

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

4 ERIK HRABOVSKY¹, CSILLA S. MOLNÁR¹, ROBERT NAGY¹, BARBARA VIDA¹, BEÁTA Á.

5 BORSAY², KÁLMÁN RÁCZ², LÁSZLÓ HERCZEG², MASAHIKO WATANABE³, IMRE KALLÓ^{1,4},

6 ZSOLT LIPOSITS^{1,4}

7 ¹Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine, Hungarian Academy of
8 Sciences, Budapest, 1083 Hungary;

9 ²Department of Forensic Medicine, Faculty of Medicine of the University of Debrecen, Debrecen, 4012
10 Hungary;

11 ³Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Japan;

12 ⁴Department of Neuroscience, Faculty of Information Technology, Pázmány Péter Catholic University,
13 Budapest, 1083 Hungary

14
15 Corresponding authors:

16 **Erik Hrabovszky, MD, PhD, DSc**

17 Department of Endocrine Neurobiology

18 Institute of Experimental Medicine

19 Hungarian Academy of Sciences

20 43 Szigony St.

21 Budapest, 1083 Hungary

22 Phone: 36-1-2109400, ext.: 366

23 Fax: 36-1-2109944

24 E-mail: hrabovszky.erik@koki.hu

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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.

68 **Introduction**

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

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

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

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

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

102

103 **Materials and methods**

104 *Human subjects*

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

110 *Section preparation*

111 Following dissection, the hypothalamic tissue blocks were rinsed briefly with running tap water and
112 then, immersion-fixed with 4% formaldehyde in 0.1M phosphate buffer saline (PBS; pH 7.4) for 7 days
113 at 4°C. Following fixation, the blocks were trimmed further to include the optic chiasma rostrally, the
114 mammillary bodies caudally and the anterior commissure dorsally (20). Bilateral sagittal cuts were made
115 2cm lateral from the midline. The blocks were finally bisected and then, infiltrated with 20% sucrose for
116 5 days at 4°C. The right hemihypothalami were placed in a freezing mold, surrounded with Jung tissue

117 freezing medium (Leica Microsystems, Nussloch GmbH, Germany; diluted 1:1 with 0.9% sodium
118 chloride solution), snap-frozen on powdered dry ice, and sectioned coronally at 30µm with a Leica SM
119 2000R freezing microtome (Leica Microsystems).

120 *Pretreatments*

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

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

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

135 Specificity control experiments either used primary antibodies that were preabsorbed with 1µg/ml of
136 the relevant immunization antigen or immunohistochemical procedures from which the primary antibody
137 step was omitted. Positive control experiments used a different set of primary antibodies against GST-
138 fusion constructs of the three vesicular transporters: guinea pig anti-mouseVIAAT (N82; aa 31-112;
139 1:2000), rabbit anti-mouseVGLUT1 (C30; aa 531-560; 1:2000) and mouse anti-humanVGLUT2 (aa 542-
140 582; #228; 1:8000). As described for the goat primary antisera, these control antibodies were reacted
141 with appropriate biotinylated secondary antibodies (Jackson ImmunoResearch) and then, with the ABC

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

144 *Detection of GnRH neurons*

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

151 *Section mounting and coverslipping*

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

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

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

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

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

175 *Double-labeling fluorescent immunohistochemistry*

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

192 scan. The digital images were processed with the Adobe Photoshop CS software (Adobe Systems, San
193 José, CA, USA) at a resolution of 300 dpi. Brightness and contrast were adjusted when needed. Neuronal
194 appositions were illustrated on single 0.7 μ m optical slices.

195

196 **Results**

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

207 The immunohistochemical detection of GnRH revealed a few cell bodies and dendrites per section
208 that were scattered over large areas in the human hypothalamus. To eliminate area-dependent variations,
209 the comparative analysis of the different types of amino acidergic inputs to GnRH neurons was carried
210 out region-specifically in two distinct hypothalamic nuclei, the Inf and the Pa. The most dense input to
211 GnRH neurons of the Inf was by VIAAT-IR axons. These fibers formed numerous contacts onto the cell
212 bodies and dendrites of GnRH-IR neurons (Fig. 3A). Glutamatergic axons of both the VGLUT1 (Figs.
213 3C, D) and VGLUT2 (Figs. 3E, F) phenotypes were also juxtaposed to GnRH neurons. Overall, both the
214 VGLUT1-IR and the VGLUT2-IR innervation appeared less heavy, compared with the VIAAT-IR input.
215 Confocal microscopic analysis of dual-immunofluorescent specimens confirmed that GnRH neurons

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

218 The quantitative analysis of neuronal contacts onto GnRH cell bodies of the Inf revealed that the
219 mean incidence of axo-somatic contacts (contacts/perikaryon) was 72.4% lower in case of VGLUT1 and
220 59.8% lower in case of VGLUT2 than the incidence of VIAAT-IR contacts. These differences were
221 statistically significant by one-way ANOVA, followed by Newman-Keuls test (VGLUT1 *vs.* VIAAT:
222 $P=0.003$; VGLUT2 *vs.* VIAAT: $P=0.009$; Fig. 4A). The mean incidence of VGLUT2-IR contacts was
223 somewhat higher compared with the mean incidence of VGLUT1-IR appositions, but statistical
224 difference was not detected ($P=0.35$).

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

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

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

238

239 **Discussion**

240 **GABAergic regulation of human GnRH neurons**

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

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

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

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

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

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

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

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

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

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

334 While the primary goal of the present study was to analyze the amino acid neurotransmitters in
335 neuronal afferents to the human GnRH neuronal system, the confocal analysis of dual-
336 immunofluorescent specimens also allowed us to address the presence of VGLUT2 immunoreactivity in

337 GnRH neurosecretory axon terminals targeting the postinfundibular eminence (55). In this study we have
338 found no evidence for any VGLUT2 signal in GnRH-IR neurosecretory axons. This somewhat
339 unexpected negative finding may suggest a species difference and raises the possibility that GnRH
340 neurons in the human, unlike in the rat (52), do not express the glutamatergic marker VGLUT2.
341 Alternatively, the colocalization of the two signals could have failed because of technical reasons.
342 VGLUT2 expression might be of too low levels in human GnRH neurons to be detected with the
343 immunofluorescent detection method. Electron microscopic studies provided evidence that VGLUT2 is
344 localized to small-clear vesicles in the rat median eminence (56). It might be technically difficult to find
345 GnRH-IR axon segments that co-contain small clear vesicles with VGLUT2 and large dense-core
346 granules with GnRH. It is interesting to note that so far we have not been able to detect VGLUT2
347 immunoreactivity in GnRH-IR axon terminals of the mouse median eminence either (unpublished
348 observation), despite recent evidence for the VGLUT2 phenotype of mouse GnRH neurons from the
349 VGLUT2-GFP transgenic mouse model (57).

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

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

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

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

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

373 **Technical considerations**

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

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

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

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

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407

408

409 **Legends**

410

411 **Figure 1. Identification of GABAergic and glutamatergic fibers in different hypothalamic nuclei.**412 Most hypothalamic sites receive GABAergic innervation (immunoreactive to VIAAT; **A, D, G, J**) as well413 as glutamatergic innervation of both the VGLUT1 (**B, E, H, K**) and VGLUT2 (**C, F, I, L**) phenotypes.

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

415 DAB chromogen) is in accordance with the accumulation of the vesicular neurotransmitter transporters in

416 small synaptic vesicles within amino acidergic axon terminals. The differential distribution of the three

417 types of fibers in distinct anatomical regions is illustrated from the hypothalamic infundibular (Inf; **A-C**),418 ventromedial (VMH; **D-F**), supraoptic (SO; **G-I**) and paraventricular (Pa; **J-L**) nuclei. Scale bar=50µm.

419

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

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

430

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

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

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

449

450

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

454 High-power light microscopic analysis of dual-immunolabeled sections was used to determine the
455 relative incidences of GABAergic and glutamatergic contacts onto the somata (**A**) and dendrites (**B**) of
456 GnRH-IR neurons in the infundibular nucleus (Inf). The counts were obtained from 1-3 sections per
457 subject and expressed as the mean of the 5-8 individuals \pm SEM. Quantitative analysis of axo-somatic
458 contacts established that the main input to the cell bodies of GnRH neurons is provided by VIAAT-IR
459 GABAergic axons (**A**). Glutamatergic axons of both the VGLUT1 and VGLUT2 phenotypes also
460 innervate GnRH-IR perikarya, although the mean incidences of these contacts are significantly lower and
461 only reach 27.6% and 40.2%, respectively, of the incidence of VIAAT-IR contacts (** $P < 0.01$ by
462 ANOVA, followed by Newman-Keuls). The relative incidence of the three types of contacts on GnRH-IR
463 dendrites (expressed as the mean number of contacts/10 μ m dendrite \pm SEM of 5-8 individuals; **B**)
464 exhibits a similar trend, but glutamatergic inputs here have higher relative contributions (VGLUT1-IR
465 inputs representing 42.2% and VGLUT2-IR inputs representing 66.5% of the VIAAT-IR contacts). The
466 combined contribution of VGLUT1-IR and VGLUT2-IR inputs exceeds that of VIAAT-IR inputs by
467 8.6%.

468

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

471 High-power light microscopic analysis of neuronal contacts was also carried out in the paraventricular
472 nucleus (Pa) to see if the innervation pattern is different in this region. The relative incidences of
473 GABAergic and glutamatergic appositions to the somata (**A**) and dendrites (**B**) of GnRH-IR neurons
474 show similar tendencies to those observed in the infundibular nucleus (Figure 4). Quantitative analysis
475 of axo-somatic contacts established that the main input to the cell bodies of GnRH neurons is provided
476 by VIAAT-IR GABAergic axons (**A**). Glutamatergic axons of both the VGLUT1 and VGLUT2
477 phenotypes also innervate GnRH-IR perikarya, although the mean incidence of their afferent contacts are
478 significantly lower (29.8% and 46.6%, respectively, of the incidence of VIAAT-IR contacts; * $P < 0.05$ and
479 ** $P < 0.01$ by ANOVA, followed by Newman-Keuls. The incidence of the three types of contacts on
480 GnRH-IR dendrites (expressed as the mean number of contacts/10 μ m dendrite \pm SEM of 5-8 individuals;
481 **B**) changes similarly, but glutamatergic inputs here have higher relative contributions (VGLUT1 input:
482 57.0% of VIAAT-IR contacts; VGLUT2 input: 69.3% of VIAAT-IR contacts). The combined
483 contribution of VGLUT1-IR and VGLUT2-IR inputs exceeds that of VIAAT-IR inputs by 26.3%.

484

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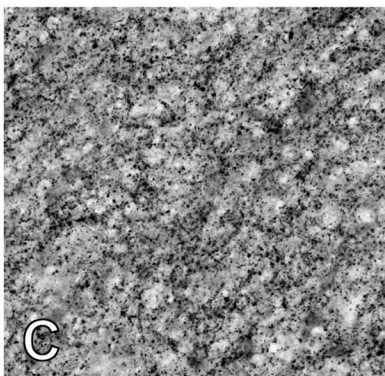
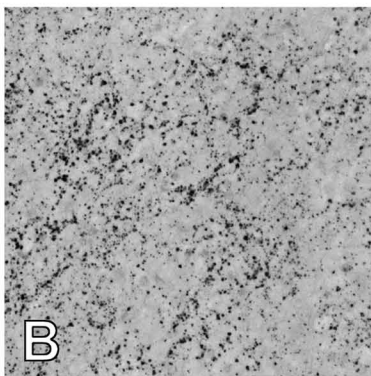
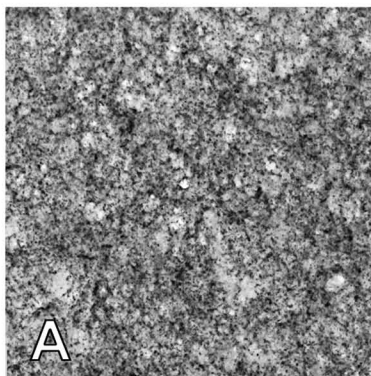
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VIAAT

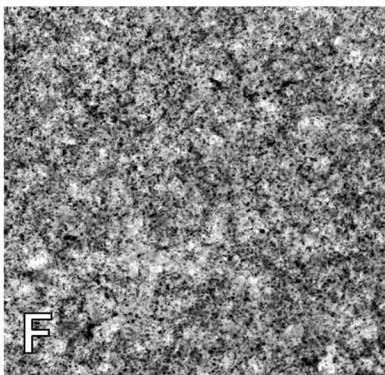
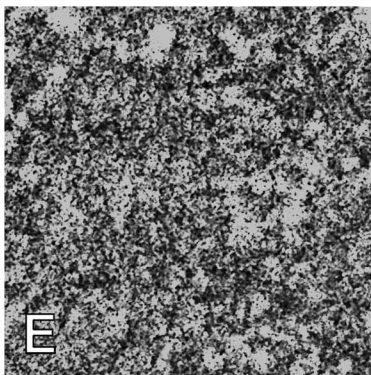
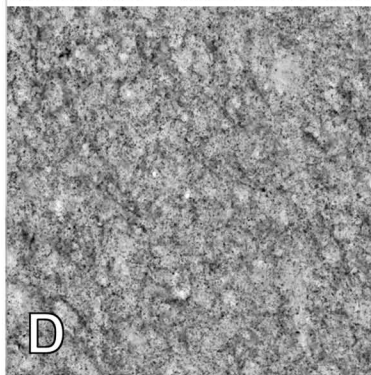
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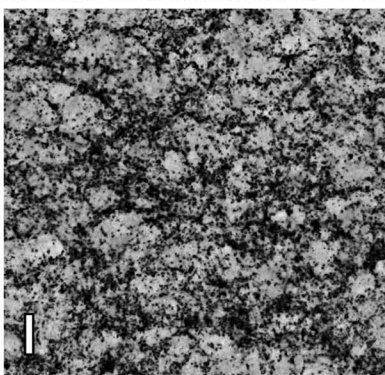
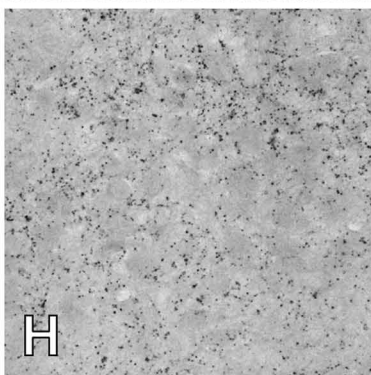
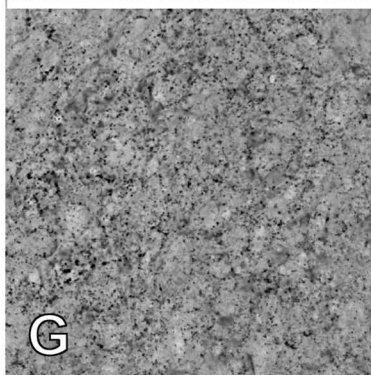
Inf



VMH



SO



Pa

