Hypophysiotropic Thyrotropin-Releasing Hormone and Corticotropin-Releasing Hormone Neurons of the Rat Contain Vesicular Glutamate Transporter-2

Erik Hrabovszky, Gábor Wittmann, Gergely F. Turi, Zsolt Liposits, and Csaba Fekete
Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest 1083, Hungary

TRH and CRH are secreted into the hypophysial portal circulation by hypophysiotropic neurons located in parcellular subdivisions of the hypothalamic paraventricular nucleus (PVH). Recently these anatomical compartments of the PVH have been shown to contain large numbers of glutamatergic neurons expressing type 2 vesicular glutamate transporter (VGLUT2). In this report we presented dual-label in situ hybridization evidence that the majority (>90%) of TRH and CRH neurons in the PVH of the adult male rat express the mRNA encoding VGLUT2. Dual-label immunofluorescent studies followed by confocal laser microscopic analysis of the median eminence also demonstrated the occurrence of VGLUT2 immunoreactivity within TRH and CRH axon varicosities, suggesting terminal glutamate release from these neuroendocrine systems. These data together indicate that the hypophysiotropic TRH and CRH neurons possess glutamatergic characteristics. Future studies will need to address the physiological significance of the endogenous glutamate content in these neurosecretory systems in the neuroendocrine regulation of thyroid and adrenal functions. (Endocrinology 146: 341–347, 2005)

GLUTAMATE IS a major excitatory synaptic transmitter in neuroendocrine regulation (1, 2). The recent discovery of vesicular glutamate transporters (VGLUTs) 1–3, which selectively accumulate glutamic acid into synaptic vesicles, has provided histochemical markers for the identification of glutamatergic neurons. The three molecular forms of VGLUT appear to be expressed by distinct populations of glutamatergic neurons; VGLUT2 represents the dominant isoform synthesized in hypothalamic excitatory neurons (3–12). The occurrence of VGLUT2 mRNA and immunoreactivity has been observed in hypothalamic regions that play crucial roles in neuroendocrine regulation (10, 11), including the parcellular compartments of the hypothalamic paraventricular nucleus (PVH), which comprise hypophysiotropic TRH and CRH neurons, among other peptidergic neuronal phenotypes. The observations of glutamate- (13) and VGLUT2-immunoreactive (IR) (11, 14) axons in the external zone of the median eminence (ME) have raised the possibility that some of the parcellular neurosecretory systems may accumulate glutamic acid into synaptic vesicles for terminal corelease with hypophysiotropic neuropeptides. Also in support of this idea, we have recently shown that GnRH neurons forming the final common pathway in the neuroendocrine control of reproduction express VGLUT2 mRNA in their perikaryon and exhibit VGLUT2 immunoreactivity in their axons terminating in the organum vasculosum of the lamina terminalis and the ME (14).

In these studies we addressed the putative glutamatergic phenotype of hypophysiotropic TRH and CRH neurons in the adult male rat. First, we performed dual-label in situ hybridization histochemistry (ISHH) to analyze VGLUT2 mRNA expression in TRH and CRH neurons of the PVH. In addition, we carried out dual-label immunofluorescent studies followed by confocal laser microscopic analysis of the ME to localize VGLUT2 immunoreactivity within neurosecretory TRH and CRH axon terminals.

Materials and Methods

Animals

Adult male Wistar rats (n = 8; 200–225 g body weight) were purchased from Charles River Hungary Ltd. (Isaszeg, Hungary) and maintained in a light- and temperature-controlled environment (lights on 0900–1900 h; 22 C) with free access to food and water. Experimental protocols were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine.

Dual-label in situ hybridization experiments

Four rats were decapitated and their brains were rapidly removed and frozen on powdered dry ice. Coronal, 12-μm-thick sections were cut with a CM 3050 S cryostat (Leica Microsystems Nussloch GmbH, Nussloch, Germany) and collected serially on gelatin-coated microscope slides. Mounted sections were processed either for the simultaneous detection of VGLUT2 with TRH mRNAs or VGLUT2 with CRH mRNAs. Dual-label ISHH was carried out as described recently for the demonstration of VGLUT2 mRNA expression in GnRH neurons (14). Briefly, sections were hybridized at 52 C with a mixture of a 35S-labeled cRNA hybridization probe to VGLUT2 mRNA and a digoxigenin-labeled

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cRNA probe to either proTRH or proCRH mRNA. After hybridization and posthybridization treatments, the nonisotopic probes were reacted with horseradish peroxidase (POD)-conjugated antidigoxigenin antibodies (1:200; Roche Diagnostics Co., Indianapolis, IN). The POD signals were enhanced with biotin tyramide amplification using a procedure (15) adapted from Adams (16) and finally visualized with diaminobenzidine (DAB) chromogen (14, 15). To reduce autoradiographic background, we added 1000 mM diethytothiol to the hybridization solution (17). To enhance autoradiographic hybridization signal for VGLUT2 mRNA, we also applied high concentrations of radioisotopic probe (80,000 cpm/μl) and dextran sulfate (25%) to the sections and extended the time of hybridization from 16 to 40 h. The advantages of these changes have been formally established in a methodological report (17) and confirmed in recent dual- and triple-label ISHH studies from our laboratory (14, 15, 18, 19). The autoradiographs were detected on NTB-3 nuclear track emulsion (Kodak, Rochester, NY) after 2 wk of exposure (14).

Procedures to label radioisotopic and nonisotopic cRNA probes and details of the dual-label ISHH method have been described elsewhere (15). The cDNA templates to generate proTRH (1.2 kb) and proCRH (1.1 kb) probes were kindly provided by Drs. R. M. Lechan (New England Medical Center, Boston, MA) and K. Mayo (Northwestern University, Evanston, IL), respectively, and used in previous nonisotopic ISHH experiments (15, 20). The mRNA encoding VGLUT2 was detected with a 35S-labeled probe to bases 522-1400 of VGLUT2 mRNA (GenBank accession no. NM 053427) (14). TRH and CRH neurons exhibiting DAB labeling were analyzed individually for the autoradiographic VGLUT2 signal at high power. The percent ratios of TRH and CRH neurons with VGLUT2 mRNA were determined using two sections per animal for each region of interest. Results of dual-labeling were expressed as mean ± SEM of four animals.

In situ hybridization control experiments

Test sections from the PVH were hybridized using a distinct antisense probe to VGLUT2 mRNA (VGLUT2–734; complementary to bases 1704–2347). The plasmid template for in vitro transcriptions of this probe (10) was a kind gift from Dr. J. P. Herman (University of Cincinnati Medical Center, Cincinnati, OH), and results served as positive control for hybridization specificity. Furthermore, control sections were hybridized with the combined application of the digoxigenin-labeled antisense TRH probe and the isotopically labeled sense-strand VGLUT2–879 or VGLUT2–734 transcripts.

Tissue preparation for immunocytochemistry

Four rats were anesthetized with pentobarbital (35 mg/kg body weight, ip) and perfused transcardially with 150 ml fixative solution containing 2% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) and 4% acrolein (Aldrich Chemical Co., Milwaukee, WI) in 0.1 M PBS (pH 7.4). The hypothalami were dissected and soaked in 25% sucrose overnight for cryoprotection. Then 20-μm-thick free-floating coronal sections were cut from the hypothalami using a cryostat. The sections were rinsed in Tris-buffered saline [0.1 M Tris-HCl/0.9% sodium chloride (pH 7.8)] and treated with 0.5% sodium borohydride (Sigma, 30 min), then 0.5% H2O2 + 0.5% Triton X-100 (15 min), and finally 2% normal horse serum in Tris-buffered saline (30 min) (15).

Immunocytochemical procedures

After pretreatments, the sections were incubated in a polyclonal VGLUT2 antiserum raised in a guinea pig (AB5907; 1:1000; Chemicon, Temecula, CA) for 72 h at 4 C. The primary antibodies were reacted with donkey, biotin-SP-antiguiena pig IgG (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h and then with streptavidine-conjugated Cy3 fluorochrome (1:200; Vector Laboratories, Burlingame, CA) for 2 h. Subsequently immunoreactivities for either TRH or CRH were detected with a rabbit antiserum against TRH (no. 31) at 1:2500 dilution (a gift from Dr. R. M. Lechan) and a rabbit CRH antiserum (1:300; Peninsula Laboratories Inc., San Carlos, CA), respectively, which were applied to the sections for 48 h (4 C) and then reacted with fluorescein isothiocyanate (FITC)-conjugated antirabbit IgG (1:200; 12 h; Jackson ImmunoResearch). Dual-immunofluorescent specimens were analyzed at high power (×60 objective lens) 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 560/500–530 nm for FITC and 560–610 nm for Cy3. The analysis of individual optical layers (<0.7 μm) was used for the demonstration of the axonal colocalization phenomenon and its photographic illustration (14).

Immunocytochemical control experiments

Negative and positive control studies to assess specificity of VGLUT2 immunolabeling were carried out as described recently (14). Primary antibodies (AB5907) preabsorbed with 10 μM of the immunization antigen (AG209; Chemicon) were applied to test sections in parallel with the experimental specimens (14). In addition, the AB5907 guinea pig VGLUT2 antibodies (Chemicon) were used in combination with rabbit VGLUT2 antibodies (AB 135103; 1:5000; Synaptic Systems, Göttingen, Germany) for dual-immunofluorescent experiments. The different primary antibodies were reacted with appropriate secondary antibody-fluorochrome conjugates (antiguinea pig-FITC and antirabbit-Cy3, respectively; Jackson ImmunoResearch) for 12 h, and colocalization of the two signals was assessed in the hypothalamus with confocal microscopy (14).

Results

Dual-label in situ hybridization results

Silver grain clusters were abundant in the diencephalon after 2 wk of autoradiographic exposure (Fig 1A). Virtually all anatomical compartments of the PVH through its rostrocaudal extent (Fig. 1, B–D), including the anterior parvicellular (ap) and periventricular (pv) subnuclei as well as the dorsal subdivision of the medial parvicellular subnucleus (mpd), exhibited numerous cells expressing VGLUT2 mRNA. Very heavy grain clusters accumulated in the lateral hypothalamic area (LHA; Fig. 1A), which is known to contain nonhypophysiotropic TRH neurons in large numbers. In contrast, the reticular nucleus of the thalamus (RT), which exhibits cells containing proTRH mRNA but not TRH tripeptide (21), was devoid of VGLUT2 hybridization signal (Fig. 1A).

Using bright-field illumination of dual-labeled sections, the majority of DAB-labeled TRH neurons in the PVH, including nonhypophysiotropic TRH neurons in the ap subnucleus (Fig. 2A) and hypophysiotropic TRH neurons in the pv (Fig. 2B) and mpd (Fig. 2C) subnuclei, exhibited moderate levels of isotopic hybridization signal for VGLUT2 mRNA. In sections hybridized for CRH and VGLUT2 mRNAs, most CRH neurons in the PVH also expressed the signal for VGLUT2 mRNA (Fig. 2D). The counted ratio of dual-labeled TRH neurons was 97.4 ± 1.1% in the ap, 93.8 ± 1.4% in the pv, and 94.3 ± 1.4% in the mpd subnuclei of the PVH. Furthermore, 90.5 ± 1.3% of CRH neurons in the mpd subnucleus, which includes most hypophysiotropic CRH neurons, exhibited autoradiographic signal for the glutamatergic marker, VGLUT2. In addition to occurring within TRH and CRH neurons in the PVH, it is worth noting that TRH neurons with the heaviest cellular levels of VGLUT2 signal were identified in the LHA (Fig. 2E). Of these cells, 98.6 ± 0.7% exhibited VGLUT2 labeling. In contrast, only 18.9 ± 4.5% of the TRH neurons in the perifornical region (Fig. 2F) contained the VGLUT2 signal, and proTRH mRNA-expressing
neurons in the RT (Fig. 2G) did not accumulate silver grains. Sections dual labeled for CRH and VGLUT2 mRNAs also included CRH neurons in the central amygdaloid nucleus. In contrast to CRH neurons of the PVH, these cells remained unlabeled for VGLUT2 (Fig. 2H). In control experiments, application of the sense VGLUT2 probes (Fig. 2, J and L) did not generate any patterned labeling of sections exceeding background labeling. TRH and CRH neurons in these sections were devoid of silver grain clusters, also indicating lack of potential positive chemography on the DAB chromogen. Furthermore, the distribution of cells labeled for VGLUT2 mRNA was identical using either the VGLUT2–879 probe (Fig. 2I; also used in Fig. 1 and Fig. 2, A–H) or the control probe (VGLUT2–734; Fig. 2K) that recognized nonoverlapping segments of VGLUT2 mRNA; dual-labeled neurons were also clearly identifiable with the latter (Fig. 2K), in strong support of hybridization specificity.

Dual-label immunofluorescent results

Immunofluorescent studies revealed a dense plexus of VGLUT2-immunoreactive axons in the external zone of the ME (Fig. 2O), which overlapped with the distribution of hypophysiotropic peptidergic axons. Confocal laser microscopic analysis established an extensive terminal coexpression of VGLUT2 with TRH (Fig. 2M) and VGLUT2 with CRH (Fig. 2N) immunoreactivities. The two types of signal showed a clear segregation within individual terminal varicosities (Fig. 2N), supporting the assumption that VGLUT2 (and the glutamate transmitter pool) and the neuropeptides are contained in distinct vesicular components. Specificity control experiments showed that preabsorption of VGLUT2 antibodies (AB5907) with 10 μM of the immunization antigen (AG209) eliminated the axonal labeling from the ME (Fig. 2O). Furthermore, VGLUT2 antibodies from Chemicon and
FIG. 2. Histochemical detection of VGLUT2 mRNA expression and immunoreactivity in TRH and CRH neurons of the diencephalon. Black arrows in dual-label in situ hybridization images (A–L) point to TRH or CRH neurons (brown DAB chromogen) that also express VGLUT2 mRNA (silver grain clusters), whereas white arrows indicate TRH or CRH neurons that lack the autoradiographic signal. A–C, The VGLUT2 hybridization signal is expressed at moderate levels by the majority of nonhypophysiotropic TRH neurons (brown DAB chromogen) in the anterior parvicellular subnucleus of the paraventricular nucleus (PVHap; A). Similarly, most hypophysiotropic TRH neurons in the periventricular subnucleus (PVHp; B) and the dorsal subdivision of the medial parvicellular subnucleus (PVHmpd; C) contain VGLUT2 mRNA. D, The mpd compartment also includes a large number of CRH neurons with VGLUT2 mRNA. E–G, TRH neurons in the LHA (E) are labeled heavily for VGLUT2, whereas more than 80% of TRH neurons in the perifornical region (pf; F) and all proTRH mRNA-expressing neurons in the RT (G) are devoid of the VGLUT2 hybridization signal. H, Similarly, VGLUT2 mRNA is not detectable in CRH neurons of the central amygdala (Ac). I–L, Serial sections from the PVH used for control (Ctrl) purposes were hybridized in parallel using two distinct antisense (AS; I, K) and sense (S; J, L) VGLUT2 transcripts (VGLUT2–879 and VGLUT2–734) together with the digoxigenin-labeled antisense probe to TRH mRNA. The autoradiographic VGLUT2 signal and dual-labeled TRH neurons (clustered silver grains over brown DAB deposits) are present using either one of the two antisense (I, K) but none of the two sense (J, L) VGLUT2 probe sequences, providing strong support for hybridization specificity. M and N, Dual-label immunofluorescent studies using confocal laser microscopy reveal a dense plexus of VGLUT2-IR axons (red fluorochrome) in the external layer of the ME, which overlaps with the distribution of hypophysiotropic TRH (M) and CRH (N) axons (green fluorochrome). The high-power confocal images of single optical layers (<0.7 μm) demonstrate an extensive axonal colocalization of VGLUT2 with TRH (M; merged figure) and VGLUT2 with CRH (N) immunoreactivities. Arrows delineate dual-labeled terminal varicosities. The three panels in N also illustrate the intraxonal segregation of organelles containing VGLUT2 (red; left panel) and CRH (green; middle panel) immunoreactivities.
those from Synaptic Systems consequently labeled identical axon varicosities in the hypothalamus (Fig. 2P), serving as positive control for the specificity of axonal VGLUT2 immunolabeling (14).

Discussion

In this report we present evidence that the majority of TRH and CRH neurons in the PVH express the mRNA for VGLUT2. Furthermore, their neurosecretory axon terminals in the ME contain VGLUT2 immunoreactivity.

Classical nonpeptide neurotransmitters including l-glutamate are transported into synaptic vesicles by vesicular neurotransmitter transporters before their quantal release by exocytosis (22). Recently it has been established that the brain-specific Na⁺-dependent inorganic phosphate cotransporter (23) fulfills the criteria required for the vesicular glutamate transporter, and hence, the molecule was renamed VGLUT1 (3, 24). Subsequently several laboratories have shown that differentiation-associated Na⁺-dependent inorganic phosphate cotransporter represents a second member of the VGLUT family, VGLUT2 (4, 6, 25–28). Although VGLUT1 and VGLUT2 occur in distinct classes of glutamatergic neurons establishing asymmetric synapses typical of excitatory neurons (4–6, 29), the roles of the third member of the VGLUT family, VGLUT3 (7–9), are less well understood given its unexpected occurrence in serotonergic (7–9), cholinergic (7–9), and even γ-aminobutyric acid (GABAergic) (7) neurons, presence in neuronal cell bodies and dendrites, in addition to axon terminals (7–9), and involvement in symmetric, in addition to asymmetric synaptic (7, 8). The VGLUT2 isoform we identified in various groups of diencephalic TRH and CRH neurons is considered an authentic marker for the glutamatergic neuronal phenotype.

Together the expression of the mRNA for the glutamatergic marker VGLUT2 in the majority of TRH- and CRH-synthesizing neuronal perikarya in hypophysiotropic subdivisions of the PVH and the occurrence of VGLUT2 immunoreactivity in TRH and CRH IR terminals in the ME indicate that the hypophysiotropic TRH and CRH neurosecretory systems release glutamate, in addition to neuropeptides, into the pericapillary space of hypophyseal portal vessels. The physiological role of this glutamate cosecretion with TRH and CRH remains to be investigated.

One possibility is that glutamate released from hypophysiotropic terminals reaches the anterior pituitary at high enough concentrations to exert physiological effects. This putative hypophysial site of glutamatergic actions would be compatible with the reported presence of glutamate receptors on thyrotrrophs (30). However, it is even more likely that glutamate acts centrally at the level of the ME. Central glutamatergic mechanisms are clearly crucial in thyroid regulation, which is indicated by decreased serum TSH and thyroid hormone levels in response to intracerebroventricular administration of antagonists to 2-amino-3-hydroxy-5-methyl-4-isoxazol propionic acid and N-methyl-D-aspartate (NMDA) receptors (31). In contrast, exclusively central glutamatergic mechanisms appear to be involved in adrenal regulation. Plasma ACTH levels rise after systemic (32–34) or central (35) administration of NMDA and kainate, whereas NMDA, kainate, or glutamate does not directly elicit ACTH release from incubated pituitaries (34). Moreover, the observation that ACTH release induced by systemic NMDA or kainate injections can be blocked by antisera to CRH also suggests that the central actions of glutamate may involve CRH terminals in the ME (34). It is reasonable to speculate that glutamate actions in the ME can be partly exerted directly on neurosecretory terminals. Indeed, some of these axon terminals have been shown to express immunoreactivity for the KA2 and NR1 ionotropic glutamate receptor subunits (13, 36) Such receptors, assuming they are also present on TRH and CRH terminals, may be involved in autocrine/paracrine regulatory mechanisms on binding glutamate secreted from endogenous sources in TRH and CRH axon terminals. In addition, a further possibility is that the secreted glutamate regulates important glial functions at the sites of release. Tanyocytes and astrocytes in the ME were found to contain mRNAs and immunoreactivity for kainate receptors (37–39) and to express c-Fos immunoreactivity on kainate stimulation (37). Because tanyctic processes regulate the neurohemal junction in the ME, it is an intriguing possibility that glutamate induces plastic changes in this cell type to regulate the access of secretion products to the hypophysial portal vessels. Finally, intrinsic glutamate released by hypophysiotropic TRH and CRH terminals may also act on vascular elements. Although somewhat controversial (40), the presence of functional metabotropic (41, 42) and ionotropic (42, 43) glutamate receptors has been described on cerebral microvascular endothelial cells. It is interesting to note that the stimulation of NMDA receptors increases nitric oxide production within the ME (44), which is considered to be of endothelial origin (45). Nitric oxide, in turn, is an important regulator of CRH release from the ME (45), proposed to act via increasing cGMP and/or prostaglandin E2 production in hypophysiotropic axon terminals (45). Actions mediated by the soluble nitric oxide may also propagate the glutamatergic signal to a larger group of neurosecretory terminals and to a higher distance from the site of glutamate release.

The observation of VGLUT2 in functionally and neurochemically diverse neurosecretory endings also suggests that intrinsic glutamate fulfills similar regulatory functions in several neuroendocrine systems. A putative general mechanism would be its contribution to regulate episodic secretion, which equally characterizes the TRH and CRH systems (46, 47) Whereas the concept requires experimental support, it is tempting to speculate that the endogenous glutamate

The yellow color in merged figure (right panel) likely occurs at sites containing both small clear and dense core vesicles. Q, Specificity of VGLUT2 immunostaining in the ME (upper panel) is indicated by the lack of labeled axons (lower panel) after adding 10 μM of the immunization antigen to the working dilution of AB 5907. P, Furthermore, glutamatergic axons in the external layer of the ME can be dual immunolabeled with two distinct VGLUT2 antisera (AB 5907 and AB 135103) and using green and red fluorochromes, respectively. Presence of mostly dual-labeled axon varicosities (yellow color; arrows) serves as a strong evidence for the authenticity of VGLUT2 immunolabeling. Scale bars, 1 μm in M, N; 10 μm in P; and 20 μm in other panels.
content of these systems contributes to common signaling mechanisms that generate the pulsatile patterns in neurohormone output.

It is worth noting that, whereas VGLUT2 mRNA was identifiable in the vast majority of the hypophysiotropic TRH and CRH neurons in the PVH, the ME contained both TRH and CRH axon terminals that were not immunoreactive for VGLUT2. A methodological explanation for this discrepancy may be the superior detection sensitivity of the ISHH vs. the immunocytochemical technique. Alternatively, the nonhomogeneous intraaxonal distribution of VGLUT2 vs. TRH/CRH immunoreactivities in our confocal microscopic studies raises the possibility that the segregation of vesicular pools containing VGLUT2 and the hypophysiotropic neuropeptides could partly account for the observation of VGLUT2-immunonegative TRH and CRH terminals. Finally, of particular interest to our observation that VGLUT2 mRNA is expressed in the vast majority of CRH neurons in the PVH is a previous report on glutamic acid decarboxylase and GABA immunoreactivities in a small subset of parvicellular CRH neurons in this nucleus (48). Whether these apparently GABA-synthesizing CRH neurons constitute a subpopulation of the CRH/VGLUT2 neurons or they correspond to the small subset (<10%) of CRH neurons that showed no VGLUT2 mRNA expression in our present study remains to be established.

In addition to being expressed in hypophysiotropic TRH and CRH neurons, VGLUT2 mRNA also occurred in several nonhypophysiotropic TRH neuronal populations. Hybridization results have shown that nonhypophysiotropic TRH neurons in the anterior PVH and the LHA (49, 50) exhibit common glutamatergic characteristics. In contrast, proTRH mRNA synthesizing neurons in the RT region were devoid of VGLUT2 mRNA, in accordance with the predominant GABAergic phenotype of this brain region (51). We were similarly unable to find VGLUT2 mRNA expression in the majority (>80%) of TRH neurons in the perifornical region. It remains unclear whether perifornical TRH cells consist of glutamatergic and nonglutamatergic (putative GABAergic) subpopulations or else their majority contains VGLUT2 mRNA at levels below the detection threshold of our isotopic ISHH approach. Evidence exists that the perifornical TRH neurons are neurochemically and functionally distinct from the TRH neurons of the PVH in that they contain enkephalin and project to the lateral septum (52). A neurochemical diversity also exists for distinct CRH-containing neuronal cell groups with regard to their amino acid transmitter cocontent. CRH has been localized to GABAergic neurons in the bed nucleus of the stria terminals and central amygdaloid nucleus (53), which appears to be in agreement with our present data on the absence of VGLUT2 hybridization signal in CRF neurons of the central amygdala.

In summary, in the present ISHH and immunocytochemistry studies, we provide evidence that hypophysiotropic TRH and CRH neurons of the PVH synthesize VGLUT2 mRNA and protein in the male rat. The functional significance of this marked glutamatergic phenotype, which is shared by several neurosecretory systems, requires clarification.


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