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GLUTAMATERGIC INNERVATION OF THE HYPOTHALAMIC MEDIAN EMINENCE AND POSTERIOR PITUITARY OF THE RAT

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Abstract—Recent studies have localized the glutamatergic cell marker type-2 vesicular glutamate transporter (VGLUT2) to distinct peptidergic neurosecretory systems that regulate hypophysial functions in rats. The present studies were aimed to map the neuronal sources of VGLUT2 in the median eminence and the posterior pituitary, the main terminal fields of hypothalamic neurosecretory neurons. Neurons innervating these regions were identified by the uptake of the retrograde tract-tracer Fluoro-Gold (FG) from the systemic circulation, whereas glutamatergic perikarya of the hypothalamus were visualized via the radioisotopic *in situ* hybridization detection of VGLUT2 mRNA. The results of dual-labeling studies established that the majority of neurons accumulating FG and also expressing VGLUT2 mRNA were located within the paraventricular, periventricular and supraoptic nuclei and around the organum vasculosum of the lamina terminalis and the preoptic area. In contrast, only few FG-accumulating cells exhibited VGLUT2 mRNA signal in the arcuate nucleus. Dual-label immunofluorescent studies of the median eminence and posterior pituitary to determine the subcellular location of VGLUT2, revealed the association of VGLUT2 immunoreactivity with SV2 protein, a marker for small clear vesicles in neurosecretory endings. Electron microscopic studies using pre-embedding colloidal gold labeling confirmed the localization of VGLUT2 in small clear synaptic vesicles.

These data suggest that neurosecretory neurons located mainly within the paraventricular, anterior periventricular and supraoptic nuclei and around the organum vasculosum of the lamina terminalis and the preoptic area secrete glutamate into the fenestrated vessels of the median eminence and posterior pituitary. The functional aspects of the putative neuropeptide/glutamate co-release from neuroendocrine terminals remain to be elucidated.

Key words: glutamate, hypophysiotropic, neurosecretion, portal circulation, synaptic vesicle, VGLUT2.

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Abbreviations: ARH, arcuate nucleus of the hypothalamus; BSA, bovine serum albumin; FG, Fluoro-Gold; ME, median eminence; OVLT, organum vasculosum of the lamina terminalis; PBS, phosphate-buffered saline; Pe, periventricular nucleus of the hypothalamus; POA, preoptic area; PP, posterior pituitary; PVH, paraventricular nucleus of the hypothalamus; SO, supraoptic nucleus of the hypothalamus; SSC, standard saline citrate; TBS, Tris-buffered saline; VGLUT, vesicular glutamate transporter.

The major excitatory synaptic transmitter L-glutamate (van den Pol et al., 1990; Brann, 1995) is accumulated into synaptic vesicles by three molecular forms of vesicular glutamate transporters (VGLUT1-3) which are expressed by distinct subsets of glutamatergic excitatory neurons (Bellocchio et al., 2000; Fremeau et al., 2001, 2002; Fujiyama et al., 2001; Herzog et al., 2001; Sakata-Haga et al., 2001; Gras et al., 2002; Schafer et al., 2002; Ziegler et al., 2002; Lin et al., 2003). The *in situ* hybridization detection of VGLUT2 mRNA has established high expression levels of the VGLUT2 transporter isoform in several anatomical regions that are critically important in neuroendocrine regulation; these include the preoptic area (POA), the organum vasculosum of the lamina terminalis (OVLT) and the paraventricular (PVH), supraoptic (SO) and anterior periventricular (Pe) hypothalamic nuclei (Ziegler et al., 2002; Collin et al., 2003; Lin et al., 2003; Eyigor et al., 2004; Hrabovszky et al., 2004, 2005a,b,c, 2006). As opposed to these hypophysiotropic regions populated densely by excitatory glutamatergic neurons, the hypothalamic arcuate nucleus (ARH), which plays a crucially important role in the regulation of growth hormone and prolactin secretion, is dominated by inhibitory GABA containing neurons over glutamatergic cells (Hrabovszky et al., 2005a). Results of recent colocalization experiment by dual-label *in situ* hybridization and dual-label immunocytochemistry have also demonstrated that some glutamatergic cells in hypophysiotropic regions are identical with the known hypophysiotropic neurons secreting classical peptide releasing and release-inhibiting hormones into the hypophysial portal circulation. Parvicellular neurosecretory systems synthesizing VGLUT2 mRNA and protein, include gonadotropin-releasing hormone neurons in the POA and the OVLT (Hrabovszky et al., 2004), thyrotropin-releasing hormone (Hrabovszky et al., 2005c) and corticotropin-releasing hormone (Hrabovszky et al., 2005c) neurons in the PVH and somatostatin (Hrabovszky et al., 2005b) neurons in the PVH and the anterior Pe. In addition, magnocellular oxytocin and vasopressin neurons in both the PVH and the SO share the VGLUT2 phenotype with these parvicellular systems (Kawasaki et al., 2005; Hrabovszky et al., 2006). In contrast, tubero-infundibular dopaminergic (Meister and Hokfelt, 1988) and growth hormone-releasing hormone synthesizing (Meister and Hokfelt, 1988; Hrabovszky et al., 2005b) neurons of the ARH appear to exhibit a GABA amino acid neurotransmitter phenotype.

The goal of the present studies was to map the distribution of VGLUT2-synthesizing glutamatergic neurons that regulate hypophysial functions via innervating the median eminence (ME) and the posterior pituitary (PP). To this

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aim, we have used the neuronal uptake of the retrograde tracer, Fluoro-Gold (FG) from the systemic circulation, as a marker of neurosecretory systems which project their axon outside of the blood–brain barrier (Schmued and Fallon, 1986; Merchenthaler, 1991b). The immunofluorescent visualization of FG was combined with the *in situ* hybridization detection of VGLUT2 mRNA in histological sections, in order to identify the hypophysiotropic glutamatergic neuronal systems. The subcellular distribution of VGLUT2 protein in neurosecretory terminals was also addressed with a series of immunofluorescent and pre-embedding immunoelectron microscopic studies of neuroendocrine axon terminals in the ME and PP.

EXPERIMENTAL PROCEDURES

Animals

Prepubertal male Wistar rats (N=10; 150 g bw) were purchased from Charles-River Hungary Ltd. (Isaszeg, Hungary) and maintained in a light- and temperature-controlled environment (lights on 05:00–19:00 h; 22 °C) with free access to standard laboratory food (Sniff Spezialdiaeten GmbH, Soest, Germany) and tap water. All experiments were carried out in accordance with the Council Directive of 24 November 1986 of the European Communities (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine (47/003/2005). All protocols were designed to minimize both the total number of animals used and their suffering.

Localization of glutamatergic cell bodies projecting outside the blood–brain barrier

Retrograde labeling of hypophysiotropic neurons with FG. To label hypophysiotropic neurons via the uptake and retrograde transport of FG from the systemic circulation (Merchenthaler, 1991b), four rats at the age of 6 weeks were injected i.p. with 16 mg/kg, bw of FG (hydroxystilbamidine methanesulfonate; Molecular Probes, Eugene, OR, USA; dissolved in distilled water) and allowed to survive for 2 weeks.

Tissue collection for combined immunocytochemical/in situ *hybridization studies.* The FG-injected rats were anesthetized with pentobarbital (35 mg/kg bw, i.p.) and perfused transcardially with 150 ml fixative solution containing 4% paraformaldehyde (Sigma Chemical Company, St. Louis, MO, USA) in 0.1 M phosphate-buffered saline (PBS; pH 7.4). The hypothalami were dissected and soaked in 20% sucrose overnight for cryoprotection. Then, they were snap-frozen on powdered dry ice and 20-μm-thick free-floating coronal sections were prepared from the preoptic-hypothalamic region using a Leica CM 3050 S cryostat (Leica Microsystems Nussloch Gmbh, Nussloch, Germany).

Immunofluorescent detection of FG. The tissue mRNAs were protected against enzymatic degradation by adding 1000 U/ml of heparin sodium salt (Hofler et al., 1987) to the immunocytochemical reagents and by using diethyl pyrocarbonate-pretreated and autoclaved 0.1 M PBS (pH 7.4) as a rinsing solution between the incubation steps. The sections were blocked against non-specific antibody binding with 1% bovine serum albumin (BSA; fraction V; Sigma) in PBS for 30 min and transferred into anti-FG antiserum (AB153; Chemicon, Temecula, CA, USA; 1:5000) for 24 h. The primary antibodies were reacted with biotin-SP-anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:1000) and streptavidin conjugated to the red fluorochrome Cy3 (Jackson; 1:250) for 4 h. The sections were mounted from sterile buffer solution onto microscope slides coated with (3-aminopropyl) triethoxy-silane (Sigma), air-dried and processed for the radioisotopic *in situ* hybridization detection of VGLUT2 mRNA, as described elsewhere for fresh-frozen tissue sections (Hrabovszky et al., 2004, 2005c).

Detection of VGLUT2 mRNA with radioisotopic in situ *hybridization.* Prior to hybridization, the section were re-fixed in 4% paraformaldehyde for 5 min, acetylated with 0.25% acetic anhydride in 0.9% NaCl/0.1 M triethanolamine (Sigma Chemical Company; pH 8.0) for 10 min, rinsed in standard saline citrate solution (2× SSC; 1× SSC=0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 2 min, dehydrated in 70, 80, 95 and 100% ethanol (2 min each), delipidated in chloroform for 5 min, and finally, rehydrated partially in 100%, followed by 95% ethanol, for 2 min each. The slides were then air-dried on slide trays and hybridized with a ^{35}S-labeled complementary RNA hybridization probe targeting bases 522–1400 of VGLUT2 mRNA (GenBank acc. # NM 053427); the "VGLUT2-879" complementary DNA template to transcribe this probe has been prepared in our laboratory and validated in previous *in situ* hybridization experiments (Hrabovszky et al., 2004, 2005c). To prevent the formation of high autoradiographic background, 1000 mM dithiothreitol has been added to the hybridization solution (Hrabovszky and Petersen, 2002). Further, an enhanced hybridization signal has been achieved via using high concentrations of radioisotopic probe (80,000 cpm/μl) and dextran sulfate (20%) in the hybridization solution, as formally established in previous experiments (Hrabovszky and Petersen, 2002). After the overnight hybridization at 52 °C, the non-specifically bound probe was digested with 50 μg/ml ribonuclease A (Sigma; dissolved in 0.5 M NaCl/10 mMTris–HCl/1 mM EDTA; pH 7.8) for 60 min at 37 °C, followed by a stringent treatment step to further clear the signal (55 °C in 0.1× SSC solution for 60 min). The slides were dipped into MQ water for 2 s, rinsed in 70% ethanol for 5 min and air-dried. The autoradiographs were visualized on Kodak NTB-3 nuclear track emulsion (Kodak; Rochester, NY, USA) with Kodak processing chemicals after 2 weeks of exposure at 4 °C in the dark. The sections were finally dehydrated with graded ethanol (95%, followed by 100%; 5 min each), cleared in xylene (2×5 min), coverslipped with DPX mounting medium (Fluka Chemie; Buchs, Switzerland) and examined with a Zeiss Axiophot epifluorescent microscope (Zeiss; Göttingen, Germany) using an epifluorescent filter set with the following parameters: excitation of 540–590 nM, bandpass of 595 nm, and emission of 600–660 nm. The autoradiographic signal was examined under dark-field illumination. The fluorescent and autoradiographic images of the same section area were captured with an RT Spot digital camera (Diagnostic Instruments, Sterling Heights, MI, USA), processed as separate layers using the Adobe Photoshop 7.0.1 software (Adobe Systems Inc., Berkeley, CA, USA). The colocalization of the signals for FG and VGLUT2 mRNA was demonstrated on merged digital images.

In situ *hybridization control experiments.* For positive control, test sections from the hypothalami were hybridized using a distinct antisense probe to VGLUT2 mRNA ("VGLUT2-734"; complementary to bases 1704–2437), as described earlier (Hrabovszky et al., 2004, 2005c). The complementary DNA template to prepare this probe was kindly donated by Dr. J. P. Herman (University of Cincinnati Medical Center, Cincinnati, OH, USA). As a negative control approach, sense RNA transcripts were applied to the sections.

Immunofluorescent analysis of glutamatergic axon terminals in the ME and PP

Tissue preparation for immunocytochemistry. Three adult male rats were anesthetized with pentobarbital (35 mg/kg bw, i.p.) and perfused transcardially with 150 ml fixative solution containing 4% paraformaldehyde. The hypothalami were dissected and soaked in 25% sucrose overnight for cryoprotection. The pituitaries were first