Independent inputs by VGLUT2- and VGLUT3-positive glutamatergic terminals onto rat sympathetic preganglionic neurons

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Received 26 November 2003; accepted 2 December 2003

DOI: 10.1097/01.wnr.0000113866.62539.71

To characterize glutamatergic axon terminals onto sympathetic preganglionic neurons (SPNs), we visualized immunohistochemically three vesicular glutamate transporters (VGLUTs) in the intermediolateral cell column (IML) of rat thoracic spinal cord. VGLUT2 and VGLUT3 immunoreactivities but not VGLUT1 immunoreactivity were distributed in the IML and found in terminals making asymmetric synapses and apposed to dendrites immunopositive for choline acetyltransferase, an SPN marker. VGLUT2 and VGLUT3 immunoreactivities were not co-localized with each other. A population of VGLUT2-immunoreactive but not VGLUT3-immunoreactive terminals were adrenergic or noradrenergic. Some of VGLUT3-immunoreactive but not VGLUT2-immunoreactive terminals contained serotonin. These results indicate at least two independent glutamatergic terminal populations, which include a distinct monoaminergic subpopulation, making excitatory inputs onto SPNs. *NeuroReport* 15:431–436 © 2004 Lippincott Williams & Wilkins.

Key words: Cardiovascular control; Dopamine β-hydroxylase; Double immunofluorescence microscopy; Fever; Immunoelectron microscopy; Intermediolateral cell column; Lateral horn; Sympathetic nervous system; Thermoregulation; Vesicular glutamate transporter

INTRODUCTION

Sympathetic preganglionic neurons (SPNs), which are located in the intermediolateral cell column (IML) of the thoracic spinal cord, receive information processed in the brain via bulbospinal pathways and generate integrated sympathetic output leading to the control of peripheral organs. Electrophysiological evidence has indicated that glutamate is an important excitatory neurotransmitter to mediate the inputs onto SPNs [1,2]. In understanding the roles of glutamate in the central circuitry of the sympathetic nervous system, it is essential to analyze histochemically glutamatergic synapses onto SPNs. Glutamatergic axon terminals in the IML have been detected with glutamate [3–5] or phosphate-activated glutaminase immunoreactivity [6]. However, glutamate also serves as the precursor of inhibitory neurotransmitter GABA. Although glutaminase has been considered as a main synthetic enzyme for transmitter glutamate in the cerebral cortex, it is located in some GABAergic neurons in other brain regions, probably supplying the GABA-precursor glutamate [7]. Thus, neither glutamate nor glutaminase immunoreactivity is suitable as a definitive marker of glutamatergic terminals.

Recently, several laboratories have identified three vesicular glutamate transporters (VGLUTs): VGLUT1, VGLUT2 (for review, see [8]) and VGLUT3 [9–12], which have been shown to accumulate glutamate as a transmitter into synaptic vesicles. These transporters are localized in axon terminals of largely distinct populations of excitatory neurons in the central nervous system [8,9,11–13]. The segregated expression pattern of the VGLUTs indicates that excitatory transmissions known to be mediated by the glutamatergic nervous system can be further categorized into at least three populations, which might have distinct selectivity in glutamatergic functions. To examine which VGLUTs are expressed in the glutamatergic axon terminals onto SPNs, in the present study, we performed immunohistochemistry in the IML of the rat thoracic spinal cord with antibodies specific to VGLUTs and investigated the histochemical characteristics of respective populations of VGLUT-positive terminals as a component of the sympathetic nervous system.

MATERIALS AND METHODS

All experiments were performed in accordance with the rules of animal care by Institute of Laboratory Animals, Faculty of Medicine, Kyoto University.

Immunohistochemistry: Immunohistochemical procedures basically followed our previous studies [14,15]. Briefly, male Wistar rats (200–300 g; Japan SLC, Shizuoka, Japan), under deep anesthesia with chloral hydrate (280 mg/kg body weight, i.p.), were perfused transcardially with 150 ml 50 mM phosphate-buffered saline (PBS), followed by 300 ml 2% formaldehyde in 0.1 M phosphate buffer. Thoracic spinal cord was removed and divided into three parts: upper (T1–T4), middle (T5–T9) and lower (T10–L1) segments. The tissue was post-fixed in the same fixative at 4°C for 2 h, cryoprotected with 30% sucrose in PBS, and cut into 20 μm transverse or horizontal sections on a freezing microtome.

The transverse sections were incubated overnight with 0.2 μg/ml anti-VGLUT1, 0.1 μg/ml anti-VGLUT2 [13] or 0.5 μg/ml anti-VGLUT3 [16] guinea pig antibody and then for 1 h with 10 μg/ml biotinylated donkey antibody to guinea pig IgG (Jackson, West Grove, PA, USA). The sections were further incubated for 1 h with avidin-biotinylated peroxidase complex (ABC-Elite; Vector, Burlingame, CA, USA). Bound peroxidase was visualized by incubation of the sections with 0.02% 3,3′-diaminobenzidine tetrahydrochloride, 0.0002% hydrogen peroxide, and 0.5% ammonium nickel sulfate hexahydrate in 50 mM Tris-HCl (pH 7.6).

Double immunofluorescence study: The horizontal sections were incubated overnight with a mixture of one of the anti-VGLUT guinea pig antibodies and anti-dopamine β-hydroxylase (DBH) mouse monoclonal IgG (1:1000; Chemicon, Temecula, CA, USA), anti-choline acetyltransferase (ChAT) goat antibody (1:200; Chemicon) or anti-serotonin rabbit serum (1:5000; Sigma, St. Louis, MO, USA). The sections were incubated for 1 h with 10 μg/ml biotinylated donkey antibody to mouse IgG, goat IgG or rabbit IgG (Chemicon), and then blocked for 30 min with 10% normal mouse, goat or rabbit serum, respectively. Under the presence of the normal serum, the sections were further incubated for 1 h with 10 μg/ml Alexa488-conjugated goat antibody to guinea pig IgG and 5 μg/ml Alexa546-conjugated streptoavidin (Molecular Probes, Eugene, OR). The sections were observed under a confocal laser-scanning microscope (LSM 5 PASCAL; Zeiss, Oberkochen, Germany).

For double fluorescence staining for VGLUT2 and VGLUT3, anti-VGLUT2 rabbit antibody (0.1 μg/ml) [17] and anti-VGLUT3 guinea pig antibody (0.5 μg/ml) were used as primary antibodies. The antibodies bound to sections were detected by incubating with Alexa488-conjugated goat antibody to rabbit IgG and Alexa546-conjugated goat antibody to guinea pig IgG (10 μg/ml; Molecular Probes).

Immunoelectron microscopy: The procedure for immunoelectron microscopy followed our previous study [13]. The concentrations of the primary antibodies used were 1 μg/ml for anti-VGLUT2 guinea pig antibody and 0.2 μg/ml for anti-VGLUT3 guinea pig antibody. For the staining of VGLUT3, preincubation and primary antibody incubation of sections were performed under the presence of 0.03% Triton X-100 to enhance the penetration of the antibody.

Table I. Localization of DBH or serotonin in VGLUT2- and VGLUT3-immunoreactive axon swellings.

		Percentage in VGLUT-positive axon swellings[a]	
Part in thoracic cord		DBH +	Serotonin +
VGLUT2	Upper	11.7 ± 0.2	N.D.
	Middle	11.3 ± 0.1	N.D.
	Lower	11.3 ± 0.2	N.D.
VGLUT3	Upper	N.D.	16.9 ± 1.7
	Middle	N.D.	6.0 ± 0.9[b]
	Lower	N.D.	22.0 ± 0.9

[a]Percentage of DBH- or serotonin-immunoreactive axon swellings in VGLUT-positive ones for each thoracic part is shown as the mean ± s.e.m. of three animals (see Materials and Methods).
[b]Significant difference from both upper and lower thoracic parts ($p < 0.01$). N.D., no double-labeled axon swellings detected.

Data analysis: To statistically analyze the expression of DBH or serotonin immunoreactivity in VGLUT2- and VGLUT3-immunoreactive axon terminals, seven square fields (18.3 × 18.3 μm) were randomly selected in the IML for each upper, middle or lower part of the thoracic cord in the double-immunofluorescence-stained horizontal sections, and were taken as confocal images with a confocal depth of 0.8 μm. We counted the numbers of VGLUT2- and VGLUT3-immunoreactive axon swellings with or without DBH or serotonin immunoreactivity in the images. With the total numbers collected from the seven images, percentages of DBH- and serotonin-immunoreactive subpopulations in VGLUT-positive axon swellings were calculated. In this analysis, we examined 300–700 VGLUT-positive axon swellings per thoracic part in one immunostaining set. We repeated this statistical procedure for three rats and the data are presented in Table 1 as the means ± s.e.m. Statistical analysis was performed using the unpaired Student's *t*-test (Instat 2.00 program; Graph Pad, San Diego, CA, USA).

RESULTS

Prominent accumulation of VGLUT3-immunoreactive terminals was observed in the IML of the thoracic spinal cord. As shown in Fig. 1c, VGLUT3-immunoreactive terminals were distributed around somata of putative SPNs (arrow) and along extending fibers into the lateral funiculus and commissural fibers (arrowheads). In double immunofluorescence staining for VGLUT3 and ChAT, a marker for SPNs, VGLUT3-immunoreactive terminals were distributed in and around ChAT-immunoreactive cell clusters (Fig. 2c) and found to be making close contact with ChAT-immunoreactive somata and dendrite-like fibers (arrowheads in Fig. 2e). VGLUT2-immunoreactive terminals were distributed evenly throughout the gray matter of the spinal cord, including the IML (Fig. 1b, Fig. 2b). Like in VGLUT3 staining, VGLUT2-immunoreactive terminals in close apposition to ChAT-immunoreactive somata and dendrite-like structures were observed (arrowheads in Fig. 2d). VGLUT1-immunoreactive terminals were sparsely distributed throughout most of the gray matter in the spinal cord, but were rarely observed in the IML (Fig. 1a, Fig. 2a), in agreement with a recent report [18]. Thus, we further analyzed VGLUT2- and VGLUT3-immunopositive terminals in this sympathetic region.

To reveal the precise subcellular localization of VGLUT2 and VGLUT3 immunoreactivities, we performed electron

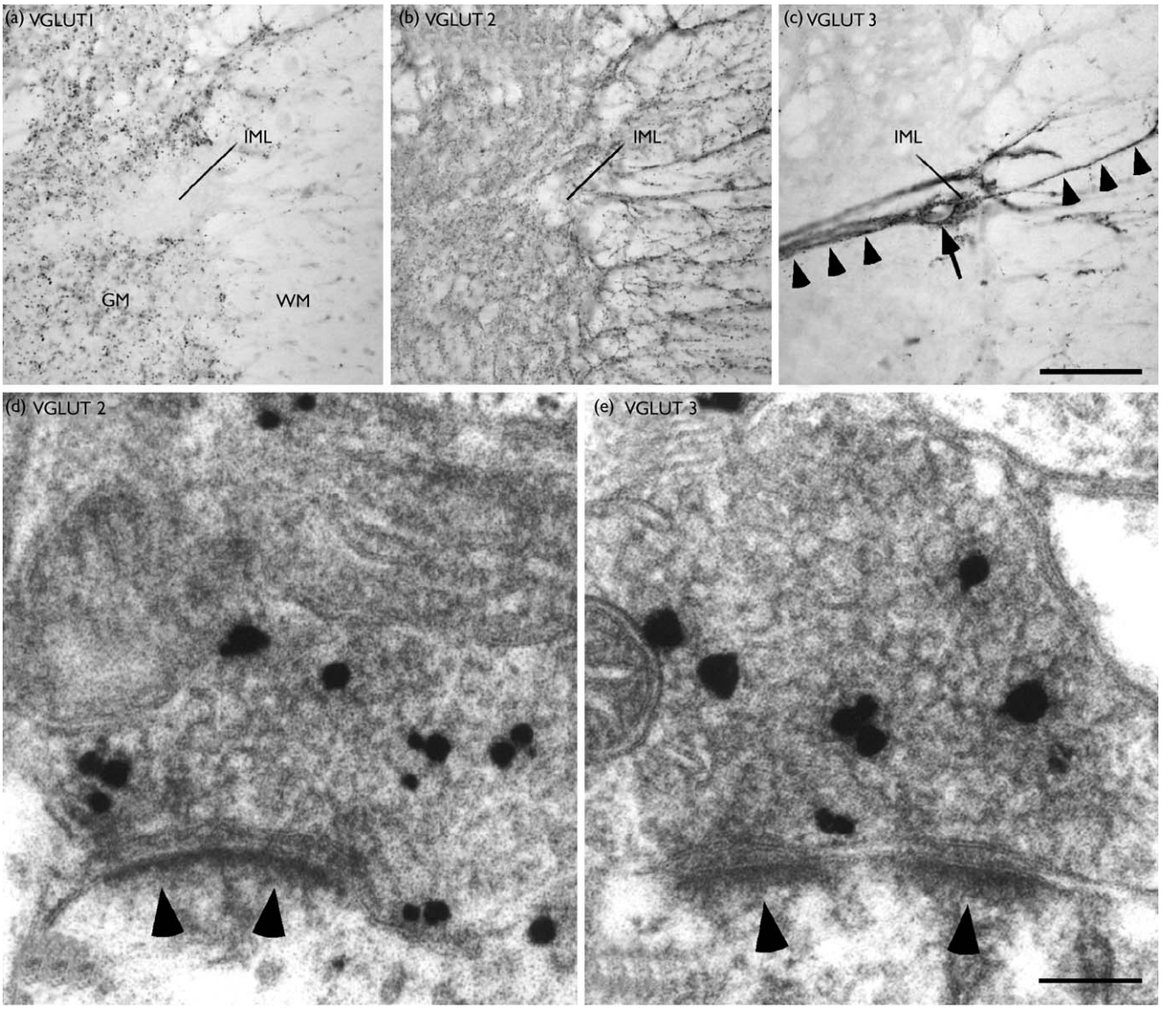

Fig. 1. Light (**a**–**c**) and electron (**d**,**e**) microscopic views of the distributions of immunoreactivities for three vesicular glutamate transporters (VGLUTs) in the intermediolateral cell column (IML). Intense accumulation of VGLUT3-immunoreactive terminals (c) and dispersed distribution of VGLUT2-immunoreactive terminals (b) were observed in the IML of upper thoracic segments. This region lacked VGLUT1 immunoreactivity (a). As shown in (c), VGLUT3-immunoreactive terminals were distributed around somata of putative sympathetic preganglionic neurons (SPNs) (arrow) and along extending fibers into the lateral funiculus and commissural fibers (arrowheads). The photomicrographs shown in (a-c) were taken from adjacent sections. VGLUT2 (d) and VGLUT3 (e) immunoreactivities, which were enhanced with silver grain, seemed associated with synaptic vesicles within axon terminals making asymmetric synapses in the IML of the third thoracic segment. Arrowheads in (d) and (e) indicate postsynaptic densities. GM, gray matter; WM, white matter. Bar = 50 μm (a– c); 100 nm (d,e).

microscopy in the IML. Silver-intensified grains indicating VGLUT2 and VGLUT3 immunoreactivities were closely associated with the membranes of synaptic vesicles in axon terminals forming asymmetric synapses (arrowheads in Fig. 1d,e), suggesting the excitatory characteristics of the synapses. All the VGLUT2- and VGLUT3-immunopositive axon terminals that we found were accompanied by the asymmetric synaptic structure. As seen in Fig. 2f, no co-localization of VGLUT2 and VGLUT3 immunoreactivities was found from our confocal microscopic analysis in 937 of VGLUT2-immunoreactive axon swellings and 805 of VGLUT3-immunoreactive ones in the IML of three animals. This result indicates that VGLUT2- and VGLUT3-containing axon terminals are provided by projections from distinct populations of glutamatergic neurons. In light of a previous spinal transection study showing that glutamate immunoreactivity was eliminated in the IML region caudal to the transection [3], a large part of glutamatergic terminals in the IML are likely derived from supraspinal neurons. Because medullary catecholaminergic and serotonergic neurons send their massive projections to the IML, we next tested the possibility of the expression of the VGLUTs in monoaminergic projections. Double immunofluorescence staining revealed that immunoreactivity for DBH, a marker for adrenergic and noradrenergic axon terminals, was localized in 11% of VGLUT2-immunoreactive axon swellings in the

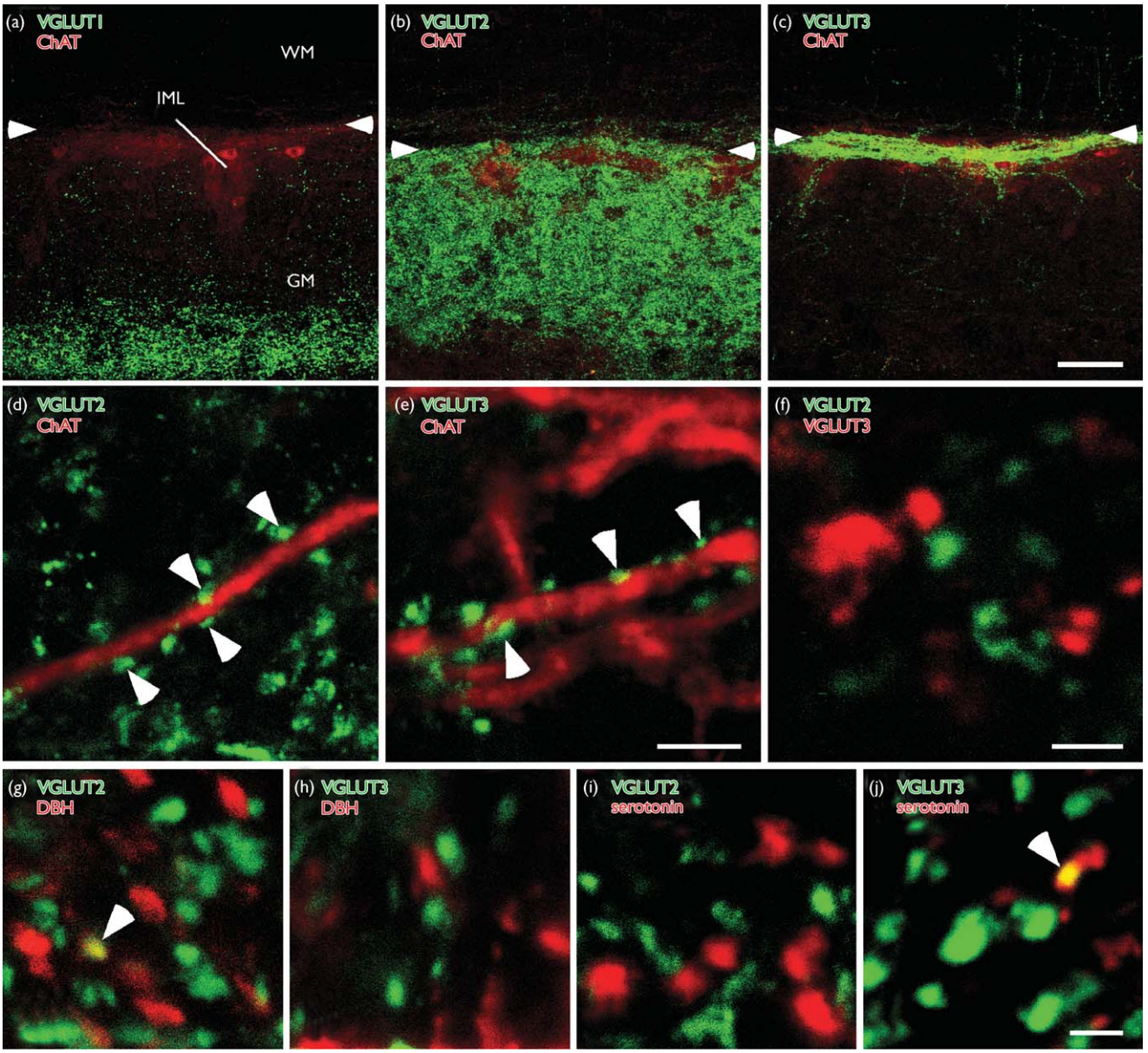

Fig. 2. Confocal laser-scanning microscopic analyses of VGLUT-immunoreactive axon terminals in the IML of upper thoracic segments. (**a**–**c**) Double immunofluorescence staining for VGLUTs (green) and choline acetyltransferase (ChAT; red), a marker for SPNs, showed accumulation of VGLUT3-immunoreactive terminals in and around ChAT cell clusters in the IML (c) and even distribution of VGLUT2-immunoreactive terminals throughout the GM, including the IML (b). VGLUT1-immunoreactive terminals were hardly distributed in the IML (a). Arrowheads indicate the boundary between the GM and WM. (**d**,**e**) VGLUT2- (d, green) and VGLUT3- (e, green) immunoreactive terminals formed close apposition (arrowheads) to ChAT-immunoreactive dendrite-like fibers (red). (**f**) VGLUT2 (green) and VGLUT3 (red) immunoreactivities showed no co-localization in the IML. (**g**) A population of VGLUT2-immunoreactive axon swellings (green) exhibited immunoreactivity for dopamine ß-hydroxylase (DBH; red), a marker for adrenergic and noradrenergic axon terminals (arrowhead). (**h**) VGLUT3-immunoreactive axon swellings (green) showed no co-localization with DBH immunoreactivity (red). (**i**) VGLUT2-immunoreactive axon swellings (green) showed no co-localization with serotonin immunoreactivity (red). (**j**) A population of VGLUT3-immunoreactive axon swellings (green) exhibited serotonin immunoreactivity (red, arrowhead). Bar = 100 μm (a–c); 5 μm (d,e); 3 μm (f–j).

IML (arrowhead in Fig. 2g, Table 1). The size of the DBH-immunoreactive population in VGLUT2-positive swellings was constant through the upper, middle and lower thoracic parts (Table 1). VGLUT3-immunoreactive axon swellings in the IML did not show DBH immunoreactivity (Fig. 2h, Table 1). Serotonin immunoreactivity was detected in some VGLUT3-immunoreactive axon swellings in the IML (arrowhead in Fig. 2j). As shown in Table 1, the percentage of serotonin-immunoreactive swellings in VGLUT3-positive ones in the middle thoracic part was significantly low compared with those in the upper and lower parts. VGLUT2-immunoreactive axon swellings in the IML did not show serotonin immunoreactivity (Fig. 2i, Table 1).

DISCUSSION

The present study has revealed intriguing distribution patterns of VGLUTs in the sympathetic IML. VGLUT3-

immunoreactive axon terminals formed dense aggregations in and around SPN cell clusters and VGLUT2-immunoreactive terminals showed a dispersed distribution in the IML, while this sympathetic region was vacant of immunoreactivity for VGLUT1. Furthermore, immunoreactivities for VGLUT2 and VGLUT3 showed no co-localization. These observations indicate the existence of at least two (VGLUT2- or VGLUT3-positive) independent populations of glutamatergic terminals in the IML. As evidenced by asymmetric synapses observed in the present immunoelectron microscopy, VGLUT2- and VGLUT3-positive terminals in the IML probably form excitatory synapses. Our double immunofluorescence study showed close apposition of VGLUT2- and VGLUT3-immunoreactive terminals to ChAT-immunoreactive dendrite-like fibers and somata. Taken together, these findings propose that VGLUT2- and VGLUT3-positive populations of glutamatergic projections independently make excitatory inputs to SPNs.

As shown by a previous spinal transection study [3], the majority of glutamatergic terminals in the IML are thought to be derived from supraspinal neurons. One of the major supraspinal regions originating glutamatergic projections to the IML is the rostral ventrolateral medulla (RVLM) [4], which contains adrenergic C1 and non-catecholaminergic vasomotor neurons that project to the spinal cord [19]. In the present study, we found a DBH-containing population of VGLUT2-immunoreactive axon swellings in the IML. Consistent with our result, VGLUT2 mRNA was recently shown to be expressed by both adrenergic C1 and non-catecholaminergic vasomotor RVLM neurons that project to thoracic spinal cord [18]. These results indicate that VGLUT2-positive glutamatergic projections, part of which likely use adrenaline as a co-transmitter, mediate RVLM-derived vasomotor inputs directly onto SPNs. On the other hand, VGLUT3-positive projections appear to be unrelated with the catecholaminergic system, because our observations detected no DBH immunoreactivity in VGLUT3-positive terminals, consistent with previous reports that VGLUT3 expression was not found in catecholaminergic neurons in the brain [11,12].

Another candidate for glutamatergic sympathetic premotor neurons is considered to be distributed around the rostral raphe pallidus nucleus (rRPa), which is one of the medullary raphe regions providing massive serotonergic and non-serotonergic projections to the spinal cord [20]. Disinhibition of neurons around the rRPa produced increases in the sympathetic nerve activity to brown adipose tissue [21] and inhibition of rRPa neurons blocked the sympathetic thermogenesis in brown adipose tissue induced by pyrogenic stimulations [22]. Most of the rRPa neurons involved in the thermogenesis are considered to be non-serotonergic [22]. In addition, there are few VGLUT1-positive terminals in the IML as shown in the present study and neurons around the rRPa do not seem to express VGLUT2 (see Fig. 2F,G in [23]). These lines of evidence suggest that the excitatory effect by the rRPa neurons on the sympathetic output leading to thermogenesis is mediated by VGLUT3-positive glutamatergic projections, most of which are possibly non-serotonergic, to the IML, although the expression of VGLUT3 in medullary neurons should be examined. As shown in our present study and a very recent report [24], there is a serotonergic population in VGLUT3-immunoreactive projections to the IML. Although VGLUT3 expression by serotonergic neurons in the dorsal and median raphe nuclei has been reported [9,11,12], most of their projections terminate in the forebrain [25]. Thus, VGLUT3-positive serotonergic terminals in the IML are likely provided by medullary raphe nuclei rather than by the midbrain and pontine raphe nuclei. Of interest is the functional significance of the co-transmitters, adrenaline/noradrenaline and serotonin in VGLUT2- and VGLUT3-positive terminals, respectively. The co-released monoamines might exert slow modulatory effects on the activity and/or efficiency of the fast glutamatergic transmission.

CONCLUSION

The present study shows the existence of two independent types of glutamatergic axon terminals, which are selectively labeled with anti-VGLUT2 and anti-VGLUT3 antibodies, making inputs onto SPNs. It is proposed that the two populations of glutamatergic projections have distinct medullary origins and show a clear difference in the sympathetic functions on which they exert influences. VGLUT2-positive glutamatergic terminals, which are derived from the RVLM, are likely involved in the cardiovascular control and VGLUT3-positive ones, which are from the rRPa, could be involved in the control of thermoregulation and the induction of fever.

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Acknowledgements: This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 12308039 and No. 15029224). K.N. is a Research Fellow of the Japan Society for the Promotion of Science (No. 3814).