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Short communication

Vesicular glutamate transporter immunoreactivity in the central and peripheral endings of muscle-spindle afferents

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Abstract

Expression of vesicular glutamate transporters (VGLUTs: VGLUT1, VGLUT2 and VGLUT3) in muscle spindle afferents was examined in rats. VGLUT1 immunoreactivity was detected in the sensory endings on the equatorial and juxta-equatarial regions of intrafusal fibers as well as in many axon terminals within lamina IX of the spinal cord. VGLUT1 might be expressed not only in the central axon terminals but also in the peripheral sensory endings of muscle-spindle afferents.

Theme: Sensory systems
Topic: Somatic and visceral afferents

Keywords: VGLUT; Primary sensory afferent; Intrafusal muscle; Rat; Immunohistochemistry

Glutamate is transported into synaptic vesicles by specific vesicular glutamate transporters (VGLUTs) before exocytotic release. VGLUT1, VGLUT2 and VGLUT3 have so far been identified [2–4,6,22,25]. It appears that most of the known glutamatergic neurons express VGLUT1 or VGLUT2, and that the expression patterns of VGLUT1 and VGLUT2 are complementary in many brain regions including the retina [3,5,7,9,10,15]; VGLUT3 exhibits a more restricted expression pattern [4,6,21,23].

In the spinal cord, VGLUT1 immunoreactivity is observed mainly in deep part of the dorsal horn, intermediate gray and ventral horn, whereas VGLUT2 immunoreactivity is seen throughout the grey matter [11,12,24,25]; VGLUT3 immunoreactivity is marked in the intermediolateral column and scattered in the other laminae [11,16]. VGLUT1 and VGLUT2 immunoreactivity has also been observed in sensory ganglion neurons [11,13,19,24]. In the present study, we examined light- and electron-microscopically VGLUTs immunoreactivity in muscle-spindle afferents in the lower limb of the rat.

The present experiments were performed in 16 male Wistar rats (200–300 g body weight; Japan SLC, Shizuoka, Japan and China SH, Xi'an, China). All experimental procedures were in accordance with the rules of animal care and use of Institute of Laboratory Animals, Graduate school of Medicine, Kyoto University (Japan) and the Fourth Military Medical University (China).

In six rats anesthetized by intraperitoneal injection of chloral hydrate (35 mg/100 g body weight), dorsal rhizotomy was done unilaterally from the second to the sixth lumbar cord segments. The operated rats were allowed to survive for 7 days. The six operated rats and a further six normal rats were deeply anesthetized by intraperitoneal injection of an overdose of chloral hydrate (70 mg/100 g body weight) and perfused transcardially with 100 ml of 5 mM phosphate-buffered saline (PBS; pH 7.3), which was followed by 400 ml of 0.1 M phosphate buffer (PB; pH 7.3) containing 3% (w/v) paraformaldehyde and 75% (v/v) saturated picric acid. After perfusion, the lumbar spinal cord and the triceps surae muscle were removed and then cryoprotected with 30% (v/v) sucrose

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in PBS overnight at 4 °C. The fourth lumbar cord segments were cut into 30-μm-thick cross sections on a freezing microtome. The triceps surae muscles were cut into 20-μm-thick longitudinal sections on a cryostat and the sections were mounted onto gelatin-coated glass sides.

The sections of the triceps surae muscles were incubated at room temperature sequentially with (1) 0.2 μg/ml anti-VGLUT1 rabbit IgG [7], 0.1 μg/ml anti-VGLUT2 rabbit IgG [7] or 0.2 μg/ml anti-VGLUT3 guinea pig IgG [8] overnight; (2) 1 μg/ml biotinylated anti-rabbit IgG or anti-guinea pig IgG donkey antibody (Jackson, West Grove, PA, USA) for 2 h; (3) 1/100-diluted avidin-biotinylated peroxidase complex (Vector, Burlingame, CA, USA) for 1 h. The sections were incubated in steps (1) and (2) with 0.05 M PBS (pH 7.3) that contained 0.3% (v/v) Triton X-100, 0.25% (w/v) λ-carrageenan, 0.02% (w/v) NaN_3 and 1% (v/v) normal donkey serum, and in step (3) with 0.05 M PBS (pH 7.3) containing 0.3% Triton X-100. Subsequently, the sections were reacted with 0.02% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (DAB), 20 mM nickel ammonium sulfate and 0.0003% (v/v) H_2O_2 in 0.05 M Tris–HCl buffer (pH 7.6), and counterstained with 1% (w/v) eosin.

Dual immunofluorescence histochemistry for VGLUT1, VGLUT2 or VGLUT3 and choline acetyltransferase (ChAT) was done on sections of the lumbar cord segments and the triceps surae muscles. The sections were incubated at room temperature sequentially with (1) a mixture of anti-ChAT goat antibody (1:100; Chemicon, Temecula, CA, USA) and 1 μg/ml rabbit or guinea pig IgG against VGLUT1, VGLUT2 or VGLUT3 overnight; (2) 1 μg/ml biotinylated anti-goat IgG donkey antibody (Jackson) for 2 h; (3) a mixture of 1 μg/ml Alexa594-conjugated streptavidin (Molecular Probes, Eugene, OR, USA) and 4 μg/ml Alexa488-conjugated goat antibody against rabbit or guinea pig IgG (Molecular Probes) in the presence of 10% (v/v) normal goat serum for 1 h. The sections were then coverslipped with 50% (v/v) glycerol and 2.5% (w/v) triethylenediamine in PBS and observed under confocal microscope LSM5 Pascal (Zeiss, Oberkochen, Germany).

Four rats were anaesthetized with an overdose of chloral hydrate and perfused transcardially with 100 ml of PBS followed by 400 ml of 4% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde in 0.1 M PB. The lumbar cord segments and the triceps surae muscles were removed and postfixed in 4% (w/v) paraformaldehyde for 4 h. The fourth lumbar cord segments were cut into 50-μm-thick transverse sections on a vibratome. The triceps surae muscles were placed in PBS containing 30% (w/v) sucrose for 72 h and then cut into 20-μm-thick transverse sections on a cryostat.

Dual immunohistochemistry was performed on the lumbar cord sections. VGLUT1 and ChAT immunoreactivity was detected, respectively, with the immunogold-silver method and with the immunoperoxidase method. The sections were placed in 20% (v/v) normal donkey serum in PBS for 1 h and incubated at 4 °C overnight with a mixture of 0.5 μg/ml anti-VGLUT1 rabbit IgG and 1/100-diluted anti-ChAT goat IgG in PBS containing 2% (v/v) normal donkey serum (PBS-G). Then the sections were incubated at 4 °C overnight with 1 μg/ml of biotinylated anti-goat IgG donkey antibody in PBS-G, then placed in 10% normal goat serum for 1 h at room temperature, and further incubated at 4 °C overnight with 1/100-diluted anti-rabbit IgG goat antibody conjugated with gold particles (Nanoprobes, Stony Brook, NY, USA) in PBS-G. After postfixation for 10 min with 1% (v/v) glutaraldehyde in 0.1 M PB, silver enhancement was done in the dark with HQ Silver Kit (Nanoprobes). The sections were incubated at room temperature with 1/60-diluted ABC-Elite (Vector) for 4 h, and then with 0.02% (w/v) DAB and 0.001% (v/v) H_2O_2 in 50 mM Tris–HCl (pH 7.6). After washing, the sections were placed for 40 min in 1% (w/v) OsO_4 in 0.1 M PB, counterstained with 1% (w/v) uranyl acetate, embedded in epoxy-resin (Nacalai Tesque, Kyoto, Japan), and cut into ultrathin sections. The ultrathin sections were examined with an electron-microscope H-7100 (Hitachi, Tokyo, Japan).

VGLUT1 immunoreactivity in the triceps surae muscles was also examined electron-microscopically by the immunogold-silver method or the immunoperoxidase method, as described above.

Many axonal components in the spinal cord were observed to show immunoreactivity for VGLUT1, VGLUT2 or VGLUT3 (Fig. 1). It has been known that group Ia afferents are the major primary afferents to make synaptic contact with motoneurons in the ventral horn. Thus we examined VGLUTs immunoreactivity in axon terminals on ventral horn motoneurons, which were identified by ChAT immunoreactivity. In dual immunofluorescence histochemistry for VGLUT (VGLUT1, VGLUT2 or VGLUT3) and ChAT, the majority of VGLUT-immunopositive axon terminals on motoneurons were distributed on ChAT-immunopositive dendritic profiles; they were also seen occasionally on ChAT-immunopositive somatic profiles (Fig. 1A–C). The axon terminals on motoneuronal profiles often showed VGLUT1 or VGLUT2 immunoreactivity, but rarely exhibited VGLUT3 immunoreactivity.

After the unilateral dorsal root rhizotomy from the second to the sixth lumbar cord segments, the density of VGLUT1-immunopositive axonal components in the ventral horn of the fourth lumbar cord segment were markedly reduced on the side ipsilateral to the rhizotomy (Fig. 1D,D′); no such changes were observed in VGLUT2 or VGLUT3 immunoreactivity (Fig. 1E,E′ and F,F′). The data indicated that the primary afferents in synaptic contact with motoneurons express VGLUT1.

It was further examined whether the VGLUT1-immunopositive axon terminals in close apposition to motoneurons under light-microscope might really constitute synapses. The electron-microscopic dual immunohistochemistry revealed that VGLUT1-immunopositive axon terminals containing synaptic vesicles were in asymmetric synaptic contact with ChAT-immunopositive dendritic profiles in the ventral horn (Fig. 1G,H).

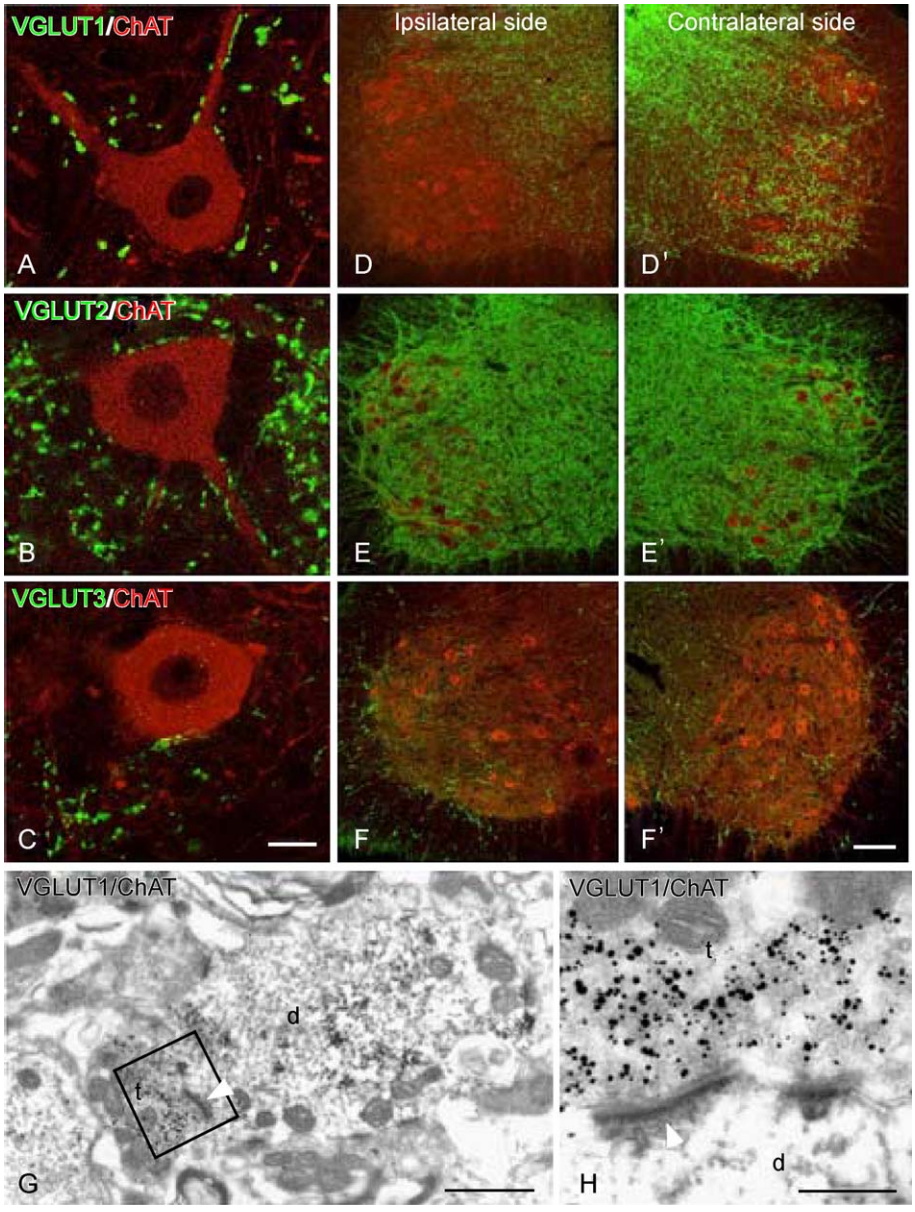

Fig. 1. Dual immunofluorescence histochemistry for VGLUTs (VGLUT1, VGLUT2 or VGLUT3) and ChAT (A–C, D–F, D′–F′) and immunoelectron-microscopic dual immunohistochemisry for VGLUT1 and ChAT (G, H) in the ventral horn of the fourth lumbar cord segment. A–C: VGLUT1—(A), VGLUT2—(B) and VGLUT3—(C) immunoreactivity (green) is seen in axon terminals on ChAT-immunopositive motoneuronal profiles (red). D–F and D′–F′: VGLUTs immunoreactivity in lamina IX on the side ipsilateral (D, E, F) and contralateral (D′, E′, F′) to the unilateral dorsal rhizotomy from the second to the sixth lumbar cord segment. VGLUT1 immunoreactivity is markedly reduced on the side ipsilateral to the rhizotomy (compare D with D′); no such obvious changes are seen for VGLUT2 (E, E′) or VGLUT3 (F, F′) immunoreactivity. G and H: Electron-micrographs show that a VGLUT1-immunopositive (silver grain-labeled) axon terminal (t) is in asymmetric synaptic contact (arrowheads) with a ChAT-immunopositive (peroxidase reaction product-labeled) dendritic profile (d). A part indicated with a rectangle in G is enlarged in H. Scale bars = 20 μm (in C for A–C), 100 μm (in F′ for D–F and D′–F′), 1 μm (G), 0.25 μm (H).

In the longitudinal sections of the triceps surae muscles, VGLUT1-immunopositive fibers ran through the capsule of the muscle spindles to form endings of ribbon-like spirals, which appeared to encircle the equatorial region of the intrafusal muscle fibers (Fig. 2A,B). Some VGLUT1-immunopositive fibers also formed knob-like endings on the juxta-equatarial regions of the intrafusal muscle fibers (Fig. 2A,B). These VGLUT1-mmunopositive endings showed no ChAT immunoreactivity in dual immunofluorescence histochemistry for VGLUT1 and ChAT (Fig. 2B). Neither VGLUT2 nor VGLUT3 immunoreactivity was detected in the triceps surae muscles.

The VGLUT1-immunopositive fiber endings on the intrafusal muscle fibers were further observed electron-microscopically (Fig. 2C–F). The endings contained many spherical vesicles, which were associated with the immuno-

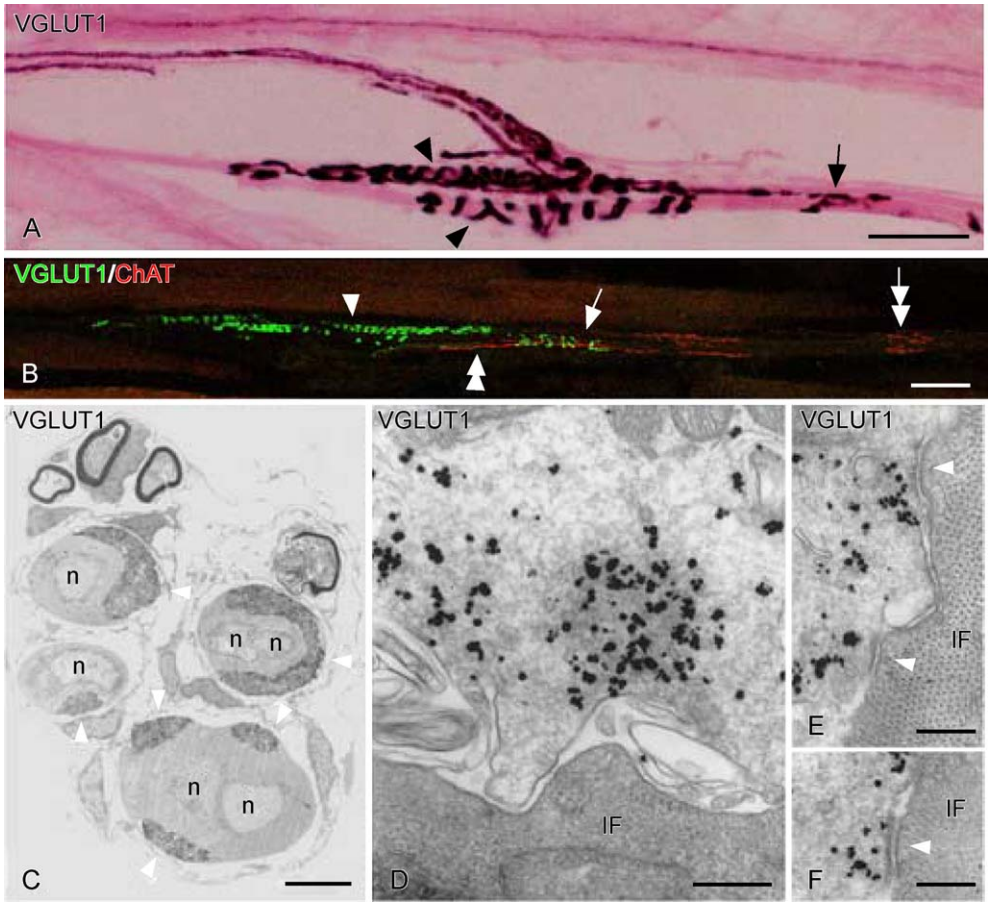

Fig. 2. VGLUT1 immunorreactivity in muscle-spindle of the triceps surae muscle. (A) Muscle spindle afferent fibers that constitute the sensory endings in the equatorial (arrowheads) and the juxta-equatarial regions (arrow) on the intrafusal muscle fibers are immunostained by the immunoperoxidase method. Counterstained with eosin. (B) Dual immunofluorescence histochemistry shows VGLUT1 immunoreactivity (green) in the equatorial (arrowhead) and the juxta-equatarial regions (arrow) of muscle spindle afferents, as well as ChAT-immuonoreactvity (red) in the possible γ-efferent fibers (double arrowhead) and their endings (double arrow) on the intrafusal muscle fibers. No co-expression of VGLUT1 and ChAT is indicated in the neuronal fibers or endings. (C) A low-power immunoelectron micrograph, showing VGLUT1-immonopositive (peroxidase reaction product-labeled) endings (arrowheads) of muscle-spindle afferents in apposition to the cross-sectional profiles of the intrafusal muscle fibers; "n" indicate nuclear profiles of the intrafusal muscles. (D–F) Immunoelectron micographs, showing that the endings of muscle-spindle afferents contain VGLUT1-immunopositive (silver grain-labeled) synaptic vesicle-like structures, and that the junctional couplings (arrowheads) are often seen between the plasma membrane of the VGLUT1-immunopositive endings and the sarcolemma of the intrafusal muscle fibers (IF). Scale bars = 50 μm (A), 100 μm (B), 5 μm (C), 0.1 μm (D–F).

reaction products (Fig. 2D). Multiple sites of junctional adhesion were also observed occasionally between the plasma membrane of the VGLUT1-immunopositive endings and the sarcolemma of the intrafusal muscles near the accumulation of VGLUT1 immunoreactivity (Fig. 2E,F).

Thus, the present data indicate that muscle-spindle afferents express VGLUT1, but not VGLUT2 or VGLUT3. Although myelinated primary afferent fibers, including proprioceptive primary afferent fibers, have already been indicated to express VGLUT1 [24], the present data have further indicated that VGLUT1 is expressed not only in the central axon terminals but also in peripheral sensory endings of muscle-spindle afferents.

Group Ia afferents release glutamate as a fast transmitter from the central axon terminals on motoneurons in the ventral horn [1,14,17,20]. The present study revealed that the peripheral sensory endings of muscle-spindle afferents also contained VGLUT1-immunopositive synaptic vesicle-like structures. However, it is not certain whether glutamate might be released from the peripheral sensory endings of group Ia muscle-spindle afferents, although the assumption has been made that glutamate released from the peripheral sensory endings of group Ia afferents might have an autogenic effect on excitability of the sensory endings [1].

In the present study, multiple sites of junctional adhesion were occasionally observed between the plasma membrane of the sensory endings on the intrafusal muscle fibers and the sarcolemma of the intrafusal muscle fibers. Such structures have also been reported in the tenuissimus muscles of the hamster [18]. However, the functional significance of such membrane specialization at the sensory endings of muscle-spindle afferents still remains to be settled.

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