

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

3 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.

67 **Introduction**

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

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

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

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

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

101

102 **Materials and methods**

103 *Human subjects*

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

109 *Section preparation*

110 Following dissection, the hypothalamic tissue blocks were rinsed briefly with running tap water and
111 then, immersion-fixed with 4% formaldehyde in 0.1M phosphate buffer saline (PBS; pH 7.4) for 7 days at
112 4°C. Following fixation, the blocks were trimmed further to include the optic chiasma rostrally, the
113 mammillary bodies caudally and the anterior commissure dorsally (20). Bilateral sagittal cuts were made
114 2cm lateral from the midline. The blocks were finally bisected and then, infiltrated with 20% sucrose for 5
115 days at 4°C. The right hemihypothalami were placed in a freezing mold, surrounded with Jung tissue

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

119 *Pretreatments*

120 The tissues were permeabilized and endogenous peroxidase activity reduced using a mixture of 0.2%
121 Triton X-100 and 0.5% H₂O₂ in PBS for 30 min. Antigen epitopes were unmasked by antigen retrieval
122 using a 0.1M citrate buffer (pH 6.0) treatment at 80 °C for 30 min.

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

124 To detect GABAergic terminals, every 60th section from each block was incubated in polyclonal antisera
125 against VIAAT for 48 h at 4°C. Another two series of sections were used similarly to visualize VGLUT1
126 and VGLUT2 immunoreactivities, respectively. As described previously (21, 22), the affinity-purified
127 primary antibodies were raised in goats against GST-fusion constructs, which included mouseVIAAT (aa
128 31-112), mouseVGLUT1 (aa 531-560) and mouseVGLUT2 (aa 559-582) sequences. The antibodies were
129 diluted at 1:2000 in normal horse serum (NHS) and reacted sequentially with biotin-SP-antigoat IgG
130 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:500) and the ABC Elite reagent (Vector
131 Laboratories, Burlingame, CA, USA; 1:1000) for 60 min each. The peroxidase signal was visualized with
132 nickel-intensified diaminobenzidine chromogen and then, post-intensified with silver-gold (23).

133 Specificity control experiments either used primary antibodies that were preabsorbed with 1 μ g/ml of the
134 relevant immunization antigen or immunohistochemical procedures from which the primary antibody step
135 was omitted. Positive control experiments used a different set of primary antibodies against GST-fusion
136 constructs of the three vesicular transporters: guinea pig anti-mouseVIAAT (N82; aa 31-112; 1:2000),
137 rabbit anti-mouseVGLUT1 (C30; aa 531-560; 1:2000) and mouse anti-humanVGLUT2 (aa 542-582;
138 #228; 1:8000). As described for the goat primary antisera, these control antibodies were reacted with
139 appropriate biotinylated secondary antibodies (Jackson ImmunoResearch) and then, with the ABC reagent.

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

142 *Detection of GnRH neurons*

143 Following the visualization of amino acidergic fibers with the black silver-gold-intensified nickel-
144 diaminobenzidine chromogen, GnRH immunoreactivity was detected. First, the sections were incubated
145 overnight with a guinea pig primary antiserum against the mammalian form of GnRH (GnRH-I; #1018;
146 1:5000), followed by biotin-SP-antiguinea pig IgG (Jackson ImmunoResearch Laboratories; 1:500) and the
147 ABC Elite reagent (1:1000). The peroxidase signal was developed with the brown diaminobenzidine
148 chromogen. For characterization and specificity testing of this guinea pig GnRH antiserum, see (24).

149 *Section mounting and coverslipping*

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

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

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

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

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

172 *Double-labeling fluorescent immunohistochemistry*

173 To demonstrate neuronal appositions in confocal images, a set of sections was treated with a mixture of
174 0.5% H₂O₂ and 0.2% Triton X-100 for 30 min. To reduce tissue autofluorescence caused by neuronal
175 lipofuscin deposits, the sections were pretreated with Sudan black (24). For immunofluorescent labeling,
176 the sections were incubated in the goat anti-VIAAT, anti-VGLUT1 or anti-VGLUT2 antisera (diluted at
177 1:2000 with 2% NHS in PBS) for 48h at 4°C, then, in biotin-SP-antigoat IgG (Jackson ImmunoResearch
178 Laboratories; 1:500) for 60 min and in ABC Elite reagent (Vector Laboratories; 1:1000) for 60 min. Then,
179 biotinylated tyramide was deposited on peroxidase-containing sites according to the manufacturer's
180 instructions (TSA kit; NEN Life Science Products, Boston, MA). Biotin-tyramide deposits were finally
181 reacted with Cy3-conjugated-streptavidin (Jackson ImmunoResearch; 1:1000) for 60 min. Subsequently,
182 immunoreactivity for GnRH was detected using the guinea pig GnRH antiserum (1:5000; 48h) which was
183 reacted with FITC-conjugated donkey anti-guinea pig IgG (Jackson ImmunoResearch; 1:250; 2h).
184 Photographic illustrations were prepared with a Radiance 2100 confocal microscope (Bio-Rad
185 Laboratories, Hemel Hempstead, UK) using laser excitation lines 488 nm for FITC and 543 nm for Cy3
186 and dichroic/emission filters 560 nm/500–530 nm for FITC and 560–610 nm for Cy3. To eliminate
187 emission cross-talk, single optical slices were collected in “lambda strobing” mode in a way that only one
188 excitation laser and the corresponding emission detector were active during a line scan. The digital images
189 were processed with the Adobe Photoshop CS software (Adobe Systems, San José, CA, USA) at a

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

192

193 **Results**

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

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

213 IR and glutamatergic afferent inputs, without visible gaps between the juxtaposed neuronal profiles (Figs.
214 3B, G).

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

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

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

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

235

236 **Discussion**

237 **GABAergic regulation of human GnRH neurons**

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

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

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

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

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

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

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

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

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

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

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

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

344 **Relative incidences of GABAergic and glutamatergic appositions to human GnRH cells**

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

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

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

357 The VIAAT-IR GABAergic appositions also represented the most frequently encountered type of axo-
358 dendritic contact. However, the combined incidence of VGLUT1-IR+VGLUT2-IR inputs on the dendritic
359 compartment somewhat exceeded that of the VIAAT-IR inputs (by 8.6% in the Inf and by 26.3% in the
360 Pa). This glutamatergic dominance on GnRH dendrites is in accordance with the general tendency of
361 glutamatergic inputs to target dendrites and also with the specific observation on rats that VGLUT2-IR
362 axons preferentially innervate the dendritic compartment of GnRH neurons (17). Although excitatory PSCs
363 generated by these dendritic inputs might be undetectable in GnRH cell bodies using whole-cell patch-
364 clamp electrophysiology (15), their physiological importance may still be crucial considering that most of
365 the action potentials, at least in mice, appear to originate from the dendritic compartment of GnRH neurons
366 (58).

367 **Technical considerations**

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

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

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

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

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399

400

401 **Legends**

402

403 **Figure 1. Identification of GABAergic and glutamatergic fibers in different hypothalamic nuclei.**404 Most hypothalamic sites receive GABAergic innervation (immunoreactive to VIAAT; **A, D, G, J**) as well405 as glutamatergic innervation of both the VGLUT1 (**B, E, H, K**) and VGLUT2 (**C, F, I, L**) phenotypes.

406 Note that the fine punctate appearance of the immunohistochemical signals (silver-gold intensified Ni-DAB

407 chromogen) is in accordance with the accumulation of the vesicular neurotransmitter transporters in small

408 synaptic vesicles within amino acidergic axon terminals. The differential distribution of the three types of

409 fibers in distinct anatomical regions is illustrated from the hypothalamic infundibular (Inf; **A-C**),410 ventromedial (VMH; **D-F**), supraoptic (SO; **G-I**) and paraventricular (Pa; **J-L**) nuclei. Scale bar=50µm.

411

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

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

422

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

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

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

441

442

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

446 High-power light microscopic analysis of dual-immunolabeled sections was used to determine the relative
447 incidences of GABAergic and glutamatergic contacts onto the somata (**A**) and dendrites (**B**) of GnRH-IR
448 neurons in the infundibular nucleus (Inf). The counts were obtained from 1-3 sections per subject and
449 expressed as the mean of the 5-8 individuals \pm SEM. Quantitative analysis of axo-somatic contacts
450 established that the main input to the cell bodies of GnRH neurons is provided by VIAAT-IR GABAergic
451 axons (**A**). Glutamatergic axons of both the VGLUT1 and VGLUT2 phenotypes also innervate GnRH-IR
452 perikarya, although the mean incidences of these contacts are significantly lower and only reach 27.6% and
453 40.2%, respectively, of the incidence of VIAAT-IR contacts (** $P < 0.01$ by ANOVA, followed by Newman-
454 Keuls). The relative incidence of the three types of contacts on GnRH-IR dendrites (expressed as the mean
455 number of contacts/10 μ m dendrite \pm SEM of 5-8 individuals; **B**) exhibits a similar trend, but glutamatergic
456 inputs here have higher relative contributions (VGLUT1-IR inputs representing 42.2% and VGLUT2-IR
457 inputs representing 66.5% of the VIAAT-IR contacts). The combined contribution of VGLUT1-IR and
458 VGLUT2-IR inputs exceeds that of VIAAT-IR inputs by 8.6%.

459

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

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

475

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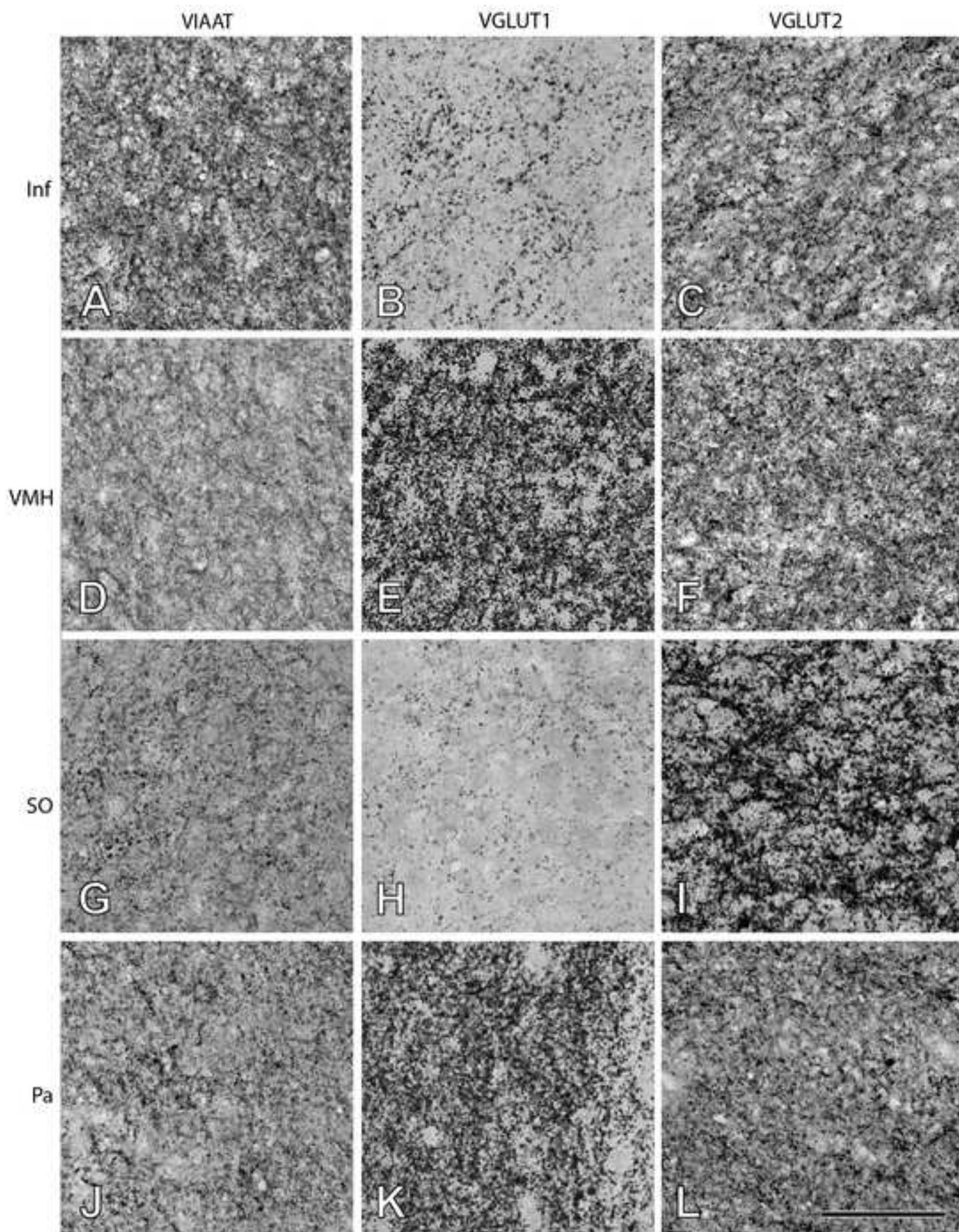


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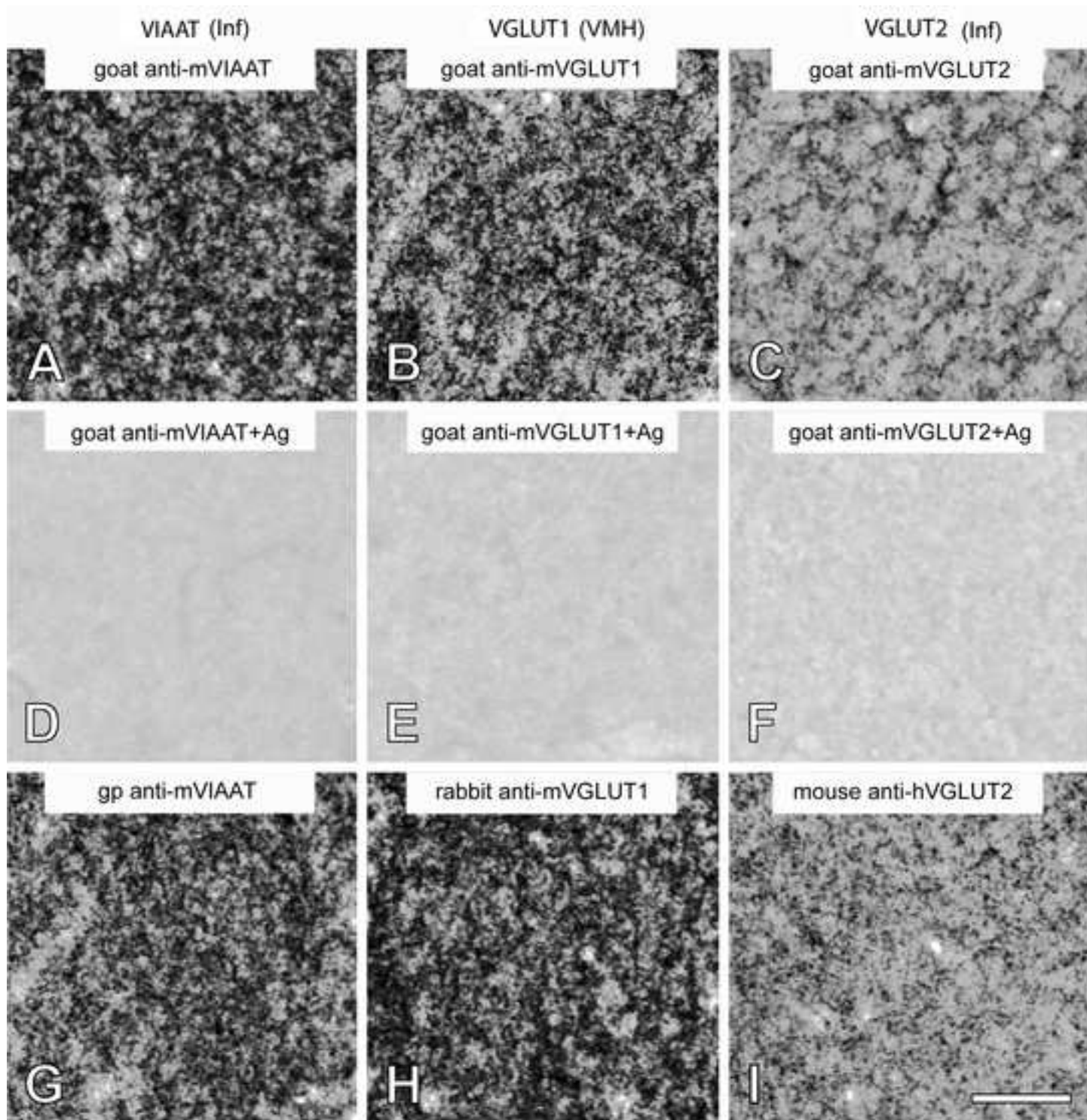
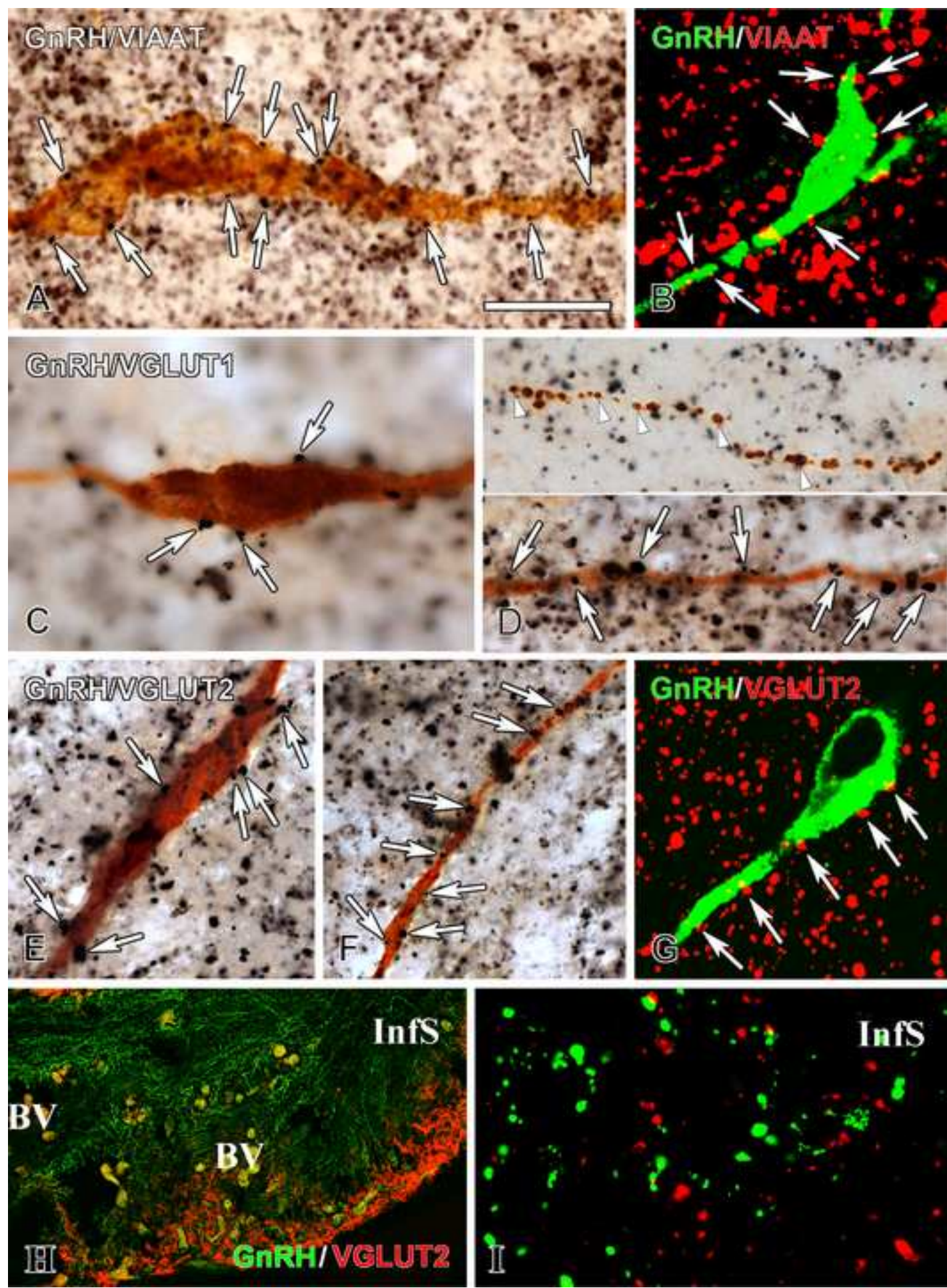


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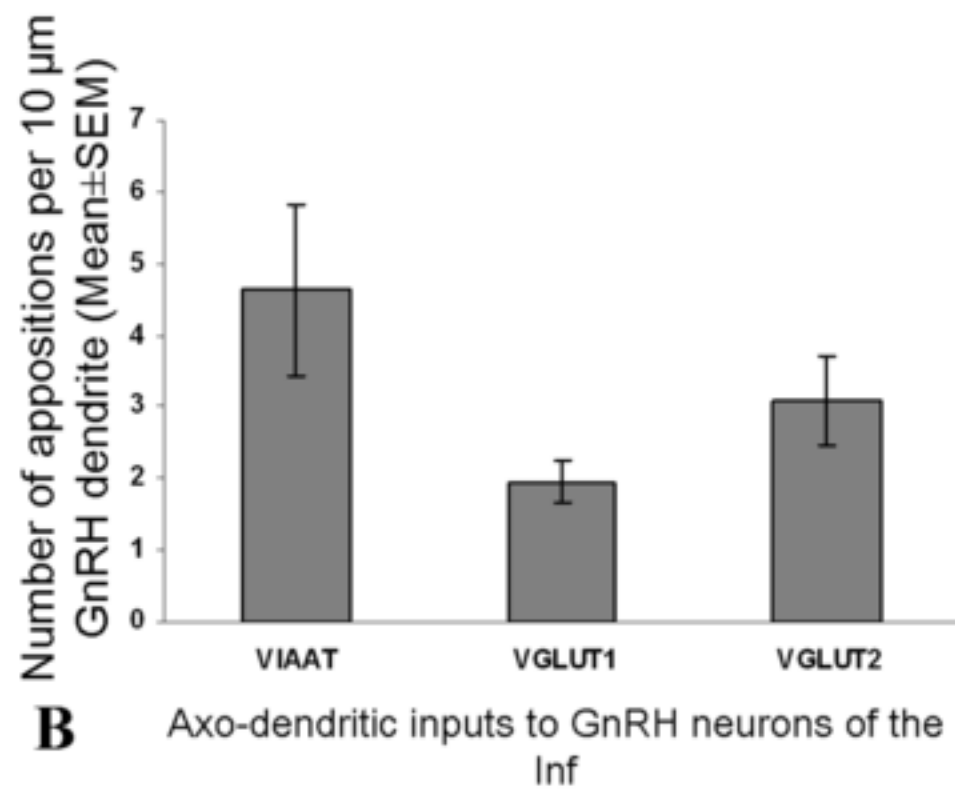
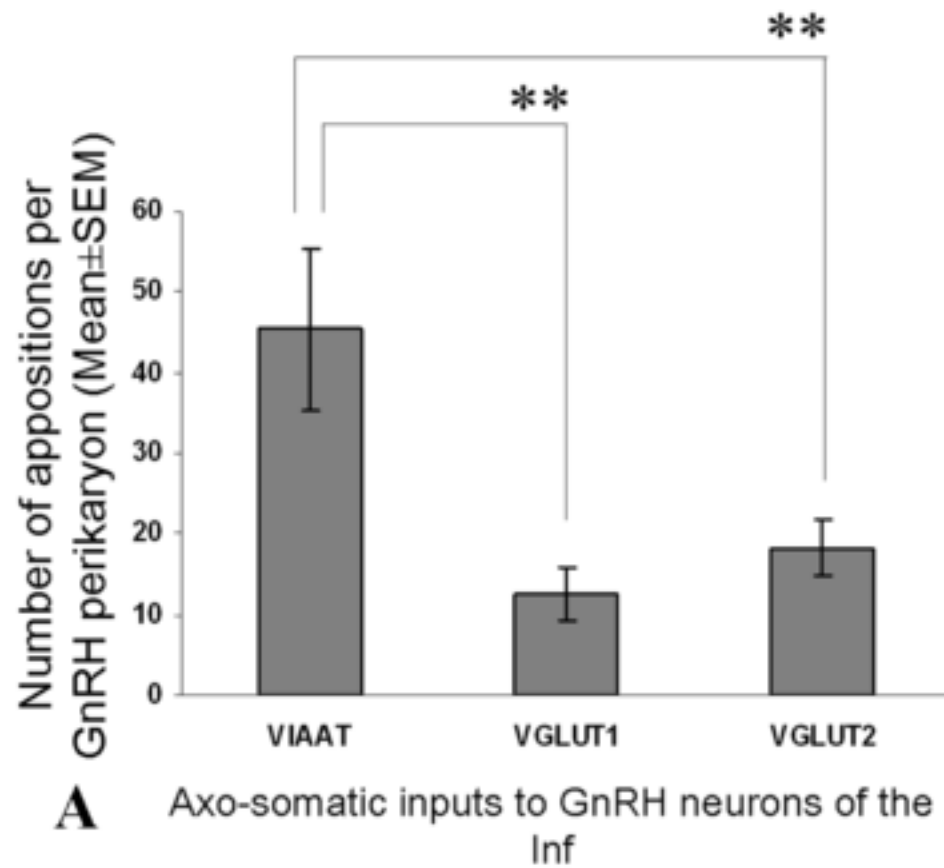


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