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Effects of Microgravity on Vestibular Development and Function in Rats: Genetics and Environment

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Our anatomical and behavioral studies of embryonic rats that developed in microgravity suggest that the vestibular sensory system, like the visual system, has genetically mediated processes of development that establish crude connections between the periphery and the brain. Environmental stimuli also regulate connection formation including terminal branch formation and fine-tuning of synaptic contacts. Axons of vestibular sensory neurons from gravistatic as well as linear acceleration receptors reach their targets in both microgravity and normal gravity, suggesting that this is a genetically regulated component of development. However, microgravity exposure delays the development of terminal branches and synapses in gravistatic but not linear acceleration-sensitive neurons and also produces behavioral changes. These latter changes reflect environmentally controlled processes of development.

This review will focus on the mammalian vestibular system in the ear, its connections with the brain, and how microgravity affects the development of these connections and their related behaviors. We will first present an overview of the organization and function of the vestibular system as a foundation for understanding the subsequent anatomical and behavioral studies. The vestibular labyrinth is a part of the inner ear and contains five sensory epithelia (Fig. 1). Three of these areas respond to angular stimuli, i.e., velocity of head rotation (semicircular canals) and two respond to gravitational stimuli, i.e., linear acceleration (maculae). The response properties are determined by specialized accessory structures that extract different information from stimuli that reach the vestibular labyrinth. Each of these sensory epithelia contains a group of specialized cells that each has an apical specialization consisting of a single kinocilium and multiple, actin filament-rich, microvilli, known as stereocilia. These receptor cells are called "hair cells" because of these ciliated apical specializations.

Semicircular canals

The three fluid-filled semicircular canals contain swellings (ampulla). Within each ampulla is a sensory epithelium, or crista, that contains the hair cells. The cilia of the hair cells are covered by a gelatinous cupula. During a rapid head rotation, the movement of the fluid (endolymph) within a canal will initially lag behind the movement of the head. This causes the endolymph to push against the cupula, moving the cilia of the hair cells and causing excitation or inhibition of the hair cells, depending on the direction of movement. The excited hair cells subsequently excite the sensory afferent neurons that contact them. Rotational head movements should occur fairly normally in microgravity and so stimulation of these receptors and their central projections would be expected to occur fairly normally as well.

Gravistatic receptors

The hair cells of the two gravistatic maculae are covered by many small calcium crystals, called otoconia. The high density of the otoconia causes them to lag behind during head movements that cause linear acceleration. On Earth, tilting the head causes the otoconia to slide towards the center of gravity. This movement of the otoconia splays the stereocilia, which opens mechanically-gated ion channels, and thus stimulates the hair cells. However, tilting the head in microgravity would not change the gravitational pull on the otoconia, and therefore would not stimulate the gravistatic-responsive hair cells. Nevertheless, rapid head rotation will result in some linear acceleration of otoconia. Thus most, but not all, sensory stimuli are eliminated in microgravity for these macular receptors.

Sensory afferent neurons

The hair cells within the sensory epithelia of both semicircular canals and gravistatic receptors synapse with dendrites of vestibular sensory neurons. The cell bodies of both semicircular- and gravistatic-responsive sensory neurons are located within the vestibular

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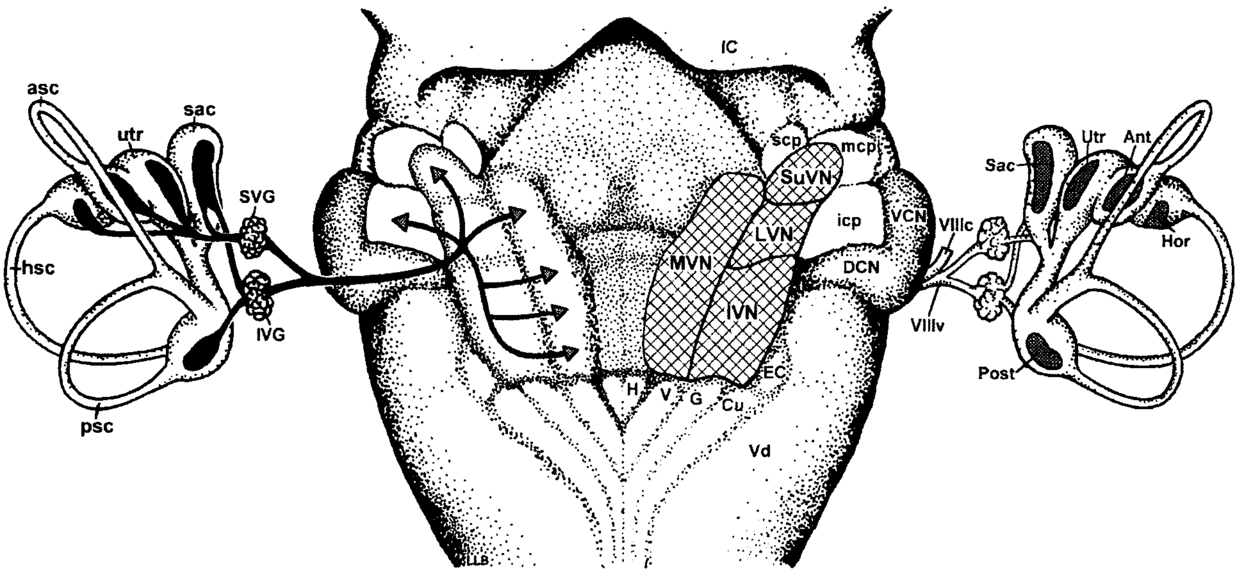

Fig. 1. Illustration of the dorsal view of a rodent brainstem showing the relationships between the vestibular apparatus with the sensory epithelia, the vestibular component of the eighth nerve (cochlea is omitted for clarity), and the vestibular nuclei. Ant, anterior semicircular epithelium; asc, anterior semicircular canal; Cu, cuneate nucleus; DCN, dorsal cochlear nucleus; EC, external cuneate nucleus; G, gracile nucleus; H, hypoglossal nucleus; Hor, horizontal semicircular epithelium; hsc, horizontal semicircular canal; IC, inferior colliculus; icp, inferior cerebral peduncle; IVG, inferior vestibular ganglion; IVN, inferior vestibular nucleus; LVN, lateral vestibular nucleus; mcp, middle cerebellar peduncle; MVN, medial vestibular nucleus; Post, posterior semicircular epithelium; psc, posterior semicircular canal; sac, saccule; Sac, saccular epithelium; scp, superior cerebellar peduncle; SuVN, superior vestibular nucleus; SVG, superior vestibular ganglion; utr, utricle; Utr, utricular epithelium; V, vagal area; VCN, ventral cochlear nucleus; Vd, descending trigeminal nucleus; VIIIc, cochlear division of eighth cranial nerve; VIIIv, vestibular division of eighth cranial nerve.

ganglion. These neurons receive synaptic input from hair cells via their dendritic fibers. Their axons project into the brain to synapse predominantly with the vestibular nuclei and the flocculonodular lobe of the cerebellum. Interestingly, the sensory neurons within the vestibular ganglia are incompletely segregated according to their peripheral targets (Maklad and Fritzsch, 1999) and their central projection areas are partially overlapping (Kevetter and Perachio, 1986; Dickman and Fang, 1996).

Gravity and Vestibular Development

Effects of gravity on vestibular receptors

Morphological changes in the vestibular labyrinth have been studied in adult rodents exposed to microgravity but not, to our knowledge, in developing rodents. Ultrastructural studies of the utricular maculae demonstrated a 41-55% increase in the number of synapses between hair cells and afferent dendrites in rats exposed to microgravity compared to controls (Ross, 1993). The number of synapses remained high in flight animals even 9 days after landing (Ross, 1994). These data suggest that the macular hair cells respond to weightless otoconia by increasing the number of their connections with sensory neuron dendrites. An effect of microgravity on synaptic development in the gravistatic maculae remains to be determined.

Role of gravity on development of vestibular connections

To study the effects of gravity on the formation of connections between gravity receptors of the inner ear and brain, pregnant rats were launched aboard the shuttle Atlantis, flight STS-66. The embryos were exposed to weightlessness from gestation day 9 (where the day of conception is considered gestation day 1) to gestation day 20, for a total of 11 days. In a subsequent flight of the shuttle Discovery, STS-70, embryos were exposed to weightlessness from gestation day 11 to 20. This period encompasses the developmental period before and during which the vestibular ganglion neurons make peripheral or central contacts with hair cells or neurons in vestibular nuclei as well as the time the vestibular system becomes somewhat functional. Some of these fetuses were used to study the developmental state of their gravistatic and angular vestibular connections at the time of landing (Bruce and Fritzsch, 1997), and others were allowed to develop to term and their vestibular behaviors were studied postnatally (Ronca and Alberts, 1997). The following paragraphs summarize our findings.

Connectional changes: Within 3 h of shuttle landing embryos from each rat were deeply anesthetized and fixed in 4% paraformaldehyde by perfusion through the heart and the heads were hemisected sagittally. Controls were exposed to Earth's gravity but otherwise were kept in a comparable environment. The neuronal

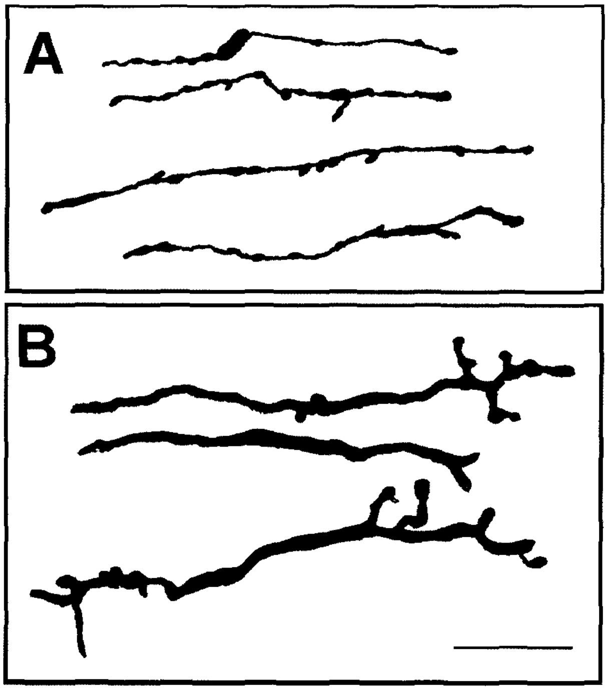

Fig. 2. Line drawing of DiI-labeled axons in the medial vestibular nucleus after small applications to the saccule in Flight (A) and control (B) rat pups aged G20. Medial is to the right. Note that the saccular (gravistatic) axon arbors of flight animals have fewer long and short side branches than do those of controls. This difference suggests that the neurons that receive reduced gravistatic stimulation develop more slowly compared to those that receive normal, Earth gravity stimulation. Scale bar=50 µm.

tracer DiI (Molecular Probes, Oregon) was dissolved in dimethylformamide and nylon-filter strips were soaked in this solution. After evaporation of the solvent, the nylon strips were cut into appropriate-sized pieces and inserted into the desired sensory epithelium or specific areas of vestibular nuclei, specifically either the posterior vertical canal of the semicircular canals (angular acceleration) or the saccule (gravistatic receptor). The hemisected heads were then incubated in a 37℃ oven for accelerated diffusion of the dye (2-4 weeks) to obtain filling of the fibers of the appropriate receptors in the brain. The brains were then dissected from the bony capsule, embedded in 10% gelatin, and hardened in 4% paraformaldehyde overnight. The blocks were sectioned on a vibratome at 100 µm, the sections mounted on a slide, coverslipped and examined with an epifluorescence microscope.

Central branching patterns from gravistatic receptors: Analyses of labeled axonal projections from the saccule (gravistatic receptors) into the medial vestibular nucleus showed that in flight animals most axons ended in small growth cones and rarely had side branches. In control animals the axons had greater numbers of side branches and extensive arborization patterns that terminated with swellings suggestive of forming synapses (Fig. 2). When these sections were photoconverted and examined with transmission electron microscopy, no structures were observed in the flight animals that could unequivocally be identified as a synapse.

Central branching patterns from semicircular canals: Stimuli that produce linear acceleration are rare in microgravity, whereas stimuli that produce angular acceleration would occur at least as frequently as on Earth (1 g). Hence, the connections of the posterior vertical canal should develop comparably in microgravity and in 1 g, and so comparing the connections of the posterior vertical canal in flight and control embryos serves as an excellent internal control for possible maternal factors (such as reduced nourishment to the embryo) that may affect overall embryonic brain development. Our data showed that, in both flight and control embryos, labeled projections from the posterior vertical canal had elaborate arborizations within the medial vestibular nucleus. When these labeled axons were processed for electron microscopy, numerous labeled profiles were observed forming well-developed synapses in both flight animals and control animals.

Role of gravity on development of behavior

Development of motor behaviors: The unmanned Soviet Cosmos-1514 satellite carried the first payload designed for studies of microgravity on fetal development. Pregnant Wistar rats were exposed to microgravity for about 5 d, from G13-G18. Postnatal evaluations revealed that the animals developed at about normal rates and were morphologically intact (Alberts et al., 1986; Serova et al., 1993a). Three behavioral tests to measure vestibular and motor function were performed: ability to turn over from a supine to a prone position (righting response), turning against gravity on a tilted plane (negative geotaxis), and response on a rotating platform (head turns in the opposite direction of rotation) (Serova et al., 1993b). The results of these tests showed that the vestibular and sensorimotor systems functioned comparably in the flight and control animals.

Similar results were obtained from rats that were exposed prenatally (G9-G20) to microgravity aboard the space shuttle flight STS-66. These rats were cross fostered with ground-based dams at birth and the postnatal development of motor control patterns related to walking was compared to ground-based control groups (Wong and DeSantis, 1997). They found that walking began and progressed normally in flight and control rats. Together these results indicate that prenatal exposure to microgravity does not effect postnatal somatosensory and motor control.

Development of vestibular behaviors: Vestibular-mediated responses of offspring derived from the STS-66 flight were analyzed in neonatal rats derived from the same study and applied from Postnatal day (P) 0 until P5

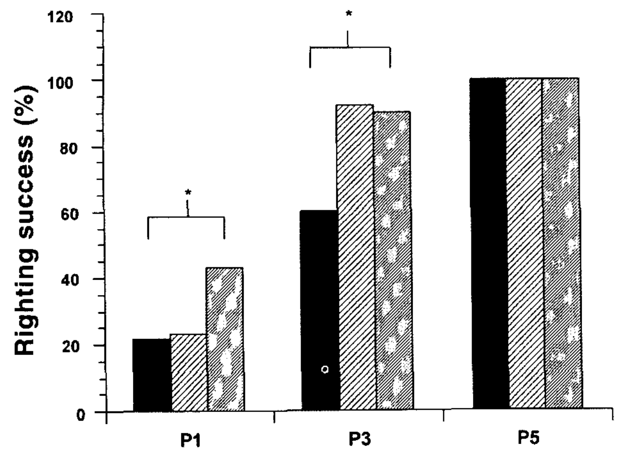

Fig. 3. Percentage of P1, P3 and P5 rats that achieved the prone posture during vestibular head righting in the water immersion test. The data presented are comprised of a total of 34 neonates from 24 dams (Flight, N=8; Synchronous, N=7; Vivarium, N=9).

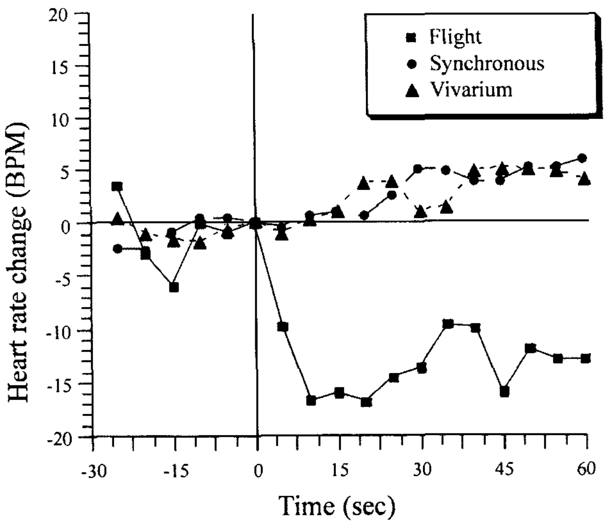

Fig. 4. Average HR response of Gestational Day (GD) 20 fetuses in Flight (N=4) and Synchronous Control (N=3) and Vivarium Control (N=3) conditions to 70 head-up tilt (roll). Heart rate (bpm) is plotted as the difference from pre-stimulus baseline (time=0). The horizontal axis at time zero corresponds to stimulus onset.

(Ronca and Alberts, in press, 2000). Contact righting and water immersion righting tested the pups' vestibular responsivity with and without tactile or proprioceptive cues. Based on these findings, we identified modality-s pecific deficits in vestibular function following prenatal exposure to spaceflight.

Contact righting: Spaceflight and exposure to microgravity during gestation had no apparent effect on contact righting. Both controls and Flight neonates displayed similarly high frequencies of successful righting; they achieved the prone position with similar latencies. In the contact righting test, the neonates receive tactile and proprioceptive cues from the solid substrate and these cues may have compensated for altered vestibular sensitivity. For this reason, we also studied the water immersion righting test, which involves vestibular, but not tactile or proprioceptive, function.

Water-immersion righting: In this test, as in surface righting, rat pups are initially held in a supine position but here they are released in a bath of warm water. Their buoyancy provides sufficient time for righting responses and alleviates contact with a substrate, making the test more purely vestibular, without additional tactile and proprioceptive stimulation. Using this test, we observed clear deficits in righting abilities of Flight pups. Fig. 3 illustrates the results of the tests with neonates that underwent prenatal development in space. On P1, the day on which the response is first observed in the neonatal rat, Flight neonates made no attempt to right themselves during a time period twice as long as that required by Synchronous controls. Similar proportions of animals made righting attempts in the two conditions, but fewer Flight neonates succeeded in righting prior to reaching the bottom of the tank. On P3, Flight animals again performed poorly compared to Controls. Whereas successful righting was observed in over 90% of the control animals, only 60% of the Flight animals were observed to right themselves completely. In addition, 10% of the Flight neonates (as compared to 0% of the controls) made no righting attempts. Response latencies were also significantly longer among pups in the Flight condition. These findings suggest that Flight animals were relatively insensitive to the vestibular cues in this postnatal, post-flight test. The vestibular deficit was transient, however, as evidenced by the complete success with which Flight neonates responded on P5, when their performance matched that of the control subjects.

The results of the contact righting test demonstrate that Flight pups were capable of righting themselves. Thus, the observed righting deficiency in the water immersion test was not a motor deficiency. In addition, 60% of the Flight neonates righted themselves in the water, suggesting that fetuses were either affected differentially by prenatal spaceflight or that they recovered at different rates. The observation that, by P5, water righting responses of the Flight animals were indistinguishable from those of controls suggests developmental recovery or readaptation in the presence of Earth-normal gravitational cues. The pups' postflight changes reflect a shift from in-flight profiles of stimulation to those imposed by the 1G, Earth-normal environment.

In this study, microgravity exposure during gestation appears to have selectively diminished vestibular responsivity around the time of developmental emergence of this sensory modality.

Vestibulo-autonomic responses: We also analyzed the responses of fetal rats to vestibular perturbation (tilt) following prenatal spaceflight from G11 until G20 (Ronca and Alberts, 1997). Using a classic psychophysiological measure of sensory detection that relies on changes in cardiac rate (Sokolov, 1963), we applied the following procedure. Live, unanesthetized fetuses were externalized from the uterus and the mother's abdomen following chemomyelotomy, which completely blocked discomfort and disruptive movements in the dam. While still attached by the umbilical cord to the mother, and submerged in a warm bath, each fetus was fitted with EKG electrodes, positioned on its right side, and then exposed to a series of vestibular perturbations consisting of 70° head- up rolls, each lasting 10 sec. Flight fetuses responded to the first presentation of the roll stimulus (predominantly angular acceleration) with large, long-lasting bradycardia, whereas Control subjects did not respond to the roll stimulus (Fig. 4). The robust cardiac decelerations of Flight fetuses closely resemble those observed in ground studies using older G20 fetuses (Ronca and Alberts 1994). This suggests that Flight fetuses displayed more pronounced, if not precocial, responses to angular acceleration. Successive presentations of the roll stimulus did not elicit a response. The appearance of habituation, rather than continued responsiveness to the roll stimulus, indicates that the bradycardiac response was not a by-product of cardiovascular reflexes. Rather, evoking the cardiac orienting response (OR) and its subsequent habituation provide evidence for central processing of sensory information.

Videorecordings of behaviors: In-flight videorecordings of the Flight dams and corresponding videos of the Synchronous controls were compared to evaluate maternally-instigated sensory input to prenatal semicircular canals during space flight. Individual dams were identified using a kinematic coding scheme to classify and quantify their movements in microgravity and the control condition, earth gravity. A self-referencing framework, one in which movements were encoded in relation to the posture of the body at an immediately preceding time point, was used. We found that movements involving pitch and yaw were about equivalent in Flight and Synchronous animals. In contrast, Flight dams displayed about seven times more rolling movements than did controls. We believe that this dramatic difference was a consequence of the increased number of surfaces available during microgravity for walking and crawling ("walls" and "ceiling" are more like "floors" in a weightless environment). Many of the movements from surface-to-surface involve rolling movements along the dam's longitudinal axis (the z axis) and provide additional angular acceleration stimulation to the fetuses residing within her body.

Thus, during spaceflight, the fetus' gravistatic receptors are effectively unloaded, whereas the fetus' angular acceleration detectors (the semicircular canals) are hyperstimulated by the dams' increased rolling movements. This contrasting distinction, to our knowledge, has been overlooked in previous analyses of spaceflight results. Recent ground-based studies of fetal sensory function indicate that late-term rat fetuses (Gestation Day 20) detect the angular accelerations generated by normal movements of the mother's body, such as ambulation, turning and rearing (Ronca and Alberts, 1994). Combined with the present data and observations, it seems likely that behaviorally-derived stimulation from maternal movements may be a significant variable in future studies of mammalian development in altered gravity fields. Taken together with the anatomical findings presented above, these studies provide the first evidence that gravity and angular acceleration shape prenatal organization and function within the mammalian vestibular system.

Genetic and Environmental Regulation of Vestibular Development

Factors affecting vestibular development: The general corpus of experimental embryological data suggests that genes regulate the initial development of vestibular morphology and connections and environment has no direct effects (Fritzsch et al., 1998; Fekete, 1999). Once the vestibular apparatus has developed to the point that sensory neurons and hair cells can respond to environmental vestibular stimuli (several days before birth), this information can modify the expression of genes that regulate connectivity of the sensory neurons with hair cells and the brain. This two-step approach seems to be a conserved developmental sequence of events that occurs not only in the vestibular, but also in the auditory and visual systems (Batkin et al., 1970; Hubel and Wiesel, 1970; Blakemore and Van Sluyters 1975; Fritzsch et al., 1998; Fekete, 1999; Maurer et al, 1999; Rubinstein and Miller, 1999). The results from our spaceflight provide the first evidence that environmental vestibular stimuli do, in fact, modify vestibular connections and related behaviors. We will concentrate on these findings and their implications in the paragraphs below.

Gravity appears to be a critical stimulus in normal vestibular development during perinatal stages. For example, prenatal exposure to microgravity causes temporary deficits in gravity-dependent righting behaviors (Alberts et al., 1995), and exposure to hypergravity from conception to weaning causes permanent deficits in gravity-dependent righting behaviors (Sondag, 1997). Several parts of the vestibular system may contribute to these altered behaviors. In particular, the parts of the vestibular system that monitor the position with respect to gravity (saccule and utricle) may require exposure to gravity for normal, functional development. Data on hypergravity and microgravity exposure suggest some change in the otolith formation during development, in particular the size (Pedrozo and Wiederhold, 1994; Sondag, 1997). The details, however, are not consistent between different studies (Hara et al., 1995) and may actually vary with the species involved. In adults exposed to microgravity there is a change in the synaptic density in sensory epithelia (Ross, 1993; Fermin et al., 1996) suggesting that some adaptation may occur there. Effects have also been reported in the brainstem. Several studies have shown synaptic changes in the lateral vestibular nucleus (Johnson et al., 1976) and in the nodulus of the cerebellum (Krasnov, 1991) after neonatal exposure to hypergravity. Our own data indicate that synaptogenesis in the medial vestibular nucleus and the ability to respond to gravistatic stimuli are retarded in developing rat embryos that were exposed to microgravity from gestation days 9 to 20 (Bruce and Fritzsch 1997; Ronca and Alberts 1997). Thus, in the absence of gravity the projections from the angular acceleration receptors receive adequate stimulation to form synapses in the vestibular nuclei, whereas those from gravistatic receptors do not and will not be able to compete for synaptic space. Thus, we expect that after longer periods of exposure to microgravity the angular acceleration axons will project far more extensively to the vestibular nuclei than those of the gravistatic axons.

These data suggest that development of the vestibular sensory connections, like the visual (Hubel and Wiesel, 1970; Blakemore and Van Sluyters 1975; Maurer et al, 1999) and auditory systems (Batkin et al., 1970; Rubinstein and Miller, 1999) are determined by an intrinsic, gene-mediated process for axonal target finding, as well as an environmental modulation of genetic expression that regulates fine-tuning of synaptic contacts and other aspects of function and phenotype. These two sources of influence are essential for creating the orderly connections during development that allow proper integration of the linear and angular acceleration information by the vestibular system. Our data demonstrate that the axons of all vestibular sensory neurons reach their specified targets in the brain in both microgravity and normal gravity. Thus, target finding appears to be genetically (internally) specified rather than under environmental influence. It is likely that some of the specific genes that participate in these formative processes include the ephrin family of ligands and their receptors (Cowan et al., 2000). During the subsequent stages, axon terminals form arbors and synapses. In microgravity as in normal Earth gravity, the neurons connected to the semicircular canals appear to receive the amounts and kinds of stimulation that support the growth of axonal branches and regulation of synaptogenesis. We believe that this is because the fetuses continue to receive and perceive forces of angular stimulation while in microgravity. We know that many of these angular accelerations are derived from the mother's behavior (Ronca et al., 1993; Ronca and Alberts, 1994). Nevertheless, in microgravity conditions, neurons connected with the gravity receptors receive minimal stimulation; functionally this component of the vestibular system is down-regulated relative to the afferent neurons of the semicircular canals. Thus, there is a reasonable basis to appreciate the finding that the axons carrying gravistatic information have fewer branches and synapses with their targets in the brain, compared to those in normal gravity.

Patterning of the vestibular connections is not entirely specified by genes. Our data show that its projections are molded by environmental stimuli such as different levels of gravity and the stimulating effects associated with microgravity and the balance of stimulation between emerging gravistatic and angular systems. The exposure to microgravity is associated with altered projections of the gravistatic and angular acceleration sensory neurons into the brain. This altered projection may result in atypical responses and novel phenotypes, especially in systems that require or involve gravity.

It is tempting to speculate that the effects of gravity deprivation on the vestibular system parallel those of visual deprivation on the visual system and auditory deprivation in the auditory system (Batkin et al., 1970; Hubel and Wiesel, 1970; Blakemore and Van Sluyters 1975; Maurer et al., 1999; Rubinstein and Miller, 1999). This parallel suggests that there is a critical period of development during which exposure to gravity is essential to form the necessary connections that will allow sensation of gravity and properly directed responses.

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