Exposure to reduced gravity impairs junctional transmission at the semicircular canal in the frog labyrinth

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Rossi ML, Rubbini G, Gioglio L, Martini M, Fesce R. Exposure to reduced gravity impairs junctional transmission at the semicircular canal in the frog labyrinth. Am J Physiol Regul Integr Comp Physiol 298: R439–R452, 2010. First published December 2, 2009; doi:10.1152/ajpregu.00673.2009.—The effects of microgravity on frog semicircular canals have been studied by electrophysiological and morphological approaches. Reduced gravity (microG) was simulated by a random positioning machine (RPM), which continually and randomly modified the orientation in space of the anesthetized animal. As this procedure stimulates the semicircular canals, the effect of altered gravity was isolated by comparing microG-treatment with an identical rotary stimulation in the presence of normal gravity (normoG). Electrophysiological experiments were performed in the isolated labyrinth, extracted from the animals after the treatment, and mounted on a turntable. Functional activity was measured by recording quantal events (mEPSPs) and spikes from the afferent fibers close to the junction, at rest and during rotational stimulation. MicroG-treated animals displayed a marked decrease in the frequency of resting and evoked mEPSP discharge, vs. both control and normoG (mean decrease ~50%). Spike discharge was also depressed: 57% of microG-treated frogs displayed no spikes at rest and during rotation at 0.1 Hz, vs. 23–31% of control or normoG frogs. Among the firing units, during one cycle of sinusoidal rotation at 0.1 Hz microG-treated units emitted an average of 41.8 ± 8.06 spikes, vs. 77.2 ± 8.19 in controls. Patch-clamp analysis on dissociated hair cells revealed altered Ca2+ handling, after microG, consistent with and supportive of the specificity of microG effects. Marked morphological signs of cellular suffering were observed after microG, mainly in the central part of the sensory epithelium. Functional changes due to microgravity were reversible within a few days.

resting and evoked sensory discharge; quantal analysis; hair cells; ionic currents; reperfusion; multiple organ damage

INFORMATION ON HEAD POSITION and movement is elaborated in the central nervous system by integrating the input from labyrinthine organs with visual and somesthetic information. Most vestibular functions are directly or indirectly affected by gravity; thus, changes in gravity conditions are bound to interfere with sensation, perception, and central elaboration of vestibular information. Many common and evident disturbances of this system arise during central processing and integration of sensory information, due to incoherence between visual and gravity (vestibular, neck muscle tension, etc.) sensory inputs. However, little is known about possible primary alterations of vestibular function under altered gravity, due to significant and measurable interference with transduction pro-

cesses at the vestibular organs and with neural encoding of the static and dynamic stimuli the head is exposed to.

A series of structural (4, 5, 13, 14, 19), biochemical (12), genetic (for a review see ref. 11), behavioral (2), and developmental (3) modifications have been reported to occur in several cellular systems following exposure to conditions of altered gravity. Functional data are scarce: an increased response sensitivity of toadfish utricular afferents has been described shortly after a space flight (1) and, more recently, alterations have been reported in the biophysical properties of hair cell K+ and Ca2+ currents at the frog labyrinth, after conditioning the surviving animal in a microG environment (10).

To explore the interference of gravity with vestibular functioning, a condition of reduced gravity (microG) was simulated in this study by placing the anesthetized frog in a random positioning machine (RPM), which continuously changed the orientation of the animal with respect to the gravitational axis. This is considered a standard way to simulate the absence of gravity, especially to study cell culture and tissue preparations (6). Linear acceleration-sensitive organs are most likely affected by the true absence of gravity, but simulation in the RPM is far from reproducing that condition, as otoliths are continuously subjected to random accelerations. Conversely, the effects of gravity on the semicircular canals can be isolated by applying similar protocols of random rotation in the presence of either a fixed or a continuously varying gravitational vector. Electrophysiological recording during microG simulation would be excessively demanding and complicated by the concomitant application of random angular accelerations by the RPM. Thus, it is more practical and informative, and equally pertinent, to study the possible long-lasting changes induced in the function of vestibular organs by a prolonged exposure to altered gravity conditions. In this work, appropriate protocols of prolonged treatment in the RPM were set up to isolate possible long-lasting effects of gravity from those of mere mechanical stimulation.

MicroG’s effects were studied a posteriori by quantitatively analyzing: 1) the mEPSP and spike discharge at the posterior canal in the intact frog labyrinth; 2) the ionic currents in hair cells dissociated from the posterior ampulla; and 3) the morphology of the posterior canal sensory epithelium.

MATERIALS AND METHODS

Experimental treatments. All procedures for animal handling and surgery were approved by the Animal Care and Use Committee of the University of Ferrara and authorized by the National Ministry of Health. The experiments were performed on frogs (Rana esculenta, 25–30 g body wt) at 20–22°C. Animals were of the same geograph-
FROG LABYRINTH AFTER EXPOSURE TO MICROGRAVITY

MicroG. The anesthetized frog (tricaine methane sulfonate, 200 mg/kg ip) was exposed to MicroG conditioning by using a desktop RPM. The absence of gravity is simulated by coupling two rotating square frames; the outer frame rotates around a horizontal axis; the inner frame rotates within the outer one, around a perpendicular axis. The combined rotation of both frames produces a continuous repositioning of the preparation with respect to the gravity vector (vertical axis), and a condition of virtual absence of gravity in the geometrical centre of rotation. The frog was mounted on a custom-made table fixed to the internal frame of the apparatus so that 1) the rotation axis of the inner frame was coincident with the antero-posterior (cranio-caudal) midline vector (thus, parallel and close to the axis of the posterior canal) and 2) the rotation axis of the outer frame passed through the centers of the two acoustic membranes. This way, rotation axes crossed at the midpoint of the line that connects the centers of the acoustic membranes of the frog, and both labyrinths were positioned ~5 mm apart from the precise centre of rotation.

NormoG. The procedure used to simulate the absence of gravity (microG, above) was performed on a second group of animals, with the difference that the external frame of the RPM was kept fixed, and the inner frame was rotated around the vertical axis. In particular, in a first series of experiments, the path of the inner frame (acceleration-deceleration pattern) was reproduced unaltered. This was aimed at producing a similar degree of stimulation of the posterior canal as that produced by microG simulation. Subsequently, to be sure that the overall stimulation of the preparation under this condition was not less than that in microG simulation, the values of inner frame velocity were scaled up so that their root-mean-square value was comparable to that of the combined velocities of the inner and outer frames in microG simulation. By this procedure, the animal was exposed to a normal, steady gravitational field, and the posterior canal was subjected to the simulation. By this procedure, the animal was exposed to a normal, steady gravitational field, and the posterior canal was subjected to the simulation.

This condition, referred to as normoG (4 h stimulation in simulated zero gravity).

During this treatment, the three semicircular canals were subjected to continuous changes in rotational velocity, and therefore to a continuous mechanical stimulation. It was, therefore, necessary to set up adequate control conditions to dissect possible effects of reduced gravity per se from the effects of the mechanical stimulation.

No stimulation, anesthesia, and null control. Two further control groups were examined: frogs subjected to 4 h of anesthesia alone or no treatment at all.

Pithed animals. To verify whether important contribution to the observed effects arose from anesthesia (that prevents afferent spike discharge and consequently the peripheral effrent control of the receptors), three groups of animals were pithed (spinalized) and thereafter exposed to no treatment, microG, or normoG conditioning.

Recovery. A subgroup of the animals conditioned to microG were not killed at the end of the conditioning period and were rested for 1, 6, 8, or 10 days under normal housing conditions (4–5°C).

Electrophysiology on the isolated intact labyrinth. Electrophysiological experiments were performed on the isolated and intact frog labyrinth after exposure to the appropriate treatment. Frogs not previously anesthetized by the intraperitoneal injection of the anesthetic
When spikes were present, they were automatically detected, counted, and subtracted from the recording before proceeding with the analysis of mEPSPs. Subtraction of each spike left a 3-ms portion of the recording, which could not be corrected and analyzed. This implied that some elementary events were unavoidably missed. Analysis of mEPSP rates at times immediately preceding or following the spikes, the observation that momentary rates up to 800 mEPSPs/s could be occasionally estimated in spike-free portions of the recordings, and the consideration that the coincidence of several mEPSPs must occur to generate a spike, led us to estimate that 4–6 mEPSPs were missed for each subtracted spike. Therefore, mEPSP counts were corrected by adding 5 mEPSPs in the 3-ms interval corresponding to each subtracted spike.

All routines for quantal analysis have been developed in Matlab software environment (Version 5.3. The MathWorks, Natick, MA, USA).

Patch clamp of isolated hair cells. For patch-clamp experiments, the animal was anesthetized by immersion in tricaine methane sulfonate solution (1 g/l in water) and subsequently decapitated: immediately, to obtain control hair cells, or after 4 h of conditioning in the absence of gravity. The head was pinned down at the bottom of the dissection chamber and submerged in a dissection solution of the following composition (in mM): 120 NaCl, 2.5 KCl, 0.5 EGTA, 5 HEPES, 3 glucose, and 20 sucrose. The final pH was 7.2, and the osmolality was 260 mOsmol/kg. The ampullas were isolated from both labyrinths and treated for a period of 20–30 s with subtilisin A, type VIII (50 μg/ml, Sigma); thereafter, they were transferred, in the presence of trypsin inhibitor type II-S (Sigma) (final concentration 0.7 mg/ml), into the experimental chamber (500 μl volume) submerged in the standard extracellular solution (in mM): 120 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 5 glucose, 5 Tris buffer (pH 7.3); 245 mOsmol/kg. The hair cells were mechanically dissociated from the ampullas by gently scraping the epithelium with fine forceps. Recordings were carried out by using the patch-clamp recording technique in the “whole-cell” configuration. Pipettes were pulled from 50-μl glass capillaries, and fire-polished to a final resistance of 3–4 MΩ. The pipette was filled with the following solution (in mM): 110 NaCl, 2 MgCl2, 1 ATP (K salt), 0.1 GTP (Na salt), 5 EGTA-NaOH, 10 HEPES-NaOH (pH 7.2; 235 mOsmol/kg). When calcium currents were recorded, the composition of the chamber solution was (in mM): 100 NaCl, 6 CsCl, 20 tetraethyl ammonium chloride (TEACI), 4 CaCl2, 10 HEPES, 6 glucose (pH 7.2); 260 mOsmol/kg. Pipette solution was as follows (in mM): 90 CsCl, 20 TEACI, 2 MgCl2, 1 ATP (K salt), 0.1 GTP (Na salt), 5 EGTA-NaOH, 10 HEPES-NaOH, (pH 7.2; 235 mOsmol/kg).

Cd2+ (200 μM) was used as blocker of voltage-dependent calcium channels. It was applied by rapidly (typically <50 ms) changing the external solution via a horizontal shift (computer-controlled stepping motor) of a multibarrelled perfusion pipette positioned in front of the recorded cell. For further details, see previous studies (8–10).

Morphology. Morphometric analyses were performed on the cristalampullaris of control preparations and frogs exposed to stimulation in normogravity and microG. The membranous labyrinth was extracted from the bone by careful dissection, and the posterior ampulla was isolated and processed for light microscopy. Specimens were dehydrated in alcohol, treated with xylene, and included in paraffin. Serial longitudinal sections, about 5 μm thick, were obtained from each ampulla using a Leitz microtome (Wetzlar, Germany). The sections were then freed from paraffin, rehydrated, and colored with hematoxylin-eosin. The three central sections were observed in a Nikon microscope, equipped with a video camera. Photographs were taken at a fixed 100× magnification, taking care to capture the entire ampullar epithelium length (10–13 photographs per section).

Morphometric analysis was performed using computerized image analysis software (Nis Elements AR 2.30) for measuring areas and perimeters of the cells and the nuclei, and the resulting measurements were analyzed in the Matlab software environment.

Data analysis. When not otherwise specified, all data are reported as means ± SE. Comparisons among different conditions were performed by chi-squared (binary frequencies), by Kolmogorov-Smirnov test (continuously but not normally distributed parameters), by Student’s t-test (normally distributed parameters and paired observations) or by ANOVA (functional recovery and morphometric measurements in different portions of the crista). Values of P < 0.05 were considered significant.

RESULTS

Afferent spike activity. Recordings from the primary afferent axon at the posterior canal display quantal synaptic events (mEPSPs), which occur at a variable rate and generate action potential firing at variable frequency. About 20–30% of the units do not display any spike at rest, and when the spikes are present, their rate very rarely exceeds 20/s. When the preparation is sinusoidally rotated, both mEPSP and spike rates are clearly modulated by rotational acceleration (Fig. 1). Both spontaneous and mechanically evoked activities appeared to be attenuated following exposure to microG conditions (Fig. 2).

The anesthetic effect of tricaine is associated with the impairment of action potential conduction. Therefore, it is reasonable to suppose that the vestibular afferent fibers of the frog, anesthetized by intraperitoneal injection of tricaine before the conditioning period on the RPM, were deprived of their spiking activity, and the peripheral efferent control of the afferent discharge was also impaired. The tricaine effect is fully and rapidly reversible, so that the time required to isolate the labyrinth in normal Ringer solution (about half an hour) is sufficient to restore the spike discharge completely. The effect of tricaine and its reversibility were directly tested in single afferent units by adding tricaine 10–4 M (a concentration comparable to that circulating in the anesthetized animal) to Ringer’s solution. The effect was complete within 2–3 min: both the resting and mechanically evoked spike discharges were abolished. On the contrary, mEPSP emission rate remained unaltered and regularly modulated by the excitatory and inhibitory phases of the sinusoidal rotation. The tricaine effect was completely and rapidly reversible upon returning to the normal Ringer solution. This property was also exploited to verify the reliability of the mEPSP automated counting (and spike subtraction) procedure in a population of variably responsive units (336 to 2,790 mEPSPs during the first rotation 0.1 Hz cycle): mEPSP rates computed before or after tricaine were consistent (an average difference of 7.7 ± 16.7%) in nonspiking units; the average difference before and after tricaine in spiking units (i.e., without or with spike subtraction by the counting algorithm) was strictly comparable (12.3 ± 9.8%).

Spiking activity in the distinct experimental groups of animals was evaluated by counting the number of units that did or did not display spikes at rest and during the first complete cycle of rotational stimulation at 0.1 Hz. No significant differences were observed among the various control conditions: i.e., null control, anesthetized, pithed, and normogravity groups.

Conversely, a significantly higher fraction of units were silent (no spikes, mEPSPs during the first cycle only) in the microG groups stimulated for 4 h in the simulated absence of gravity (Table 1). It should be noted that the depression of the spike discharge rate was observed not only in the pithed frogs, in which the
afferent-efferent central connections were permanently active, but also in anesthetized animals (spiking and cross-talk with central structures presumably blocked during microG conditioning), though the depression appeared slightly milder in the latter condition. This suggests that the effect of microG con-

Fig. 1. A: electrophysiological recording of spontaneous miniature excitatory post synaptic potential (mEPSP) and spike discharge in a control preparation from a posterior canal fiber, with expanded time resolution. Action potentials are truncated. B, C, D: recordings on a compressed time scale of the responses evoked from the same unit by rotational canal stimulation at increasing frequencies: 0.1, 0.2, and 0.5 Hz (peak accelerations 12.5, 25.14, and 62.83 deg/s², respectively). The turntable angular velocity is displayed under each tracing. Note the wide modulation of synaptic activity, which at this compressed time scale, appears as a thickening of the trace, and of spike discharge, by the excitatory and inhibitory phases of the stimulus.

Fig. 2. The effect of microgravity (microG) on the resting and evoked discharge of a posterior canal unit. The panels display the same kind of information as in Fig. 1. A: spontaneous mEPSP, and spike discharge. The action potential is truncated. B, C, D: responses evoked from the same unit by rotational canal stimulation at 0.1, 0.2, 0.5 Hz (peak accelerations 12.5, 25.14, and 62.83 deg/s², respectively). The turntable angular velocity is displayed under each tracing. Note the reduced resting discharge and mechanically evoked responses in this typical unit.
2-min sinusoidal rotation at 0.1 Hz was compared with the neural fatigue was considered in detail.

Fig. 5; the comparison of the cumulative distributions (dotted lines, n = 88; Kolmogorov-Smirnov test D = 0.22, P < 0.001).

Fig. 6: cumulative distributions of spontaneous spike frequency evaluated in control (continuous lines, n = 160) and microG-conditioned units (dotted lines, n = 88; Kolmogorov-Smirnov test D = 0.44, P < 0.001).

Fig. 3: A: cumulative distributions of spontaneous mEPSP frequency evaluated in control (continuous lines, n = 160) and microG-conditioned units (dotted lines, n = 88; Kolmogorov-Smirnov test D = 0.22, P < 0.001). B: cumulative distributions of spontaneous spike frequency evaluated in control (continuous lines, n = 92) and microG-conditioned units (dotted lines, n = 88; Kolmogorov-Smirnov test D = 0.44, P < 0.001).

Table 1. Comparison of the number of units that did or did not display spikes at rest and during the first complete cycle of rotational stimulation at 0.1 Hz, in the various experimental groups

<table>
<thead>
<tr>
<th>Treatments and/or Preconditioning</th>
<th>No. of Frogs</th>
<th>No. of Units</th>
<th>No. of spiking Units</th>
<th>No. of Silent Units*</th>
<th>No. of Spiking Units, %</th>
<th>No. of Silent Units, %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null control</td>
<td>13</td>
<td>60</td>
<td>41</td>
<td>19</td>
<td>77.0</td>
<td>23.0</td>
</tr>
<tr>
<td>Anesthetized</td>
<td>5</td>
<td>38</td>
<td>29</td>
<td>9</td>
<td>68.5</td>
<td>31.5</td>
</tr>
<tr>
<td>normoG</td>
<td>6</td>
<td>85</td>
<td>61</td>
<td>24</td>
<td>71.8</td>
<td>28.2</td>
</tr>
<tr>
<td>microG</td>
<td>8</td>
<td>66</td>
<td>28</td>
<td>38</td>
<td>42.4†</td>
<td>57.6†</td>
</tr>
<tr>
<td>Pithed</td>
<td>4</td>
<td>8</td>
<td>7</td>
<td>1</td>
<td>87.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Pithed after normoG</td>
<td>4</td>
<td>35</td>
<td>28</td>
<td>7</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>Pithed after microG</td>
<td>5</td>
<td>13</td>
<td>2</td>
<td>11</td>
<td>15.4</td>
<td>84.6§</td>
</tr>
</tbody>
</table>

normoG, normal gravity; microG, reduced gravity. *Miniature excitatory post synaptic potentials (mEPSPs) only. †Significantly different from all other conditions (nonpithed): Chi-squared (1 degree of freedom) = 16.6, P < 0.001. §Significantly different from all pithed controls: Chi-squared (1 degree of freedom) = 16.6, P < 0.001.

Conditioning mainly occurs directly at the peripheral end organ, although central mechanisms (the efferent systems among others) might increase the sensitivity.

A more detailed analysis of the sensory discharge was then performed, and the spike and mEPSP discharges were evaluated by measuring the following parameters: 1) average rates at rest; 2) distribution of the units in terms of number of total discharge during the first complete cycle of rotational stimulation at 0.1 Hz; and 3) ratio between the discharges during the last vs. first cycles during a 2-min rotational stimulation at 0.1 Hz.

Resting activity. A significant difference in mEPSP and spike emission frequency, between control and microG units, was detected during spontaneous activity (Fig. 3). Mean mEPSP rates were 129 ± 5.8 Hz in control (n = 160) and 97 ± 5.6 Hz after microG (n = 88; Kolmogorov-Smirnov test D = 0.22, P < 0.001); corresponding spike rates were 5.4 ± 0.78 Hz (control, n = 92) and 0.4 ± 0.12 Hz (microG, n = 88, Kolmogorov-Smirnov test D = 0.44, P < 0.001).

Evoked activity. The distribution of the number of mEPSPs detected during the first full cycle of rotational stimulation at 0.1 Hz in units exposed to microG (956 ± 87.3, n = 25) was compared with the corresponding distribution in the control group (1,589 ± 84.6, n = 25). The result is displayed in Fig. 4, where the comparison of the cumulative distributions is also depicted (C), indicating a significant decrease in quantal activity for the microG group (Kolmogorov-Smirnov test; D = 0.56, P < 0.01).

A similar analysis was performed on spike activity. The distribution of the number of action potentials occurring during the first full cycle of rotational stimulation at 0.1 Hz in the spiking units exposed to microG (41.8 ± 8.06, n = 27) was compared with the corresponding distribution in the pooled control groups (77.2 ± 8.19, n = 27). The result is displayed in Fig. 5; the comparison of the cumulative distributions (C) indicates a significant decrease in spiking activity for the microG group (Kolmogorov-Smirnov test, D = 0.485, P < 0.001).

An example of the measurements of the sensory activity at rest and during mechanical stimulation at 0.1 Hz in two typical units (a control and a microG unit), is graphically depicted in Fig. 6.

Fatigability. The effect of prolonged stimulation on cyto-neural fatigue was considered in detail.

The number of spikes counted during the last full cycle of a 2-min sinusoidal rotation at 0.1 Hz was compared with the corresponding number during the first full cycle. Because of the high percentage of nonspiking units in microG, this comparison was relevant in control units only. The last cycle displayed a reduced total number of spikes (74.0 ± 23.6 vs.
A similar comparison was performed for the number of mEPSPs, in 14 control and 14 microG-conditioned units. In control units, the last cycle displayed a reduced total number of mEPSPs (1,171 ± 108.3 vs. 1,417 ± 106.0, i.e., 82.4 ± 4.97%, P < 0.01, paired t-test). Conversely, the numbers of mEPSPs during the first and last cycles were comparable in microG-conditioned units (872 ± 85.8 vs. 861 ± 72.1, i.e., 103 ± 6.4%, not significant).

Fig. 4. Histograms of the number of mEPSPs discharged during the first cycle of sinusoidal rotation at 0.1 Hz by control units or by units from microG-conditioned animals. Histograms are fitted by maximum-likelihood γ-distribution functions of the form: \( p(n) = \left(\frac{1}{\Gamma(\nu)}\right) \frac{\nu^n}{\gamma^{\nu n}} n^{\nu n - 1} \exp\left(-\frac{n}{\gamma}\right). \) In control (1,589 ± 84.6; n = 25), the parameters of the distribution are \( \nu = 177, \gamma = 8.5. \) After microG (956 ± 87.3, n = 25), the corresponding values are \( \nu = 280, \gamma = 2.5. \) The cumulative distributions, as used in Kolmogorov-Smirnov test to compare the two populations, are shown in C \( (D = 0.56, P < 0.01). \)

Fig. 5. Histograms of the number of spikes recorded at posterior canal afferent fibers during the first stimulation cycle at 0.1 Hz, in control units or in units from microG-conditioned animals. Histograms are fitted as in Fig. 4. In control (A), the distribution is slightly skewed to the right (77.2 ± 8.19, n = 27), \( \nu = 41.8, \gamma = 1.1. \) In microG (B), the distribution approaches an exponential \( (41.8 ± 8.06, n = 27), \nu = 44.2, \gamma = 0. \) The cumulative distributions, as used in Kolmogorov-Smirnov test to compare the two populations, are shown in C \( (D = 0.485, P < 0.001). \)
Thus, the reduced synaptic activity in microG-conditioned units appears to be accompanied by a decreased susceptibility to fatigue during prolonged moderate stimulation.

At higher rotation frequencies, a decrease in mEPSP rates in the last vs. the first cycle of rotation was generally observed in control preparations (−13.4% and −4.8% in the average, at 0.2 and 0.5 Hz, respectively), but the differences were not statistically significant. Conversely, a significant decrease was observed at all rotation frequencies in spike rates during the last cycle (measured in controls only) (−29.3%, −21.4%, −21.5% at 0.1, 0.2, and 0.5 Hz, respectively, P < 0.05). Frequency dependence of synaptic activity during rotational stimulation. The responses to sinusoidal rotation of the posterior canal were studied in control and microG preparations by counting the number of mEPSPs and spikes discharged during a 20-s period of rotation at different frequencies.

Figure 7 shows a comparison of the average responses to rotational stimulation at 0.1, 0.2, and 0.5 Hz in control and microG groups. The time courses of mEPSP and spike rates were obtained by averaging the measurements from n = 20–43 units at each value of time. The most apparent feature is the reduction in rates after microG. This is particularly apparent for spikes, although in this case the average value is significantly reduced by the relatively high number of units, which did not show any spikes. Careful inspection of the time courses at 0.1 Hz reveals that mEPSP rate is slightly delayed after microG conditioning (−47.5 degrees phase delay vs. −56.8 in control, t = 2.37, P < 0.05); furthermore, spike rate anticipates mEPSP rate by 21 ± 6.3 degrees (P < 0.01), and this phase lead appears to be due to an anticipated and rapid drop during the second half of the cycle in microG-conditioned units.

The delay in mEPSP rise during rotational stimulation is well in agreement with the observations on isolated hair cells. In fact, calcium currents have been reported to be decreased by microG conditioning (10), which would produce both a decrease and a delay in mEPSP response. This aspect was further investigated by studying the time course of calcium current during sinusoidal voltage changes in isolated hair cells. As illustrated in Fig. 8, during hair cell depolarization, calcium currents start to appear at −54/−52 mV, reach a first maximum value at −28.5 mV (n = 5) during the rising phase of the voltage command, then they transiently decrease; they rise again during the falling phase of the voltage command, exhibiting a second peak value at −29.0 mV, and eventually wane upon complete repolarization. The biphasic time course of the Ca²⁺ current during the sinusoidal voltage migration reflects the fact that membrane potential trespasses to and from the peak of the I-V curve for ICa (in fact, biphasicity disappears with smaller sinusoidal deflections that do not reach this critical value); the second peak usually is of lower amplitude, possibly due to Ca²⁺ accumulation in the cytosol (reduced driving force) and/or partial Ca²⁺-dependent ICa inactivation (8). The standard ICa I-V curves, computed in the classical way by applying instantaneous voltage steps, display a peak at −10 mV (10). The current peaks at more negative values, observed in Fig. 8, are presumably generated by the dynamical changes brought about by the slow continuous protocol, which produces a higher intracellular Ca²⁺ load.

MicroG-conditioning does not affect either the threshold or the peak in standard ICa I-V curves (10). During sinusoidal voltage migration the threshold is unaffected by microG, but the peak in the dynamic I-V relation appears to be shifted (Fig. 8E: −17.5 mV during the sinusoidal rising phase; −19.2 mV during the repolarizing phase; n = 5). This is presumably due to lower Ca²⁺ accumulation because of a reduced Ca²⁺ influx: this would shift the driving force for Ca²⁺ toward more positive values. Because under these conditions, the ICa re-
quires a longer time to reach its peak value, the time during which the membrane is held above the I-V peak, at constant sinusoidal voltage amplitude, is reduced, and this should contribute to the attenuated biphasicity of the ICa tracing under microG conditions (Fig. 8E). A decreased degree of Ca$^{2+}$-dependent inactivation may further contribute to the decreased depth of the saddle in current time course. The difference between the I-V peak potential (controls vs. microG), therefore, would suggest that not only the simple ICa amplitude, but the entire Ca$^{2+}$/H11001 handling by the hair cell is affected by microG conditioning.

The relationship between mEPSP and spike rates: input-output relation of the spike encoder. The time courses of mEPSP and spike rates in response to 0.1 Hz rotation have been replotted in Fig. 9 after rescaling, so that mEPSP--spike dynamic relationship is more easily perceived. As it has been generally observed, spike rate declines earlier than mEPSP rate, after the peak, which produces a slight phase lead for spike vs. mEPSP signal. Following microG treatment, the decline in spike rate consistently appears to be further anticipated. This observation suggests that microG treatment may specifically affect the properties of the spike encoder.

Whereas the frequency response of mEPSPs to rotational stimulation reflects bioelectrical events in the hair cell, the relationship of spike rates with mEPSP rates depends on bioelectrical properties of the first afferent neuron, which could not be investigated by single cell recording and current-conductance measurements. The dynamic behavior of the spike encoder can be described by examining spike rate as a function of mEPSP rate. This kind of analysis is illustrated in Fig. 10 for mechanical rotation at 0.1 and 0.2 Hz, using the averaged data from the same groups of units as in Fig. 9, but neglecting all units that did not display any spikes. When silent encoders are discarded, the gain of the spike-vs.-mEPSP relation becomes comparable in control (solid symbols) and treated preparations (open symbols), but the dynamic behavior remains clearly different. In these graphs, the spike frequency is plotted vs. mEPSP frequency for each value of time during 2–3 rotation cycles. The input-output relation is in all cases markedly nonlinear, as it displays a threshold value at about 50 mEPSP/s, below which no spikes are generally detected, and a smoothly increasing slope toward a relatively fixed ratio $\Delta$ (spikes/s) $\propto$ 0.2$\cdot$Δ(mEPSP/s). The marked effect of microG on spike rate is likely to be at least partly due to such nonlinearity, as a
moderate decrease in mEPSP rate is able to completely cancel spike activity. The most evident difference between control and microG preparations concerns the dynamic behavior of the encoder input-output relation. In controls, spike rate slightly anticipates mEPSP rate, giving rise to a slight hysteresis (separation of spike-vs-mEPSP relation in the rising and falling portions of the stimulation). The phase lead of spikes vs. mEPSP discharge appears to be markedly enhanced by microG treatment, as illustrated by the wide departures of the rising and falling portions (indicated by the arrows in Fig. 10) of the spike-vs-mEPSP relations after microG conditioning, with respect to control preparations: a marked hysteresis is thus observed, i.e., for each value of mEPSP frequency, spike rate is markedly higher during the ON than during the OFF phase.

Recovery of the afferent discharge after microG conditioning. The resting and evoked posterior canal sensory discharge was evaluated after 1, 6, 8, and 10 days after the microG treatment. mEPSP and spike discharge rates were estimated at rest (10-s period preceding sinusoidal rotation); the evoked mEPSP and spike numbers were counted during the first complete cycle of sinusoidal rotation at 0.1 Hz. The values were compared with the corresponding values in controls, as reported in Table 2.

The data indicated significant changes in mEPSP and spike emission after microG treatment, during the 10-day recovery period (one-way ANOVA, \( P < 0.05 \) for all parameters). In particular, resting mEPSP and spike frequency were significantly lower than in control experiments 1 day after microG treatment, and the same was true for the numbers of mEPSPs and spikes emitted during one rotation cycle at 0.1 Hz (t-test,
see Table 2 for values of t and P for the various contrasts). Recovery of mEPSP emission, either resting or evoked, was complete by 6 days after microG conditioning, and a statistically significant rebound was observed in the evoked mEPSP response after 10 days. Spike discharge recovery also occurred, and a significant rebound was already observed after 8 days.

Quantum size. In our experience, the sizes of the mEPSPs noticeably vary among the units and the experiments, also due to the high variability of the input resistance of the postsynaptic fibers. Under each of the conditions here examined, 1,500–6,000 single events have been counted; the average mEPSP peak sizes ranged 0.44 to 0.78 mV (mean = 0.57 mV) and displayed no clear relation with treatment, pithing, or recovery time. The dispersions of peak amplitudes in the various units were also comparable and uncorrelated with treatment (coefficient of variation 1.1 ± 0.48). Possible differences in mEPSP size, however, did not contaminate the mEPSP automated counting procedure.

Morphology. The complete epithelium of the posterior crista ampullaris from a preparation exposed to normoG conditioning is reproduced as an example in Fig. 11, to illustrate how the photograms were acquired for morphometric analysis. A similar reconstruction from a microG-treated preparation is presented in Fig. 12, for comparison.

<table>
<thead>
<tr>
<th></th>
<th>No. of Evoked mEPSPs [F = 5.75, P &lt; 0.01]</th>
<th>No. of Evoked Spikes [F = 7.52, P &lt; 0.01]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>129.5 ± 116.1</td>
<td>102.2 ± 26.1</td>
</tr>
<tr>
<td>n = 14 (160 rest)</td>
<td></td>
<td></td>
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<tr>
<td>1-day recovery</td>
<td>93.8 ± 13.0 [t = -2.25, P &lt; 0.05]</td>
<td>1,034 ± 115 [t = -2.03, P &lt; 0.05]</td>
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<tr>
<td>n = 16</td>
<td></td>
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<tr>
<td>6-day recovery</td>
<td>144.3 ± 23.0 [t = -2.03, P &lt; 0.05]</td>
<td>1,446 ± 226 [t = -2.03, P &lt; 0.05]</td>
</tr>
<tr>
<td>n = 9</td>
<td></td>
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<tr>
<td>8-day recovery</td>
<td>137.0 ± 31.2 [t = -2.03, P &lt; 0.05]</td>
<td>1,697 ± 179 [t = -2.03, P &lt; 0.05]</td>
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<tr>
<td>n = 8</td>
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<tr>
<td>10-day recovery</td>
<td>187.8 ± 31.6 [t = -2.03, P &lt; 0.05]</td>
<td>1,882 ± 100 [t = -2.03, P &lt; 0.05]</td>
</tr>
<tr>
<td>n = 10</td>
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</table>

*One-way ANOVA. Values are given as mean ± SE; t and P values vs. controls.
intermediate-central portion. In particular, cell area significantly increased toward the center of the crista \((P < 0.001\) for all comparisons), and a small overall increase in cell perimeter was detected in the central portion. No significant differences were instead observed for nuclear parameters. The same analysis indicated that the prolonged stimulation in the RPM under normoG produced a significant increase in cell area, and, in particular, toward the central and intermediate regions of the crista. However, the increases were relatively small \((+16 \mu m^2 = +14\% \text{ in the average}; +28 \mu m^2 = +24\% \text{ in the center of the crista})\) and may be compatible with the absence of detectable functional alterations. Treatment in microG appeared to induce significant increase in cell area with respect to both control and normoG preparations, in the center of the crista (where a significant increase of cell perimeter was also observed), and with respect to the control in the intermediate region. The overall cell area increase was quantitatively more marked in microG \((+38 \mu m^2 = +32\% \text{ in the average}; +87 \mu m^2 = +74\% \text{ in the center of the crista})\) than in normoG-treated preparations, which may indicate a moderate degree of cellular stress and may account for the observed reduced secretory capability. No significant alterations were observed in nuclear area or perimeter, under the various conditions examined.

Inspection by eye revealed that normoG and microG treatments did not produce morphological alterations of supporting and basal cells. No morphometric analysis was performed on these cells.

DISCUSSION

MicroG has been shown to affect the morphology, development, and function of several systems and tissues. Vestibular organs, which are specifically dedicated to perception and neural coding of linear and angular accelerations, constitute a particularly important target for altered gravitational conditions. Indeed, significant morphological modifications in the labyrinth of vertebrates have been reported in response to prolonged exposure to microG \((4, 5, 13, 14, 19)\), as well as biochemical \((12)\), genetic \((\text{for a review see Ref. 11})\), behavioral \((2)\), and developmental \((3)\) alterations. Functional aspects have been little investigated. Changes of the biophysical properties of K\(^{+}\) and Ca\(^{2+}\) currents in the vestibular hair cell have been reported in the frog, after conditioning the surviving animal in an artificial microG environment \((10)\). Still, no morphofunctional correlations have been attempted so far, and the effects on the peripheral vestibular organs remain poorly understood. This study constitutes the first report describing specific effects of temporary cancellation of the gravitational vector on the functional properties, and the morphological features of the intact labyrinth, and points out an impairment in afferent sensory information processing, which is not promptly reversible, but is recovered from within 6–8 days and gives rise to a rebound in both resting and evoked mEPSP and spike discharge at 8–10 days.

Experimentally, reduced gravity can be simulated in the RPM, by continuously and randomly changing the orientation
of the preparation with respect to the gravitational axis (microG). The problems in applying this procedure to an alive animal, and, in particular, in studying labyrinthine function, are twofold: on the one hand, the frog must be anesthetized, and anesthesia might produce by itself specific effects both locally and in terms of cross-talk with central nervous structures; on the other hand, the microG simulation procedure intrinsically implies a remarkable degree of long-lasting stimulation of vestibular organs, as the head is subjected to continuously changing angular accelerations around variable spatial axes. Because of the latter problem, a series of control procedures should be accomplished to carefully dissect any possible effect of microG from those related to animal handling and prolonged stimulation of the vestibular organs during the microG simulation protocol.

At first, saccule and utricle might appear the most appropriate sensors to be studied, as they are in charge of the detection of linear accelerations and the perception of the orientation of the head with respect to the gravitational axis. However, exposure to simulated microG in the RPM implies crucial differences from true absence of gravity. Whereas in the absence of gravity, the labyrinth is not exposed to any external linear acceleration or mechanical stimulation, in the RPM machine, the average linear acceleration is null along any axis in space, but the preparation—and the otolithic membrane in the saccule and utricle—are continually exposed to a linear acceleration with the “normal” module of 9.8 m/s² and a continuously varying direction. Thus, in these organs the stimulation of zero gravity is not fully appropriate; it is not possible to devise a control procedure to separate possible effects of the stimulation in the RPM from the effect of an average null gravity.

On the contrary, semicircular canals do not sense, in principle, linear accelerations. At difference with true absence of gravity, the semicircular canals are exposed, in the RPM simulation, to continuously changing angular accelerations; however, if one of the canals is positioned so that its axis coincides with the axis of rotation of the internal frame of the RPM, then it can be exposed to exactly the same kind of stimulation in the presence of a normal gravitational field, provided that only the internal frame of the RPM is rotated, in the same way, keeping its axis still and vertical.

The semicircular canals have thus been selected as the most interesting target for this study, because in this system, the effects of the physical manipulations required to simulate the absence of gravity can be isolated and do not contaminate the estimate of the effects of microG per se.

In particular, the quantal secretory capability of the hair cell was evaluated by counting the miniature excitatory postsynaptic potentials (mEPSPs) recorded near the terminal of the primary afferent neuron, at rest, and during mechanical rotational stimulation of the posterior canal; the properties of the neuronal encoder were evaluated by simultaneously counting the action potentials in the afferent nerve fiber under the same conditions. Finally, changes in the structure of the crista were studied by analyzing the number and morphometric features of the cells in the receptor epithelium of the crista ampullaris. The analysis was completed by a patch-clamp study of membrane currents in hair cells isolated from the semicircular canals, with particular attention to calcium currents evoked by membrane voltage fluctuations that mimicked the receptor potential time course during sinusoidal rotation of the canal in the intact labyrinth.

The main result of this study is the observation that junctional and afferent activity at the posterior canal are significantly reduced after microG treatment, and this functional impairment is accompanied by detectable biophysical and morphological changes, the latter being mostly evident in the central region of the crista.

The rather intense stimulation of the semicircular canals entailed by microG simulation does not appear to significantly contribute to any of these effects. No clear-cut changes were observed in mEPSP or spike rates at rest or during mechanical stimulation in the various different conditions used as controls in this study: untreated animals, anesthetized and unstimulated animals, and anesthetized or pithed animals exposed to nor-
moG stimulation. The microG conditioning was usually performed under conditions in which most neuronal firing activity was suppressed by the anesthetic (afferent sensory spiking was actually blocked by the tricaine concentration used). On the contrary, the afferent-efferent connections of the labyrinth presumably were operative in the pithed animals. Although synaptic impairment and spike suppression appeared to be more marked in the latter condition, suggesting that central mechanisms might contribute to the intensity of the observed changes, the effects of microG were constantly reproduced and clearly discernible from those of simple stimulation in normoG under both of these conditions, namely, overall conduction block or normal peripheral-central connections. The observed effects are thus likely related to peripheral changes and may not require a major central component.

Prolonged exposure to microG impairs the synaptic machinery, as indicated by the decrease in spontaneous mEPSP rates; interference with sensory transduction may also occur, as suggested by the lower magnitude of mEPSP frequency oscillations during mechanical stimulation but does not appear to be a major aspect, as the response to rotational stimulation is essentially preserved, and scaled down by a factor similar to spontaneous mEPSP discharge; furthermore, the spike encoder mechanism appears to be somehow impaired, since the moderate decrease in mEPSP rates is associated with a dramatic reduction in spike firing; actually, spikes totally disappear in a large number of fibers. The reduction in spike firing is accompanied by a faster decay of spike rate after the excitatory peak during rotational stimulation (see Fig. 9). It is not clear whether these two aspects are mutually related, i.e., whether the encoder displays some kind of enhanced “fatigability,” which may account for both the anticipated off response and the decreased average spike rate.

These observations on the whole intact labyrinth are in good agreement with the biophysical properties of individual hair cells. The decreased synaptic activity might be related either to an intrinsic impairment of the secretory machinery or to a reduced stimulus to secretion, i.e., a reduced Ca\(^{2+}\) signal. Indeed, patch-clamp recording from hair cells isolated from the Ka tickling device (gating) provides some indication that the K\(^+\) and Ca\(^{2+}\) current components are affected. As concerns the K\(^+\) currents, the IA is systematically involved during the sinusoidal cycles shown in Fig. 8, which presumably reflect the physiological excitatory-inhibitory bending of hair cell cilia (9). The IA kinetic changes result in a prolonged duration of this current and in an overall increase of the hair cell repolarizing power (10). As concerns the Ca\(^{2+}\) currents, they have been reported to be generally reduced by the microG treatment (10), whereas the shape of the related I-V curves, and therefore the voltage dependent gating kinetics, apparently were not modified (10). Here, we present a comparative study of the time course of Ca\(^{2+}\) currents in normal and microG-treated cells during membrane potential oscillations imposed to mimic the effects of rotational stimulation on the receptor: in addition to the overall decrease of Ca\(^{2+}\) currents, the dynamics of calcium fluxes appear to be altered during such sinusoidal activation pattern. The peak in Ca\(^{2+}\) current is rapidly reached in control hair cells, at a membrane potential (−28.5 mV) during sinusoidal stimulation well negative with respect to the reported peak in I-V relations (−10 mV, obtained via voltage steps; 10); this is presumably due to the intense inflow of calcium ions, which further reduces the driving force across the channel, and/or to the Ca-dependent ICa inactivation after increased [Ca\(_i\)] (8). In microG cells, the peak, which has an amplitude about 40% lower, is reached at a later time and at a more positive value of membrane potential (−17.5 mV), suggestive of a shift of the peak of the ICa-I-V curve. Because the shape of the I-V, measured in the standard way (voltage steps), is not modified by microG (10), this effect is likely to ensue from the altered dynamics of Ca\(^{2+}\) fluxes and accumulation. Thus, reduced Ca\(^{2+}\) inflow characterizes the whole depolarizing phase of the sinusoidal cycle. The delay in Ca\(^{2+}\) current dynamics is consistent with the observed delay in mEPSP rise during the on phase of the cycle; conversely, the increased repolarizing power due to the modified IA kinetics during the off phase of the stimulation cycle is consistent with the accelerated decrease in mEPSP and spike discharge rate during the off phase of the cycle. The constant dimension of the elementary synaptic event (quantum size), which is apparently unaffected by the gravity-related manipulations, also supports the prevailing involvement of a Ca-related interference with the secretion process.

The semicircular canal sensory discharge frequency recovered its control values with a consistent delay, after microG treatment (6–8 days). Later on (8–10 days), it displayed a rebound effect (i.e., mEPSP and spike frequency significantly higher than in control preparation). To our knowledge, a single study in the literature reported long-lasting changes in cellular function of a labyrinthine organ, in particular, the toadfish utricle following a “Neuralab” mission on the Shuttle (1). On landing, the utricular first-order neuron discharge was examined. The permanence in microG environment was consistently longer than in our experiments. The utricular afferent discharge proved hypersensitive to translational acceleration 10–16 h after landing, compared with control animals, and slowly recovered to control values (on average 2–4 days are required). To explain their results, the authors hypothesized alterations in either or both 1) mechanoreception, i.e., changes in otolith-stereociliary coupling might cause an enhanced bundle deflection for a given movement; and 2) junctional transmission, based mainly on the morphological changes described in the hair cell synaptic apparatus shortly following microG exposure (13, 14). They pointed out that the time course of these changes was too long to be explained by adaptation of the hair cell receptor potential. Our data are obtained with a different experimental protocol, in a different species and in another labyrinthine organ. We report a decrease in synaptic efficiency after a 4-h exposure to simulated microG, and decreased Ca\(^{2+}\) currents in hair cells. The main common aspect in the two studies regards the long time courses of recovery. The prolonged time constants of the recovery, and the morphological changes we report, suggest that the effects produced by microG in our preparation are not limited to momentary functional regulation but rather involve long-term complex resetting, such as upregulation/downregulation of expression of channels (typically Ca\(^{2+}\) channels) and/or other proteins implied in synaptic transmission. Recovery from such regulatory processes is generally characterized by rebound phenomena: consistently, we observed a functional rebound at 8–10 days.

The methodological refinements introduced here to be able to measure quite high mEPSP rates, even in the presence of significant spike rates, have made it possible to compare both
mEPSP and spike frequencies among the various experimental conditions here examined; in addition, they have opened the possibility of analyzing the input-output function of the encoder, by examining the spike rate vs. mEPSP rate relation, to compare the various experimental conditions and to pinpoint dynamical aspects of the encoder activity. As illustrated in Fig. 10, the encoder input-output relation is clearly nonlinear, displays a functional threshold for spike generation at 50–80 mEPSP/s, and approaches a slope of about 0.2 spikes/mEPSP at high rates. The dynamic analysis during sinusoidal rotation reveals a slight hysteresis of the input-output relation, which corresponds to a slight phase lead of spike rate vs. mEPSP rate; the hysteresis becomes more apparent with increasing rotation frequency (i.e., the phase lead angle tends to increase with frequency). Most relevant, it appears to be markedly more pronounced after microG, consistent with the observed anticipated drop of spike rate during the off phase of rotational stimulation.

Perspectives and Significance

The data reported in this paper achieve two main relevant goals: on the one hand, they show that a simultaneous and powerful analysis of synaptic activity and encoder performance can be performed at the cytoneural junction, which makes it possible to investigate in more depth the respective contributions of the hair cell and the first afferent neuron to overall signal processing at labyrinthine organs; on the other hand, they reveal unexpected, clear-cut effects of microgravity on the morphology and functioning of vestibular organs. These effects, which are fully reversible in the particular preparation here considered, are consistent with the changes here and previously reported in the biophysical properties of hair cells isolated from similar preparations and examined by the patch-clamp technique.

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GRANTS

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


