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Localization of vesicular glutamate transporters in the peripheral vestibular system of rat

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Abstract: Objective To examine the vesicular glutamate transporters (VGluTs: VGluT1-VGluT3) in the peripheral vestibular system. **Methods** The vestibular structures, including Scarpa's ganglion (vestibular ganglion, VG), maculae of utricle and saccule, and ampullary cristae, from normal Sprague-Dawley rats were processed immunohistochemically for VGluTs, by avidin-biotinylated peroxidase complex method, with 3-3'-diaminobenzidine (DAB) as chromogen. **Results** (1) VGluT1 was localized to partial neurons of VG and to the putative primary afferent fibers innervating vestibular end-organs. (2) Intense VGluT3 immunoreactivity was detected in large number of sensory epithelia cells, and weak labeling of VGluT3-positive afferent fibers was in the maculae and ampullary cristae. (3) No or very weak VGluT2 immunoreactivity was observed in the VG and acoustic maculae. **Conclusion** These results provide the morphological support that glutamate exists in the peripheral vestibular system, and it may play an important role in the centripetal vestibular transmission.
Keywords: vesicular glutamate transporter; acoustic maculae; Scarpa's ganglion; immunohistochemistry; rat

1 Introduction

Glutamate plays an important role in neurotransmission in both central and peripheral nervous system (CNS and PNS, respectively). Recently, three vesicular glutamate transporter (VGluT) isoforms (VGluT1-VGluT3) located on the vesicular membrane were cloned[1,2]. They transport glutamate from the axoplasm into the synaptic vesicles. Morphological studies have revealed that these VGluTs are expressed in distinct populations of neurons in the brain[1-3]. VGluT1 and VGluT2 were considered to specifically label glutamatergic neurons, whereas VGluT3 was identified in perikarya and terminals of some modulatory neurons such as cholinergic, serotonergic and GABAergic neurons[1,2]. Using VGluTs as markers for glutamatergic neurons, we can characterize and analyze the excitatory neuron(s) involved in a network or pathway of a particular sensory and/or motor system.

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Article ID: 1673-7067(2007)03-0175-05
CLC number: Q427
Document code: A
Received date: 2007-04-10

Peripheral vestibular system is responsible for transferring the signals encoding head orientation in space from the macula and ampullary crista to the brain via Scarp's gangalion (vestibular ganglion, VG). Previous physiological and morphological data indicated that VG neurons could be activated by the excitatory transmitter of the hair cells at the peripheral afferent nerve endings, and could release glutamate to excite the central otolith neurons in the brain[4-6]. Up to date, VGluTs have received wide investigations in various brain regions, spinal cord, and peripheral structures such as dorsal root ganglion, trigeminal ganglion, cochlea, and retina[7-12]. It is noteworthy that although the vestibular-related brain structures have been studied[13,14], the expression patterns of VGluTs in the peripheral vestibular system, however, remains unclear. The present immunocytochemical study is performed to address this issue.

2 Materials and methods

2.1 Animals and tissue preparation Five adult Sprague-Dawley rats, weighing 230-250 g, were used in the present study. All animals were deeply anaesthetized with pentobarbital sodium and perfused with 0.1 mol/L phosphate buffered saline (PBS, pH 7.2-7.4) via the ascending aorta. The rats were immediately decapitated, and the brains to-

gether with the connected the temporal bones were removed, immersed in 75% (V/V) saturated picric acid–0.1 mol/L sodium phosphate buffer (pH 7.2-7.4) containing 0.5% paraformaldehyde for 10 h, and postfixed in 1% paraformaldehyde instead for 5 h. Under the stereo microscope, the Scarpa's ganglion, which is interposed between the brain cochlear nucleus and the internal auditory meatus in the temporal bone, and the membrane labyrinth containing otolith organs and ampullary cristae were carefully removed by fracturing the bone with fine tweezers. And then these structures were cryoprotected with PBS containing 30% sucrose overnight at 4 °C. Every fourth sections at 20 μm thickness, serially cut through the ganglion in cryostat, were directly mounted on gelatine-coated slides. Four series of sections were collected. One series was used for control experiment while the others were used for VGluT receptor immunohistochemistry.

2.2 Immunohistochemistry for VGluTs Sections were incubated in fully humidified chamber overnight in PBS at room temperature. Three series of brain sections were immunohistochemically stained for the three transporters, respectively. The sections were sequentially incubated through the following solutions at room temperature: (1) a mixture of 2% normal goat serum and 0.3% Triton X-100 containing either of the following primary antibodies (gifts from Kaneko T, Japan): rabbit anti-VGluT1 (0.5 μg/mL), rabbit anti-VGluT2 (0.4 μg/mL) or rabbit anti-VGluT3 (0.5 μg/mL); (2) biotinylated goat anti-rabbit IgG (1:200; Vector) in PBS containing 2% normal goat serum and 0.3% Triton X-100 for 24 h; and (3) avidin-biotin complex (Vector, 1:200) in PBS for 24 h. Following each step, the sections were rinsed with PBS for 3 ×10 min. Finally, the signal was visualized with 3-3'-diaminobenzidine (DAB) as chromogen, and the sections were air-dried, dehydrated, cleared and coverslipped for observation. The fourth series of sections was used as negative control, where the first antibody was replaced by PBS. No staining was observed on the brainstem sections when the primary antibody was omitted from the protocol.

3 Results

VGluT immunoreactivities were examined in detail in the VG, maculae of utricle and saccule, and ampullary cristae. Excepting that VGluT2 showed no or very weak labeling (if any), both VGluT1 and VGluT3 proteins were clearly observed in these peripheral vestibular structures. The labeling patterns, however, displayed great difference between the two transporters (Fig. 1).

Both superior and inferior ganglia showed VGluT1 immunoreactivity, and VGluT1-immunoreactive grains demonstrated a relatively homogeneous distribution in the cytoplasm. The labeling clearly delineated the neuronal configuration and the round nucleus (Fig. 1A). A close examination revealed that the larger dots of VGluT1-immunostaining, each of which were observed at higher resolution to be the congregation of fine immunopositive particles, scattered or gathered to form dark brown labeling in the cytoplasm. In the VG, the immunostaining for VGluT1 was restricted only to part of the neurons, and the labeling intensity varied among neurons. No positive fibers were observed in the VG. In the vestibular end organs, many VGluT1-immunolabeled fibers with different lengths were observed in the connective tissues underlying the cell layer of macula, and a VGluT1-labeled plexus was shown in the stalk of the ampullary crista (Fig. 1B, C). In the cell layer of the end organs, circles of VGluT1 labeling could be seen, and in some cases the circle was continuous with the immunopostive fibers extending from under the cell layer (the arrow in Fig. 1C).

VGluT3 immunoreactivity was absent in the VG, but was seen in large number of cells, presumably hair cells, in the sensory epithelium of both the macula and the ampullary crista. The immunostained products were localized to the cellular cytoplasm, in striking contrast with the round nucleus which lacks the immunoreactivity. The VGluT3 immunostaining intensity was different from cell to cell, spanning from weak to intense, and was heterogeneously distributed in the cytoplasm. The labeled sensory cells in the macula or ampullary crista were similar in size and shape. In our sections (Fig. 1E, F), the labeled cells displayed a multiple instead of single layer pattern. This can be easily understood by the oblique section plane which was not perpendicular to the epithelium cell layer. In addition, many weakly VGluT3-immunostained fibers in connective tissues of maculae or stalk of ampullary cristae were observed (Fig. 1E, F). In the connective tissue of otolith organs the VGluT3-labeled fibers showed a labeling plexus, but the individual fibers were shown to be orientated towards the cell layer (Fig. 1E). Similarly, the VGluT3-immunostained fibers in the crista ampularis coursed toward the labeled cells (Fig. 1F).

No VGluT2-immunoreactive fibers or cells were observed in the otolith organs or cristea ampullaris. VGluT2 immuonreactivity could be barely seen in the VG (Fig. 1D).

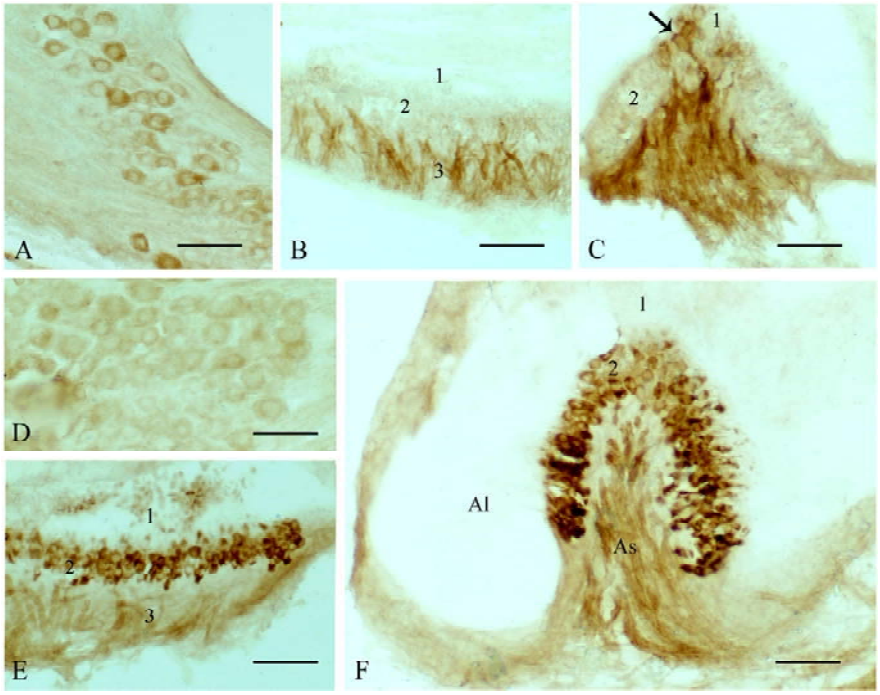

Fig. 1 Vesicular glutamate transporter immunoreactivities in the peripheral vestibular system. A-C, VGluT1 immunostaining in VG, acoustic macula and crista ampullaris, respecitively (upward is to the otolith chamber for B, and to the lumens of ampulla for C). Note the VGluT1-immunostained fibers in the connective tissue in B and C. The arrow in C points to the putative calyx formed by the afferent nerve ending of vestibular ganglion neuron. D, VG neurons showing no or weak VGluT2 immunoreactivity. E-F, VGluT3 immunoreactivities in acoustic crista (E) and crista ampullaris (F) (upward is to the otolith chamber in E, and to the lumens of ampulla in F). The numbers 1-3 indicate cupula/ otolithic membrane, cell layer, and connective tissue, respectively. Al, lumen of the ampulla; As, stalk of the ampullary crest. Scale bar, 80 μm in A and D, 60 μm in B, 90 μm in C and F, and 100 μm in E.

Most of the ganglion neurons were devoid of VGluT2 immunostaining, only very few had an extremely weak labeling which was close to the background staining. Because using the same primary antibody directed against VGluT2, a high density of VGluT2-immunopostive axon terminals was detected in the brain, as shown in the vestibular nucleus of our previous study[14]. It was reasonable to consider no or very weak labeling, if any, for VGluT2 expression in the VG.

4 Discussion

With the antibodies against the recently cloned VGluTs, the present study provided morphological data indicating that except for VGluT2, VGluT1 and VGluT3 were distributed in the peripheral vestibular system. Their labeling patterns, however, were different. These results further confirm that peripheral vestibular pathway employs glutamate for synapstic transmission.

No or very weak staining, if any, for VGluT2 was observed in the VG neurons. This contrasted with the trigeminal ganglion where about 80% neurons expressed VGluT2, and with the dorsal root ganglia in which a few of neurons expressed VGluT2[9,10].

A low percent of VG neurons expressing VGluT2, as shown in the present study, implies that the subset of these neurons is different from others. The similar cases occurred in the trigeminal and dorsal root ganglia, where not all the neurons were VGluT1-immunoreactive[9-11]. The physiological function of this particular group of VG neurons, and the significance underlying their expression of VGluT1 are interesting issues. The lack of VGluT1-immunoreactivity in other VG neurons does not necessarily mean that they are not glutamategic, because there is convincing evidence that many types of neurons which are well known to use glutamate as neurotransmitters do not express VGluT1. For instance, the glutamatergic mesencephalic trigeminal nucleus neurons just transiently express VGluT1 during early postnatal development and stop the expression in adult[15].

In CNS, VGluT1 mostly immunostained axon terminals of glutamatergic neurons, although occasionally they labeled the short lengths of fibers[9-11,15,16]. In agreement with

this is our previous data demonstrating that the central axons of VG neurons immunoreactive for VGluT1 mostly showed punctate labeling[14]. However, in contrast to the substantial VGluT1 punctate labeling in the CNS, we observed VGluT1-immunostained nerve fibers with different lengths in the acoustic maculae and ampullary cristae. It is reasonable to presume that these VGluT1-positive fibers represented the peripheral axons of VG neurons. This speculation is derived not only from the well known anatomical knowledge that the peripheral vestibular afferent nerves are oriented in a similar way as we shown here, but from the comparable VGluT1 labeling pattern in muscular tissue as well[15]. In masseter, long fibers of muscle spindle afferents were immunostained by VGluT1, different from their dot-like central labeling in the brain[15]. Finally, it is well known that calyx is the structure formed between peripheral nerve ending and type I hair cell[6]. In the present study, the immunostained circles in the cell layer of ampullary crista and its associated VGluT1-immunostained fibers in the stalk of ampullary crista (Fig. 1C, arrow) strongly implied that they were most likely to be an afferent nerve ending forming a calyx in the sensory epithelium.

The presently observed VGluT3-immunoreactive sensory epithelia cells and fibers in the acoustic maculae and ampullary cristae implied these structures containing glutamate. We presumed these VGluT3-immunoreactive cells in the sensory epithelium to be hair cells, which can be supported by the general view that the inner ear hair cells, instead of supporting cells, contain much glutamate and are responsible for the excitatory centripetal neruotransmission[6,8]. Similar to those fibers observed in the case of VGluT1-immunoreacitvity, the weak VGluT3-immunostained fibers may be the afferent fibers of the VG neurons. Previous studies reported that VGluT3 was localized to the perikarya/dendrites as well as the axon terminals of neurons and co-localized with acetylcholine, GABA/glycine or monoamine in neurons or other types of cells[1,7,12]. In the vestibular end organs, whether the putative VGluT3-labeled hair cells contain neuroactive substances such as monoamines and choline is another interesting issue which needs to be addressed.

Acknowledgments: This work was supported by the National Natural Science Foundation of China (No. 30470562, No. 30470907, No. 30600339).

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囊泡膜谷氨酸转运体在前庭外周系统中的分布

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摘要：目的 检查囊泡膜谷氨酸转运体(vesicular glutamate transporter，VGluT)在前庭外周系统中的分布特征。**方法** 采用 ABC (avidin-biotinylated peroxidase complex)免疫组织化学方法，二氨基联苯作为染色剂，观察 VGluT1-3 在正常成年 SD 大鼠前庭外周系统，包括球囊、椭圆囊、壶腹嵴和前庭神经节 (Scarpa 神经节)的表达。**结果** (1) VGluT1样免疫阳性产物位于前庭神经节和传入纤维支配的前庭外周终末感受器。(2)大部分感觉上皮细胞表达高密度的 VGluT3 样免疫阳性产物，但在球囊斑和椭圆囊斑，VGluT3 样免疫阳性传入纤维表达较弱。(3)在上述部位, 没有或仅有很弱的VGluT2样免疫反应。**结论** VGluT1和VGluT3参与了初级前庭传入纤维和毛细胞中将谷氨酸转运入囊泡的过程，可能在调节前庭终末感受器通过前庭核向大脑传递信息的传导通路中具有重要作用。

关键词： 囊泡膜谷氨酸转运体；位觉斑；前庭神经节；免疫组织化学；大鼠