Glutamatergic innervation of corticotropin-releasing hormone- and thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus of the rat

Gábor Wittmann, Ronald M. Lechan, Zsolt Lipóts, Csaba Fekete

Abstract

Glutamate plays a role in the central regulation of the hypothalamic–pituitary–adrenal (HPA) and thyroid (HPT) axes. Until the recent discovery of vesicular glutamate transporters (VGLUT1–3), there was no specific tool for the examination of the putative morphological relationship between the glutamatergic and the hypophysiotropic systems. Using antisera against VGLUT2, corticotropin-releasing hormone (CRH), and prothyrotropin-releasing hormone (proTRH) (178–199), we performed double-labeling immunocytochemistry at light and electron microscopic levels in order to study the glutamatergic innervation of the CRH- and TRH-synthesizing neurons in the hypothalamic paraventricular nucleus (PVN). Fine VGLUT2-immunoreactive (IR) axons very densely innervated the parvocellular subdivisions of the PVN. VGLUT2-IR axons established juxtapositions with all parvocellular CRH- and TRH-synthesizing neurons. The innervation was similarly intense in all parvocellular subdivisions of the PVN. At ultrastructural level, VGLUT2-IR terminals frequently established synapses with perikarya and dendrites of the CRH- and proTRH-IR neurons. These findings demonstrate that glutamatergic neurons directly innervate hypophysiotropic CRH and TRH neurons in the PVN and, therefore, support the hypothesis that the glutamate-induced activation of the HPA and HPT axes may be accomplished by a direct action of glutamate on hypophysiotropic CRH and TRH systems.

1. Introduction

The hypothalamic–pituitary–adrenal (HPA) and thyroid (HPT) axes are two major endocrine regulatory systems that are important for energy homeostasis [48]. These neuroendocrine systems are governed by the hypophysiotropic corticotropin-releasing hormone (CRH)- and thyrotropin-releasing hormone (TRH)-synthesizing neurons, both residing in the paraventricular nucleus of the hypothalamus (PVN) [36,41,56]. Modulation of basal hormone synthesis of these hypophysiotropic neurons and their response to specific stimuli require both hormonal and neuronal regulation. Thus,
circular levels of glucocorticoids and thyroid hormones exert negative feedback effects on the activity of hypothalamic hypophysiotropic CRH and TRH neurons, respectively [34,49], and these neurons can be influenced by neuronal systems that establish synaptic formations with their cell bodies and dendrites. Suppression of CRH and TRH gene expression during fasting, for example, is partly attributable to inhibition of the release of α-melanocyte-stimulating hormone (α-MSH) from axons originating in the hypothalamic arcuate nucleus [16,17]. Catecholamines, released from ascending, monosynaptic projections of the lower brainstem, are responsible for upregulation of TRH gene expression and release during cold stress [46,59], and for the increase in CRH gene expression following endotoxin administration [15].

In addition to the peptidergic and catecholaminergic inputs to PVN neurons, the inhibitory and excitatory neurotransmitters, GABA and glutamate, may also contribute to the regulation of these systems [2,20,31,40]. GABA-ergic axon terminals have been recently reported to densely innervate both hypophysiotropic CRH and TRH neurons [19,43]. However, there is no morphological evidence as to whether these neurons also receive a glutamatergic innervation. Until the recent discovery of the vesicular glutamate transporters (VGLUT1–3), highly specific markers of glutamatergic neurons [3,6,22,26,53,54,57], there was no specific tool for the morphological analysis of glutamatergic neuronal systems.

Using antisera against VGLUT2, the most abundant VGLUT expressed in the hypothalamus, CRH, and proTRH (178–199), we have performed double-labeling immunocytochemistry at light and electron microscopic levels in order to study the glutamatergic innervation of the CRH- and TRH-synthesizing neurons in the PVN.

2. Materials and methods

2.1. Animals

The experiments were carried out on adult male Wistar rats, weighing 280–350 g, housed under standard environmental conditions (light between 06:00 and 18:00 h, temperature 22 ± 1 °C, rat chow and water ad libitum). All experimental protocols were reviewed and approved by the Animal Welfare Committee at the Institute of Experimental Medicine of the Hungarian Academy of Sciences and Tufts New England Medical Center.

2.2. Animal preparation for double-labeling immunocytochemistry at light and electron microscopic levels

Since our preliminary data indicated that colchicine pretreatment is necessary for the optimal detection of CRH and TRH-IR perikarya in the PVN, and low dose colchicine does not alter the immunostaining of VGLUT2-IR fibers, we used colchicine-treated animals in our studies.

Eight animals were deeply anesthetized with sodium pentobarbital (35 mg/kg BW, ip) and injected intracerebroventricularly with 60 μg of colchicine in 3 μl 0.9% saline under stereotaxic control. After 20 h of survival, the animals were perfused transcardially with 20 ml 0.01 M phosphate-buffered saline (PBS), pH 7.4, followed sequentially by 100 ml of 2% paraformaldehyde/4% acrolein in 0.1 M phosphate buffer (PB), pH 7.4, and then by 50 ml of 2% paraformaldehyde in the same buffer. The brains were rapidly removed and stored in PBS, pH 7.4, for 24 h at 4 °C.

2.3. Light microscopic double-labeling immunocytochemistry for VGLUT2, CRH, and proTRH

For light microscopy, colchicine-treated brains from 6 animals were cryoprotected in 30% sucrose in PBS at 4 °C overnight and then frozen on powdered dry ice. Serial 16 μm (for immunofluorescence; n = 3) and 10 μm (for silver intensified NiDAB/DAB staining; n = 3) thick coronal sections through the PVN were cut on a freezing microtome and collected in cryoprotectant solution (30% ethylene glycol; 25% glycerol; 0.05 M phosphate buffer) and stored at −20 °C until use. Two series of sections from each brain were treated with 1% sodium borohydride in distilled water for 30 min and with 0.5% Triton X-100/0.5% H2O2 in PBS for 15 min. To reduce nonspecific antibody binding, the sections were treated with 2% normal horse serum in PBS for 20 min.

For immunofluorescence, the sections were incubated in guinea pig VGLUT2 antiserum (AB5907; Chemicon International, Temecula, CA, USA) at 1:1000 in PBS containing 2% normal horse serum and 0.2% sodium azide (antiserum diluent) for 2 days at 4 °C. After rinses in PBS, the sections were incubated in biotinylated donkey anti-guinea pig IgG (1:500; Jackson Immunoresearch Lab, West Grove, PA) followed by the avidin-biotin-peroxidase complex (ABC Elite; 1:1000; Vector Laboratories, Burlingame, CA) in 0.05 M Tris buffer for 1 h at room temperature. The sections were then rinsed in PBS and subjected to tyramide amplification (NEN, Boston, MA) for 1 h to intensify the immunoreactivity [1]. After further rinses, the sections were incubated in fluorescein avidin DCS (1:250, Vector) for 1 h. The sections were then transferred to rabbit anti-proTRH (178–199) antiserum (a kind gift from Éva Rédei, Northwestern University, Chicago, IL; diluted 1:2500) or to rabbit anti-CRH antiserum (Peninsula Laboratories, San Carlos, CA, USA; diluted 1:300) for 2 days at 4 °C. After rinses in PBS, sections were incubated in CY3-conjugated donkey anti-rabbit IgG (1:100, Jackson) for 2 h.

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The sections were mounted onto glass slides, coverslipped with Vectashield mounting medium (Vector Laboratories), and analyzed using a Radiance 2100 confocal microscope (Bio-Rad Laboratories, Hemel Hempstead, UK) using the following laser excitation lines: 488 nm for FITC, 543 nm for CY3 and dichroic/emission filters, 560 nm/500–530 nm for FITC, and
To confirm the results of double-labeling immunofluorescence using VGLUT2 antisera AB5907, we also performed double-labeling immunocytochemistry using a rabbit polyclonal VGLUT2 antisem (No. 135 102; Synaptic Systems, Göttingen, Germany). After pretreatment as described above, series of 10-μm sections were incubated in rabbit anti-VGLUT2 serum at 1:10,000 for 2 days at 4 °C. After rinses in PBS, the sections were incubated in biotinylated donkey anti-rabbit IgG for 2 h (1:500) and in ABC Elite complex (1:1000) for 1 h. The sections were then rinsed in PBS, and the immunoreaction product was developed with 0.05% diaminobenzidine (DAB), 0.15% nickel ammonium sulfate (Ni), and 0.005% H2O2 in 0.05 M Tris buffer, pH 7.6 (TB), and intensified using the Gallyas silver intensification technique to yield a black precipitate [38]. After visualization of VGLUT2 immunoreactivity, the sections were incubated in rabbit anti-proTRH (178–199) antisem diluted 1:20,000 or rabbit anti-CRH (C70, a kind gift from Dr. Paul E. Sawchenko) diluted 1:8,000 for 2 days at 4 °C. After washing in PBS, the tissue sections were incubated in donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch) and in ABC Elite complex (1:1000). The immunolabeling was visualized by 0.025% DAB and 0.0036% H2O2 in TB to yield a brown reaction product. Thus, the black, silver-intensified Ni-DAB-labeled VGLUT2-IR fibers and the brown, DAB-labeled proTRH-IR and CRH-IR elements could be easily distinguished in the same sections.

2.4. Electron microscopic double-labeling immunocytochemistry for VGLUT2, CRH, and proTRH

For electron microscopy, serial 25-μm-thick coronal sections were cut on a Vibratome through the rostro-caudal extent of the PVN and collected in PBS. The sections were treated with 1% sodium borohydride in 0.1 M PB for 30 min, followed by 0.5%H2O2 in PBS for 15 min. The sections were cryoprotected in 15% sucrose in PBS for 15 min at room temperature and in 30% sucrose in PBS overnight at 4 °C and then quickly frozen over liquid nitrogen to improve antibody penetration to the tissue. To reduce nonspecific antibody binding, the sections were treated with 2% normal horse serum in PBS for 20 min. Sections were incubated in the rabbit antisem to VGLUT2 (Synaptic Systems) at dilution 1:10,000 for 4 days at 4 °C, followed by biotinylated donkey anti-rabbit IgG (1:500; Jackson Immunoresearch) for 20 h at 4 °C and ABC Elite Complex (1:1000) for 1 h at room temperature. Immunoreactivity was detected in 0.025% DAB/0.0036% H2O2 in 0.05 M Tris buffer, pH 7.6. The sections were then placed into rabbit anti-proTRH 178–199 (1:8000) or rabbit anti-CRH (C70; diluted 1:3000) for 2 days at 4 °C. After rinsing in PBS and 0.1% cold water fish gelatin/1% bovine serum albumin (BSA) in PBS, the sections were incubated in goat anti-rabbit IgG conjugated with 0.8 nm colloidal gold (Electron Microscopy Sciences, Fort Washington, PA) diluted at 1:100 in PBS containing 0.1% cold water fish gelatin and 1% BSA. The sections were washed in the same diluent and PBS, followed by a 10-min treatment in 1.25% glutaraldehyde in PBS. After rinsing in 0.2 M sodium citrate, pH 7.5, the gold particles were silver intensified with IntenSE Kit (Amersham-Pharmacia Biotech UK, Buckinghamshire, UK) [8]. Sections were then osmicated, treated with 2% uranyl acetate in 70% ethanol for 30 min, dehydrated in an ascending series of ethanol followed by propylene oxide, flat embedded in Durcupan ACM epoxy resin (Fluka) on liquid release agent (Electron Microscopy Sciences)-coated slides, and polymerized at 56 °C for 2 days. Ultrathin 50–60 nm thin sections were cut with Leica ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany), collected onto Formvar-coated single slot grids, and examined with a JEOL electron microscope.

2.5. Specificity of antisera

The specificity of the Chemicon AB5907 VGLUT2 antisem has been demonstrated by Hrabovszky et al. [28] using the same immunofluorescent method. Preincubation of the working dilution of rabbit VGLUT2 antisem from Synaptic Systems with 10−6 M VGLUT2 control protein (a purified recombinant GST fusion protein provided by Synaptic Systems, Cat No. 135-1P) resulted in a total loss of immunolabeling. The specificity of antisera to CRH and proTRH (178–199) has been reported earlier [16,42,47].

3. Results

3.1. VGLUT2-immunoreactive (IR) innervation of CRH-IR and proTRH-IR neurons in the PVN

Immunolabeling by both VGLUT2 antisera showed that VGLUT2-IR axons homogeneously and densely innervated all parvocellular subdivisions of the PVN, while a slightly lower density of labeled fibers was seen in the magnocellular division. VGLUT2-IR axon varicosities were frequently juxtaposed to the perikarya and proximal dendrites of all CRH-IR neurons located in the medial parvocellular subdivision of the PVN (Figs. 1A–C and 2A, B). Similarly, in each of the major parvocellular subdivisions where proTRH neurons were identified, including the anterior, periventricular, and medial parvocellular subdivisions, numerous VGLUT2-IR axon varicosities were closely apposed to cell bodies and proximal dendrites of all proTRH neurons (Figs. 1D–1 and 2C–F). The innervation patterns were highly similar with both VGLUT2 antisera.

Ultrastructurally, VGLUT2-IR nerve terminals were observed to contain electron-dense DAB reaction product
associated with small clear vesicles. These terminals established close membrane appositions with both CRH-IR and proTRH-IR perikarya and dendrites, recognized by the presence of highly electron-dense deposits of immunogold-silver particles distributed throughout the labeled structures. Tracing the juxtaposed VGLUT2-IR terminals with CRH-IR and proTRH-IR neurons through a series of ultrathin sections demonstrated synaptic specializations on the perikarya and dendrites of both cell types (Figs. 3 and 4).

4. Discussion

Until recently, the morphological examination of the glutamatergic system has been difficult due to the lack of marker molecules specific to the glutamatergic phenotype of neurons. Two highly homologous transmembrane proteins, vesicular glutamate transporter 1 and 2, have now been proven specific for glutamatergic neurons based on the following observations. First, both VGLUT1 and VGLUT2 specifically transport glutamate into synaptic vesicles [3,6,22,25,26,53,54,57]. Second, both transporters are associated with synaptic vesicles in nerve endings that establish asymmetric synapses [5,22,26]. Third, transfection of VGLUT1 and VGLUT2 into non-glutamatergic neurons is sufficient to allow the synaptic release of glutamate [53,54]. A third member of the VGLUT family (VGLUT3) has also been recognized, however, the presence of VGLUT3 in terminals establishing inhibitory synapses [51] raises the possibility that VGLUT3 is not an appropriate marker of glutamatergic elements.

Using antisera specific to VGLUT1 and VGLUT2, we observed in preliminary studies that VGLUT2-IR nerve fibers in the PVN far exceeded those containing VGLUT1. Therefore, we used antisera against VGLUT2 in this study to elucidate the glutamatergic innervation of CRH and TRH neurons in the PVN.

VGLUT2-IR boutons established close contacts with all CRH neurons in the medial parvocellular subdivision of the PVN. Similarly, all TRH-containing neurons were contacted by VGLUT2-IR varicosities in the anterior, periventricular, and medial parvocellular subdivisions. In case of both CRH and TRH-IR neurons, VGLUT2-IR axon terminals established contacts with their somata and first order dendrites, and TRH-IR neurons, VGLUT2-IR axon terminals established contacts with their somata and first order dendrites, and TRH neurons in the medial parvocellular subdivision of the PVN.

Our findings that CRH and TRH neurons are densely innervated by glutamatergic fibers suggest that glutamate may have a direct role in the regulation of both CRH and TRH neurons in the PVN. Since many of these neurons are hypophysiotropic, particularly neurons in the medial and periventricular parvocellular subdivisions of the PVN [30,32], glutamate may be involved in the regulation of the release of CRH and TRH into the hypophyseal portal circulation. This hypothesis is supported by the observation that when glutamate is directly injected into the PVN, it increases plasma ACTH and corticosterone levels [13,20], and that corticosterone responses to restraint stress can be attenuated by bilateral injection of an ionotropic glutamate receptor antagonist into the PVN [58]. In addition, central administration of ionotropic glutamate receptor agonists increase, while antagonists decrease serum TSH levels [2]. Collectively, these data raise the possibility that glutamate may be a major driving force for CRH and TRH secretion in the basal state, and contribute to increased release of these peptides under conditions known to activate the HPA and HPT axes. Unfortunately, there are very few data about the physiological importance of the glutamatergic stimulation of CRH and TRH neurons in the PVN. So far, two mechanisms have been investigated that regulate PVN neurons by changing the intensity of the glutamatergic input. Di et al. [14] have proposed that glucocorticoids suppress the excitatory input to PVN neurons by activating a membrane receptor on parvocellular PVN neurons that leads to the release of an endocannabinoid which retrogradely acts on presynaptic glutamate terminals to inhibit glutamate release. Thus, it is conceivable that the rapid inhibition of CRH release by glucocorticoids could be mediated, at least in part, by the decrease of glutamatergic transmission [33].

The second mechanism pertains to the modulation of magnocellular neurosecretory cells in the PVN by glutamate. Daftary et al. [11] found that noradrenaline increases the frequency of spontaneous EPSPs on magnocellular oxytocinergic and vasopressinergic neurons acting via α1-adrenoreceptors on the perikarya/dendrites of glutamatergic neurons within the PVN that send projections to magnocellular neurons. Similar effects of noradrenaline were observed on 33% of recorded parvocellular neurons in the medial parvocellular subdivision of the PVN, although the location of the glutamatergic interneurons has not been revealed [12]. Were CRH and TRH neurons contained within this subset of responsive parvocellular neurons, then the stimulation of CRH and TRH release by noradrenaline via α1-receptor activation may involve the local release of glutamate from interneurons [23,45]. This hypothesis is
Fig. 2. VGLUT2-IR innervation of CRH- and proTRH-IR neurons in the PVN as revealed by using the Synaptic Systems 135 102 rabbit VGLUT2 antiserum. (A) Low power micrograph shows the association of VGLUT2-IR axons (black) with CRH containing neurons (brown perikarya) at the mid-level of the PVN. (B) Higher magnification image shows VGLUT2-IR axon varicosities in juxtaposition to CRH-IR neurons in the medial parvocellular subdivision. (C) Association of VGLUT2-IR axons with proTRH containing neurons at the caudal level of the PVN. Higher magnification images demonstrate VGLUT2-IR axon terminals closely apposed to proTRH-IR neurons in the anterior (D), periventricular (E), and medial (D) parvocellular subdivisions of the PVN. Arrows indicate the interactions between the labeled profiles. III, third ventricle. Scale bars in A and C: 100 μm; in B and D: 20 μm; scale bar in D corresponds to D–F.
Fig. 3. Electron micrographs demonstrate axosomatic (A) and axodendritic (B) synaptic contacts (arrows) between VGLUT2-containing axon terminals and CRH-IR neurons in the PVN. VGLUT2 immunoreactivity is labeled by the DAB precipitate, while CRH immunoreactivity is labeled by the deposits of immunogold-silver particles. Scale bars: A, 1 μm; B, 1 μm.

Fig. 4. Electron micrographs showing synapses (arrows) established by VGLUT2-containing axon terminals on the perikaryon of a proTRH-IR neuron (A). (B and C) Higher magnification images of the axosomatic synapses seen on A. (D) VGLUT2-IR axons establish axodendritic synapses on a distal proTRH-containing dendrite in the PVN. VGLUT2 immunoreactivity is labeled by DAB precipitate, while proTRH content is identified by the deposits of immunogold-silver particles. Scale bars (shown in A): A, 1 μm; (shown in B): B–D, 1 μm.
further supported by the observation that injection of ionotropic glutamate receptor antagonists into the PVN prevents the increase in ACTH and corticosterone levels evoked by \(\alpha_2\)-receptor agonist injection into the PVN or stimulation of the ventral noradrenergic bundle [21].

The origin of the glutamatergic innervation to CRH and TRH neurons in the PVN is yet unknown. However, electrophysiological experiments have identified glutamatergic inputs to the PVN arising from the hypothalamus including the suprachiasmatic nucleus [25], dorsomedial hypothalamus, anterior hypothalamic nucleus, perifornical area [7], and the PVN itself [11]. These sites have recently been confirmed by Csáki et al. [10] using \(^{3}H\)-d-aspartate stereotaxically injected into the PVN. Furthermore, retrogradely labeled neurons were also identified in all hypothalamic nuclei and areas known to send afferents to the PVN, and outside the hypothalamus, including the paraventricular nucleus of the thalamus, lateral septum, bed nucleus of the stria terminalis, and the amygdala [10]. Bartanusz et al. [4] have found that in organotypic PVN cultures, a local glutamate circuit is present intrinsic to or in very close proximity to the PVN that regulates CRH release and neuronal activity in the area where the hypophysiotropic CRH neurons are located. Since recent data from our laboratory demonstrated the glutamatergic nature of hypophysiotropic CRH and TRH neurons [29], and CRH/CRH, TRH/TRH contacts and interconnection of CRH and TRH elements exist in the PVN [27,37,55], we hypothesize that glutamate may be an important transmitter in these local connections.

Another possible source of the glutamatergic afferents to the CRH and TRH neurons is the cocaine- and amphetamine-regulated transcript (CART)/pro-opiomelanocortin (POMC) neuron population in the arcuate nucleus. The presence of VGLUT2 protein has been reported in these neurons [9], and earlier studies from our laboratories have demonstrated that CART/POMC neurons densely innervate hypophysiotropic CRH and TRH neurons [16,18,39]. CART/POMC neurons of the arcuate nucleus express leptin receptors and are stimulated by leptin [24,35,44]. Furthermore, both CART and \(\alpha\)-MSH stimulate HPA and HPT axes [16–18,50,52], leading us to propose that CART/POMC neurons of the arcuate nucleus are involved in the regulation of hypophysiotropic CRH and TRH neurons by leptin. The glutamatergic phenotype of the CART/POMC neurons raises the possibility that leptin may also stimulate the release of glutamate from the CART/\(\alpha\)-MSH terminals in the PVN and, therefore, glutamate may contribute to the stimulatory effects of leptin on CRH and TRH synthesis in the PVN.

Using VGLUT2 as a marker, we conclude that CRH- and TRH-synthesizing neurons in the PVN are heavily innervated by the glutamatergic nerve fibers. Glutamate, therefore, may play an important role in the regulation of the hypothalamic–pituitary–adrenal and hypothalamic–pituitary–thyroid axes.

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