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Full-length article

VGluT1- and GAD-immunoreactive terminals in synaptic contact with PAG-immunopositive neurons in principal sensory trigeminal nucleus of rat[1]

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Key words

trigeminal; vesicular glutamate transporter 1; glutamic acid decarboxylase; phosphate-activated glutaminase; rats

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Abstract

Aim: To trace the origin of abundant vesicular glutamate transporter 1-like immunoreactive (VGluT1-LI) axon terminals in the dorsal division of the principal sensory trigeminal nucleus (Vpd) and the relationships between VGluT1-LI, as well as the glutamic acid decarboxylase (GAD)-LI axon terminals, and phosphate-activated glutaminase (PAG)-LI thalamic projecting neurons in the Vpd. **Methods:** Following unilateral trigeminal rhizotomy, triple-immunofluorescence histochemistry for VGluT1, GAD and PAG and the immunogold–silver method for VGluT1 or GAD, combined with the immunoperoxidase method for PAG were performed, respectively. **Results:** After unilateral trigeminal rhizotomy, the density of VGluT1-like immunoreactivity (IR) in the Vpd on the lesion side was reduced compared to its contralateral counterpart. Under the confocal laser-scanning microscope, the VGluT1-LI or GAD-LI axon terminals were observed to be in close apposition to the PAG-LI thalamic projecting neuronal profiles, and further electron microscope immunocytochemistry confirmed that VGluT1- and GAD-LI axon terminals made asymmetrical and symmetrical synapses upon the PAG-LI neuronal structures. **Conclusion:** The present results suggest that the VGluT1-LI axon terminals, which mainly arise from the primary afferents of the trigeminal ganglion, along with the PAG-LI neuronal profiles, form the key synaptic connection involved in sensory signaling.

Introduction

The trigeminal sensory nuclear complex was originally divided into 4 nuclei: principalis (Vp), oralis (Vo), interpolaris (Vi), and caudalis (Vc) by Olszewski[1]. Vp comprises a ventral division and a dorsal division (Vpd), which are cytoarchitectonically different and have been proposed to serve the different functions of relaying and processing of sensory signals from the head, such as tactility and trigeminal proprioception[2–4]. Such functions of the Vpd can be supported by the findings that this structure receives direct primary input from intraoral structures and possesses diverse afferent and efferent fiber connections with other brain areas[5–8].

It is well known that glutamate and gamma-aminobutyric acid (GABA) are important excitatory and inhibitory neutrotransmitters in the brain, respectively. Several lines of evidence suggest that these transmitters are involved in signal transmission or processing within the Vpd[9–11]. The Vpd contains a high density of different-sized, immunohistochemically heterogeneous neurons, many of which are likely to contain glutamate[12], and constitute most of the Vpd projection neurons[11]; therefore, these neurons play essential roles in relaying mechanical sensory signals from the head. In addition, the observation that myelinated trigeminal primary afferent terminals are immunoreactive for glutamate implies the glutamatergic nature of their associated input to the Vpd[9]. However, in the central nervous system (CNS), glutamate can also serve as the substrate for GABA synthesis, and phosphate-activated glutaminase (PAG), a marker for glutamatergic structures, was expressed only in the glutamatergic cell bodies and dendrites[13]. So because of the lack of suitable immunocytochemical markers for glutamatergic axons, an understanding of the sensory trans-

mission through the glutamatergic primary afferents from the orofacial area has been hampered. Recently, 3 vesicular glutamate transporters, vesicular glutamate transporter 1 (VGluT1), VGluT2 and VGluT3, have been identified and shown to be present selectively in axons belonging to largely nonoverlapping populations of glutamatergic neurons throughout the CNS[14–16]. Previous immunocytochemical studies have provided evidence that VGluT1 is strongly expressed in the myelinated afferent fibers of the Vpd, whereas VGluT2 and VGluT3-IRs are expressed at very low levels[17,18]. On the other hand, many axon terminals in the Vpd were immunostained for GABA[9]. Based on the aforementioned studies, we infer that VGluT1-LI axon terminals, coming from trigeminal primary afferents, could activate Vpd glutamatergic projection neurons which may be modulated by GABA-LI axon terminals. Therefore, an attempt was made to examine whether or not the VGluT1-LI axon terminals of the Vpd come from the primary afferent fibers by performing a trigeminal rhizotomy in rats, and examining the relationship between the PAG-LI neurons and VGluT1/glutamate decarboxylase (GAD; a marker for GABAergic neurons)-LI axon terminals in the Vpd under the confocal laser-scanning microscope (CLSM) and electron microscope (EM).

Materials and methods

All procedures for the experiments were approved by the Animal Care and Use Committees at the Fourth Military Medical University, Xi'an, China. A total of 10 adult Sprague-Dawley male rats weighing 200–250 g were used. The rats were anesthetized with chloral hydrate (70 mg/100 g body weight) and perfused transcardially for light and electron microscopic experiments[19].

Triple-immunofluorescence histochemistry for VGluT1, GAD and PAG was performed. Briefly, the sections were incubated at room temperature sequentially with: (1) a mixture of 50 μg/mL mouse anti-PAG IgM[20] and 0.8 μg/mL guinea pig anti-VGluT1 IgG[21] or mouse anti-GAD IgG (1:500; Chemicon, Temecula, CA, USA) overnight; (2) biotinylated donkey anti-mouse IgM (1:100; Jackson ImmunoResearch, West Grove, PA, USA) for 4 h; and (3) a mixture of 10% (*v/v*) normal mouse serum, fluorescein isothiocyanate (FITC)-labeled avidin-D (1:1000; Vector Labs, Burlingame, CA, USA) and rhodamine-labeled goat anti-guinea pig IgG (1:100; Chemicon, USA) and Cy5-labeled donkey anti-mouse IgG (1:100; Chemicon, USA) for 3 h. The incubation medium in steps 1 and 2 was prepared by using 0.05 mol/L phosphate-buffered 0.9% saline (PBS) containing 0.5% (*v/v*) Triton X-100, 0.25% (*w/v*) λ-carrageenan, 0.05% (*w/v*) NaN_3 and 5% (*v/v*) normal donkey serum. The incubation medium in step 3 was prepared by using 0.05 mol/L PBS containing 0.3% (*v/v*) Triton X-100. After the incubation, the sections were rinsed in 0.05 mol/L PBS, mounted onto gelatin-coated glass slides, and then observed under the CLSM (LSM 410; Zeiss, Oberkochen,Germany) by using a laser beam of 488 nm, 543 nm and 633 nm with appropriate emission filters for FITC (510–525 nm), rhodamine (590–610 nm) and Cy5 (670–810 nm). In the control experiments for the immunofluorescence histochemistry, one of the primary antibodies was omitted or replaced with normal IgG; no immunoreactivity for the omitted or replaced antibody was found.

Electron microscopically, the immunogold-silver method for VGluT1 or GAD, combined with the immunoperoxidase method for PAG, was performed. Briefly, the sections were incubated for 24 h at room temperature with a mixture of 0.8 μg/mL guinea pig anti-VGluT1 IgG or 1/500-diluted mouse anti-GAD IgG (Chemicon, USA) and 75 μg/mL mouse anti-PAG IgM, each diluted in 50 mmol/L Tris-buffered saline (TBS; pH 7.4) containing 2% (*v/v*) normal goat serum (TBS–NGS). Then the sections were washed in TBS and further incubated overnight at room temperature with a mixture of 1/100-diluted biotinylated donkey anti-mouse IgM antibody (Jackson ImmunoResearch, USA) and 1/100-diluted anti-guinea pig IgG or anti-mouse IgG antibody conjugated to 1.4 nm gold particles (Nanoprobes; Stony Brook, NY, USA), each diluted in TBS-NGS. Subsequently the sections were processed for: (1) 1% postfixation with glutaraldehyde in 0.1 mol/L phosphate buffer PB for 10 min; (2) silver enhancement with an HQ Silver Kit (Nanoprobes, Stony Brook, NY, USA); (3) incubation with an ABC reagent (Vector Labs, Burlingame, CA, USA) diluted at 1:50 in 50 mmol/L TBS for 3 h at room temperature; (4) visualization of PAG-immunoreactivity by incubation with diaminobenzidine tetrahydrochloride and H_2O_2; (5) osmification with 1% OsO_4 in 0.1 mol/L PB for 1 h; (6) counterstaining with uranyl acetate; and (7) flat-embedding in Durcupan (Fluka, Buchs, Switzerland) after dehydration and mounting on silicon-coated glass slides. Ultrathin sections were prepared and examined as described[19].

Trigeminal rhizotomy was prepared as described[22]. Briefly, after anesthesia, unilateral trigeminal rhizotomy was performed on 3 rats. A 10-day postoperative period was allowed for degeneration, and then the rats were fixed and tissue was prepared for VGluT1 immunohistochemistry as mentioned earlier. The sections containing the Vpd were incubated sequentially at room temperature with: (1) 0.5 μg/mL guinea pig anti-VGluT1 IgG overnight; (2) 1/100-diluted biotinylated anti-guinea pig IgG donkey antibody

(Jackson ImmunoResearch, USA) for 4 h; and (3) 1/50-diluted avidin-biotinylated peroxidase complex (Vector Labs, USA) for 2 h. The incubation medium was the same as those used in the immunofluorescence histochemistry. Subsequently the sections were reacted with 0.02% (*w/v*) 3,3'-diaminobenzidine tetrahydrochloride and 0.003% H_2O_2 (*v/v*) for 10–30 min in 0.05 mol/L Tris-HCl buffer (pH 7.6).

Results

Under CLSM, the bodies of PAG-LI neurons were easily identified with FITC fluorescence (green channel); VGluT1-LI axon terminals with rhodamine fluorescence (red channel) and GAD-LI axon terminals with Cy5 fluorescence (blue channel). The PAG-IR was seen in small or medium-sized cell bodies of the Vpd neurons. On the other hand, VGluT1-IR was found mostly in the varicosities' characteristic of axon terminals. We also found many GAD-LI axon varicosities distributed in the Vpd. Both the VGluT1- and GAD-LI axon terminals were frequently seen in close apposition to PAG-LI cell bodies, and there were some VGluT1- and GAD-LI axonal terminals which formed close appositions on the same PAG-LI cell body (Figure 1).

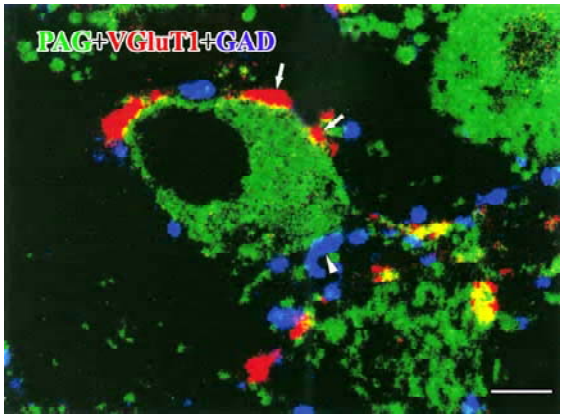

Figure 1. Digital images of one section through the lower brainstem part containing the Vpd, which were taken with confocal laser-scanning microscope. Immunoreactivities for phosphate activated glutaminase (PAG), vesicular glutamate transporter 1 (VGluT1) and glutamic acid decarboxylase (GAD) are visualized with fluorescein isothiocyanate (FITC; green), tetramethyl rhodamine (Rh; red) and indodicarbo cyanine (Cy5; blue), respectively. Arrow or arrowhead indicated VGluT1 or GAD varicosities in close apposition with PAG-LI cell body, respectively. Scale bar=4 μm.

Under the EM, some terminals were labeled with immunogold-silver grains indicating VGluT1-LI axon terminals. Most of the VGluT1-LI terminals contained abundant small clear round vesicles, and a number of VGluT1-LI terminals made asymmetric synaptic contacts with PAG-positive dendritic profiles (Figure 2A). No VGluT1-LI axon terminals containing flattened vesicles and forming symmetrical synapses were found. On the other hand, in the material processed for dual immunohistochemical labeling of GAD/PAG, most of the GAD-LI terminals made symmetric synapses with PAG-LI dendritic profiles (Figure 2B).

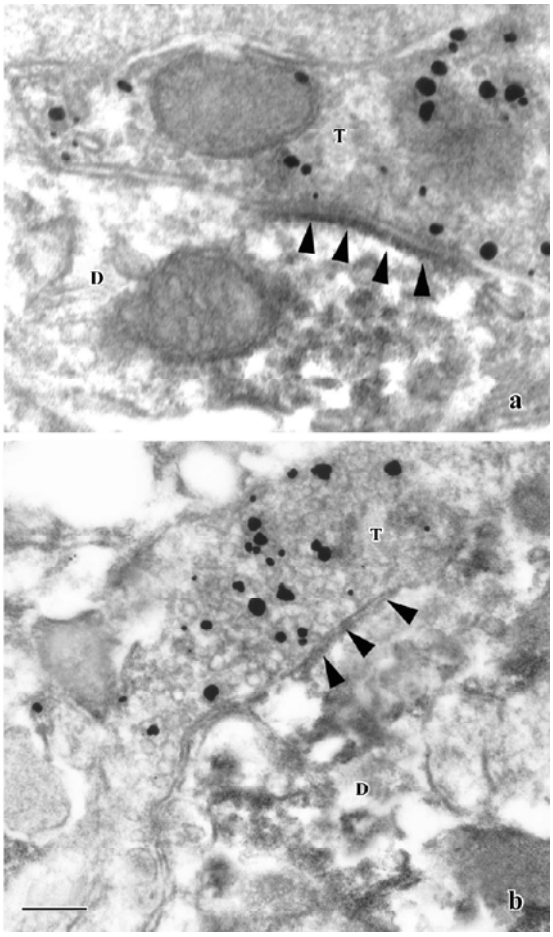

Figure 2. Photomicrographs illustrating synapses between VGluT1- or GAD-LI axon terminals (T) and PAG-LI dendritic profile (D) in the Vpd. a: an axon terminal (T), which contains silver grains (VGluT1-LI), made an asymmetric synaptic contact with a PAG-LI dendritic profile (D, identified by fine granular and homogeneously distributed electron dense reaction product). b: an axon terminal (GAD, containing silver grains), which is in symmetric synaptic contact with a PAG-LI dendritic profile (D). Scale bar=0.15 μm.

It was examined whether or not VGluT1-IR is expressed in primary afferent fibers. After unilateral trigeminal rhizotomy, it was clearly shown that VGluT1-IR was less

intense in the Vpd (Figure 3A) on the side ipsilateral to the trigeminal rhizotomy compared to the contralateral side (Figure 3B, 3C). Thus, the trigeminal rhizotomy experiments indicated that VGluT1 was expressed in primary afferent fibers terminating within the Vpd.

Discussion

The results of present study show that PAG-LI neurons receive orofacial sensory information transmitted from the VGluT1-LI axon terminals, mainly arising from primary afferent fibers of the trigeminal ganglion. It has been reported that most of the neurons in the Vp projecting to the ventroposterior medial nucleus of the thalamus are glutamatergic[9,11] and that glutamate-IR is present in some primary afferent terminals and functions as an important excitatory transmitter involved in the relay of sensory information to the Vp[10]. Recently, Varoqui *et al* suggested that mechanoreception at the Vp predominantly involved VGluT1 expressing axonal terminals[17]. Combined with the previous studies, our present results suggest that glutamatergic axon terminals might act on the PAG-LI neurons in the Vpd and transmit the orofacial region non-nociceptive sensory information to them.

Bae *et al*[9] tested whether GABA could act on the glutamatergic primary afferents through the axoaxonic synapses to modulate the transmission of the orofacial sensory information in the Vpd. In the present study, we observed that the GAD-LI axon terminals formed symmetric synapses with PAG-LI neurons and inferred that the GABA functioned as an inhibitory transmitter acting on the neurons of the Vpd in addition to the primary afferent fibers.

In summary, the present results, together with the previously reported ones, provide strong anatomical evidence that Vpd neurons receive trigeminal orofacial sensory information from VGluT1 expressing glutamatergic terminals and that GABAergic postsynaptic regulation might occur at the level of the Vpd neurons.

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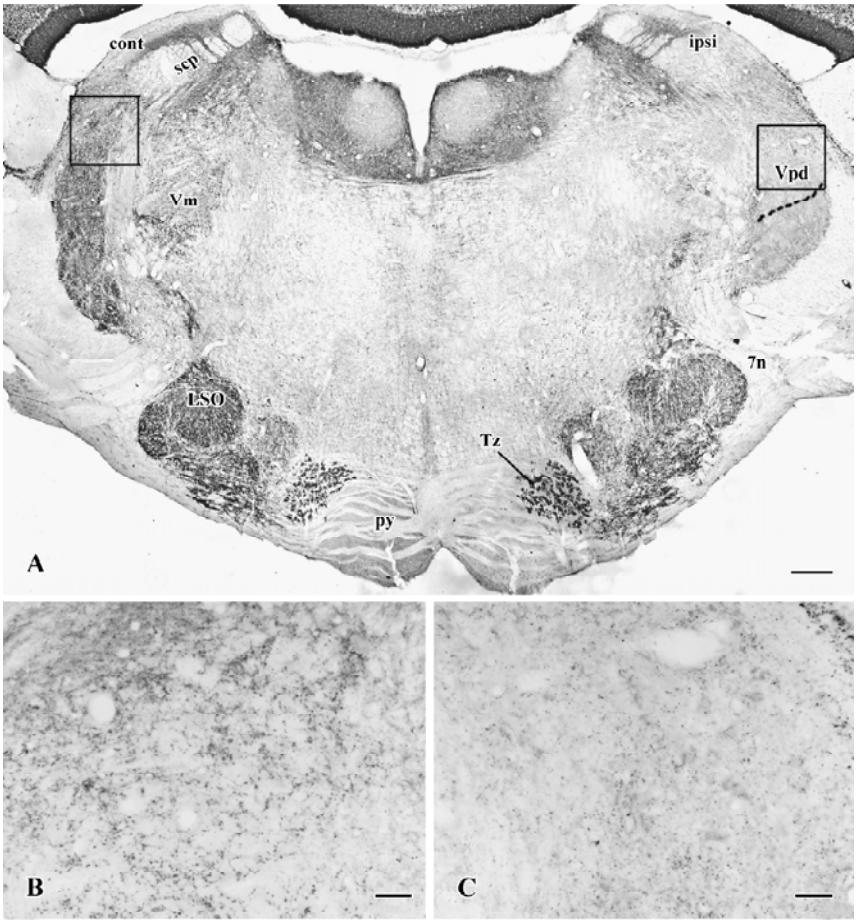

Figure 3. Vesicular glutamate transporter 1-immunoreactivity (VGluT1-IR) in the Vpd of rat, which was subjected to unilateral trigeminal rhizotomy and then was allowed to survive for 10 days. VGluT-IR is decreased on the ipsilateral side to the trigeminal rhizotomy (right) compared with that on the contralateral side (left). B, C is the amplified picture in the left or right box of A, respectively. cont, contralateral side; ipsi, ipsilateral side; LSO, lateral superior olive; py, pyramidal tract; scp, superior cerebellar peduncle; Tz, trapezoid body; Vm, motor trigeminal nucleus; Vpd, the dorsal division of principal sensory trigeminal nucleus. Scale bars=600 μm in A, 50 μm in B and C.

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