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VESICULAR GLUTAMATE TRANSPORTERS TYPE 1 AND 2 EXPRESSION IN AXON TERMINALS OF THE RAT NUCLEUS OF THE SOLITARY TRACT

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Abstract—The nucleus of the solitary tract is the site of termination of primary afferent fibers running in the facial, glossopharyngeal and vagus nerves. The present study was performed to map the distribution of glutamatergic axons terminals in the rat nucleus of the solitary tract using immunodetection of vesicular glutamate transporter 1 and vesicular glutamate transporter 2. The two vesicular glutamate transporters were differentially distributed among nucleus of the solitary tract subdivisions. Vesicular glutamate transporter 1 immunoreactivity was mostly found in the lateral part of the nucleus (ventrolateral, interstitial and intermediate subdivisions) whereas vesicular glutamate transporter 2 labeling was distributed throughout the nucleus of the solitary tract. Electron microscope examination indicated that vesicular glutamate transporter immunoreactivity was localized in axon terminals filled with round synaptic vesicles. After injection of cholera toxin B subunit in sensory ganglia, anterograde labeling was found in vesicular glutamate transporter 1, as well as vesicular glutamate transporter 2-immunoreactive boutons. Double immunolabeling experiments allowed distinctions between terminals expressing either vesicular glutamate transporter 1 or vesicular glutamate transporter 2 or both vesicular glutamate transporter 1 and vesicular glutamate transporter 2 immunoreactivities. The latter population, expressing both transporters immunolabeling, completely disappeared after deafferentation induced by removal of sensory ganglia.

This study indicates that vesicular glutamate transporter content identifies three different subpopulations of glutamatergic boutons in the nucleus of the solitary tract and provides definitive evidence that primary afferent neurons contribute glutamatergic terminals to the nucleus of the solitary tract.

Key words: visceral sensory neurons, vagus nerve, glossopharyngeal nerve, glutamatergic neurotransmission, immunocytochemistry

The nucleus of the solitary tract (NTS) is the main site of termination for the primary visceral afferent fibers running in the facial, trigeminal, glossopharyngeal and vagus nerves (Contreras et al., 1982). These afferent fibers convey information from sensory endings located in various parts of the cardiovascular, respiratory and digestive systems. After entering the medulla, they course in the solitary tract and segregate into the different subdivisions of the NTS with a loose viscerotopy (Norgren and Smith, 1988; Altschuler et al., 1989; see also Blessing, 1997 for review). The NTS also receives projections from several hindbrain and forebrain regions including the paraventricular and arcuate hypothalamic nuclei, the central nucleus of the amygdala and the medial prefrontal and insular cortex (Van der Kooy et al., 1984; Blessing et al., 1991).

Numerous pharmacological studies performed *in vivo* and *in vitro* indicate that glutamate is the major excitatory neurotransmitter in the NTS (reviewed by Andresen and Mendelowitz, 1996; Lawrence and Jarrott, 1996; Sapru, 1996; Talman et al., 2001). Presumptive glutamatergic nerve terminals have been probed in the rat and cat NTS using post-embedding electron microscope detection of glutamate immunoreactivity (Saha et al., 1995a,b; Sykes et al., 1997). However, the method is questionable since glutamate is involved in several metabolic and biosynthetic pathways. Especially, being the precursor of GABA, it may also be present in GABAergic terminals. The recent identification of vesicular glutamate transporters (VGLUTs) has provided new tools to probe glutamatergic axon terminals. Currently, 3 VGLUTs have been identified (see Fremeau et al., 2004 for review). VGLUT1 and VGLUT2 are brain specific and selectively located in typical glutamatergic terminals establishing excitatory asymmetric synaptic contacts (Bellocchio et al., 2000; Takamori et al., 2000, 2001; Fremeau et al., 2001; Herzog et al., 2001). Hybridization studies indicate that distinct neuronal populations express VGLUT1 and VGLUT2 (Fremeau et al., 2001; Herzog et al., 2001). VGLUT3 is also present in peripheral tissues (Fremeau et al., 2002; Gras et al., 2002). In the brain, it is found in cholinergic and serotonergic axon terminals (Gras et al., 2002; Schafer et al., 2002) as well as in a subset of hippocampal GABAergic neurons (Fremeau et al., 2002). Thus, VGLUT1 and VGLUT2, but not VGLUT3, appear as reliable markers of glutamatergic terminals. Recent immunohistochemical studies indicate that the NTS contains both VGLUT1 and VGLUT2 (Kaneko et al., 2002; Lin et al., 2004). However, no information is available as regards the origin, peripheral and/or central, of the different subsets of VGLUTs-containing NTS terminals.

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Abbreviations: CTB, cholera toxin, B subunit; NTS, nucleus of the solitary tract; PB, phosphate buffer; PBS, phosphate-buffered saline; VGLUT1, type 1 vesicular glutamate transporter; VGLUT2, type 2 vesicular glutamate transporter; X, dorsal motor nucleus of the vagus nerve; XII, hypoglossal nucleus.

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In the present study, we used immunodetection of VGLUT1 and VGLUT2 to map the distribution of glutamatergic terminals in the rat NTS. We also performed immunohistochemistry in combination with either anterograde tracing or ganglionectomy in order to identify VGLUTs expressed in visceral sensory projections.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

Experiments were performed on 10 adult Wistar rats weighing 180–200 g. All procedures were in accordance with the European Communities Council directive (86/609/EEC) for the care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering.

Animals were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (15 mg/kg) and perfused through the ascending aorta with 300 ml of cold 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). The brainstem was removed and extensively washed in PB. For immunoperoxidase staining (light and electron microscopic experiments), the medulla was dissected and coronal sections (50 μm thickness) were obtained on a vibratome and collected in PB. For immunofluorescence, the medulla was dissected, cryoprotected in 30% sucrose in PB and frozen in liquid isopentane cooled at −50 °C. Coronal medullary sections (10 μm-thick) were cut on a cryostat, thaw-mounted on gelatin-coated slides and stored at −20 °C until used.

Anterograde tracing was performed in three rats under ketamine/xylazine anesthesia. The ventral surface of the neck was incised and the cervical portion of the right vagus nerve was exposed. The nerve was traced centrally to the nodose ganglion. Cholera toxin, B subunit (CTB; List Biological Laboratories, Campbell, CA, USA; 15 mg/ml, 5 μl) was injected into the nodose and petrosal ganglia which form a single ganglionic mass in the rat (Altschuler et al., 1989). Injection was performed using a glass micropipette (tip diameter: 50 μm) glued to a Hamilton syringe. Animals were killed after 48 h survival.

Unilateral ganglionectomy was performed on three rats under ketamine/xylazine anesthesia. The nodose and petrosal ganglia were exposed as described above and removed. The animals were allowed to recover and killed after one week survival. Control rats ($n=2$) were sham-operated using the same technique except for ganglion removal.

Primary antibodies

Immunodetection of VGLUT1 and VGLUT2 was performed using commercial rabbit antisera (Synaptic System, Göttingen, Germany). These antisera were raised against fusion proteins containing glutathione-*S*-transferase and fragments from the carboxy-terminus of rat VGLUT1 (amino-acid residues 456–561; Takamori et al., 2000) or VGLUT2 (amino-acid residues 506–582; Takamori et al., 2001). A commercial guinea-pig anti-VGLUT1 antiserum (Chemicon, Temecula, CA, USA) was also used for double labeling experiments. CTB was detected using a goat anti-serum to CTB (List Biological Laboratories).

Immunoperoxidase staining for light and electron microscopy

Free-floating medullary sections were blocked 1 h at room temperature in 10% normal goat serum diluted in 0.1 M phosphate-buffered 0.9% saline (PBS, pH 7.4). The sections were then incubated overnight at 4 C° with the anti-VGLUT1 or VGLUT2 antiserum (1/500 in PBS). After extensive washes in PBS, the sections were exposed to an anti-rabbit biotinylated secondary antibody (1/200, Jackson Immunoresearch, West Grove, PA, USA) for 1 h at room temperature. Sections were washed in PBS, and incubated for 1 h at room temperature in the avidin–biotin–peroxidase complex (ABC kit, Vector Laboratory, Burlingame, CA, USA). Peroxidase activity was revealed using 3,3′-diaminobenzidine tetrahydrochloride (0.0025% in PB). Some sections were processed as described but without the primary antibody to evaluate the non-specific staining. For light microscopy observation, selected sections were mounted on gelatin-coated glass slides, dried at room temperature, dehydrated in ascending alcohols, cleared in xylene and coverslipped with DPX (BDH Laboratory Supplies, Poole, UK). Sections were observed on a Nikon Optiphot 2 microscope and photographed using a ×20 or a ×63 oil immersion objectives and a digital camera (Nikon Coolpix 950). Sections used for electron microscopy were fixed with 1% osmium tetroxide in PB (45 min), stained with 1% uranyl acetate in distilled water (45 min), dehydrated in ascending alcohols and propylene oxide and flat embedded in Durcupan ACM Fluka (Sigma-Aldrich, St. Quentin, France). Ultrathin sections (70–80 nm thick) were obtained on a Reichert Ultracut ultramicrotome and photographed on a Philips CM10 electron microscope. Negatives were digitized at 1200 ppi using an Epson Perfection scanner. Area measurement of immunoreactive profiles was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Ultrathin sections from six different blocks (three animals×two types of label) were used. Due to the limited penetration of the antibodies (no more than 3–4 μm from the surface of the tissue), ultrathin sections were taken from the most superficial portions of tissue blocks. Data were pooled by type of label (VGLUT1 or VGLUT2) and the mean area of labeled profiles was calculated for each type of label. Statistical comparison was carried out using the Mann-Whitney test.

Double immunofluorescence labeling

Section were first blocked with 10% normal goat serum (1 h) and incubated overnight at 4 °C with rabbit anti-VGLUT2 primary antiserum (1/1000) and guinea-pig anti-VGLUT1 antiserum (1/2000) in PBS. Anti-VGLUT2 immunolabeling was detected with an Alexa-488-conjugated goat anti-rabbit secondary antibody (1/200; Molecular Probes, Eugene, OR, USA, 1 h). Anti VGLUT1 immunolabeling was detected using either and a Cy3-conjugated goat anti-guinea-pig secondary antibody (1/200; Jackson Immunoresearch, 1 h) or a biotinylated goat anti-guinea-pig secondary antibody (1/200; Jackson Immunoresearch, 1 h) followed by Cy5-conjugated avidin (1/200; Jackson Immunoresearch, 1 h).

For simultaneous detection of VGLUTs and CTB, sections were first blocked with 10% normal donkey serum (1 h) and incubated overnight at 4 °C with goat antiserum to CTB (1/10,000) and rabbit anti-VGLUT1 or Anti-VGLUT2 antiserum (1/1000). After rinsing, they were incubated (1 h) with an Alexa-488-conjugated donkey anti-rabbit secondary antibody (1/200; Molecular Probes) and a Cy5-conjugated donkey anti-goat secondary antibody (1/200, Jackson Immunoresearch).

After incubation, sections were washed in PBS and coverslipped using glycerol-PB (80:20) as mounting medium.

Confocal analysis

Confocal analysis was performed on a Leica TCS SP2 confocal microscope using the 488 nm band of an Ar laser for excitation of Alexa 488 (spectral detection 500–535 nm) and either the 543 nm or the 633 nm band of He–Ne lasers for excitation of Cy3 (spectral detection >560 nm) or Cy5 (spectral detection >650 nm), respectively. High magnification images of medullary sections were acquired using a ×63 oil immersion objective (numerical aperture 1.32). Pinhole size was set to "Airy one" to achieve the best possible resolution (theoretical lateral and axial limits: 165 nm and 330 nm, respectively) without reducing luminosity. Pixel size was