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On the role of the central nervous system in regulating the mineralisation of inner-ear otoliths of fish

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Summary. Stato- or otoliths are calcified structures in the organ of balance and equilibrium of vertebrates, the inner ear, where they enhance its sensitivity to gravity. The compact otoliths of fish are composed of the calcium carbonate polymorph aragonite and a small fraction of organic molecules. The latter form a protein skeleton which determines the morphology of an otolith as well as its crystal lattice structure. This short review addresses findings according to which the brain obviously plays a prominent role in regulating the mineralisation of fish otoliths and depends on the gravity vector. Overall, otolith mineralisation has thus been identified to be a unique, neuronally guided biomineralisation process. The following is a hypothetical model for regulation of calcification by efferent vestibular neurons: (1) release of calcium at tight junctions in the macular epithelia, (2) macular carbonic anhydrase activity (which in turn is responsible for carbonate deposition), (3) chemical composition of matrix proteins. The rationale and evidence that support this model are discussed.

Keywords: Mineralisation; Neuronal control; Utricle; Saccule; Lapillus; Sagitta.

Introduction

Stato- or otoliths are calcified structures in the organ of balance and equilibrium of vertebrates, the inner ear, where they enhance its sensitivity to gravity. With respect to the function of vertebrate inner-ear otolithic organs as gravity sensors, numerous studies have addressed the question of whether altered gravity may have an impact on the formation of the earstones themselves (Lim et al. 1974, Ballarino and Howland 1984, Ross and Donovan 1986, Hara et al. 1995). These investigations were focused on the qualitative evaluation of the morphology of amphibian, avian, or mammalian otoconial masses, yielding contradictory results (Ballarino and Howland 1984, Hara et al. 1995). Quantitative analyses of inner-ear otoconial masses after altered-gravity exposure have never been satisfactorily performed. The area covered with otoconia cannot be methodologically correlated with otoconial total mass or "physical capacity" since the otoconia can be redistributed in the direction of the gravitational force by hypergravity experiments (Lim et al. 1974). The actual mass of an otoconial assemblage is difficult to determine because some otoconia can be overlooked in the course of a dissection, and the dissected material may contain additional tissue which cannot be separated readily. Moreover, most of the studies cited deal with adult specimens, where the ontogenetic development of otoconial masses is finished and thus cannot be affected anymore by altered gravity.

The compact otoliths of bony fish are composed of the calcium carbonate polymorph aragonite and a small fraction of organic molecules. The latter form a protein skeleton which determines the morphology of an otolith as well as its crystal lattice structure. In contrast to the otoconial masses of higher vertebrates, the physical capacity of compact fish otoliths can be directly assessed by weighing, measuring their size, or by determining their calcium content. Last but not least, fish otoliths continue growing during the entire life span of the animal. Fish are therefore highly suitable to investigate if altered gravity affects otolith growth and in which way an appropriate adaptation is effected.

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Evidence for a neuronal control of otolith growth and calcification

Calcium

Fish otoliths (from the cichlid fish *Oreochromis mossambicus* as well as from the swordtail fish *Xiphophorus helleri*) grow slower under 3 **g** hypergravity than under normal 1 **g** earth gravity, as determined by measurements of their size (Anken et al. 1998a, 2000c, 2002b) and calcium incorporation (Anken et al. 2000c, 2001b). Additionally, their asymmetry (i.e., differences between the otoliths from the left and the right side of the body) becomes diminished (Anken et al. 1998a, 2001b, 2002b). Opposite effects are obtained when fish are kept under orbital microgravity (Wiederhold et al. 2000) or under simulated microgravity within a fast-rotating clinostat (Baur 2004).

There are further, ground-based studies which support the hypothesis that size (and thus symmetry) of otoliths must be regulated (Anken et al. 1998b, Helling et al. 2005, Lychakov and Rebane 2005).

In order to gain further insights into a possible neuronal regulation of otolith growth and mineralisation, the vestibular nerve was unilaterally transected, and the otolith growth was then assessed by measuring the calcium incorporation. Calcium incorporation and thus otolith mineralisation stopped after vestibular nerve transection (Anken et al. 2000a, 2001a, 2002a, c; Edelmann et al. 2004). This clearly indicates that the otolithic calcium uptake is regulated neurally.

Carbonate

The enzyme carbonic anhydrase (CAH) occurs in the inner-ear epithelium, where it regulates the provision of carbonate for calcium carbonate incorporation into otoliths (Fermin et al. 1998, Tohse and Mugiya 2001). Experiments showed that nerve transection or altered gravity affect – depending on the force of linear-acceleration (gravity) dose applied – calcium carbonate incorporation via the efferent vestibular system (determined via acetylcholine esterase histochemistry; Feucht 2004) by a stimulation (deprivation) of inner-ear CAH activity leading to an increased (decreased) provision of endolymphatic carbonate (Beier et al. 2002b, 2004a, b).

Protein

Further studies provided mostly indirect evidence that the environmental gravity vector guides not only the provision of calcium and carbonate to the otoliths but also the selection of the lattice structure of calcium carbonate incorporated. Thus the protein skeleton of otoliths can be adjusted in order to adapt otoliths (mass, structure, fine morphology) to altered gravity (Anken et al. 2000b).

It was shown that altered gravity alters CAH reactivity (see above). CAH and a particular otolith protein, calbindin D28K, are likely coexpressed (Parmentier et al. 1991). It may therefore be assumed that altered gravity will also affect the expression of calbindin D28K. Since the calcium carbonate polymorph aragonite is possibly based on calbindin D28K, a down-regulation of this protein will result in a higher amount of vaterite. This hypothesis is strengthened as follows. Employing inductively coupled plasma mass spectrometry, the absolute calcium and strontium contents of otoliths of fish larvae were measured who had developed under hypergravity (Anken et al. 2000c, 2001b). The contents of calcium were massively decreased in comparison with 1 **g** controls, whereas significantly more strontium had been incorporated (Anken et al. 2000c, 2001b). This finding clearly indicates that the hypergravity otoliths contained a higher ratio of the polymorph vaterite in comparison with 1 **g** controls, and thus, suggests that the mineralisation sites of the matrix protein had been altered towards a preference of strontium salt nucleation. This higher amount of vaterite is in complete agreement with the above suggestion of a down-regulation of calbindin D28K (in conjunction with the down-regulation of CAH) due to hypergravity.

In the course of another set of experiments, larval animals were subjected to parabolic aircraft flights and individuals swimming kinetotically (i.e., exhibiting sensorimotor disorders which are homologous to human space sickness [Anken and Rahmann 1999]) were analysed separately from individuals who swam normally during the phases of diminished gravity (i.e., 3×10^{-2} to 5×10^{-2} **g**). The inner ears of the kinetotic animals revealed a (predispositioned) highly asymmetric CAH reactivity in the macular epithelia (Beier et al. 2002b) and thus a highly asymmetric incorporation of calcium carbonate (Beier et al. 2002a) as well as highly asymmetric distribution of otolith sizes (Hilbig et al. 2002, 2003) (independently, the expression of calbindin D28K will have been asymmetrical if it is indeed coexpressed with carbonic anhydrase, see above). Laser scanning microscopy moreover showed that the otoliths' internal distribution of calcium (visualised with the fluorescent calcium tracer Alizarin Complexone, applied after the experiment) varied between kinetotic and normal individuals (A. Forster, R. Anken, and R. Hilbig, Zoological Institute, University of Hohenheim, Stuttgart, unpubl.), which

clearly indicates that the composition of the otoliths' proteinaceous matrix was different between the two groups.

Taken together, these findings strongly indicate the existence of a gravity-induced adaptation process affecting otolith mineralisation in order to maintain the afferent excitation from the otolithic organs in a range that allows neuronal vestibular compensation.

Presumed regulatory pathways of neuronal control of otolith growth and calcification

Neuronal control of calcium supply

Studying the inner ear of the cichlid fish by electron spectroscopic imaging and electron energy loss spectra, discrete calcium precipitations were found to be accumulated at the macular tight junctions, providing evidence that the endolymph is supplied with calcium via a paracellular pathway (Ibsch et al. 2004a, b) towards the proximal endolymph (Beier et al. 2004c). Concerning the blood–brain barrier, there is evidence that neuronal activity alters the expression of particular claudins (components of the tight junctional complex) and thus the permeability of tight junctions (Lippoldt et al. 2000). It is hypothesized that the regulated, paracellular release of calcium into the endolymph is based on the permeability of such junctions altered by neuronal activity.

Neuronal control of carbonate supply

As has been addressed above, the provision of otolithic carbonate is based on CAH activity (Beier et al. 2002b, 2004a, b). Factors regulating CAH in the inner ear have so far not been investigated, but the enzyme's regulation in other tissues seems to involve a FOS/cAMP pathway (Dragon et al. 2003, Karaki and Kuwahara 2004) which can be mediated by acetylcholine esterase (AChE) activity (Takahashi et al. 2001). Since altered gravity affects both CAH (Beier et al. 2004a, b) and AChE (Feucht 2004) activity, it is proposed that the activity of CAH in the vestibular system is regulated via a signal transduction chain involving the action of the efferent vestibular system and AChE.

Neuronal control of lattice structure and protein composition

In several cases, a neuronal control of gene expression has been observed. The current model for the neuronal control of synaptic gene transcription in skeletal muscle, e.g., involves two distinct mechanisms. On the one hand, neurally evoked electrical activity in muscle fibres represses transcription of synaptic genes in extrasynaptic areas. On the other hand, nerve-derived signals specifically activate synaptic gene expression in subsynaptic myonuclei (for a review, see Schaeffer et al. 2001).

A factor involved in the otoliths' lattice selection and protein composition in the zebrafish is the *starmaker* gene (Söllner et al. 2003). This gene is homologous to the human dentin sialophosphoprotein gene *dspp* (DSPP is required for mineralisation of teeth and is expressed in the inner ear), and it is required for a normal crystal lattice structure and thus a normal shape of otoliths (Söllner et al. 2003). Principally, it is conceivable that the transcription of a gene like *starmaker* is under neuronal control.

In conclusion, otolith mineralisation hitherto is the only biomineralisation process known that is neuronally regulated in adaptation to an environmental parameter (i.e., gravity).

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