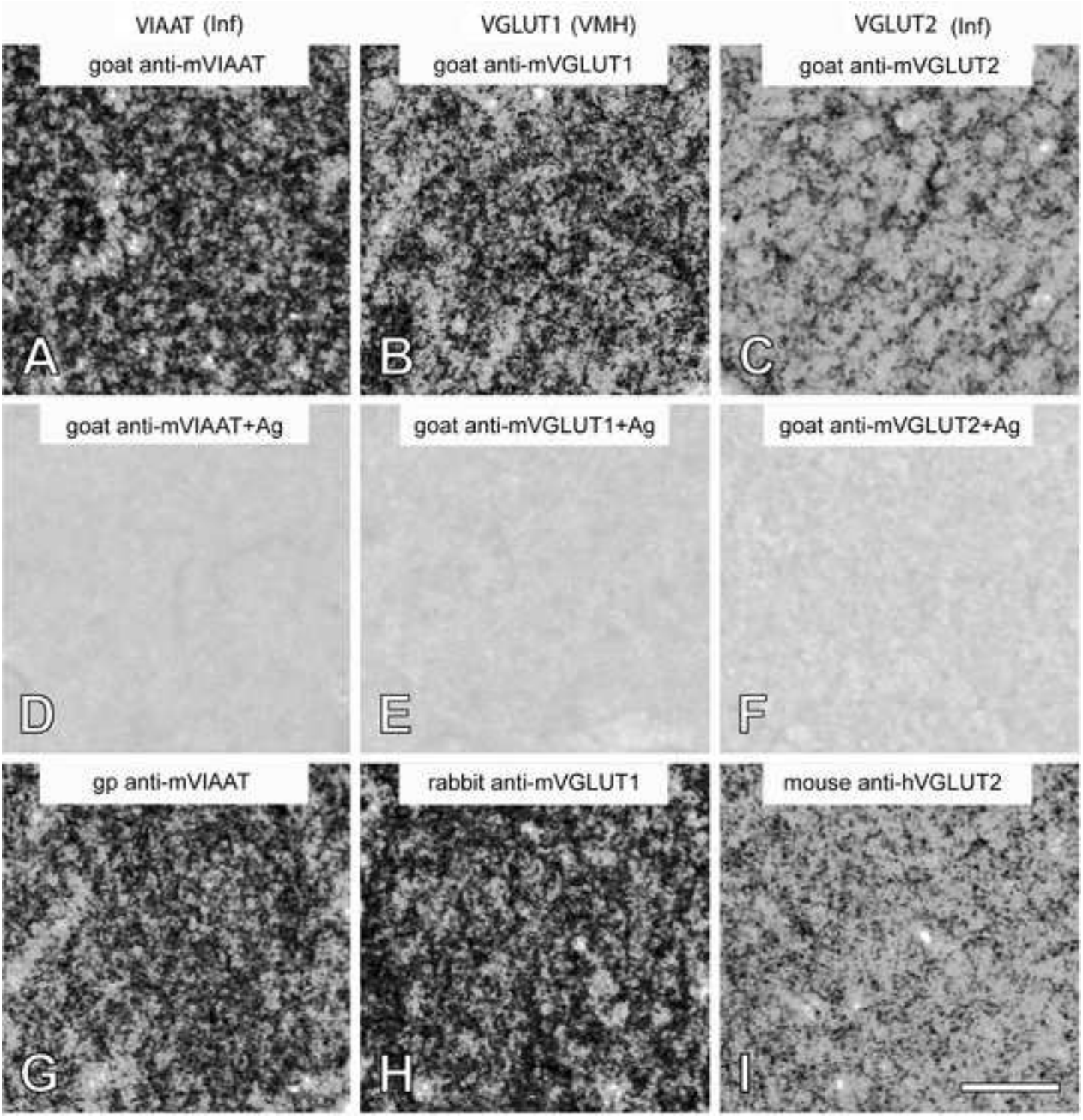

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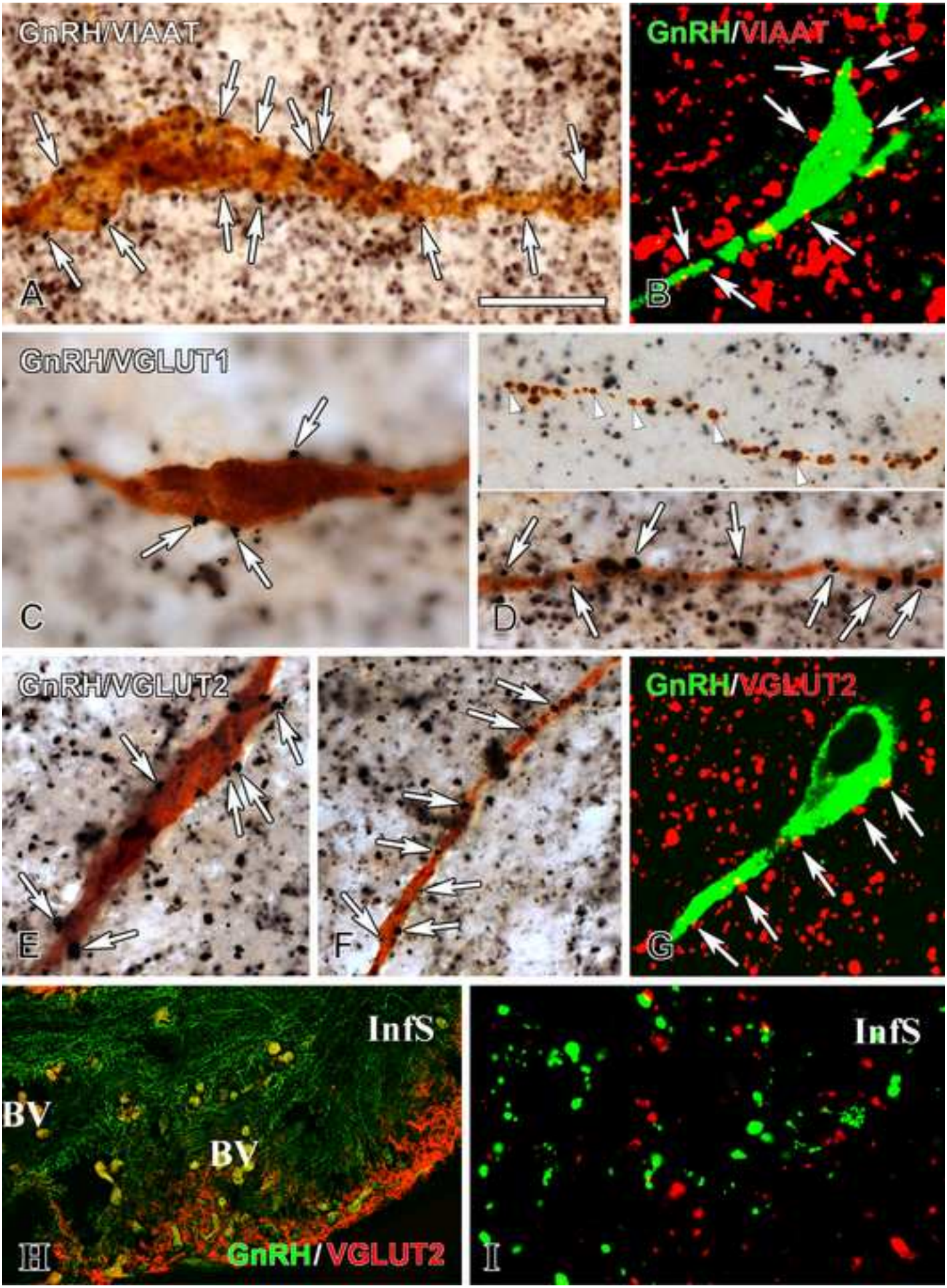

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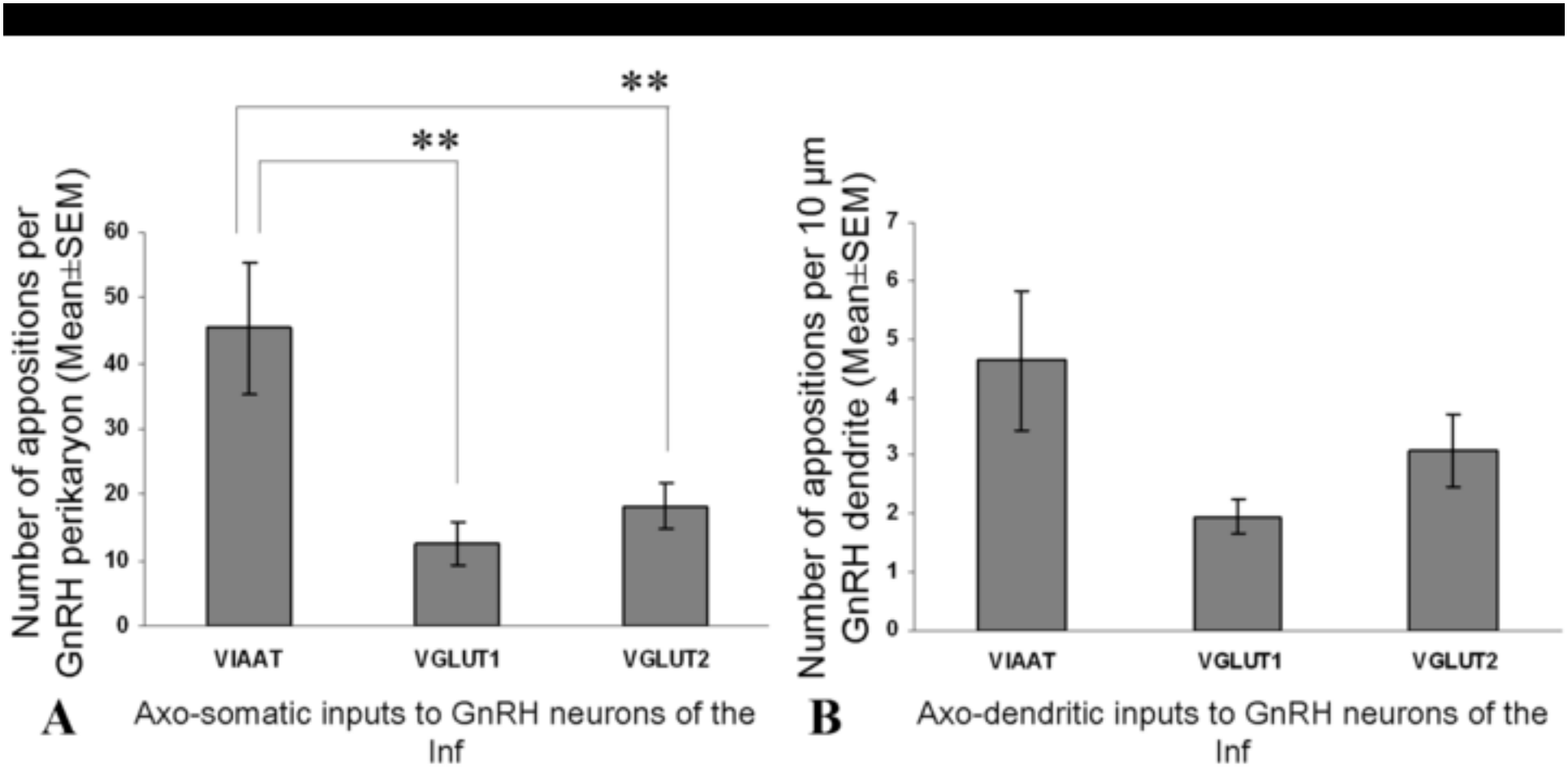

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