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Expression of Vesicular Glutamate Transporter-2 in Gonadotropin-Releasing Hormone Neurons of the Adult Male Rat

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Isoforms of the recently cloned vesicular glutamate transporters (VGLUT1–3) selectively accumulate glutamic acid into synaptic vesicles in excitatory axon terminals and are viewed as reliable markers for glutamatergic neurons. Our present studies provided dual-label *in situ* hybridization evidence that virtually all (99.5%) GnRH neurons express VGLUT2 mRNA in the preoptic region of the adult male rat. Dual-label immunofluorescent experiments were carried out to examine the presence of VGLUT2 protein in GnRH axon terminals. Confocal laser microscopic analysis of the organum vasculosum of the lamina terminalis and the external zone of the median eminence, the major termination fields for GnRH-secreting axons, demonstrated the frequent occurrence of VGLUT2 immunoreactivity in GnRH axon terminals. Together these mRNA hybridization and immunocytochemical data indicate that GnRH neurons of the adult male rat possess marked glutamatergic characteristics. The physiological significance of endogenous glutamate in the regulation of gonadotropin secretion requires clarification. (*Endocrinology* 145: 4018–4021, 2004)

RECENT DISCOVERY OF the three distinct vesicular glutamate transporter isoforms (VGLUT1–3) that selectively accumulate glutamic acid into synaptic vesicles has enabled the histochemical identification of excitatory neurons that use glutamate for neuronal transmission. The abundance of glutamatergic neurons expressing VGLUT2 mRNA in the hypothalamic paraventricular nucleus (1, 2) and the high density of VGLUT2-immunoreactive (VGLUT2-IR) axon terminals (1) and ionotropic glutamate receptors (3, 4) in the external zone of the median eminence (ME) raised the possibility that peptidergic neuroendocrine cells regulating anterior pituitary functions secrete glutamate as an autocrine/paracrine modulator of their neurohormone output.

Hypophysiotropic GnRH neurons regulating reproduction differentiate from the olfactory placode and migrate to the forebrain during fetal development (5, 6); migrating GnRH cells in rodents and humans exhibit immunoreactivity to γ-aminobutyric acid (GABA) (7). In postnatal rodents, the majority of GnRH-containing neuronal perikarya occupy their final position in the organum vasculosum of the lamina terminalis (OVLT), medial preoptic area (MPO), and medial septum, regions also populated by high numbers of glutamatergic neurons containing VGLUT2 (1, 2). In the present studies we examined the possibility that mature GnRH neurons in these regions possess glutamatergic characteristics. Dual-labeling experiments using *in situ* hybridization histochemistry (ISHH) investigated the expression of VGLUT2 mRNA in GnRH neurons of the adult male rat. In addition, dual-label immunofluorescent studies using confocal laser scanning microscopy addressed the putative VGLUT2 content of GnRH-IR axon terminals in the OVLT and ME, the major projection fields of GnRH-secreting neurons.

Abbreviations: FITC, Fluorescein isothiocyanate; GABA, γ-aminobutyric acid; IR, immunoreactive; ISHH, *in situ* hybridization histochemistry; ME, median eminence; MPO, medial preoptic area; OVLT, organum vasculosum of the lamina terminalis; VGLUT, vesicular glutamate transporter.

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Materials and Methods

Adult male Wistar rats (n = 8; 220–240 g body weight) were purchased from Charles River Hungary Ltd. (Isaszeg, Hungary) and housed in a light- and temperature-controlled environment with food and water *ad libitum*. Experimental procedures were approved by the animal welfare committee at the Institute of Experimental Medicine.

Dual-label ISHH studies

Four adult rats were decapitated, and their brains were snap-frozen on powdered dry ice. Twelve-micrometer thick coronal sections through the OVLT and the MPO were cut with a CM 3050 S cryostat (Leica, Deerfield, IL) and collected serially on gelatin-coated microscope slides. Every sixth section was processed for dual-label ISHH detection of the GnRH and VGLUT2 mRNAs. The ^{35}S-labeled VGLUT2–879 probe corresponded to bases 522-1400 of VGLUT2 mRNA (GenBank accession no. NM_053427). As a positive control for hybridization specificity, a second series of dual-label ISHH experiment was carried out with a distinct VGLUT2 probe (VGLUT2–734, gift from Dr. J. P. Herman, University of Cincinnati Medical Center, Cincinnati, OH) that was complementary to bases 1704–2437. Negative control experiments were performed with the combined use of the GnRH probe and the sense strand VGLUT2 RNA transcripts. According to a recently introduced novel approach to enhance hybridization sensitivity (8), we applied unusually high radioisotopic probe (80,000 cpm/μl), dextran sulfate (25%), and dithiothreitol (1000 mM) concentrations in the hybridization solution and extended the time of hybridization from 16 to 40 h. After hybridization (52 C) and posthybridization treatments (9), the digoxigenin-labeled cRNA probe to GnRH mRNA (transcribed from a 330-bp cDNA template; a gift from Dr. J. P. Adelman, Vollume Institute, Oregon Health Science University, Portland, OR) was detected immunocytochemically as detailed previously (9), using sequential incubations with antidigoxigenin antibodies conjugated to horseradish peroxidase (1:200; antidig-POD, Roche, St.

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Louis, MO) for 48 h, biotin tyramide amplification solution for 1 h, and ABC Elite working solution (Vector Laboratories, Inc., Burlingame, CA) for 1 h. The signal was visualized with diaminobenzidine chromogen in the peroxidase developer. Subsequently, the ^{35}S-labeled cRNA probe to VGLUT2 mRNA was detected on autoradiographic emulsion (NTB-3; Eastman Kodak Co., Rochester, NY) (8, 9) after a 2-wk exposure.

Dual-label immunofluorescent studies

Four rats were anesthetized with pentobarbital (35 mg/kg body weight, ip) and perfused transcardially with 150 ml fixative solution containing 2% paraformaldehyde (Sigma-Aldrich Corp., St. Louis, MO) and 4% acrolein (Sigma-Aldrich Corp.) in 0.1 M PBS (pH 7.4) (9, 10). Tissue blocks were dissected out and infiltrated with 25% sucrose overnight. Then 20-μm thick, free floating coronal sections were prepared from the OVLT/MPO region and the mediobasal hypothalamus with a cryostat. The sections were rinsed in Tris-buffered saline (0.1 M Tris-HCl/0.9% sodium chloride, pH 7.8), treated with 0.5% sodium borohydride (Sigma-Aldrich Corp.; 30 min) and 0.5% H_2O_2 (15 min), and finally treated with a mixture of 0.2% Triton X-100 (Sigma-Aldrich Corp.) and 2% normal horse serum in Tris-buffered saline (30 min). After these pretreatments, the sections were incubated (72 h; 4 C) in anti-VGLUT2 primary antibodies raised in a guinea pig (AB 5907; Chemicon International, Temecula, CA; 1:1000). Steps to enhance the fluorescent signal included sequential incubations with biotinylated antiguinea pig IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:1000; 2 h), ABC Elite solution (Vector Laboratories, Inc.; 1 h), biotin tyramide working solution prepared and used as described previously (9) (1:1000; 1 h), and streptavidin-conjugated fluorescein isothiocyanate (FITC) fluorochrome (Jackson ImmunoResearch Laboratories; 1:200; 12 h). Immunoreactivity for GnRH was detected with rabbit LR-1 primary antibodies (1:30,000; gift from Dr. R. Benoit, Montréal, Canada). This antiserum was applied to the sections for 48 h (4 C) and then reacted with Cy3-conjugated antirabbit IgG (Jackson ImmunoResearch Laboratories; 1:200; 12 h). The colocalization of VGLUT2 and GnRH immunoreactivities was examined 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 distinguish axonal colocalizations from cases of overlap, individual optical slices of minimal thickness (<0.7 μm) were obtained using a ×60 objective lens (with immersion oil) and an optimized pinhole. Parallel control experiments used VGLUT2 antibodies preabsorbed with 10 μM immunization antigen (AG209, Chemicon International). As a positive control for the specificity of VGLUT2 immunostaining, dual-immunofluorescent experiments were carried out with the combined use of guinea pig anti-VGLUT2 primary antibodies (AB 5907, Chemicon) and rabbit anti-VGLUT2 primary antibodies (1:5000; AB 135103; SYnaptic SYstems, Gottingen, Germany). The primary antibodies were detected with antiguinea pig-FITC (Jackson ImmunoResearch Laboratories; 1:200) and antirabbit-Cy3 (Jackson ImmunoResearch Laboratories; 1:200) conjugates, respectively, and were analyzed by confocal microscopy.

Results

Dual-label ISHH results

Development of emulsion autoradiographs exposed for 2 wk resulted in strong hybridization signal for VGLUT2 mRNA in the horizontal and vertical limbs of the diagonal band of Broca, the OVLT, the MPO, and the median preoptic nucleus (Fig. 1A). Heavy accumulation of silver grains was observed frequently above glutamatergic cells in the vicinity of GnRH neurons (Fig. 1, A_1, A_2, and B). Virtually all (mean ± SEM, 99.5 ± 0.2%) of the total 438 GnRH neurons analyzed (Fig. 1, A_1–A_3) also contained VGLUT2 hybridization signal, usually at moderate levels. Confirmative results obtained with a distinct VGLUT2 probe (Fig. 1B) and the lack of VGLUT2 signal using the sense strand VGLUT2–879 RNA transcript (Fig. 1C) provided support for hybridization specificity.

Dual-label immunofluorescent results

Both glutamatergic and GnRH-containing fibers formed dense plexus in the OVLT (Fig. 1D) and the external zone of the ME (Fig. 1F). High power confocal images demonstrated extensive terminal coexpression of VGLUT2 with GnRH immunoreactivities in both circumventricular organs (Fig. 1, E and G). Although most dual-labeled axons occurred laterally in the ME, a dense VGLUT2 plexus was also observed medially (Fig. 1F). VGLUT2-IR axons (Fig. 1H) were eliminated (Figs. 1I) from the ME when using primary antibodies preabsorbed with 10 μM immunization antigen. In addition, simultaneous use of the two different primary antisera against VGLUT2 labeled identical axons throughout the hypothalamus, in further support of labeling specificity (Fig. 1, J–L).

Discussion

Demonstration of VGLUT2 mRNA expression and VGLUT2 immunoreactivity in GnRH neurons provides conclusive evidence that these cells possess a marked glutamatergic phenotype in the adult male rat.

Glutamate is a critically important neurotransmitter in the regulation of the GnRH neuronal system. Intravenous *N*-methyl-D,L-aspartate infusion can induce precocious puberty in immature rats (11) and activation of ionotropic glutamate receptors plays a crucial role in both pulse (12) and surge (13) modes of GnRH secretion. Although glutamate can regulate GnRH neurons at the level of GnRH cell bodies and dendrites that receive VGLUT2-IR synapses (1, 14) and exhibit immunoreactivity for ionotropic glutamate receptors (15, 16), compelling evidence indicates that an additional major site of action for glutamate is the ME. GnRH terminals in the ME are apposed to glutamatergic axons (1, 4) and express immunoreactivity for the KA2 and NR1 ionotropic glutamate receptor subunits (4). Further, glutamate and agonists of ionotropic glutamate receptors can induce Ca^{2+}-dependent release of GnRH from superfused ME fragments (3). Our ISHH finding that virtually all GnRH neurons expressed VGLUT2 mRNA in the adult male rat strongly suggests that the glutamatergic chemotype is a critically important feature of the GnRH neuronal system. The molecular mechanisms by which glutamate endogenous to GnRH neurons regulates reproduction require future clarification.

The reason why previous confocal and electron microscopic studies (1, 3, 4, 14) failed to reveal the glutamatergic phenotype of the GnRH neuronal system is not clear. In view of the only moderate VGLUT2 mRNA and protein levels we found in GnRH cells, the use of amplification methods could be essential for our colocalization studies to succeed. Furthermore, although VGLUT2 mRNA was present in virtually all GnRH neurons, VGLUT2 immunoreactivity often remained undetectable in GnRH terminals. This discrepancy may be attributable to a general limitation of the immunocytochemical detection method. Another possibility is the heterogeneity of GnRH axon varicosities, in that some may mostly contain dense core vesicles with GnRH and only few small clear vesicles with VGLUT2.

It is important to note that the ME also contained a large number of VGLUT2-IR terminals that were devoid of im-

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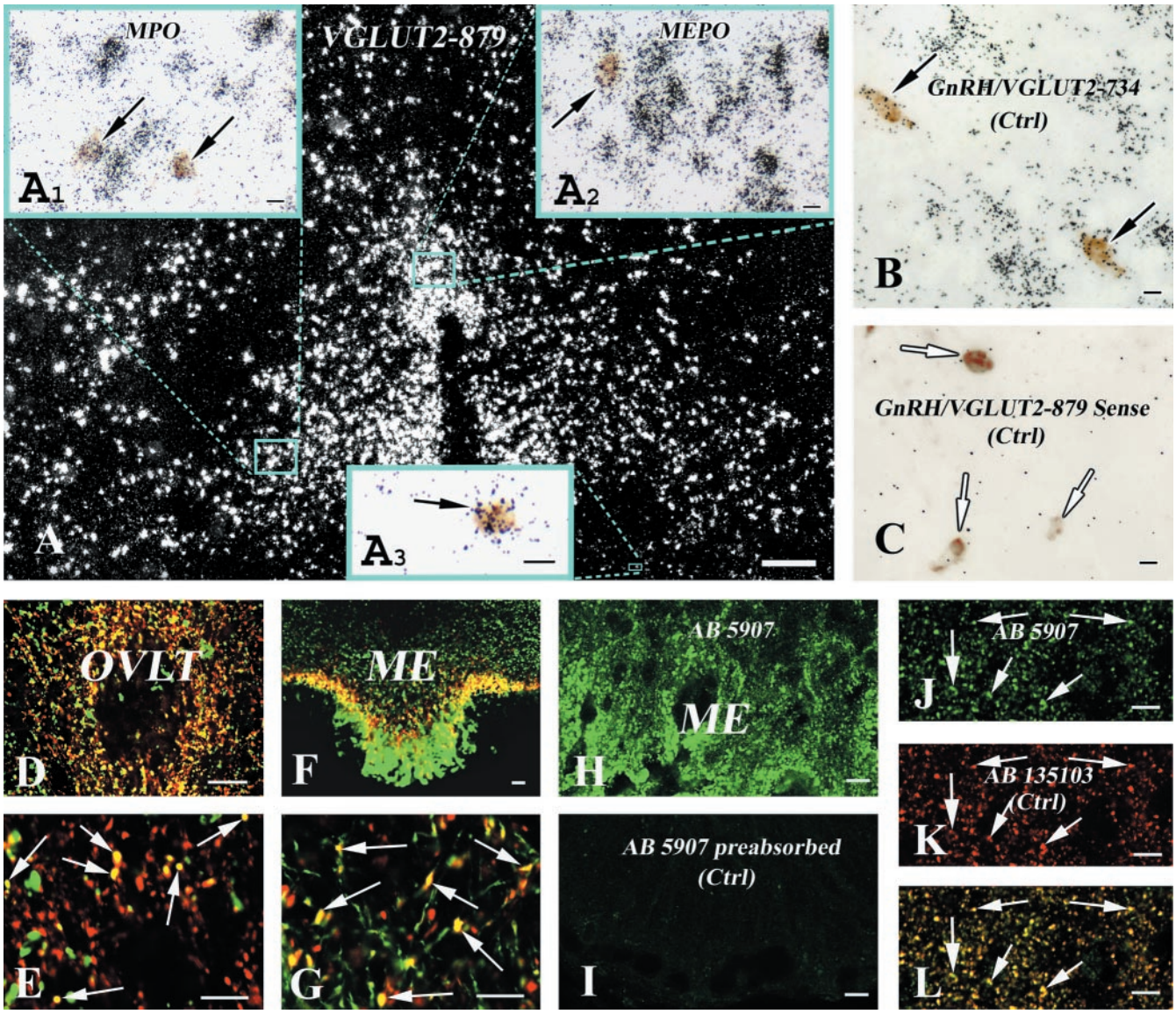

FIG. 1. GnRH neurons of the adult male rat contain VGLUT2. Dual-label ISHH experiments reveal high expression levels of VGLUT2 mRNA (autoradiographic grain clusters in A) in regions also populated by GnRH neurons (*brown* histochemical staining in A_1–A_3), including the medial preoptic area (MPO) and the median preoptic nucleus (MEPO). High power insets (A_1–A_3) illustrate moderate levels of VGLUT2 mRNA in GnRH neurons (*arrows*) from frames in A after use of the VGLUT2–879 probe. Demonstration of VGLUT2 mRNA-containing GnRH neurons with a distinct antisense probe (VGLUT2–734; B) and lack of such dual-labeled GnRH neurons after use of the sense strand VGLUT2–879 RNA transcript (C) serve as controls for hybridization specificity. Dual-immunofluorescent images (D and F) illustrate the overlapping distribution of fibers immunoreactive for VGLUT2 (*green* fluorochrome) and GnRH (*red*) in the OVLT (D) and the ME (F). Although sites of overlap (*yellow*) occur mostly in the lateral part of the ME, the dense VGLUT2-IR plexus is also present medially (F). *Arrows* in high power confocal images (E and G) point to dual-labeled axon varicosities (*yellow*) immunoreactive for both GnRH and VGLUT2. The specificity of VGLUT2 immunostaining in the ME (H) is indicated by the lack of labeled axons after adding the immunization antigen (10 μM) to the working dilution of AB 5907 (I). In addition, dual-immunofluorescent labeling of hypothalamic axons (*arrows* in confocal images J, K, and merged panel L) using two distinct primary antibodies for VGLUT2 further supports the authenticity of VGLUT2 immunoreactivity. *Scale bars*, 200 μm in A, 30 μm in D and F, and 10 μm in the other panels.

munostaining for GnRH. A likely source of some of these fibers is the parvicellular part of the hypothalamic paraventricular nucleus, where VGLUT2-expressing neurons occur in high numbers (1, 2).

A large subset of GnRH neurons in rats, mice, and humans exhibit GABA immunoreactivity (7) during fetal migration from the olfactory placode to the forebrain (5, 6). Tobet and co-workers (7) found no GABA immunoreactivity in GnRH neurons that migrated further caudal to the olfactory bulbs, suggesting that GnRH neurons may switch from the GABAergic to the glutamatergic phenotype perinatally. If the concept of this developmental conversion is valid, then its biological significance and exact time of onset need to be investigated. The possibilities also remain that VGLUT2 is already expressed prenatally in the GnRH system when these neurons still display GABA-ergic features and/or that

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GABA synthetic enzymes and the vesicular GABA transporter continue to be expressed together with VGLUT2 in adult GnRH neurons.

In summary, in the present studies we provide conclusive evidence for a marked glutamatergic phenotype of GnRH neurons in the adult male rat by demonstrating VGLUT2 mRNA expression in the perikaryon and VGLUT2 immunoreactivity in the main axon projections of these cells. The physiological significance of endogenous glutamate in the regulation of GnRH secretion requires clarification.

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