Neurotransmission in the vestibular endorgans - Glutamatergic transmission in the afferent synapses of hair cells -

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Abstract In the sensory pathways the first synapse is that between hair cells and primary afferent neurons and its most likely neurotransmitter candidate has long been thought to be glutamate. A number of pharmacological and electrophysiological studies have lent credence to this theory (reviewed by Bledsoe *et al.* 1988, Bobbin 1979, Ehrenberger and Felix 1991, Puel *et al.* 1991; Puel 1995) as has recent neurochemical and immunocytochemical work (reviewed by Ottersen *et al.* 1998; Usami *et al.* 2000). These recent studies reveal that the afferent hair cell synapse resembles the central glutamate synapses in many ways. Of the proteins confirmed to be involved in signal transduction and transmitter metabolism at most central synapses, many are also seen in the afferent hair cell synapse, and have an analogous compartmentation. On the other hand, there are also important differences, especially those related to the molecular mechanisms that underlie transmitter release.

Key words: glutamate, aspartate, receptor, transporter, immunocytochemistry

Introduction

Electrophysiological and immunocytochemical investigations have demonstrated that a variety of neuroactive substances are neurotransmitters and/or neuromodulators in the vestibular endorgans. Molecular biology has also elucidated the structure of various neurotransmitter receptors and transporters and shown that a receptor/transporter complex plays a role in neurotransmission in the inner ear. This review focuses on neurotransmission between hair cells and primary afferents, with an emphasis on immunocytochemical findings of the localization of key molecules that are involved in afferent transmission (receptors, transporters, and related enzymes).

Presynaptic localization of glutamate

Presynaptic localization must be demonstrated for the determination of a neurotransmitter. In the rat cochlea and vestibular endorgans localization of glutamate at the cellular and subcellular levels has been shown by postembedding immunocytochemistry (Usami *et al.* 1992; Usami & Ottersen 1995). Glutamate immunoreactivity in both the organ of Corti and vestibular endorgans, is distributed mostly in the hair cells, suggesting compatibility with hair cell transmitters. In the CNS, postembedding immunogold

cytochemistry has demonstrated that gold particles representing glutamate are highly correlated with synaptic vesicles in the glutamatergic synapse (Ji *et al.* 1991). On the other hand, this is not the case in hair cells in the inner ear (Usami *et al.* 1992; Usami & Ottersen 1995). It is likely then, that hair cells in the inner ear have a different release mechanism than that in the CNS, although different methodologies may account for the different results. Vesicular enrichment of glutamate in these cells has not been demonstrated but the results of our immunocytochemical investigations indicate that glutamate may be a transmitter in both types of hair cell.

Aspartate in hair cells

In spite of much investigation into the role of glutamate as a hair cell transmitter, the excitatory amino acid that serves as an endogenous ligand of glutamate receptors has not been identified. In the CNS, endogenous compounds with affinity to glutamate receptors, such as glutamate and aspartate, are candidates, as are homocysteic acid and cysteine sulphonic acid and other sulfur-containing amino acids. High levels of aspartate immunoreactivity in neuronal compartments, including all types of hair cells, and afferent and efferent nerve fibers, indicate that aspartate may be involved in neurotransmission in the inner ear (Usami & Ottersen 1996). On the other hand, strong immunoreactivity in sub-basilar tympanic cells, interdental cells, and the mesothelial cells in the Reissner's membrane suggests that aspartate has a metabolic role (Usami & Ottersen 1996).

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A glutamate-glutamine cycle in the inner ear

Glutamine is a major precursor and breakdown product of glutamate and a glutamate-glutamine cycle plays a crucial role in glutamatergic transmission in the CNS (Fonnum 1984). In this cycle, glial cells uptake glutamate, which is then converted to glutamine by the glial enzyme glutamine synthetase. This glutamine is then transferred back to the neurons and converted to glutamate by phosphate activated glutaminase (Takumi *et al.* 1999), an enzyme thought to reside mostly in neurons. This cycle probably assists the quick removal of glutamate following release, preventing interference with synaptic transmission and toxic buildup of glutamate. As shown by immunocytochemistry, glutamate immunoreactivity is primarily found in the hair cells, while glutamine immunoreactivity is enriched in supporting cells (Usami *et al.* 1992; Usami & Ottersen 1995). The glutamate-glutamine ratio is different in different cell types, and is higher in hair cells than in various types of supporting cells. Metabolic compartmentation resembles that of the glutamatergic nerve terminals and adjacent glial processes in the central nervous system. Enzymes are synthesized by glutamate and glutamine, and the gradient is maintained by phosphate activated glutaminase and glutamine synthetase. A complementary localization pattern in which glutamate synthetize enzyme is enriched in hair cells, whereas glutamine synthesizing enzyme is predominantly localized in the supporting cells, has been demonstrated by immunocytochemistry (Takumi *et al.* 1997; Ottersen *et al.* 1998). This finding is consistent with a glutamate-glutamine cycle between hair cells and supporting cells, similar to the relationship in the CNS between neuron and glial cells.

Glutamate receptors in the inner ear

The cellular response to glutamate is determined by the type of glutamate receptor, therefore the various glutamate receptors have been well-studied. Molecular techniques and antibodies specific for different receptor subunits have indicated that different glutamate receptors, including AMPA, Kainate, and NMDA receptor subunits, express in the spiral and vestibular ganglia (Kuriyama *et al.* 1993; Kuriyama *et al.* 1994; Fujita *et al.* 1994; Niedzielski & Wenthold 1995; Dememes *et al.* 1995; Usami *et al.* 1995). Metabotropic receptors also contribute to hair cell/afferent neurotransmission of the inner ear (Safieddine & Eybalin 1995). We have also shown immunocytochemical distribution of NMDA receptor subunits (NMDAR1) and AMPA-selective glutamate receptor subunits (GluR1, GluR2/3, GluR4) in the spiral and vestibular ganglion, lending credence to the idea that glutamate serves as a hair cell transmitter. In addition, our high resolution immunogold analysis has shown that concentrations of GluR2/3 and 4 of the AMPA type glutamate receptors, occur at the postsynaptic specialization of afferent synapses established by inner hair cells in the organ of Corti (Matsubara *et al.* 1996). In the vestibular

sensory epithelium, immunoreactivities for GluR2/3 and GluR4 were concentrated in the synapses between type I hair cells and the surrounding chaliceal nerve endings and the bouton type nerve endings contacting type II hair cells, indicating that AMPA receptor subunits play a role in afferent transmission. AMPA receptor immunoreactivity was not detected in the synaptic region of the outer hair cells, suggesting a great dissimilarity in glutamatergic transmission of inner, and of outer hair cells. We were also unable to localize NMDA receptors on the postsynaptic membrane at the ultrastructural level (unpublished data), even though significant mRNA signalling and immunolabeling in the ganglion cell cytoplasm has been shown (Kuriyama *et al.* 1993; Fujita *et al.* 1994; Niedzielski & Wenthold 1995). We still do not know how transmission is carried out via NMDA receptors but it is possible that they contribute to synaptic plasticity, which generally takes place during development and nerve injury healing periods.

Glutamate transporters in the inner ear

The glutamate transporter is required to finalize glutamate action. Confocal microscopy has demonstrated colocalization of GLAST and glutamine synthetase in supporting cells apposed to the immunonegative hair cells (Takumi *et al.* 1997). In addition, immunoelectron microscopy showed that GLAST is heterogeneously distributed along the plasma membranes of the supporting cells, and is more highly concentrated basally (at the level of the afferent synapses) than apically (Takumi *et al.* 1997). Our findings indicate that glutamate that has been released at the afferent synapse of vestibular hair cells is taken up by adjacent supporting cells and then converted to glutamine. It may be that at this peripheral synapse the supporting cells perform roles similar to those of glial cells in the CNS. GLAST, a glial type glutamate transporter, is distributed in supporting cells and we assume that this uptake system helps to quickly remove glutamate after release, preventing interference with synaptic transmission in addition to toxic buildup of glutamate.

Distribution of phosphate activated glutaminase in the inner ear

Phosphate activated glutaminase (PAG) is considered responsible for the majority of glutaminase activity in the CNS (Kvamme 1984) and has been found in a numerous neuronal pathways, a portion of which are regarded as glutamatergic for other reasons (Kaneko, 2000). Postembedding immunogold cytochemistry has shown that PAG is almost completely restricted to mitochondria (Laake *et al.* 1999) but there was a wide range of mitochondrial labelling intensity in the different cell compartments. There were strongly immunoreactive mitochondria in cerebellar mossy fiber terminals but the mitochondria in other putative glutamatergic fibers (such as the parallel fibers) had much lower particle densities (Laake *et al.* 1999). Therefore, the mechanisms for transmitter replenishment may not be as uniform as was thought and some glutamatergic terminals

Fig. 1 Glutamate-glutamine cycle in the inner ear. After being concentrated in the hair cells glutamate is released. On the postsynaptic membrane afferent dendrites are equipped with several glutamate receptors. Glutamate transporters, such as GLAST, assist the released glutamate to escape from the synaptic cleft and move into adjacent supporting cells. Glutamate may be transported by supporting cells in the inner ear across the membrane and converted to glutamine in the cytoplasm. The hair cells may use glutamate again as a neurotransmitter. This glutamate-glutamine cycle between hair cells and supporting cells resembles that in the CNS.

may gain glutamate mostly from sources other than PAG activity. As expected from the classic concept of the glutamate-glutamine cycle, there was no specific PAG labelling in glial cell mitochondria.

Takumi *et al.* (1999) showed that PAG is selectively enriched in cochlear and vestibular hair cell mitochondria. In supporting cell mitochondria the gold particle density was less than 15% of the density in hair cells, emphasizing the resemblance of metabolic compartmentation between the CNS and the inner ear.

A positive correlation between the glutamate-glutamine ratio and PAG immunoreactivity level for glutamate, glutamine, and PAG was shown by triple immunogold labelling (Takumi *et al.* 1999), suggesting that PAG antibodies identify a functional pool of this enzyme. Small gold particles were used for optimum resolution to show that PAG is associated with the inner mitochondrial membrane, but it is possible that there is another enzyme pool in the mitochondrial matrix. The outer mitochondrial membrane was never labelled.

There were no differences in PAG immunolabelling intensity of mitochondria between basal and apical parts of hair cells (Takumi *et al.* 1999), indicating that the hair cells can not maintain an intracellular gradient of PAG, and that before vesicular transmitter uptake the synaptic

pole of these cells is not defined.

Conclusions

The synapse between sensory hair cells in the inner ear and the afferent dendrites has been characterized, and is an established model of glutamatergic synapse, although not fully clarified. Key molecules involved in glutamate transmission (receptors, transporters, and related enzymes) are present in this synapse (Fig. 1), which is structurally and evolutionally distinct and shares a number of similarities as well as having dissimilarities with the glutamatergic synapse in the CNS. The pathogenetic relation of perturbation of glutamatergic synapse to various inner ear diseases requires further investigation.

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Fig. 1 Glutamine-glutamate cycle in the inner ear. After being concentrated in the hair cell glutaminase (PAG) in the supporting cell, glutamine is transported back to the hair cell. The hair cell contains a nucleus (N) and a phosphate-activated glutaminase (PAG) enzyme. The supporting cell contains a nucleus (N) and a glutamate transporter (GLAST). Glutamate (Glu) is released from the hair cell into the synaptic cleft and taken up by the supporting cell. Glutamine (Gln) is then transported from the supporting cell back to the hair cell. The diagram also shows the presence of glutamine synthetase (Gln synthetase) in the supporting cell.

may gain glutamine mostly from sources other than PAG activity. As expected from the classic concept of the glutamine-glutamate cycle, there was no specific PAG labelling in glial cell mitochondria. Takumi et al. (1999) showed that PAG is selectively enriched in cochlear and vestibular hair cell mitochondria. In supporting cell mitochondria the gold particle density was less than 15% of the density in hair cells, emphasizing the resemblance of metabolic compartmentation between the CNS and the inner ear. A positive correlation between the glutamine-glutamate ratio and PAG immunoreactivity level for glutamine and PAG was shown by triple immunogold labelling (Takumi et al. 1999), suggesting that PAG antibodies identify a functional pool of this enzyme. Small gold particles were used for optimum resolution to show that PAG is associated with the inner mitochondrial membrane, but it is possible that there is another enzyme pool in the mitochondrial matrix. The outer mitochondrial membrane was never labelled. There were no differences in PAG immunolabelling intensity or mitochondrial between basal and apical parts of hair cells (Takumi et al. 1999), indicating that the hair cells can not maintain an intracellular gradient of PAG and that before vesicular transmitter uptake the synaptic