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Presence of vesicular glutamate transporter-2 in hypophysiotropic somatostatin but not growth hormone-releasing hormone neurons of the male rat

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Abstract

Recent evidence indicates that hypophysiotropic gonadotropin-releasing hormone (GnRH), corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) neurons of the adult male rat express mRNA and immunoreactivity for type-2 vesicular glutamate transporter (VGLUT2), a marker for glutamatergic neuronal phenotype. In the present study, we investigated the issue of whether these glutamatergic features are shared by growth hormone-releasing hormone (GHRH) neurons of the hypothalamic arcuate nucleus (ARH) and somatostatin (SS) neurons of the anterior periventricular nucleus (PVa), the two parvicellular neurosecretory systems that regulate anterior pituitary somatotrophs. Dual-label *in situ* hybridization studies revealed relatively few cells that expressed VGLUT2 mRNA in the ARH; the GHRH neurons were devoid of VGLUT2 hybridization signal. In contrast, VGLUT2 mRNA was expressed abundantly in the PVa; virtually all (97.5 ± 0.4%) SS neurons showed labelling for VGLUT2 mRNA. In accordance with these hybridization results, dual-label immunofluorescent studies followed by confocal laser microscopic analysis of the median eminence established the absence of VGLUT2 immunoreactivity in GHRH terminals and its presence in many neurosecretory SS terminals. The GHRH terminals, in turn, were immunoreactive for the vesicular γ-aminobutyric acid (GABA) transporter, used in these studies as a marker for GABA-ergic neuronal phenotype. Together, these results suggest the paradoxic cosecretion of the excitatory amino acid neurotransmitter glutamate with the inhibitory peptide SS and the cosecretion of the inhibitory amino acid neurotransmitter GABA with the stimulatory peptide GHRH. The mechanisms of action of intrinsic amino acids in hypophysiotropic neurosecretory systems require clarification.

Introduction

L-Glutamate is the major excitatory neurotransmitter in the central nervous system (Monaghan *et al*., 1989; Collingridge & Singer, 1990; Headley & Grillner, 1990), whereas γ-aminobutyric acid (GABA) functions as the primary mediator of inhibitory synaptic transmission (Decavel & Van den Pol, 1990). The palisade zone of the hypothalamic median eminence (ME) represents the termination field for hypophysiotropic axons which secrete releasing and release-inhibiting hormones into the pericapillary space of the hypophysial portal system. This region receives a dense GABA-ergic innervation (Meister & Hokfelt, 1988); moreover, the marker enzyme for GABA, glutamic acid decarboxylase, has been localized specifically to growth hormone-releasing hormone (GHRH)-, tyrosine hydroxylase (dopaminergic marker)-, neurotensin- and galanin-immunoreactive (IR), but not to somatostatin (SS)-IR hypophysiotropic axon terminals (Meister & Hokfelt, 1988). The hypothalamus also hosts a large number of glutamatergic cell bodies that express the mRNA for type-2 vesicular glutamate transporter (VGLUT2) selectively (Herzog *et al*., 2001; Takamori *et al*., 2001; Lin *et al*., 2003). A subset of these VGLUT2-IR neurons are located in hypophysiotropic regions and innervate the ME (Lin *et al*., 2003; Varoqui *et al*., 2002); recent studies have shown that glutamatergic fibres of the ME are partly identical with hypophysiotropic gonadotropin-releasing hormone (GnRH; Hrabovszky *et al*., 2004b)-, thyrotropin-releasing hormone (TRH; Hrabovszky *et al*., 2005)- and corticotropin-releasing hormone (CRH; Hrabovszky *et al*., 2005)-IR terminals.

The putative cosecretion of glutamate from additional hypophysiotropic neurosecretory systems, including GHRH and SS neurons, has not been addressed. The synthesis of growth hormone (GH) and its release from the somatotrophs is under the dual control of the stimulatory GHRH and the inhibitory SS; these two peptide neurohormones are synthesized in neuronal perikarya located in the arcuate and the anterior periventricular nuclei (ARH; PVa), respectively (Tannenbaum *et al*., 1990). In the present studies, we used dual-label *in situ* hybridization histochemistry (ISHH) to address the expression of VGLUT2 mRNA in the perikaryon of hypophysiotropic GHRH and/or SS neurons. In addition, we conducted dual-label immunofluorescent studies to investigate VGLUT2 immunoreactivity in GHRH and/or SS axon terminals of the ME. Based on a previous report indicating that hypophysiotropic GHRH neurons might be GABAergic (Meister & Hokfelt, 1988), instead of glutamatergic, we also examined the putative occurrence of the vesicular GABA transporter (VGAT) in GHRH terminals. Although VGAT is used in common for vesicular neurotransmitter uptake by glycinergic and GABAergic inhibitory neurons, it represents an authentic GABA marker within hypophysiotropic GHRH terminals which originate in the ARH, a

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region devoid of glycinergic (Zeilhofer *et al*., 2005) and rich in GABAergic (Meister & Hokfelt, 1988) cell bodies.

Recently, we have used similar *in situ* hybridization and immunocytochemical approaches to demonstrate the occurrence of VGLUT2 mRNA and protein, respectively, in the hypophysiotropic GnRH, TRH and CRH neurosecretory systems (Hrabovszky *et al*., 2004b, 2005).

Materials and methods

Animals

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 available *ad libitum*. Experimental procedures were approved by the Animal Welfare Committee at the Institute of Experimental Medicine.

In situ *hybridization studies*

Tissue preparation

Four rats were decapitated and their brains snap-frozen on powdered dry ice. Then 12-μm thick coronal sections through the PVa and the ARH regions were cut with a Leica CM 3050 S cryostat, collected serially on gelatin-coated microscope slides and processed for dual-label ISHH studies using procedures adapted from recent work (Hrabovszky *et al*., 2004a). Sections from the PVa were processed for dual-label ISHH detection of SS and VGLUT2 mRNAs, whereas sections from the ARH were used for the simultaneous visualization of GHRH and VGLUT2 mRNAs.

Probe preparation

The preparation of ^{35}S-labelled antisense and sense 'VGLUT2-879' cRNA probes (targeted to bases 522–1400 of rat VGLUT2 mRNA; GenBank Acc. # NM053427) has been detailed elsewhere (Hrabovszky *et al*., 2004b). The 514-bp cDNA template for *in vitro* transcription of a digoxigenin-labelled SS probe to bases 3–516 of the rat somatostatin mRNA (GenBank Acc. # M2589) was cloned with polymerase chain reaction from rat hypothalamic cDNA using the TOPO TA Cloning® kit from Invitrogen (Carlsbad, CA, USA). The plasmid containing the SS amplicon was grown in TOPO cells (Invitrogen), isolated with the QIAfilter Plasmid Maxi kit (Qiagen; Valencia, CA, USA) and digested at the BamHI restriction site. The linearized transcription template was purified with phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol extractions, precipitated with NaCl and ethanol and reconstituted. The digoxigenin-labelled cRNA probe was transcribed with T7 RNA polymerase in the presence of digoxigenin-11-UTP (Roche Diagnostics Co., Indianapolis, IN, USA), as described previously (Petersen & McCrone, 1994; Hrabovszky *et al*., 2004a). A pGEM 4 plasmid containing bases 285–489 of the rat GHRH cDNA (GenBank Acc. # M73486) was kindly provided by Dr R.A. Steiner (University of Washington, Seattle, WA, USA) for the generation of the digoxigenin-labelled antisense GHRH probe. The vector was linearized at the *EcoR*I site and transcribed with T7 RNA polymerase.

Dual-label ISHH

Prehybridization, hybridization and posthybridization procedures have been adapted from similar studies that investigated VGLUT2 mRNA expression by hypophysiotropic GnRH, TRH and CRH neurons (Hrabovszky *et al*., 2004b, 2005). Based on a recently introduced methodological approach (Hrabovszky & Petersen, 2002), hybridization sensitivity for VGLUT2 has been enhanced by applying high radioisotopic probe (80 000 c.p.m./mL), dextran sulphate (25%) and dithiothreitol (1000 mM) concentrations in the hybridization solution and extending the hybridization time from 16 to 40 h. Following posthybridization treatments (Hrabovszky *et al*., 2004a), the digoxigenin-labelled cRNA probe to GHRH or SS mRNA was detected immunocytochemically using sequential incubations with anti-digoxigenin antibodies conjugated to horseradish peroxidase (1 : 200; Roche) for 48 h, biotin tyramide amplification solution for 1 h, and ABC Elite working solution (Vector; Burlingame, CA, USA; 1 : 1000 dilution of solutions 'A' and 'B' in TBS) for 1 h. The signal was visualized with diaminobenzidine chromogen in the peroxidase developer. Subsequently, the ^{35}S-labelled cRNA probe to VGLUT2 mRNA was visualized on Kodak NTB-3 autoradiographic emulsion following 2 weeks of exposure. To confirm VGLUT2 hybridization specificity in positive control experiments, the 'VGLUT2-879' probe was replaced with the 'VGLUT2-734' probe kindly provided by Dr J. P. Herman (Ziegler *et al*., 2002; Hrabovszky *et al*., 2004b, 2005), targeting a nonoverlapping segment (bases 1704–2437) of VGLUT2 mRNA. Negative control experiments for VGLUT2 hybridization specificity (Hrabovszky *et al*., 2004b, 2005) were conducted with the combined use of the nonisotopic antisense SS probe and the radioisotopically labelled sense-strand VGLUT2-879 transcript.

Dual-label immunofluorescent studies of VGLUT2 in GHRH and SS axons

To analyse the putative occurrence of VGLUT2 immunoreactivity in hypophysiotropic GHRH and SS axons, four rats were anaesthetized with pentobarbital (35 mg/kg body weight, i.p.) and perfused transcardially with 150 mL fixative solution containing 2% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA) and 4% acrolein (Aldrich Chemical Co., Milwaukee, WI, USA) in 0.1 M phosphate-buffered saline (PBS; pH 7.4). Tissue blocks were dissected out and infiltrated with 25% sucrose overnight. Then 20-μm-thick free-floating coronal sections were prepared through the mediobasal hypothalami with a cryostat. The sections were rinsed in Tris-buffered saline (TBS; 0.1 M Tris-HCl/0.9% NaCl; pH 7.8). Free aldehydes were inactivated with 0.5% sodium borohydride (Sigma; 30 min) and the tissues permeabilized and blocked against nonspecific antibody binding with a mixture of 0.2% Triton X-100 (Sigma) and 2% normal horse serum in TBS (30 min). Following pretreatments, two-thirds of the sections were incubated in anti-VGLUT2 primary antibodies raised in guinea pig (AB 5907; Chemicon; Temecula, CA, USA; 1 : 1000) for 72 h at 4 °C, then in biotinylated anti-guinea pig IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1 : 1000) for 2 h, and in streptavidine-conjugated Cy3 fluorochrome (Jackson ImmunoResearch; 1 : 200) for 12 h. Half of these sections were used to detect GHRH using sheep primary antibodies (FMS/FJL #19–4; 1 : 30 000; kind gift from Dr I. Merchenthaler; University of Maryland, School of Medicine, Baltimore, MD, USA). This antiserum was applied to the sections for 48 h at 4 °C and then, reacted with FITC-conjugated antisheep IgG (Jackson ImmunoResearch; 1 : 200) for 12 h at room temperature. The second pool of ME sections already immunostained for VGLUT2 was used for the detection of SS-IR neuronal elements. The SS antiserum (a kind gift from Dr A. J. Harmar, School of Biomedical and Clinical Laboratory Sciences, Edinburgh, UK) was generated in rabbit (R12, 1 : 4000) and recognized both the SS14 and SS28 molecular forms of SS (Pierotti

& Harmar, 1985). The primary antibodies were applied to the sections for 48 h at 4 °C and then reacted with FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch, 1 : 200) for 12 h at 4 °C. The remaining third of the mediobasal hypothalamic sections was used in control experiments and in dual-labelling studies to localize VGAT in the axon terminals of GHRH neurons. The dual-immunofluorescent procedure, used to demonstrate the GABAergic marker in GHRH terminals, was performed as described above for the VGLUT2/GHRH double-labelling, except by substituting the VGLUT2 antibodies with VGAT antibodies that were also raised in a guinea pig (AB5855; Chemicon; 1 : 2000). The fluorescently labelled specimen 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. Individual optical slices were collected for the analysis in 'lambda strobing' mode. This way, only one excitation laser and the corresponding emission detector were active during a line scan, to eliminate emission crosstalk.

Colocalization was assessed using a 60 × objective lens with immersion oil and an optimized pinhole, allowing optical slices below 0.7 μm (Hrabovszky *et al*., 2004b, 2005). Control experiments to prove the specificity of VGLUT2 immunolabelling included preabsorption of primary antibodies with 10 μM of the immunization antigen (AG209; Chemicon). In addition, the AB5907 guinea pig anti-VGLUT2 antibodies from Chemicon were used in combination with the rabbit anti-VGLUT2 antibodies from SYnaptic SYstems (AB 135103; 1 : 5000; Göttingen, Germany) in dual-immunofluorescent experiments. The primary antibodies raised in different species were reacted with secondary antibody-fluorochrome conjugates, which resulted in dual-immunofluorescent labelling of identical axonal profiles (Hrabovszky *et al*., 2004b, 2005).

Results

In situ *hybridization results*

The nonisotopic ISHH procedure visualized numerous GHRH mRNA-expressing neurons in the ARH (Fig. 1A) and SS mRNA-expressing neurons in the PVa (Fig. 1B). The development of emulsion autoradiographs exposed for 2 weeks resulted in the heavy accumulation of grain clusters in several diencephalic nuclei, including the ventromedial hypothalamic nucleus (VMH; Fig. 1A) and the PVa (Fig. 1B). The ARH contained only few VGLUT2 neurons, most of which were localized laterally within the nucleus. These glutamatergic cells were labelled lightly or moderately and their distribution overlapped with the area also containing GHRH neurons. Nevertheless, microscopic analysis of every third section through the rostro-caudal extent of the ARH of each of four rats found no evidence for the coexpression of GHRH and VGLUT2 mRNAs at this level of detection sensitivity (Fig. 1A). In contrast, silver grain clusters clearly distinct from the homogeneous background grains were associated with most SS neurons in the PVa (Fig. 1B). The analysis of 787 SS neurons (from two representative PVa sections of each of four rats) showed hybridization signal (accumulation of silver grains) for VGLUT2 mRNA in 97.5 ± 0.4% of SS neurons. The periventricular and medial parvicellular (high-power inset in Fig. 1B) subdivisions of the paraventricular nucleus (PVH) contained a further large population of SS neurons. Of 688 neurons analysed in these subnuclei (from two PVH sections of each of four rats), 96.0 ± 1.4% were clearly dual-labelled. The series of sections hybridized for SS and VGLUT2 mRNAs also included additional populations of SS neurons in the suprachiasmatic nucleus and the rostral-most part of the ARH. No VGLUT2 hybridization signal was associated with these nonhypophysiotropic SS neurons. Detection of dual-labelled SS neurons using a distinct VGLUT2 probe ('VGLUT2-734') and lack of autoradiographic signal (grain clustering) using the sense VGLUT2 transcipt provided evidence for hybridization specificity, corroborating the results of previously used control experiments (Hrabovszky *et al*., 2004b, 2005).

Immunocytochemical results

The immunocytochemical studies detected a high density of glutamatergic axons in the external zone of the ME (Fig. 1C and E); here the glutamatergic axons intermingled with peptidergic terminals containing GHRH (Fig. 1C) and SS (Fig. 1E). High-power confocal microscopic analysis found no evidence for a colocalization of VGLUT2 and GHRH immunoreactivities (Fig. 1C), whereas the GHRH terminals often contained immunoreactivity for the GABAergic marker, VGAT (Fig. 1D). In contrast with the absence of VGLUT2 from GHRH-IR axons, many of the SS-IR terminals contained VGLUT2 immunoreactivity (Fig. 1E), in accordance with the ISHH observation of VGLUT2 mRNA synthesis in SS perikarya of the PVa and the PVH. Omission of primary antibodies eliminated all labelling from the ME. Additional controls studies detailed elsewhere (Hrabovszky *et al*., 2004b, 2005) confirmed specificity of the immunocytochemical labelling for the VGLUT2 protein.

Discussion

In this report we present ISHH and immunocytochemical evidence that GHRH neurons, unlike neurons of the hypophysiotropic GnRH, TRH and CRH systems, do not appear to synthesize VGLUT2 mRNA and protein; instead, we found that their neurosecretory terminals contain immunoreactivity for the GABAergic marker, VGAT. In contrast with GHRH neurons, nearly all of the cell bodies of hypophysiotropic SS neurons in the PVa and in the medial parvicellular subdivision of the PVH express VGLUT2 mRNA and their projections to the ME contain immunoreactivity for VGLUT2. These observations indicate the capability of the inhibitory SS neurosecretory system to cosecrete the excitatory amino acid neurotransmitter, L-glutamate.

Glutamate is an important regulator of anterior pituitary functions, including regulation of GH synthesis and secretion (Brann, 1995). Subcutaneous *N*-methyl-DL-aspartic acid or kainate injections to adult male rats increases serum GH levels (Mason *et al*., 1983) and similar stimulatory effects have also been observed in other species (Estienne *et al*., 1989; Shahab *et al*., 1993). Some of the actions of glutamate on the somatotropic axis may also be exerted at the hypophysial level. Ionotropic and metabotropic glutamate receptors are expressed by anterior pituitary cells (Bhat *et al*., 1995; Caruso *et al*., 2004) and *N*-methyl-D-aspartic acid (NMDA), kainate and glutamate can stimulate dose-dependently GH secretion from perifused somatotrophs (Lindstrom & Ohlsson, 1992; Niimi *et al*., 1994). Several lines of evidence also indicate that glutamate exerts central actions on GH secretion in which GHRH neurons play a crucial role. In accordance with this idea, the *N*-methyl-D,L-aspartic acid-induced GH release can be blocked by antibodies to GHRH or prevented by the ablation of the ARH where the hypophysiotropic GHRH neurons reside (Acs *et al*., 1990). In further support of the concept that endogenous glutamate stimulates the GHRH neurosecretory system, systemic treatment of rats with an NMDA receptor antagonist can reduce hypothalamic

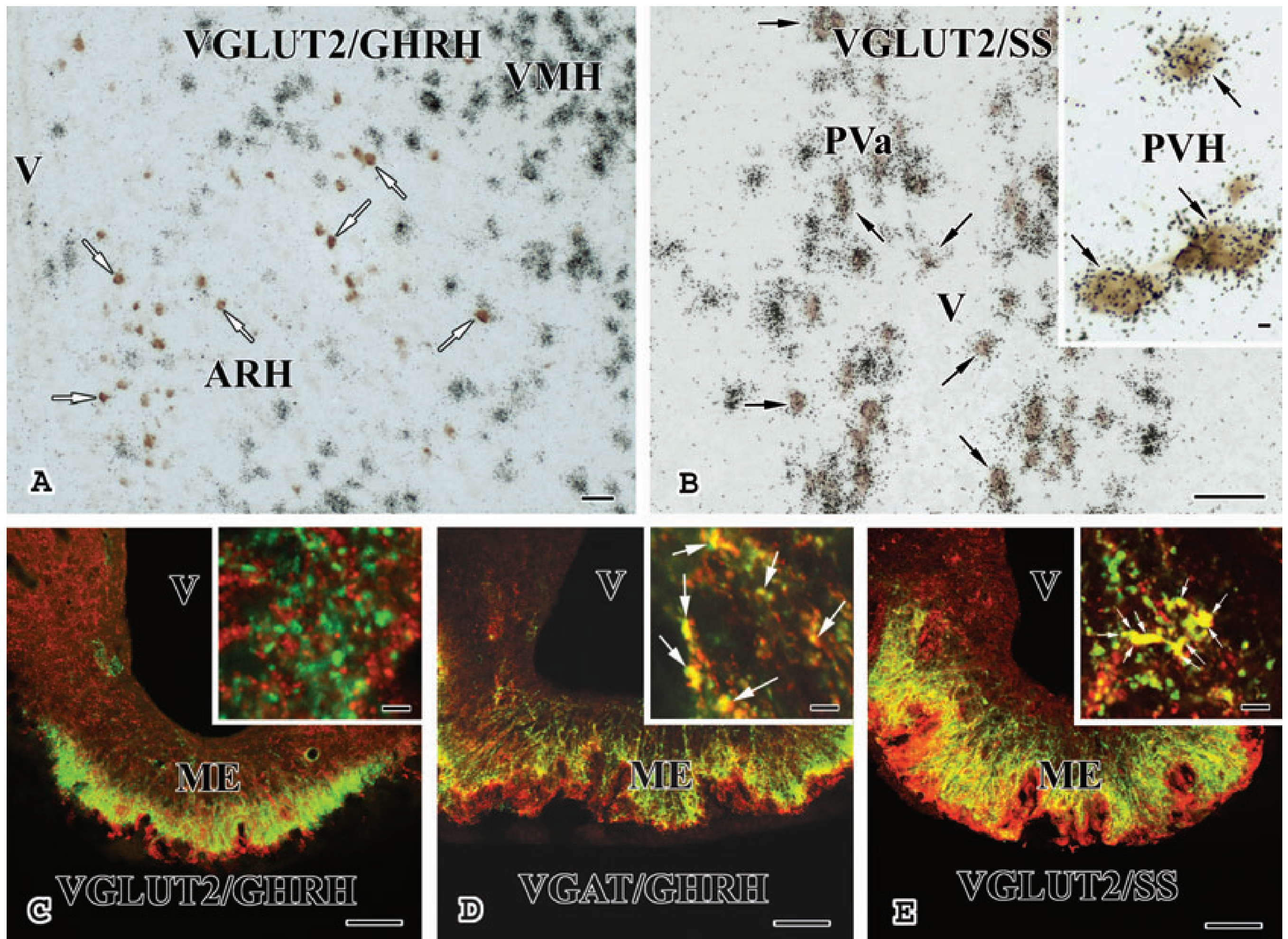

FIG. 1. Morphological evidence for the the glutamatergic phenotype of somatostatine (SS) secreting and the GABA-ergic phenotype of growth hormone-releasing hormone (GHRH) secreting hypophysiotropic neurons. (A) Results of dual-label *in situ* hybridization studies show heavy expression levels of VGLUT2 mRNA (autoradiographic grain clusters) in the ventromedial hypothalamic nucleus (VMH), whereas the arcuate nucleus (ARH) comprising hypophysiotropic GHRH neurons (brown DAB chromogen) contains relatively few and lightly labelled glutamatergic neurons. The GHRH cells (white arrows) are devoid of the autoradiographic hybridization signal for VGLUT2 mRNA. (B) The anterior periventricular nucleus (PVa) shows high levels of VGLUT2 mRNA expression on the two sides of the third cerebral ventricle (V). Virtually all hypophysiotropic somatostatin (SS) neurons (brown DAB chromogen) in this nucleus as well as in the medial parvicellular subdivision of the hypothalamic paraventricular nucleus (PVH; high-power inset) express VGLUT2 mRNA, as indicated by the presence of silver grain clusters. Black arrows point to dual-labelled SS neurons. (C) Confocal laser microscopic analysis of the median eminence (ME) shows the overlapping distribution of GHRH-immunoreactive (IR; green colour) and VGLUT2-IR (red colour) terminals in the external layer of the ME. High-power image (inset in right upper corner) of a 0.45-μm single optical slice demonstrates that VGLUT2 fibres are distinct from GHRH fibres. (D) In contrast, the vesicular GABA transporter (VGAT; red colour), used as a GABAergic marker, is often detectable in GHRH-IR axons (green colour). Arrows in high-power inset point to dual-labelled VGAT/GHRH axon varicosities (yellow colour). (E) Somatostatin-IR terminals (green colour) in the external layer of the ME exhibit an overlapping distribution with that of VGLUT2-IR (red colour) terminals. Arrows in high-power inset reveal that SS terminals cocontain immunoreactivity for VGLUT2 (yellow colour). Scale bars, 5 μm (B–E insets); 50 μm (other panels).

GHRH mRNA expression in the ARH and GHRH immunoreactivity in the ME (Cocilovo *et al*., 1992).

The central glutamatergic regulation of different neurosecretory systems, including GHRH and SS neurons, likely involves synaptic mechanisms. In addition, the dense VGLUT2-IR axon plexus observed recently in the external zone of the ME (Lin *et al*., 2003; Hrabovszky *et al*., 2004b, 2005) also raises the possibility that central glutamatergic pathways may directly act on the axon terminals of hypophysiotropic neurons. Confocal microscopic studies of the ME have revealed that many of these glutamatergic fibres are identical with the neurosecretory terminals of GnRH, TRH and CRH neurons (Hrabovszky *et al*., 2004b, 2005). To pursue the neurochemical characterization of glutamatergic axons in the ME, in the present study we investigated the putative occurrence of VGLUT2 in hypophysiotropic GHRH and SS neurons. The results of ISHH and immunocytochemical experiments established that, of these two systems, SS but not GHRH neurons are glutamatergic.

The functional importance of endogenous glutamate release from distinct types of hypophysiotropic neurosecretory systems will be difficult to determine. We found that at least four neuropeptidergic phenotypes (GnRH, TRH, CRH and SS) contribute to glutamate release in the ME and experimental tools are currently unavailable to separately manipulate the excitatory amino acid output from each of these systems. From a functional point of view, it seems more likely that glutamate acts locally in the ME, rather than influencing adenohypophysial cells as a hypophysiotropic factor. The findings that glutamatergic agents increase plasma ACTH levels *in vivo* (Makara & Stark, 1975; Farah *et al*., 1991; Jezova *et al*., 1991;

Chautard *et al.*, 1993) but do not elicit ACTH release from incubated pituitaries (Chautard *et al.*, 1993), indicate that hypophysial actions do not play a major role in the glutamatergic regulation of the adrenal axis. There is evidence that central effects also dominate in the glutamatergic regulation of the gonadotropic axis; glutamate can elevate serum luteinizing hormone levels when injected into the third cerebral ventricle (Ondo *et al.*, 1976), whereas neither its hypophysial injection (Ondo *et al.*, 1976) nor its *in vitro* application to pituitary culture (Tal *et al.*, 1983) can stimulate luteinizing hormone secretion. Despite the existing evidence that glutamate can act directly on somatotrophs (Lindstrom & Ohlsson, 1992; Niimi *et al.*, 1994), its central actions through GHRH neurons appear to be dominant (Acs *et al.*, 1990).

The target cells to the actions of glutamate in the ME and the receptorial mechanisms involved, are unclear. It is reasonable to speculate that the release of endogenous glutamate exerts autocrine actions or paracrine effects on hypophysiotropic neurosecretory axon terminals. The existence of autocrine/paracrine glutamatergic mechanisms in the central regulation of GnRH secretion received substantial support from: (i) the capability of ionotropic glutamate receptor agonists to elicit GnRH release from the mediobasal hypothalami (Donoso *et al.*, 1990; Lopez *et al.*, 1992; Arias *et al.*, 1993; Zuo *et al.*, 1996; Kawakami *et al.*, 1998a); (ii) the identification of immunoreactivity for the KA2 and the NR1 ionotropic glutamate receptor subunits on GnRH terminals (Kawakami *et al.*, 1998a, b); and (iii), our recent observation that GnRH neurons contain VGLUT2, an indication for glutamate release from endogenous stores (Hrabovszky *et al.*, 2004b). However, the putative presence and actions of glutamate receptors on SS containing and other types of neuroendocrine terminals remain to be established. Glutamate might also affect the glial and endothelial cell functions in the microenvironment of release. In strong support of this idea, tanycytes lining the ventral wall of the third ventricle and astrocytes in the ME were found to contain mRNAs and immunoreactivity for kainate receptors (Diano *et al.*, 1998; Eyigor & Jennes, 1998; Kawakami, 2000) and to express c-Fos immunoreactivity in response to stimulation by kainate (Eyigor & Jennes, 1998). Although somewhat controversial (Morley *et al.*, 1998), evidence also exists for the presence of functional metabotropic (Krizbai *et al.*, 1998; Gillard *et al.*, 2003) and ionotropic (Krizbai *et al.*, 1998; Parfenova *et al.*, 2003) glutamate receptors on cerebral microvascular endothelial cells. Endothelial cells in the ME have been implicated in the generation of nitric oxide (Aguan *et al.*, 1996; Prevot *et al.*, 2000) which is an important regulator of GnRH and CRH release from the ME (Prevot *et al.*, 2000). The stimulation of NMDA receptors within the ME, indeed, increases nitric oxide production (Bhat *et al.*, 1998). It is interesting to note that the glutamate-induced release of GnRH from mediobasal hypothalami can be blocked by a nitric oxid synthase inhibitor or a nitric oxide scavenger (Rettori *et al.*, 1994). The proposed mechanism whereby nitric oxide elicits CRH and GnRH release is by increasing the cGMP and/or prostaglandin E2 production within hypophysiotropic axon terminals (Prevot *et al.*, 2000). Nevertheless, the putative involvement of endothelial cells and nitric oxide specifically in the regulation of GHRH and SS release need to be investigated.

In accordance with the earlier observation of glutamic acid decarboxylase immunoreactivity in GHRH terminals (Meister & Hokfelt, 1988), the present confocal microscopic studies identified another GABA marker, VGAT, in GHRH axons. The putative sites of action of tuberoinfundibular GABA, secreted primarily by GHRH and dopaminergic terminals (Meister & Hokfelt, 1988), can be the anterior pituitary as well as the ME. Corroborating the hypophysial actions of GABA, GABA A receptors have been identified on anterior pituitary cells (Berman *et al.*, 1994). In addition, somatotrophs and lactotrophs, but no other types of adenohypophysial cells, are capable of internalizing [^{3}H]GABA through mechanisms that are currently unknown (Duvilanski *et al.*, 2000). Somewhat contradicting the idea that the secreted GABA regulates the anterior pituitary is the finding that GABA levels are not higher in the hypophysial portal vs. the peripheral blood (Mulchahey & Neill, 1982). Therefore, GABA released from hypophysiotropic GHRH terminals may also act primarily at the level of the ME, as we propose for glutamate. In case of both glutamate and GABA, morphological studies will need to clear whether or not, they have autoreceptors on glutamatergic and GABAergic axon terminals, respectively. Alternatively, if GABA receptors occur on glutamatergic terminals and reversely, glutamate receptors occur on GABAergic terminals in the ME, the amino acid cotransmitters could also be involved in a crosstalk among neurosecretory terminals of different amino acid phenotypes.

It is tempting to speculate that the endogenous glutamate content of GnRH, TRH, CRH and SS axon terminals contributes to the synchronized neurohormone output from individual axon terminals, a prerequisite for pulsatile neurohormone secretion. In support of this concept, the blockade of NMDA receptors is capable of abolishing the endogenous pulsatility of GnRH secretion from incubated mediobasal hypothalami (Bourguignon *et al.*, 1989). The lack of glutamate from GHRH neurons that also secrete episodically (Nakamura *et al.*, 2003) somewhat complicates this hypothesis, although it is possible that glutamatergic signals originating from other types of terminals are involved in generating GHRH secretory pulses. Although this concept requires experimental support, the synchronization in the patterned release of SS and GHRH clearly appears to exist. Recent *in vivo* studies of female monkeys using push–pull perfusion of the stalk–ME complex identified that the majority of GHRH secretory pulses either coincide with SS peaks or occur simultaneously with the SS troughs (Nakamura *et al.*, 2003). Finally, although these ISHH and immunocytochemical data reveal a clear difference between hypophysiotropic SS and GHRH neurons in their VGLUT2 content, the possibility should be recognized that GHRH neurons may contain low levels of VGLUT2 which remained undetected in the present studies.

In summary, in the present ISHH and immunocytochemical studies we provide evidence that the neurons of the hypophysiotropic SS system, similarly to GnRH, TRH and CRH neurons, express the mRNA and immunoreactivity for the glutamatergic marker, VGLUT2. Whereas GHRH-secreting neurons appeared to lack VGLUT2, their axon terminals contained the GABAergic marker VGAT. Together, these results suggest the paradoxic cosecretion of the inhibitory amino acid neurotransmitter GABA with the stimulatory peptide releasing hormone GHRH, and the cosecretion of the excitatory amino acid neurotransmitter glutamate with the inhibitory peptide SS. The physiological significance of endogenous amino acid neurotransmitter cosecretion from these hypophysiotropic neuronal systems, as well as from others, requires clarification.

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Abbreviations

ARH, arcuate nucleus of the hypothalamus; CRH, corticotropin-releasing hormone; GABA, γ-aminobutyric acid; GH, growth hormone; GHRH, growth hormone-releasing hormone; GnRH, gonadotropin-releasing hormone; mRNA, messenger ribonucleic acid; ME, median eminence; IR, immunoreactive; ISHH, *in situ* hybridization histochemistry; NMDA, *N*-methyl-D-aspartic acid; SS, somatostatin; PVa, anterior periventricular nucleus of the hypothalamus; PVH, paraventricular nucleus of the hypothalamus; TBS, Tris-buffered saline; TRH, thyrotropin-releasing hormone; VGAT, vesicular GABA transporter; VGLUT2, type 2 vesicular glutamate transporter; VMH, ventromedial nucleus of the hypothalamus.

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