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Effects of hypergravity on the morphological properties of the vestibular sensory epithelium. II. Life-long exposure of rats including embryogenesis

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ABSTRACT: Rats were exposed to a hypergravity (HG) level of 2.5 × g from conception until the age of 14 weeks. The vestibular epithelia of four of these animals and four control animals were immunohistochemically labeled for actin and tubulin. The apical cross-sectional area of epithelial cells of HG exposed rats appeared to be larger in all end organs. Area increase was 7.0% in the utricle ($p < 0.005$) and 8.2% in the crista ($p \ll 0.001$). Hair cells and supporting cells appeared to be intact. The cellular arrangement and the proportion of different cell types within the epithelia was normal.
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KEY WORDS: Gravity, Vestibular system, Hair cell, Actin, Tubulin.

INTRODUCTION

A fundamental physiological question is how external environmental conditions can modify a sensory system. It is generally acknowledged that maturation depends on the stimulus characteristics during particular developmental critical periods. Adaptation of a sensory system, by definition, depends on stimulus conditions. For the vestibular system, the magnitude of the (ever-present) gravity is such a stimulus parameter which may affect development and/or adaptation processes. Modifications of a system can be assessed by investigating either the functional or the morphological changes. Several studies on the (functional) effects of a change of gravity on vestibular-induced reflexes and behavior have shown that these are modified. Sustained hypergravity (HG) alters the airrighting reflex of hamsters [48] and rats [56,59], and the vestibulo-ocular reflex [57,58]. Spaceflight (transiently) affects the righting reflex of neonatal rats [39]. Modifications of several other types of sensori-motor interaction have been attributed to exposure to either increased or decreased gravity [17].

A sustained altered gravity level may also have a serious impact on the (morphology of the) peripheral vestibular system. In the vestibular system, hair cells are responsible for the mechano-electrical transduction process. Because this transduction mechanism is extremely sensitive, it is remarkable that the influence of altered gravity on the vestibular epithelia has received relatively little attention. Studies, which have been performed on (adult) rat [28,53], (adult and developing) fish [21,54,55], and chick embryos [15] report no significant changes of epithelial morphology.

During ontogeny of the rat vestibular end organs, terminal mitosis of progenitors into hair cells occurs between gestational day (GD) 14 and GD 19 [42]. Afferent projections to the (mouse) vestibular epithelium were found on GD 12, i.e., even before clear differentiation of sensory cells [33]. Otoconia of the otolith organs are formed between GD 16.5 and 19.5 [26]. Apoptosis of differentiated hair cells, which is part of the normal development of sensory end organs, starts on GD 19 [61]. Maturation of the vestibular epithelia is not completed before 6–14 days after birth [9,18,49], and morphological changes of the developing vestibular epithelia have been reported to occur up to the age of 1 month [11].

In principle, any of these developmental processes may be affected by a change of gravity. *A priori*, it is not predictable to what extent morphological changes will be reversible or not and, in case of irreversible changes, at what developmental stage critical periods occur. In our laboratory, we have investigated the influence of gravity on peripheral vestibular structures during different developmental stages. It was shown that (prolonged) exposure to HG has no effect on fully mature otoconia [46]. During embryogenesis, however, HG proved to alter the distribution of otoconia of different sizes [47]. Long-term exposure, starting at the age of 1 month, to HG of rats with fully mature vestibular epithelial cells caused a small decrease of their apical cross-sectional area [59,60]. Otherwise, these cells appeared to be unaffected.

Because the aim of the present study was to investigate the effect of HG exposure during embryogenesis on the organization of the vestibular sensory epithelium, rats were continuously exposed to 2.5 × g, from conception until the day the labyrinths were taken out. Vestibular epithelia were immunohistochemically labeled for actin and tubulin. These cytoskeletal proteins are characteristic for specific cellular structures in hair cells and supporting cells, and thus allow a positive identification of cell type.

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MATERIALS AND METHODS

Methods used in this study were the same as in a previous study [60]. Briefly, the following procedures were pursued.

Housing

Long–Evans rats were kept inside a centrifuge at a gravity level of $|Z| = 2.5 \times g$ [56]. Mating and gestation of the pups occurred under these HG conditions. During the first 10 postnatal days, gravity level was set at $1.8 \times g$ to increase survival rate of the pups. Thereafter, HG was increased to $2.5 \times g$ again. When the HG-born rats had reached the age of 14 weeks, they were decapitated for histological examination ($n = 4$). For comparison, a control group ($n = 4$) of the same age was used. Animal treatment was in accordance with the Dutch law and the European Communities Council Directive (86/609/EEC; November 24, 1986) on the use of animals in scientific research. Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed.

Immunohistochemistry

After fixation (4% formaldehyde + 1% glutaraldehyde + 0.1% Triton in phosphate buffer), dissection of the labyrinths yielded 5 utricles, 1 saccule, and 11 cristae from HG rats, and 8 utricles, 4 saccules, and 11 cristae from rats exposed to normal gravity (NG). After staying overnight, organs were rinsed in phosphate buffered saline (PBS; standard pH 7.4), kept in PBS (pH 8.0) + 1% $NaBH_4$ (20 min), rinsed in PBS (pH 7.4), kept in PBS + 1% Triton + 5% bovine serum albumin (BSA; 3 h), and rinsed again. Tissue stayed in PBS + 0.02% Triton + 0.1% BSA + Anti-α tubulin (primary antibody; Amersham, Little Chalfont, UK) overnight. Specimen were rinsed (5×), put in PBS + 0.02% Triton + 0.1% BSA + biotinylated anti-mouse IgG from goat (secondary antibody; Amersham, Little Chalfont, UK; 8 h), rinsed (5×) and kept in PBS + 0.02% Triton + 0.1% BSA + Streptavidine Fluorescein, overnight. After rinsing (5×), specimen were double stained for actin in PBS + Rhodamine Phalloidine (Molecular Probes, Eugene, OR, USA), overnight. After rinsing, the epithelia were mounted on glass slides.

Analysis

It was attempted to obtain samples at identical locations on the sensory epithelia for both groups of animals. Samples from the cristae were taken close to the apex, approximately halfway septum cruciatum and (lateral) canal wall. From the utricles and saccules, half of the samples were obtained at the striola region and the other half at a more peripheral location. Specimens were analyzed with a Confocal Laser Scanning Microscope (Leica Lasertechnik, Heidelberg; Ar/Kr laser). A 25× objective NA 0.75 was used with a pinhole setting of 1 Airy disc and a zoomfactor 2. Double excitation and detection was used for image acquisition (FITC: exc.: 488 nm/det.: BP520 ± 10 nm; Texas Red: exc.: 568 nm/det.: LP590). Scans were made in different focal planes (1 μm apart). Images were analyzed with QwinPro software (Leica, Cambridge, UK).

As in the previous study [60], the honeycomb-like structure due to the actin belts between hair cells and supporting cells was analyzed as a quantitative measure of tissue condition. Cross-sectional area, perimeter, and roundness (Rd) were calculated. Rd is a shape descriptor which is defined as

$$\mathrm{Rd} = \frac{P^2}{4\pi A 1.064},$$

where A = area (μm^2); P = perimeter (μm).

RESULTS

In Fig. 1, an example is shown of the sensory epithelium of the utricle of an NG rat. The actin-labeled scan (Fig. 1A) shows structural details like stereocilia bundles (s) and the actin belts (ab) which mark the tight junctions between hair cells and supporting cells (e.g. [13]). Two typical tubulin structures can be distinguished in Fig. 1B: densely packed bundles of microtubules (m-I) occurring in the 'neck' region of type I hair cells [27], and a larger (sometimes) ring-like structure (tr) which fills the apical part of supporting cells only. In more apical focal planes than the one shown in this figure, we often saw kinocilia as well. Generally, the location of a kinocilium could be matched with the location of a stereocilia bundle, thus confirming the cell's identification. The overall organization of the epithelium of hair cells separated by supporting cells is clearly visible.

In Fig. 2, the utricular epithelium of an HG rat is shown. The same cytoskeletal structures have been labeled as in the NG utricle, and also the same spatial organization of hair cells and supporting cells can be recognized. In general, we found no indications for a deterioration of the epithelium of the otolith organs of HG rats.

Figure 3 shows an example of the epithelium of the crista ampullaris of an NG rat. As in the otolith organs, hair cells and supporting cells with their characteristic cytoskeletal components can be clearly distinguished. The same cellular structures and organization can be seen in the epithelium of the crista of an HG rat (Fig. 4). Qualitatively, no differences were observed between the ampullar epithelia from both groups.

In Table 1, the mean cross-sectional area, perimeter, and Rd of the cells of seven samples from five HG utricles are listed. In

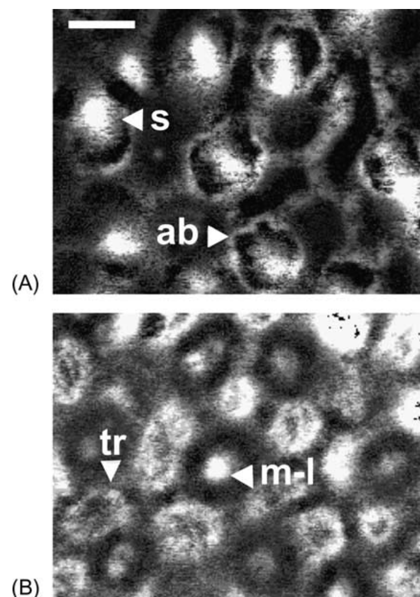

FIG. 1. Sensory epithelium from the utricle of an NG rat labeled for actin (A) and tubulin (B). The actin-labeled scan shows the epithelial structure of hair cells, with their stereocilia bundles (s), and supporting cells. Actin belts (ab) separating the epithelial cells can also be discerned. The tubulin-labeled scan is from the same area; but in order to show other cytoskeletal details, it was acquired in a 4 μm more basal focal plane. Two different tubulin structures can be distinguished: densely packed microtubules (m-I) probably located in the 'neck' region of type I hair cells, and a larger (sometimes) ring-like structure (tr) filling the apical part of supporting cells only. Bar: 5 μm.

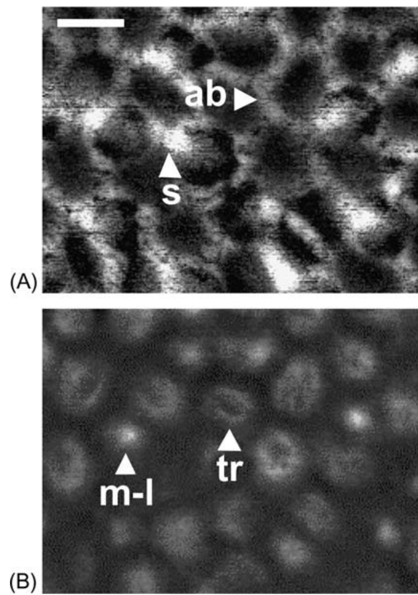

FIG. 2. Sensory epithelium from the utricle of an HG rat labeled for actin (A) and tubulin (B). Scans were obtained at approximately the same apical-basal levels as in Fig. 1; symbols as in Fig. 1.

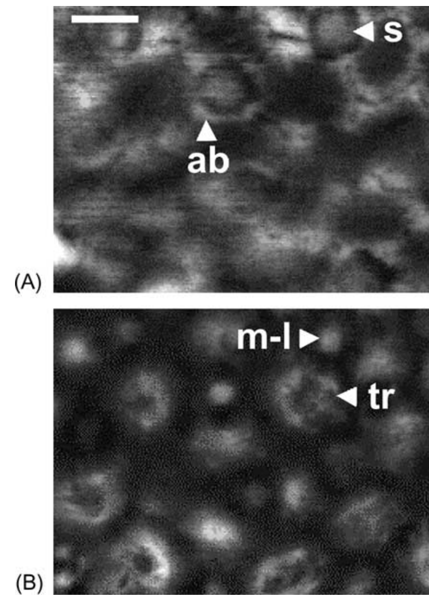

FIG. 4. Sensory epithelium from the cristae ampullaris of an HG rat labeled for actin (A) and tubulin (B). Scans were obtained at approximately the same apical-basal levels as in Fig. 3; symbols as in Fig. 1.

Table 2, the same features are listed for 12 samples from all eight NG utricles. On an average, this area is 7.0% larger for HG rats (t-test; $p < 0.005$). Perimeter is larger as well (2.8%; $p < 0.01$). Rd was same for both groups. Unfortunately, we have not been able to procure more than one HG saccule (but saccular data are not inconsistent with the rest of the data). Contrary to what might be expected, the effect of HG on the size of cells within the cristae was most prominent. Cross-sectional area within the cristae of HG rats was 8.2% larger ($p \ll 0.001$) and also the perimeter was 3.5% larger ($p \ll 0.001$). The difference in Rd, although significant, is very small. All data are summarized in Table 3.

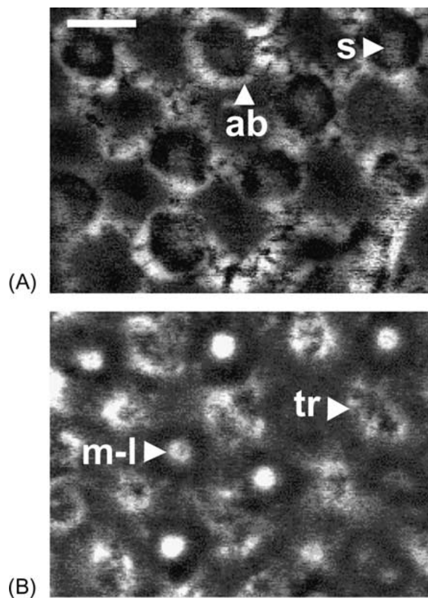

FIG. 3. Sensory epithelium from the cristae ampullaris of an NG rat labeled for actin (A) and tubulin (B). Symbols as in Fig. 1.

For both the utricle and the crista ampullaris, one HG and one NG (exemplary) specimen was inspected in greater detail. Actin- and tubulin-labeled structures were inspected in combination and in different focal planes. Almost without exception, the distinction between hair cells and supporting cells was obvious. The ring (or plate) of microtubules (‘tr’ in panel B of Figs. 1–4) is characteristic for supporting cells which have no hair bundles attached to them. The densely packed bundles of ‘m-I’ are considered to identify type I hair cells. Type II hair cells are sometimes difficult to identify positively. However, there appear to be 2–4 times more type I than type II hair cells, and the ratio of the number of supporting cells and hair cells lies somewhere between 1 and 2.

TABLE 1

CHARACTERISTICS OF THE SENSORY EPITHELIUM OF FIVE UTRICLES OF LIFE-LONG HYPERGRAVITY (HG) RATS

HG Utricle Id-code	n	$A \pm$ SD	$P \pm$ SD	Rd ± SD
1L	44	19.9 ± 5.3	18.2 ± 2.5	1.28 ± 0.12
1R	21	23.2 ± 6.5	20.2 ± 3.2	1.34 ± 0.16
2L	90	19.5 ± 6.4	18.3 ± 3.3	1.31 ± 0.13
2L	87	21.9 ± 7.3	19.3 ± 3.5	1.31 ± 0.14
2R	82	16.6 ± 5.1	17.1 ± 2.8	1.37 ± 0.23
2R	60	19.2 ± 6.7	18.1 ± 3.3	1.33 ± 0.15
3R	39	19.9 ± 4.7	18.4 ± 2.5	1.30 ± 0.13
Means	7	20.0 ± 6.0	18.5 ± 3.0	1.32 ± 0.15
‘Cells’	423	19.7 ± 6.4	18.3 ± 3.2	1.32 ± 0.16

The number in the id-code in the left-hand column refers to an individual animal. The same id-codes in different rows indicate that different parts of the same utricle were analyzed. L, left labyrinth; R, right labyrinth; n, number of cells (see text); A, area (μm^2); P, perimeter (μm); Rd = $P^2/(4\pi A 1.064)$; SD, standard deviation.

TABLE 2

CHARACTERISTICS OF THE SENSORY EPITHELIUM OF EIGHT UTRICLES OF NORMAL GRAVITY (NG) RATS

NG Utricle Id-code	n	$A \pm$ SD	$P \pm$ SD	Rd ± SD
1L	43	18.9 ± 4.8	17.9 ± 2.3	1.31 ± 0.18
1L	38	16.5 ± 6.9	16.6 ± 3.6	1.30 ± 0.12
1R	92	19.6 ± 5.8	18.3 ± 2.6	1.30 ± 0.13
1R	41	17.7 ± 6.5	17.3 ± 3.4	1.32 ± 0.13
2L	55	20.5 ± 5.7	18.7 ± 2.7	1.31 ± 0.12
2R	62	16.5 ± 6.0	17.0 ± 3.5	1.37 ± 0.17
3L	36	21.6 ± 7.3	19.4 ± 3.4	1.34 ± 0.15
3L	47	17.1 ± 6.4	17.1 ± 3.7	1.33 ± 0.14
3R	41	15.4 ± 3.6	16.1 ± 1.9	1.29 ± 0.15
4L	44	18.9 ± 5.7	18.5 ± 3.1	1.39 ± 0.21
4R	98	19.2 ± 7.0	18.3 ± 3.5	1.35 ± 0.18
4R	78	17.8 ± 6.0	17.5 ± 3.2	1.34 ± 0.18
Means	12	18.3 ± 6.0	17.7 ± 3.1	1.33 ± 0.15
'Cells'	675	18.4 ± 6.2	17.8 ± 3.2	1.33 ± 0.16

The number in the id-code in the left-hand column refers to an individual animal. The same id-codes in different rows indicate that different parts of the same utricle were analyzed. L, left labyrinth; R, right labyrinth; n, number of cells (see text); A, area (μm^2); P, perimeter (μm); Rd = $P^2/(4\pi A1.064)$; SD, standard deviation.

Total cell density is 400–500/0.01 mm^2. Hair cell density is 160–220/0.01 mm^2, which is in agreement with previous results [60] and also with our estimate of hair cell density in Fig. 3 from Dechesne et al. [11]. Similar values have been reported for the otolith organs of zebrafish [5]. However, hair cell density is not the same for all species. For instance, it is smaller in humans (60–70/0.01 mm^2 [36]) and higher in pigeon (~400/0.01 mm^2 [14]). The average apical cross-sectional area of hair cells is smaller than that of supporting cells (5–20%) but it has to be noticed that this ratio strongly depends on the depth of the focal plane in which a scan was acquired. With respect to shape features (area, perimeter, and Rd) and proportionality of numbers of different cell types, the relationship between hair cells and supporting cells appears unaltered by HG exposure.

TABLE 3

AVERAGE ± SD OF FORM FEATURES OF THE VESTIBULAR EPITHELIA OF LIFE-LONG HYPERGRAVITY (HG) RATS AND NORMAL GRAVITY (NG) RATS

	n	Area (μm^2)	Perimeter (μm)	Roundness
Utricle				
(5, 7) HG	423	19.7 ± 6.4	18.3 ± 3.2	1.321 ± 0.159
(8, 12) NG	675	18.4 ± 6.2	17.8 ± 3.2	1.332 ± 0.159
p		<0.005	<0.01	n.s.
Saccule				
(1, 1) HG	64	19.6 ± 8.1	18.5 ± 4.0	1.374 ± 0.175
(4, 6) NG	334	19.2 ± 7.2	18.0 ± 3.6	1.318 ± 0.142
p		n.s.	n.s.	0.02
Crista				
(11, 11) HG	608	25.2 ± 6.9	20.8 ± 2.9	1.313 ± 0.137
(11, 11) NG	780	23.3 ± 7.0	20.1 ± 3.2	1.334 ± 0.157
p		≪0.001	≪0.001	<0.01

The number of cells that were included in the analysis is given by 'n'. In parentheses: number of sensory organs, number of samples.

DISCUSSION

Embryogenesis under HG conditions appears to have different effects on the otolith formation of fish [2] and on the otoconia formation of birds [20] and mammals [47] (see also the review on the effects of altered gravity on vestibular receptor organs by Lychakov [29]), but the mechanism that interferes is not known [16]. Previously, we demonstrated that HG exposure, starting at the age of 1 month, decreases cell size in the fully mature vestibular epithelium [59,60]. In the present study, we show that HG exposure during ontogeny of the sensory epithelium has the opposite effect on cell size. Apparently, cell size is irreversibly increased as a result of a gravity-dependent mechanism during a particular critical period, which must occur before the age of 1 month.

At a later developmental stage, cellular maturation and/or growth is only delayed by HG exposure. When comparing the control groups of the present study and the previous one, it appears that cells within the utricular epithelium become larger with age. The mean cross-sectional area is 18.4 μm^2 at the age of 14 weeks (Table 3, this study) and 21.8 μm^2 at the age of 10 months (Table 3 from [60]; $p \ll 0.001$). For the saccular epithelium, these values are 19.2 and 21.5 μm^2 ($p \ll 0.001$), respectively. It appears, therefore, that HG exposure of mature otolith organs only delays the growth of their epithelial cells.

Apparently, after 14 weeks, aging has no effect on cell size within the epithelium of the crista ampullaris. The mean cross-sectional area of the control groups at the age of 14 weeks and 10 months is identical (23.3 μm^2). The results of our previous study [60] suggest that the growth of mature ampullar epithelial cells is also slowed down by HG exposure (mean area: 20.6 μm^2). However, the number of HG cristae is rather small ($n = 3$) and the data on cell size at the start of HG exposure (at the age of 1 month) are not available. In the present study, the number of cristae that could be retrieved was larger and there seems no doubt (but see below) that embryogenesis under 2.5 × g conditions leads to larger apical cross-sectional areas of cells in the ampullar organs.

It is a surprise that this effect is largest in the organs which are supposed to detect angular acceleration and smaller in the organs which are supposed to detect linear acceleration including gravity. It should be realized, however, that inside a rotating system the vestibular system of freely moving animals is stimulated in a rather complex way. Apart from the sustained HG component, every linear movement of the head leads to a Coriolis force [56], stimulating the otolith organs, and every rotation of the head leads to a torque on the endolymph inside the semicircular canals [51], stimulating the cristae.

When taking all vestibular epithelia into consideration, the results of the present study are consistent. The same is true for the results of the previous study [60]. However, it could well be argued that the number of animals (which is very small) should have been used in our statistical analysis instead of the number of cells. In that case, it is obvious that no significant differences have been demonstrated at all. Based upon the histological material that was collected both in the present and the previous study [60], we found no morphological indications for a deterioration of vestibular functioning. The cytoskeletal structures which are necessary for proper mechano-sensory transduction appear to be present and the arrangement of epithelial cells is normal. Scar formation, which is typical for hair cell degeneration after, for instance, application of aminoglycosides [35] was neither observed in the present study nor in the previous one [60]. Our results are in agreement with

histological results, which have been presented before, on the effects of altered gravity on vestibular epithelia [15,21,28,53–55].

The conclusion that prolonged HG exposure, including the period of embryogenesis, does not impede mechano-sensory transduction in the vestibular system of the rat is also supported by studies in which the functioning of the vestibular system was investigated. Vestibular-induced behavior and reflexes, although modified, are not eliminated by HG exposure. Both the encoding of linear acceleration, including gravity [48,56,59], and angular acceleration [57,58] continues. This indicates that the hair cells must be functionally intact. Furthermore, it has been shown that the growth of fish otoliths depends on (feedback) information about the level of gravity [2,3], which implies that mechano-sensory transduction itself is not impaired.

Several experiments have been performed to investigate the influence of increased gravity on various other cell types. Because modifications of (vestibular-induced) behavior and reflex patterns are attributed to neuronal adaptations in which interconnections between cells are altered, some investigators have focussed on possible changes of the neurons that are involved in vestibular processing. Adjustment of the number of synapses appears to be a general mechanism within the vertebrate vestibular system. Synaptic innervation of hair cells of the (adult) rat utricle increases during weightlessness [40,41]. Similar findings have been reported with respect to the number of afferent fibres in the chicken maculae [15] and the number of synapses in the fish vestibular nucleus [22]. Exposure to HG has the opposite effect: the number of synapses decreases [40]. In the vestibular nuclei of the rat, morphological changes have been observed which depend on the level of gravity [7,24].

Also, enzyme activity in fish vestibular nuclei has been shown to depend on gravity level [1]. Maturation of the efferent system, however, appears to be unaffected by exposure to microgravity between postnatal day 8 and 23 [12]. In the Mauthner cell, a large brainstem neuron receiving multimodal sensory information [62] which is believed to initiate the high-speed escape response in fish (and amphibian larvae), intracellular changes have been observed (on the endoplasmic reticulum) as a result of vestibular stimulation [43], including HG [37]. Some adaptation processes may be restricted to a (short) critical period of 'heightened developmental sensitivity to HG' [25].

The effects of HG on 'nonvestibular' cells have also been investigated. Intracellular structural changes have been reported for the cardiac muscle [34]. In skeletal muscles, the proportion of slow and fast oxidative fibres [30], the myosin content [32] and the amount of heat shock proteins [52] appear to be affected. Neuronal adaptation to HG has been demonstrated in the somatosensory cortex, where reduced GABA immunoreactivity is supposed to reflect a reprogramming of motor output [10]. The increased Golgi complex and granular endoplasmic reticulum in the parathyroid glands, when exposed to HG, suggest an augmented level of parathyroid hormone [45]. This hormone regulates the calcium level in the blood which, in turn, is believed to affect bone density. Studies on the effect of HG on bone density, however, are not altogether in agreement. Some studies report increased bone density [23] while others report no change of bone density [6,31,50]. The morphology of growth hormone producing cells of the pituitary gland is altered by HG while prolactin producing cells are not affected [44]. Changes in fibroblast cytoskeletal organization were not observed below 15 × g [8].

Thus, it appears that adaptation to an (moderate) HG level involves either a functional change in neuronal circuitry, or a modification of the organelles required for the metabolic task which is specific for the investigated system. Although we did not study the vestibular epithelial cells' cytoarchitecture in great detail, it seems plausible that the effect of HG on hair cells will be small. The only indication, so far, for intracellular adaptation of vestibular hair cells to HG appears to be an increased potassium current in type I hair cells [19]. An explanation for the small effect of HG on hair cells may be that the transduction channels, which are attached to the tip links connecting adjacent stereocilia [38], are subject to an adaptation mechanism of their own [4]. This adaptation mechanism seems perfectly suited to compensate for a physiologically small shift of the hair cell's working range.

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