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# EXPRESSION OF VESICULAR GLUTAMATE TRANSPORTERS IN PERIPHERAL VESTIBULAR STRUCTURES AND VESTIBULAR NUCLEAR COMPLEX OF RAT

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**Abstract—Glutamate transmission from vestibular end organs to central vestibular nuclear complex (VNC) plays important role in transferring sensory information about head position and movements. Three isoforms of vesicular glutamate transporters (VGLUTs) have been considered so far the most specific markers for glutamatergic neurons/cells. In this study, VGLUT1 and VGLUT2 were immunohistochemically localized to axon terminals in VNC and somata of vestibular primary afferents in association with their central and peripheral axon endings, and VGLUT1 and VGLUT3 were co-localized to hair cells of otolith maculae and cristae ampullaris. VGLUT1 and VGLUT2 defined three subsets of Scarpa's neurons (vestibular ganglionic neurons): those co-expressing VGLUT1 and VGLUT2 or expressing only VGLUT2, and those expressing neither. In addition, many neurons located in all vestibular subnuclei were observed to contain hybridized signals for VGLUT2 mRNA and a few VNC neurons, mostly scattered in medial vestibular nucleus (MVe), displayed VGLUT1 mRNA labelling. Following unilateral ganglionectomy, asymmetries of VGLUT1-immunoreactivity (ir) and VGLUT2-ir occurred between two VNCs, indicating that the VNC terminals containing VGLUT1 and/or VGLUT2 are partly of peripheral origin. The present data indicate that the constituent cells/neurons along the vestibular pathway selectively apply VGLUT isoforms to transport glutamate into synaptic vesicles for glutamate transmission.** 

**Key words: vesicular glutamate transporter, hair cell, ganglionectomy, vestibular nucleus, immunohistochemistry, rat.**

Glutamate is proposed to be the neurotransmitter at synapses along vestibular pathway, starting from vestibular sensory epithelia to brain via afferent neurons located in Scarpa's ganglion (de Waele et al., 1995; Büttner-Ennever, 2000). It was reported that glutamate released from hair cells of vestibular end-organs, by binding to its cognate receptors located on calyx- or button-shaped nerve endings, activated primary vestibular afferents (Matsubara et al., 1999; Cochran, 2000; Usami et al., 2001; Bonsacquet et al., 2006) which, in turn, applied glutamate to activate second-order neurons in vestibular nuclear complex (VNC) (Chen et al., 2000; Grassi et al., 2004, 2005; Biesdorf et al., 2008). Corroborative evidence for vestibular glutamatergic transmission also includes the findings that auditory hair cells, phylogenetically related to their vestibular counterparts, release glutamate as neurotransmitter (Glowatzki and Fuchs, 2002; Ruel et al., 2008; Seal et al., 2008). Despite these data, identifying glutamate with excitatory neurotransmitter at vestibular synapses still requires consolidation. Previous morphological approaches to defining glutamatergic neurons/hair cells rely on immunocytochemical demonstration of cytoplasm-localized glutamate or phosphate-activated glutaminase (PAG), a glutamate-synthesizing enzyme (Takumi et al., 1999; Usami et al., 2001). Since glutamate is a global metabolic substrate in protein synthesis and also serves as the precursor of inhibitory neurotransmitter GABA, glutamate or PAG presents no specific markers for glutamatergic neurons/cells (Kaneko and Fujiyama, 2002; Landry et al., 2004).

The recently cloned three isoforms of vesicular glutamate transporters (VGLUTs), named VGLUT1–3, are proton-driven carriers that can load glutamate into synaptic vesicles (Fremeau et al., 2004). VGLUT1 and VGLUT2 remain most specifically in glutamatergic neurons (Kaneko and Fujiyama, 2002; Hioki et al., 2003; Oliveira et al., 2003; Fremeau et al., 2004), and VGLUT3, though expressed by neurons that usually release a transmitter other than glutamate, such as cholinergic, serotonergic or GABAergic neurons (Fremeau et al., 2002; Gras et al., 2002; Hioki et al., 2004, 2010), has also been demonstrated to be implicated in glutamate release (Obholzer et al., 2008; Ruel et al., 2008; Seal et al., 2008).

Compared with glutamate itself or PAG, VGLUT isoforms can be applied as more specific markers for glutamatergic neurons/cells in neural networks, including those

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*Abbreviations:* As, stalk of the ampullary crest; DRG, dorsal root ganglion; g7, genu of facial nerve; icp, inferior cerebellar peduncle; LVe, lateral vestibular nucleus; MVe, medial vestibular nucleus; NLS, N-lauroylsarcosine; PAG, phosphate-activated glutaminase; PBS, phosphate buffered saline; SpVe, spinal vestibular nucleus; SSC, saline sodium citrate; SuVe, superior vestibular nucleus; TG, trigeminal ganglion; VGLUTs, vesicular glutamate transporters; VNC, vestibular nuclear complex; 2 VN, second-order vestibular neurons.

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in vestibular pathway (Kaneko and Fujiyama, 2002; Landry et al., 2004). On the other hand, distinct role for each VGLUT isoform is suggested by the mutually exclusive distribution of these transporters in the brain (Fremeau et al., 2004; Seal et al., 2008). Elucidation of the type(s) and distributional profile of VGLUTs expressed by vestibular system is, therefore, conducive to functionally understanding vestibular glutamatergic transmission. However, aside from a few recent studies revealing the presence of VGLUT3 in auditory hair cells (Furness and Lawton, 2003; Obholzer et al., 2008; Ruel et al., 2008; Seal et al., 2008) and some incidental results about VGLUTs in VNC (Hisano et al., 2002), systematic data elaborating on the expression of VGLUTs associated with vestibular pathway is yet to be obtained. We therefore performed the experiments to localize the three VGLUT isoforms in central and peripheral vestibular structures.

## EXPERIMENTAL PROCEDURES

### Tissue preparation and ganglionectomy

Thirty five adult Sprague–Dawley rats (230–250 g) of either sex, divided into intact ($n=15$), ganglionectomized ($n=10$) and sham-operated ($n=10$) groups, were used. The experimental procedures were approved by the Animal Care and Use Center at the Fourth Military Medical University (Xi'an, PR China). Ten intact animals, following perfusion with 0.1 mol/L phosphate buffered saline (PBS, pH 7.2–7.4) under pentobarbital sodium anaesthesia (40 mg/kg body weight, i.p.), were decapitated. Rat brains together with temporal bones were removed and immersed sequentially through 75% (v/v) saturated picric acid –0.1 mol/L sodium phosphate buffer (PPB, pH 7.2–7.4) containing 0.5% and 4% paraformaldehyde for 10 h and 5 h, respectively. The brains, Scarpa's ganglia, and otolith maculae and ampullary cristae were microdissected, then cryoprotected with 30% sucrose overnight at 4 °C. Frozen sections of 25 $\mu$m (brain slices) or 15 $\mu$m (peripheral tissues) were serially cut in a cryostat. The sections were divided into four series, each series consisting of every fourth sections that were collected into PBS or directly mounted on gelatin-coated slides. One series was used for control experiment while the others for immunohistochemistry for VGLUTs.

To perform *in situ* hybridization histochemistry, five intact rats were perfused with 4% (w/v) formaldehyde in 0.1 M phosphate buffer and brain blocks were further fixed in the same fixative for 3 days at 4 °C before cryoprotection with 30% (w/v) sucrose. 18-$\mu$m-thick transverse sections were cut on a freezing microtome.

For ganglionectomy, a lateral incision of skin below the opening of ear canal was made on one side from posterior part of masseter ventro-caudally to neck. Auditory bulla was then approached through microdissection of facia and deep tissues under the incision. After fracturing the bulla and removing ossicles, pterygopalatine artery caudal to the stapes was cauterized and the cochlea was entirely extirpated. Parts of bony labyrinth were removed to enlarge the surface area of the brainstem that surrounds the orifice of internal acoustic meatus, above which lies the Scarpa's ganglion. Ganglionectomy was then accomplished carefully through hard pinch of the ganglion with fine tweezers. The surgery was completed by suturing the tissues anatomically after stuffing auditory space with sterilized cotton pellets and antibiotics application. All the operated rats exhibited postural and oculomotor syndromes typical of unilateral vestibular loss after recovery from anaesthesia. For sham-operated rats which served as controls, opening of the bulla and facial nerve transection with searing of the pterygopalatine artery were performed. Both ganglionectomized and sham-operated animals were allowed to survive for 4 days, then treated for immunohistochemistry in a similar way to that of intact animals.

### Immunohistochemistry for VGLUT1–3

Immunohistochemistry for VGLUT1–3 was performed on three series of sections, respectively, following standard immunostaining procedures. Sections were incubated at room temperature sequentially with (1) either rabbit polyclonal antibodies against VGLUT1 (1 $\mu$g/ml, Hioki et al., 2003), guinea pig polyclonal antibodies against VGLUT2 (1 $\mu$g/ml, Fujiyama et al., 2001) or guinea pig polyclonal antibodies against VGLUT3 (1 $\mu$g/ml, Hioki et al., 2004) overnight; (2) biotinylated goat anti-rabbit IgG (5 $\mu$g/ml, Vector) or anti-guinea pig IgG (1:200, Chemicon) for 6 h, and (3) avidin-biotin complex (1:100, Vector) in PBS containing 0.3% Triton X-100 for 3 h. The signal was visualized with 3-3'-diaminobenzidine (DAB) as chromogen. For immunoreactive control, normal rabbit or guinea pig serum was used to replace primary antibodies for incubation of the fourth series of sections. No immunoreactivity was observed in this case.

### Double immunofluorescence histochemistry

For double immunofluorescence staining, the primary antibodies used were rabbit polyclonal antibody against VGLUT1 (1 $\mu$g/ml, Hioki et al., 2003) and guinea pig polyclonal antibody against VGLUT2 (1 $\mu$g/ml, Fujiyama et al., 2001) or VGLUT3 (1 $\mu$g/ml, Hioki et al., 2004). Secondary antibodies included Alexa 488 or Alexa 594 conjugated to donkey anti-rabbit IgG (4 $\mu$g/ml, Invitrogen) and to goat anti-guinea pig IgG (4 $\mu$g/ml, Invitrogen). Following reaction, the sections were observed under laser scanning confocal microscopy by using laser beams of 490 nm (Alexa 488) and 590 nm (Alexa 594) with appropriate emission filters, 520–530 nm and 600–630 nm, respectively.

### Fluorescence *in situ* hybridization histochemistry

Complementary DNA fragment of VGLUT1 (nucleotides 855–1788; GenBank accession number XM_133432.2; Watakabe et al., 2006) or VGLUT2 (848–2044; NM_080853.2; Nakamura et al., 2007) was cloned into a vector pBluescript II KS (+) (Stratagene, La Jolla, CA, USA). Using the linearized plasmids as template, we synthesized sense and antisense single-strand RNA probes with a digoxigenin RNA labeling kit (Roche Diagnostics, Basel, Switzerland).

The hybridization procedure was previously reported (Hioki et al., 2010). Briefly, free-floating sections were hybridized for 20–24 h at 60 °C with 1 $\mu$g/ml digoxigenin-labeled sense or antisense RNA probes for VGLUT1 or VGLUT2 in a hybridization buffer, consisting of 5× saline sodium citrate (SSC; 1×SSC=0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 2% (w/v) blocking reagent (Roche Diagnostics), 50% (v/v) formamide, 0.1% (w/v) N-lauroylsarcosine (NLS) and 0.1% (w/v) SDS. After two washes in 2×SSC, 50% (v/v) formamide and 0.1% (w/v) NLS for 20 min at 60 °C, the sections were incubated with 20 $\mu$g/ml RNase A for 30 min at 37 °C in 10 mM Tris–HCl (pH 8.0), 1 mM ethylenediamine tetraacetic acid and 0.5 M NaCl, followed by two washes with 0.2×SSC containing 0.1% (w/v) NLS for 20 min at 37 °C. Subsequently, the sections were incubated overnight at room temperature with a mixture of 1:2000-diluted peroxidase-conjugated anti-digoxigenin sheep antibody (11-207-733-910; Roche Diagnostics) in 0.1 M Tris–HCl (pH 7.5)-buffered 0.9% (w/v) saline (TS7.5) containing 1% blocking reagent (TSB). To visualize the signals for VGLUT1 or VGLUT2 mRNA efficiently, we performed the biotinylated tyramine (BT)-glucose oxidase (GO) amplification method (Kuramoto et al., 2009) with the reaction mixture containing 1.25 $\mu$M BT, 3 $\mu$g/ml GO, 2 mg/ml beta-D-glucose, and 1% bovine serum albumin (BSA) in 0.1 M PB for 30 min. The sections were then incubated with 5 $\mu$g/ml Alexa488-conjugated streptavidin (S-11223; Invitrogen, Eugene, OR, USA) in TSB for 3 h.