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Effects of hypergravity on the morphological properties of the vestibular sensory epithelium. I. Long-term exposure of rats after full maturation of the labyrinths

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ABSTRACT: The effect of prolonged exposure to hypergravity on the morphology of vestibular epithelia of rats was investigated. At the age of 1 month, i.e., when vestibular end organs are fully maturated, three rats were transferred to a hypergravity environment of 2.5 g inside a large radius centrifuge. After 9 months, vestibular epithelia of these animals and of three control animals were immunohistochemically labeled for actin and tubulin. The apical cross-sectional area of epithelial cells of hypergravity exposed rats appeared to be smaller in all end organs. Area reduction was 1.9% in the saccule (not significant), 5.0% in the utricle ($p < 0.005$), and 11.6% in the crista ($p \ll 0.001$). No indications for a deterioration of vestibular functioning were observed. © 2002 Elsevier Science Inc.

KEY WORDS: Gravity, Vestibular system, Hair cell, Actin, Tubulin.

INTRODUCTION

Since the beginning of the exploration of space, it has become clear that the vestibular system is affected by the absence of gravity (for a review see [19]). Centrifugation experiments show that hypergravity also interferes with human vestibular functioning [2,11]. It has been shown that animal behaviour is affected, both by a reduction of gravity [5,20,21] and by an increase [6,27,31,34]. Several studies, in a variety of vertebrate species, have been performed to investigate the possible relationship between a morphological change (of the otolith organs, mostly) and vestibular functioning [1,12,19,25,26]. In rodents, morphological changes induced by altered gravity have been reported for the otoconial organization [26], for the number of synapses on utricular hair cells [22,23], and for the vestibular nuclei [3,15].

The amount of data on the consequences of altered gravity exposure on the morphology of the vestibular sensory epithelia is surprisingly limited, however. In one study, 'no abnormalities were noted' in the saccular epithelium of the rat after long-term exposure to hypergravity [18]. In another study, in which rats were exposed to weightlessness for 20 days, swelling of epithelial tissue, vacuolization of cells and even degeneration of (type I) hair cells were observed [29]. In a later study, however, it has been suggested that these dramatic results were caused by accidental experimental conditions (possibly the shock of landing of the Kosmos-782) and not by weightlessness itself [30]. Two more studies on fish exposed to hypergravity [13] and chick embryos exposed to microgravity [9] report either no or 'mostly non-significant' effects on epithelial morphology.

The objective of the present study was to determine the effect of prolonged hypergravity on the organization of the sensory epithelium of the rat otolith organs. Therefore, two cytoskeletal proteins, actin and tubulin, were immunohistochemically labeled. These proteins are characteristic for specific cellular structures and, thus, make it possible to identify different cell types. In particular, the honeycomb-like structure, which is due to the actin belts of the tight junctions between hair cells and supporting cells, was analyzed as a quantitative measure of tissue condition.

The vestibular end organs of the rat appear to be morphologically complete at 6–14 days after birth [4,10,28]. Nevertheless, morphological changes of the developing vestibular epithelia have been reported to occur up to the age of 1 month [7]. Because we were interested in the effect of prolonged hypergravity on fully maturated vestibular epithelia rats were transferred from normal gravity to 2.5 g at the age of 1 month.

MATERIALS AND METHODS

Housing

One group of Long-Evans rats lived under normal gravity conditions at 1 g (NG; $n = 3$). Another group ($n = 3$) were transferred from normal conditions to the hypergravity (HG) environment inside a centrifuge at the age of 1 month. Housing consisted of acrylate boxes (0.36 × 0.44 × 0.30 m). Food and water were available *ad libitum*. The centrifuge comprises two horizontal arms (length: 1.10 m) with free-swinging gondolas

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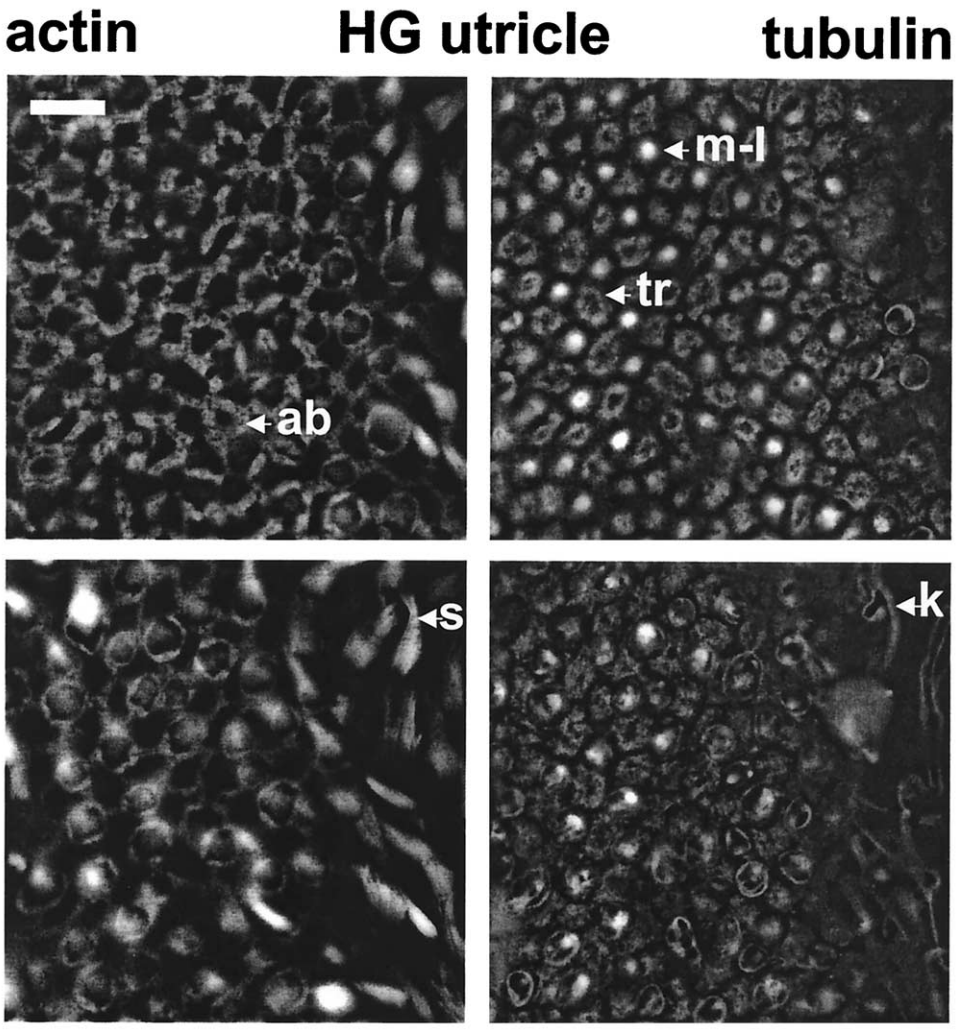

FIG. 1. Utricular sensory epithelium labeled for actin and tubulin from a hypergravity (HG) rat. The scans shown in the upper and lower panels were made in different focal planes, with the lower panels 2 μm more apical. Several cytoskeletal structures, indicated by arrowheads, can be distinguished: kinocilium (k), stereocilia (s), actin belt (ab), microtubules in the 'neck' region of type I hair cells (m–I), and tubulin rings (tr). Bar: 10 μm.

(1.10 × 0.45 × 0.725 m). The centrifuge constantly rotates at 34.3 cycles/min resulting in a hypergravity level at the bottom of the gondola of $|Z| = 2.5$ g. Daily, the centrifuge was stopped during ~20 min for animal care. Rotation was restarted in the reverse direction relative to the previous day. (Behavioral and vestibulo-ocular experiments were performed in the same period in which the animals of this study stayed inside the centrifuge. Alternating the direction of rotation was intended to prevent the development of unilateral compensation for Coriolis forces.) For details about the HG conditions, see Wubbels and de Jong [31]. For histological examination, all six animals were decapitated at the age of 10 months. In order to avoid possible effects on hair cell morphology [24], no anaesthetics were used in this study. Animal treatment was in accordance with the Dutch law and the European Communities Council Directive (86/609/EEC; 24 November 1986) on the use of animals in scientific research. Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed.

Immunohistochemistry

Labyrinths were isolated and fixated with McDowell solution (4% formaldehyde + 1% glutaraldehyde in phosphate buffer) + Triton 0.1%. Further dissection yielded a total of 5 utricles, 5 saccules and 3 cristae from HG rats, and 6 utricles, 4 saccules and 10 cristae from NG rats. (Cristae were taken from the superior and horizontal semicircular canals only.) After staying overnight, organs were rinsed in phosphate-buffered saline (PBS) with a standard pH 7.4, kept in PBS (pH 8.0) + 1% $NaBH_4$ (20 min), rinsed in PBS, kept in PBS (pH 7.4) + 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, Buckinghamshire, UK) overnight. Specimen were rinsed (5×), put in PBS + 0.02% Triton + 0.1% BSA + biotynilated anti-mouse IgG from goat (secondary antibody; Amersham; 8 h), rinsed (5×), and kept in PBS pH 7.4 + 0.02% Triton + 0.1% BSA + Streptavidine Fluorescein overnight. After rinsing (5×) specimen were double stained for actin in

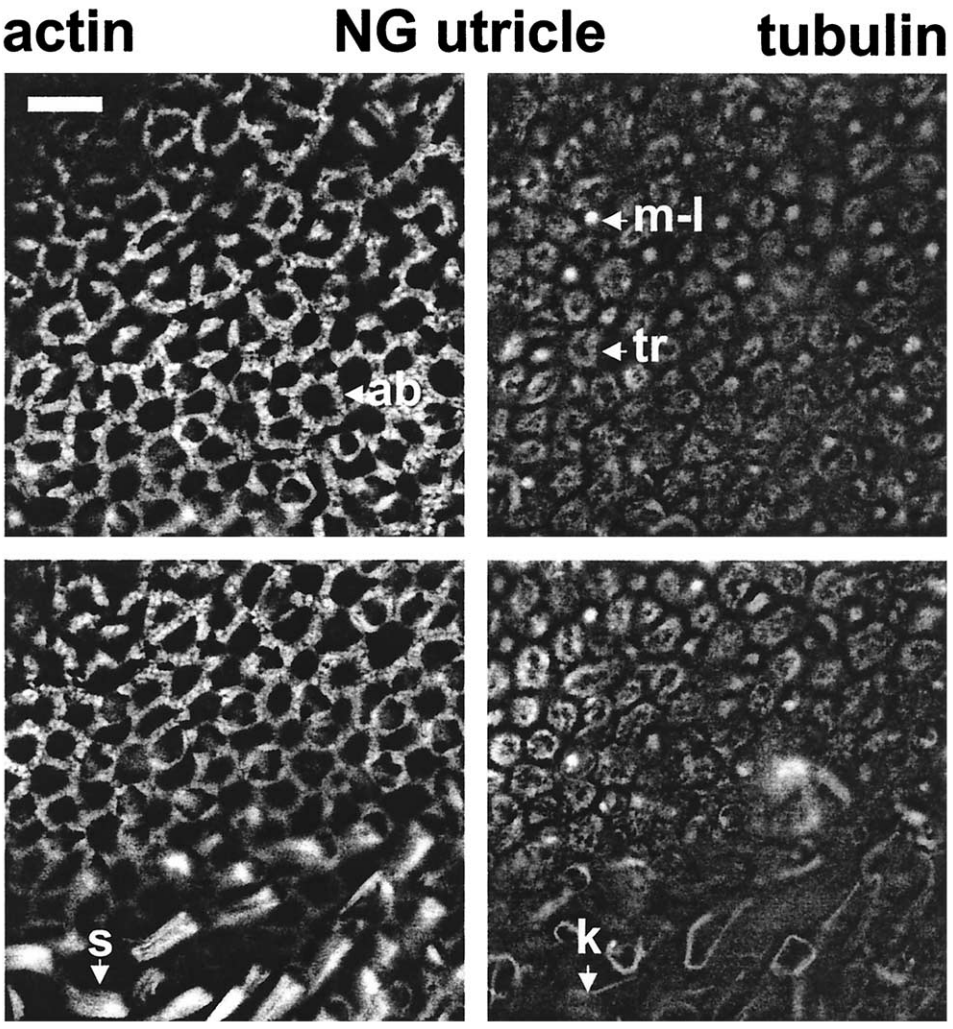

FIG. 2. Utricular sensory epithelium from a normal gravity (NG) rat. Abbreviations: ab, actin belt; k, kinocilium; m–I, microtubules in the 'neck' region of type I hair cells; s, stereocilia; tr, tubulin rings. Bar: 10 μm.

PBS + Rhodamine Phalloidine (Molecular Probes, Eugene, OR, USA) overnight. After rinsing, the organs 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 Leica CLSM (Leica Lasertechnik, Heidelberg) equipped with an Ar/Kr laser. A 25× objective NA 0.75 was used with a pinhole setting of 1 Airy disc and a zoomfactor 2. For image acquisition double excitation and detection was used (FITC: exc.: 488nm/det.: BP520 ± 10nm; Texas Red: exc.: 568/det.: LP590). Gray values within images were scaled to the full dynamic range of the system (8 bit), and images were saved to disc as tiff files.

For image analysis, we made use of QwinPro software (Leica, Cambridge, UK). Contrasts within the original scanned image were enhanced and a 'gray watershed' function was applied to separate epithelial structural elements. The contours of these structural elements (i.e., hair cells and supporting cells) were obtained, and their corresponding included area, perimeter and roundness were calculated. Roundness (Rd) is a shape descriptor that is defined as:

$$Rd = P^2/(4 \cdot \pi \cdot A \cdot 1.064)$$

A = area (μm^2); P = perimeter (μm).

RESULTS

The otolith organs function as linear acceleration detectors. Therefore, possible effects of prolonged hypergravity on the sensory tissue are primarily expected in the saccule and in the utricle. Figure 1 shows the utricle of a HG rat that has been labeled for actin (left) and tubulin (right). Scans were made in different focal planes. The lower panels show scans that are 2 μm more apical relative to the scans shown in the upper panels. Two different actin-labeled structures, which are common to all hair cells [8], can be observed: the stereocilia (s), and the belt at the apical circumference of the hair cell (ab). Tubulin labeled structures that can be distinguished are the kinocilium (k), and the microtubules in the

TABLE 1

CHARACTERISTICS OF THE SENSORY EPITHELIUM OF FIVE UTRICLES OF THREE HYPERGRAVITY (HG) RATS

HG Utricle id-Code	*n*	A ± SD	P ± SD	Rd ± SD
2L	48	20.2 ± 6.0	18.6 ± 3.0	1.31 ± 0.13
2L	47	21.7 ± 7.6	19.4 ± 3.8	1.33 ± 0.17
5L	70	22.5 ± 6.9	19.7 ± 3.3	1.32 ± 0.15
5L	60	21.1 ± 7.6	19.1 ± 3.8	1.34 ± 0.16
5R	68	20.2 ± 5.1	18.9 ± 2.9	1.34 ± 0.17
5R	71	21.4 ± 7.1	19.3 ± 3.5	1.35 ± 0.20
6L	49	22.0 ± 7.9	19.4 ± 4.0	1.32 ± 0.17
6L	80	18.3 ± 6.8	17.9 ± 3.6	1.36 ± 0.15
6R	71	20.6 ± 7.4	18.6 ± 3.4	1.31 ± 0.13
6R	50	19.7 ± 7.4	18.6 ± 3.8	1.36 ± 0.17
Means	10	20.8 ± 7.0	19.0 ± 3.5	1.34 ± 0.16
'Cells'	614	20.7 ± 7.1	18.9 ± 3.6	1.34 ± 0.16

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

apical part of the hair cells. Heavily tubulin-labeled areas (m–I), with diameters of approximately 1.5 μm, represent the 'neck' region of type I hair cells. It has been shown before that this 'neck' region is densely packed with microtubules [16]. Elsewhere in Fig. 1, rings of microtubules (tr) have been labeled. In Fig. 2, the utricle of a NG rat is shown. Qualitatively, no indications were observed for a deterioration of the utricular epithelium of HG rats.

TABLE 2

CHARACTERISTICS OF THE SENSORY EPITHELIUM OF ALL SIX UTRICLES OF THREE NORMAL GRAVITY (NG) RATS

NG Utricle id-Code	*n*	A ± SD	P ± SD	Rd ± SD
7L	61	21.8 ± 7.3	19.1 ± 3.6	1.29 ± 0.14
7L	35	24.2 ± 6.9	20.4 ± 3.0	1.33 ± 0.13
7R	53	21.8 ± 5.6	19.2 ± 2.9	1.28 ± 0.12
7R	37	18.6 ± 5.4	17.7 ± 2.7	1.29 ± 0.12
9L	94	20.7 ± 6.1	18.7 ± 3.0	1.30 ± 0.13
9L	92	19.7 ± 6.3	18.3 ± 2.7	1.32 ± 0.18
9R	49	24.5 ± 9.7	20.2 ± 4.5	1.29 ± 0.17
10L	46	23.3 ± 6.4	19.9 ± 3.2	1.29 ± 0.14
10L	77	23.0 ± 7.5	20.0 ± 3.7	1.34 ± 0.15
10R	29	22.0 ± 5.5	19.4 ± 2.8	1.30 ± 0.14
10R	42	22.5 ± 7.2	19.5 ± 3.4	1.30 ± 0.12
Means	11	22.0 ± 6.7	19.3 ± 3.2	1.30 ± 0.14
'Cells'	615	21.8 ± 7.0	19.2 ± 3.4	1.30 ± 0.15

The number in the id-code in the left-hand column refers to an individual animal; L, left labyrinth; R, right labyrinth; the same id-codes in different rows indicate that different parts of the same utricle were analyzed; n = number of cells; A, area (μm^2); P, perimeter (μm); Rd = $P^2/(4 \cdot \pi \cdot A \cdot 1.064)$; SD, standard deviation. From every utricle two different areas were scanned except from the right labyrinth of rat 9 where only one area was scanned.

In Table 1, the mean area, perimeter and roundness of the cells of 10 samples from 5 HG utricles are listed. In Table 2, the same features are listed for 11 samples from all 6 NG utricles. On average, this area is 5.0% smaller for HG rats (*t*-test; $p < 0.005$) and also the shape appears somewhat more irregular ($p \ll 0.001$). Perimeter is smaller as well (1.6%), but this difference is not significant.

In Fig. 3, the saccules of a HG and a NG rat are shown for both types of labeling. All cytoskeletal structures that were observed in the utricular epithelium were present in the saccular epithelium as well. The analysis of the honeycomb-like structure in the saccule did not show any significant differences between the epithelium of HG and NG rats (Table 3), although both area and perimeter tend to be smaller for HG rats (1.9% and 2.6%, respectively).

Although, no effects of hypergravity on the cristae ampullaris were expected this epithelium was also inspected. Qualitatively, no differences between HG and NG rats were found. To our surprise, however, size effects were most prominent here. Epithelial cells in the semicircular canals of HG rats have a 11.6% smaller area and 6.0% smaller perimeter. Their shape, however, is not significantly affected (Table 3). In general, it can be concluded that the apical cross-sectional area of vestibular sensory cells of HG rats is smaller.

By a combined inspection of actin and tubulin labeled structures in different focal planes, we tried to identify cell type. It was checked if stereocilia could be attributed to specific elements located more basally in the sensory epithelium. Thus, cytoskeletal elements could be positively identified to belong to hair cells. Due to the large variability within the data, we refrained from an extensive quantitative analysis. One HG and one NG utricle, which were both considered to be representative, were inspected. Half of the cells turned out to be hair cells. The apical cross-sectional area of hair cells appeared to be smaller than the area of supporting cells (20% for the HG and 15% for the NG sample respectively). More than half of the hairbundles belonged to cells that also contained densely packed microtubules (m–I in Figs. 1 and 2) and were thus considered to be type I hair cells. Almost without exception, cells containing a ring of microtubules (tr in Figs. 1 and 2) had no stereocilia attached to them.

Thus, a clear distinction between hair cells and supporting cells was possible, based upon the presence of stereocilia and/or a kinocilium on hair cells (and the absence of hairbundles on supporting cells), and on the presence of a ring-like tubulin structure in supporting cells (and the absence of this structure in hair cells). The hair cell density per 0.01 mm^2 was 188 for the HG and 206 for the NG utricle. Within the same HG and NG utricle, we also measured the width of the actin belts. Width was 1.32 ± 0.35 μm for the HG (n = 87) and 1.41 ± 0.47 μm for the NG sample (n = 63) respectively. This difference was not significant.

DISCUSSION

The present study shows that the fully maturated vestibular sensory epithelium is qualitatively unaffected after a prolonged period of hypergravity. The cytoskeletal elements that are typical for hair cells, like stereocilia, kinocilia, actin belts, and microtubules, appear to be intact after long-term HG exposure. The only consistent and significant result of HG exposure is a reduction of the apical cross-sectional area of vestibular epithelial cells. There is no indication, however, that this has a serious impact on the functioning of the vestibular system.

From a sensory physiological point of view, an increase from 1 g to 2.5 g (i.e., 8 dB) is, *a priori*, not expected to pose a serious problem. Like other sensory systems, the vestibular system has a large dynamic range and (probably) its adaptation mechanisms are versatile enough to compensate for a small bias of stimulus level. A study on the effect of prolonged hyper-

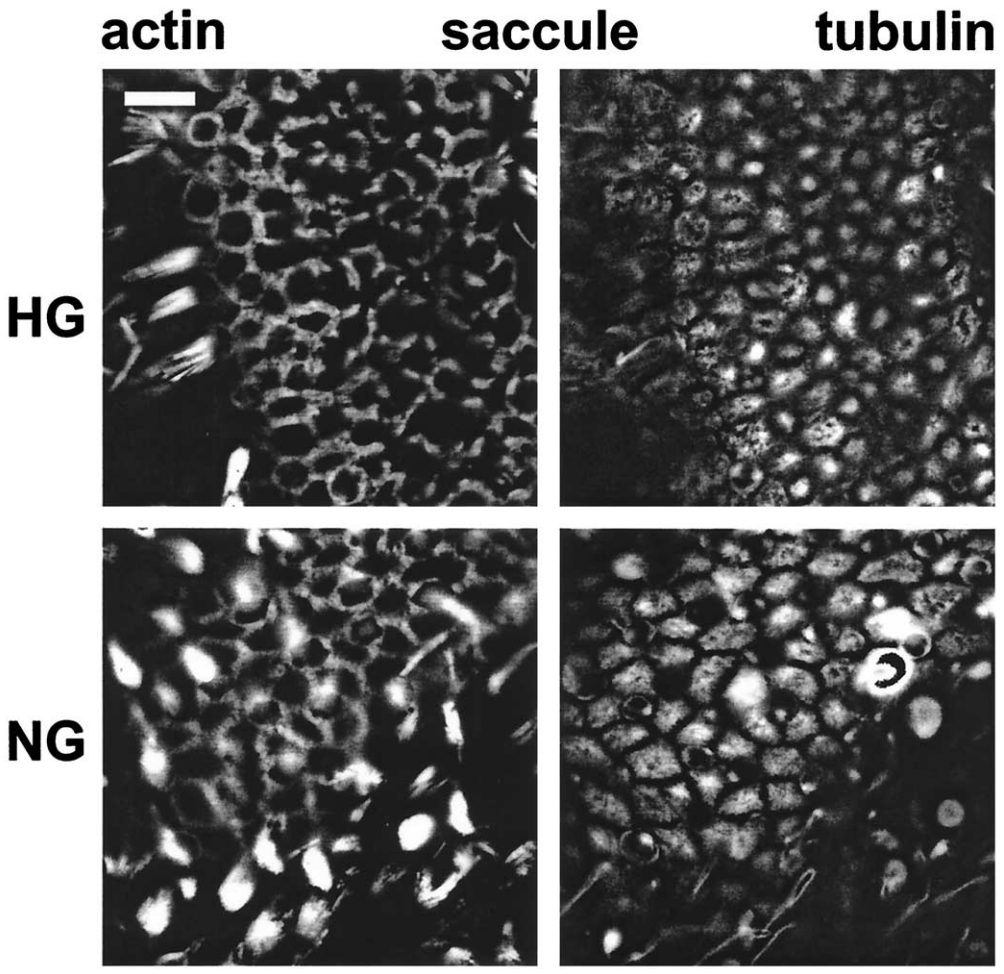

FIG. 3. Saccular sensory epithelium from a hypergravity (HG; upper panels) and from a normal gravity (NG) rat (lower panels). Epithelia were labeled for actin (left) and tubulin (right). Similar cytoskeletal structures can be recognized as in Figs. 1 and 2. Bar: 10 μm.

gravity on hamsters (transferred from 1 to 2.5 g at the age of 3 weeks) reported that the otoconia remain unaffected with respect to shape, size and chemical content [25]. Other studies have shown that ontogenetic development of the vestibular system at 2.5 g has significant effects on vestibular induced reflexes. However, this does not result in a complete loss of vestibular functioning [27,31–33].

Apparently, even the effects of development under 0 g condi-

TABLE 3

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

		n	Area (μm^2)	Perimeter (μm)	Roundness
Utricle	(5) HG	614	20.7 ± 7.1	18.9 ± 3.6	1.337 ± 0.162
	(6) NG	615	21.8 ± 7.0	19.2 ± 3.4	1.304 ± 0.145
	p		$<$0.005	Not significant	$\ll$0.001
Saccule	(5) HG	280	21.1 ± 7.6	18.8 ± 3.6	1.310 ± 0.155
	(4) NG	239	21.5 ± 6.3	19.3 ± 3.0	1.330 ± 0.137
	p		Not significant	Not significant	Not significant
Crista	(3) HG	203	20.6 ± 6.8	18.8 ± 3.3	1.338 ± 0.153
	(10) NG	720	23.3 ± 8.3	20.0 ± 3.7	1.342 ± 0.158
	p		$\ll$0.001	$\ll$0.001	Not significant

n, the number of cells that were included in the analysis.

tions (potentially, a fundamentally different situation) are moderate and do not lead to serious structural defects of the peripheral vestibular sensory organs [19]. Also, studies on neonatal rats, which were partly gestated under microgravity, show significant, but transient, behavioral changes [20,21]. Together, the results of these previous studies on the effect of altered gravity and our present data indicate that long-term HG exposure does not impair the functioning of the vestibular sensory organs themselves.

It has been shown that, at the neuronal level, adaptation to altered gravity does occur in the functionally mature vestibular system: the number of synapses on utricular hair cells increases during weightlessness [22,23], and in the vestibular nuclei morphological changes have been observed [3,15]. Because similar findings have been reported with respect to the number of afferent fibres in the chicken maculae [9] and the number of synapses in the fish vestibular nucleus [14] adjustment of the number of synapses appears to be a general adaptation mechanism within the vertebrate vestibular system. It seems plausible, therefore, that most of the changes of vestibular-related reflexes or behaviour, which have been reported in several studies on various species, mainly find their origin in this type of neuronal adaptation and not in morphological changes of the sensory epithelia.

A question that remains to be solved in the future is what effect ontogenetic development under altered gravity conditions has on the structure of the vestibular epithelia. The otoconial distribution is irreversibly modified in hypergravity [26], and central projections to the otolith organs are inhibited during weightlessness [3]. Recently, it has been shown that development of potassium channels in the hair cell membrane is delayed by hypergravity [17]. Therefore, it cannot be excluded that exposure to altered gravity has a more profound effect on the developing vestibular sensory epithelia than on the already completely maturated epithelia.

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