

# The Effect of Spaceflight on the Ultrastructure of the Cerebellum

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## ABSTRACT

In weightlessness, astronauts and cosmonauts may experience postural illusions as well as motion sickness symptoms known as the "space adaptation syndrome." Upon return to Earth, they have irregularities in posture and balance. The adaptation to microgravity and subsequent re-adaptation to Earth occurs over several days. At the cellular level, a process called neuronal plasticity may mediate this adaptation. The term plasticity refers to the flexibility and modifiability in the architecture and functions of the nervous system. In fact, plastic changes are thought to underlie not just behavioral adaptation, but also the more generalized phenomena of learning and memory. The goal of this experiment was to identify some of the structural alterations that occur in the rat brain during the sensory and motor adaptation to microgravity.

One brain region where plasticity has been studied extensively is the cerebellar cortex—a structure thought to be critical for motor control, coordination, the timing of movements, and, most relevant to the present experiment, motor learning. Also, there are direct as well as indirect connections between projections from the gravity-sensing otolith organs and several subregions of the cerebellum. We tested the hypothesis that alterations in the ultrastructural (the structure within the cell) architecture of rat cerebellar cortex occur during the early period of adaptation to microgravity, as the cerebellum adapts to the absence of the usual gravitational inputs. The results show ultrastructural evidence for neuronal plasticity in the central nervous system of adult rats after 24 hours of spaceflight.

Qualitative studies conducted on tissue from the cerebellar cortex (specifically, the nodulus of the cerebellum) indicate that ultrastructural signs of plasticity are present in the cerebellar zones that receive input from the gravity-sensing organs in the inner ear (the otoliths). These changes are not observed in this region in caged ground control animals. The specific changes include the formation of lamellar bodies, profoundly enlarged Purkinje cell mitochondria, the presence of inter-neuronal cellular protrusions in the molecular layer, and signs of degeneration in the distal dendrites of the Purkinje cells. Since these morphologic signs are not apparent in the control animals, they are not likely to be due to caging or tissue processing effects. The particular nature of the structural alterations in the nodulus, most notably the formation of lamellar bodies and the presence of degeneration, further suggests that excitotoxicity (damaging overstimulation of neurons) may play a role in the short-term neural response to spaceflight.

These findings suggest a structural basis for the neuronal and synaptic plasticity accompanying the central nervous system response to altered gravity and help identify the cellular bases underlying the vestibular abnormalities experienced by astronauts during periods of adaptation and re-adaptation to different gravitational forces. Also, since the short- and long-term changes in neural structure occurring during such periods of adaptation resemble the neuronal alterations that occur in some neurologic disorders such as stroke, these findings may offer guidance in the development of strategies for rehabilitation and treatment of such disorders.

## INTRODUCTION

Upon entering weightlessness, astronauts and cosmonauts can experience postural illusions and symptoms of motion sickness (a constellation of symptoms referred to as the "space adaptation syndrome"). After the flight, crewmembers may have irregularities in posture and balance, and motion sickness symptoms may return as they re-adapt to Earth's gravity. Behavioral adaptation to the microgravity environment (and re-adaptation to Earth) usually occurs within several days (Nicogossian, 1989).

At the cellular level, this behavioral adaptation is thought to be mediated by a process called neuronal plasticity. The term plasticity conveys the flexibility and modifiability in the architecture and functions of the nervous system. The nervous system can make new connections or use existing connections in new ways. Plastic changes in the brain are thought to underlie not just behavioral adaptation, but also the more general phenomena of learning and memory. The goal of this Neurolab experiment was to identify the structural alterations that occur in the rat brain during sensory and motor adaptation to microgravity.

One brain region in which plasticity has been studied extensively is the cerebellar cortex—a structure thought to be critical for motor control, coordination, the timing of movements, and, most relevant to the present experiment, motor learning. The anatomical and functional organization of the cerebellar cortex is well documented (Ito, 1984). The cerebellar cortex has three layers (molecular, ganglion/Purkinje, granular) and five main cell types (basket, stellate, Purkinje, granule, Golgi). Interneuronal basket and stellate cells are found in the outermost molecular layer; Purkinje cells are the only neuronal cell bodies of the ganglion or Purkinje cell layer; and granule and Golgi cells are present in the cell-dense granular layer.

The cerebellar cortex receives two main functional inputs. Climbing fibers take origin from cells in the inferior olivary complex and terminate directly on Purkinje cells. Mossy fibers, in contrast, take origin from a wide variety of brain structures and innervate cerebellar granule cells. The granule cells, in turn, give rise to long axons called parallel fibers. These axons course transversely through the molecular layer, perpendicular to the Purkinje cell dendritic trees, and provide innervation to those dendrites. The ultrastructure features of cerebellar cells have been thoroughly characterized. The Purkinje cell cytoplasm has a whorl-like arrangement of organelles surrounding the nucleus. Both granular and agranular endoplasmic reticula form a loose filigree throughout the perikaryon, including an extensive system of cisterns called hypolemmal cisternae that are located just inside the plasma membrane. The Purkinje cell mitochondria are highly variable in shape and pervasive in the cytoplasm, except in the dendritic thorns. Microtubules invade the entire dendritic tree, again except in the thorns, which have their own cytoplasmic features.

Classically, the cerebellum is subdivided into anterior, posterior, and flocculo-nodular lobes; each is composed of transversely oriented lobules. The flocculo-nodular lobe, or vestibular cerebellum, is comprised of two lobules, the midline

or vermal nodulus and the laterally placed hemispheric flocculus. Gravity information sensed by receptors in the otolith organs of the ear is conveyed to particular zones in the nodulus. These zones were the focus for the present report. In addition, the semilunar lobule (hemisphere lobule VIII) was selected as an internal tissue control, since this region does not participate directly in vestibular, postural, or balance functions.

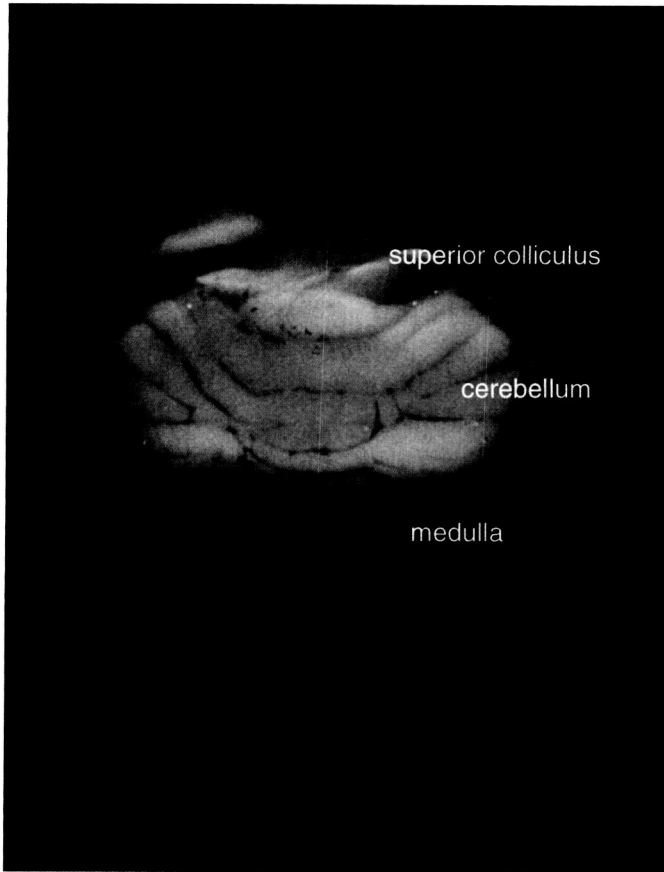
We tested the hypothesis that alterations in the ultrastructural anatomy of rat cerebellar cortex occur during the early period of adaptation to the microgravity environment. We chose to test the question of spaceflight-induced neuronal plasticity in the cerebellar cortex for three reasons: (1) various forms and manifestations of neuronal and synaptic plasticity have been clearly demonstrated in the adult rat cerebellar cortex; (2) the cerebellar cortex has been widely implicated in motor learning; and (3) there are direct as well as indirect projections from the gravity-sensing otolith organs to several regions of the cerebellar cortex.

## METHODS

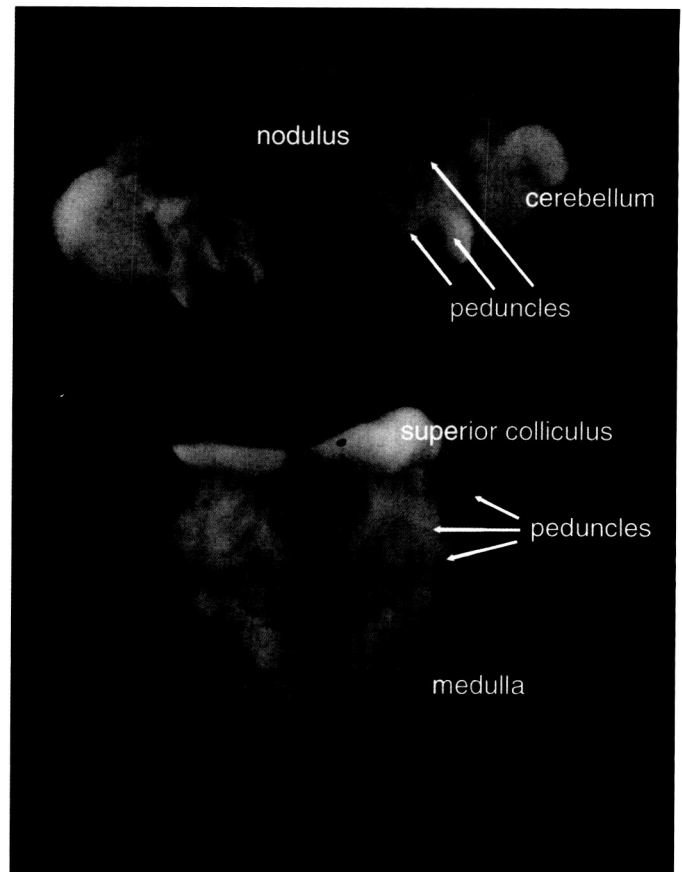
Hindbrain tissue was obtained from rats flown on the Neurolab mission (STS-90). Tissue for the present report was obtained from four adult male Fisher 344 rats on orbit during flight day 2 (FD2), 24 hours after launch, and from equal numbers of vivarium control rats and control rats housed in flight-type cages maintained on Earth (cage controls). These control rats were studied 48 and 96 hours after the flight dissections, respectively. The flight tissues could not be preserved on the Space Shuttle using vascular perfusion, which is the preferred method for this type of research. Instead, ground-based studies were conducted to establish the optimal conditions for immersion fixation of the cerebellar tissue. A technical report included in this volume provides detailed information regarding the development and verification of this fixation paradigm (see technical report by Holstein et al., in this publication). All experiments were performed in accordance with the *Principles of Laboratory Animal Care Guide* (National Institutes of Health Pub. 85-23) and were Animal Care and Use Committee approved by both NASA and the Mount Sinai School of Medicine.

Hindbrains were immersion-fixed for 45 minutes in 4% paraformaldehyde/0.1% glutaraldehyde in 0.1M phosphate buffer (pH 7.3), and were then transferred to a 4% paraformaldehyde solution in 0.1M phosphate buffer for 18 days at 4°C. After this fixation period, each cerebellum was photographed (Figure 1), dissected away from the ventral portion of the brain stem, and then re-photographed (Figure 2). All of the tissue collection and processing protocols were identical for the flight and control specimens.

After the brain stem was dissected away, the paraflocculi were separated from the cerebellum and a small notch was carved into the dorsal aspect of each paraflocculus to aid in orientating the structure in the future. Each cerebellum was then sectioned in the midline, and both halves were mounted



**Figure 1.** The brain stem of a flight rat after 18 days of immersion fixation. The hindbrain is resting in a petri dish and immersed in buffer just prior to separation of the cerebellum. At the top, the superior colliculus connects to higher brain structures, and the medulla oblongata at the bottom connects to the spinal cord.



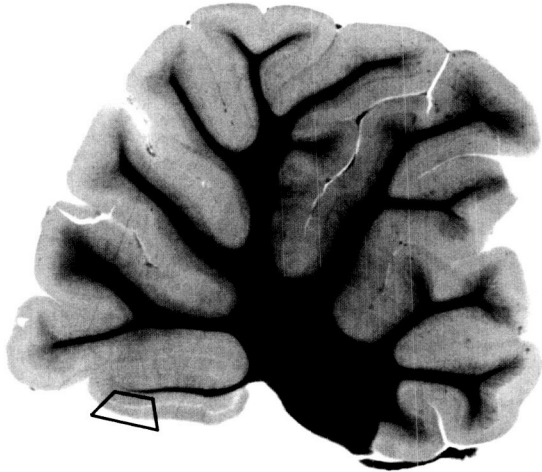
**Figure 2.** The same brain stem seen in Figure 1, immediately following the sectioning of the three pairs of cerebellar peduncles that form the attachment between the cerebellum and the brain stem. The nerve traffic from the gravity-sensing organs travels through these peduncles to the cerebellum. The cerebellum has been rotated forward, exposing the nodulus and the cut fibers of the peduncles.

together on a glass stage of a vibrating microtome. These specimens were then cut into 100- $\mu\text{m}$  sections. The sections were collected serially and processed for electron microscopy by osmication (1%  $\text{OsO}_4$  in deionized water for 30 minutes), dehydration in a graded series of methanol solutions, en bloc staining with uranyl acetate at the 70% methanol stage, infiltration with Epon-Araldite resin, and embedment in resin as tissue wafers between plastic coverslips.

The ultrastructure of the otolith-recipient zones of the cerebellar nodulus was analyzed in tissue from the FD2 flight and cage-control ground-based rats. To obtain the appropriate tissue specimens for this portion of the study, the entire series of wafers from each FD2 rat was examined to identify those sections containing the nodulus. All such wafers were photographed and then traced using a Trisimplex inverted projector. These tracings were used to reconstruct the nodulus and to determine the tissue wafer containing the middle section for each subject. Using this calculated midline as the zero

point, the wafers from two zones—1300–1400  $\mu\text{m}$  and 1600–1700  $\mu\text{m}$  bilaterally from that midline—were identified for each rat. These zones were selected because they receive indirect gravity-related otolith input via the inferior olivary complex (Voogd, 1996) and, therefore, were most likely to be affected by exposure to an altered gravitational environment. These tracings were also used to obtain estimates of the relative volume (section thickness was assumed to be 100  $\mu\text{m}$ ) of the nodulus in flight and control rats, as well as the relative volume of the molecular layer of the nodulus as a partial volume fraction (Holstein, 1999).

Specimens from the otolith-recipient zones of the nodulus were dissected from these wafers and mounted on blank resin blocks. Trapezoidal blockfaces were carved from the wafers (Figure 3), thin-sectioned (70-nm thick) by ultramicrotome onto Formvar-coated copper slot grids, and examined using a Hitachi 7500 transmission electron microscope. No post-microtomy staining was performed.



**Figure 3.** A 100-µm thick Vibratome section embedded in plastic resin and sandwiched between plastic coverslips. The section is 1300–1400 µm lateral to the midline, and it was obtained from the cerebellum of a flight rat. The trapezoid indicates the region thin-sectioned for electron microscopic observations of the nodulus from this and all other flight and control animals.

## RESULTS

Qualitative ultrastructural comparisons were conducted using tissue from the zones of the cerebellar nodulus that receive gravity-related inputs from the otoliths. Both the FD2 flight and ground-based, cage-control rats were studied. In addition, sections of the semilunar lobule (hemispheric lobule VIII) from the same FD2 flight rats were examined because this tissue provided an internal tissue control. This lobule does not receive *direct* vestibular or other inputs that could be affected by microgravity exposure. However, this internal cerebellar control tissue from the flight rats was exposed to the same launch- and Space Shuttle-related stimuli (including noise, vibration, and radiation) that the otolith-recipient cerebellar regions experienced.

The comparisons completed to date suggest that several architectural alterations occur both in the Purkinje cell cytoplasm and in the molecular layer of the nodulus of rats exposed to 24 hours of spaceflight. These structural alterations have not been apparent in the nodulus from the FD2 cage-control animals (Figure 4), and have not been observed thus far in the semilunar lobule of the FD2 flight rats.

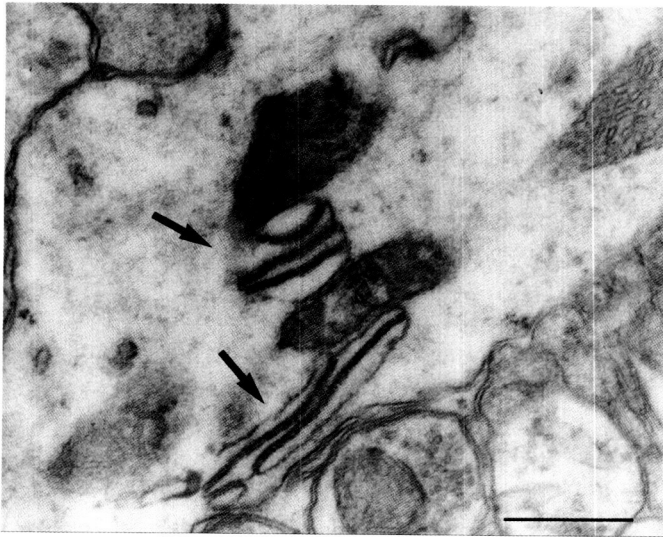
The most dramatic alteration observed in the nodular tissue from flight animals was seen in the organelles of the Purkinje cells. These cells normally contain a system of cisterns of smooth endoplasmic reticulum. In Purkinje cells of the otolith-recipient zones of the nodulus in the FD2 flight rats, such cisterns were substantially enlarged and more complex. The increased complexity of the cisterns resulted in the formation of long, stacked lamellar bodies. These were observable throughout entire Purkinje cells, including their somata, dendrites,



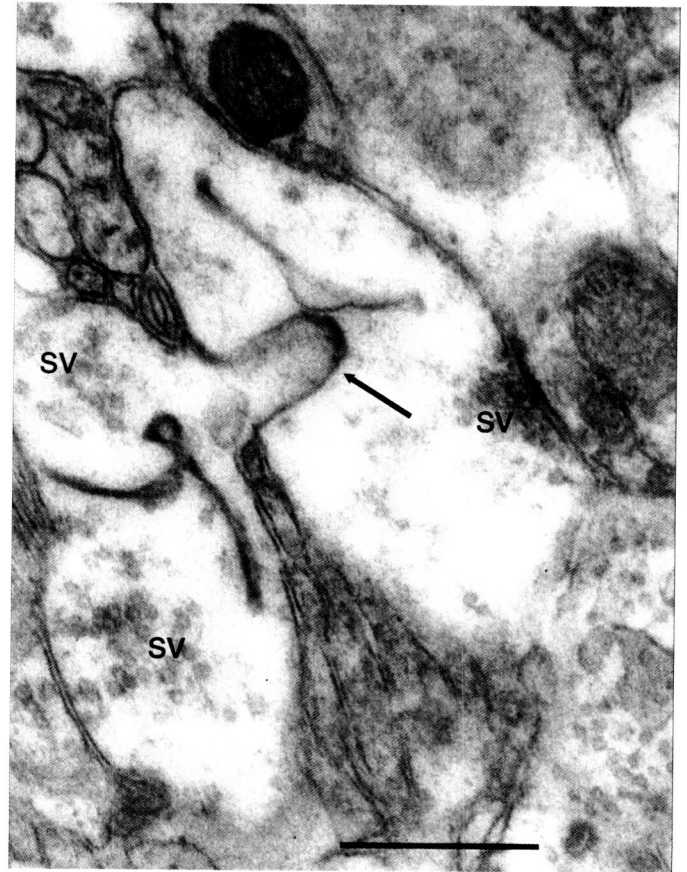
**Figure 4.** An electron micrograph of the molecular layer of the nodulus from a cage-control rat that was treated identically to the flight rats but was not exposed to spaceflight. The arrows point to examples of subsurface cisterns in the Purkinje cell dendrite. No lamellar bodies are present. Scale bar: 1 µm.

spines, thorns, and axon terminals (Figure 5). In general, these organelles were closely associated with adjacent mitochondria and the nearby plasma membrane.

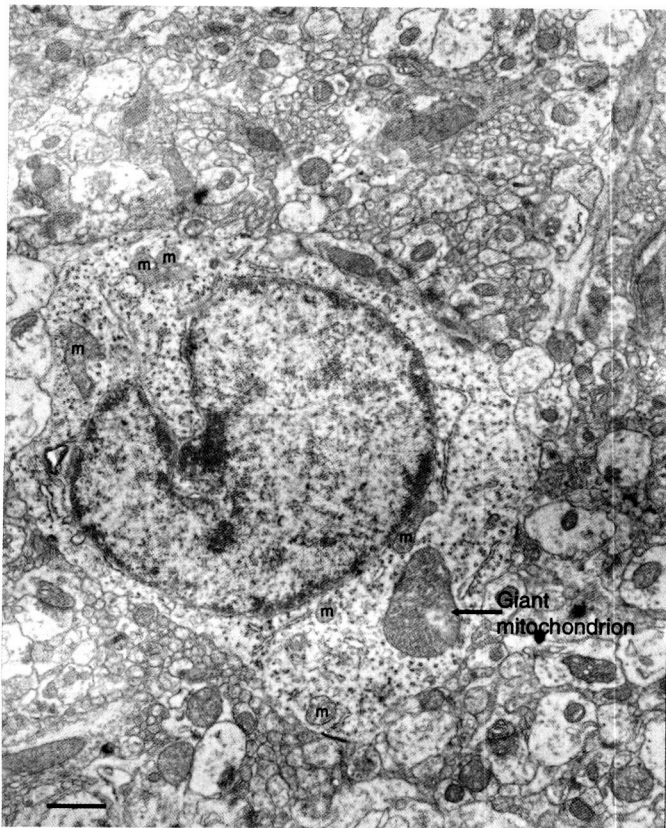
In addition, occasional enormous mitochondria, some more than 1 µm in cross-sectional diameter, were present in the Purkinje cells of flight rats (Figure 6). Alterations in the molecular layer of the nodulus included frequent and sometimes large protrusions of neuronal elements into neighboring structures (Figure 7). Lastly, ultrastructural signs of electron-dense degeneration were apparent in the dendrites of Purkinje cells from the nodulus of the flight rats. Such structures contained increased numbers of lysosomes and degenerated mitochondria, but they maintained apparently healthy synaptic contacts with parallel fiber terminals that, themselves, appeared normal (Figure 8).



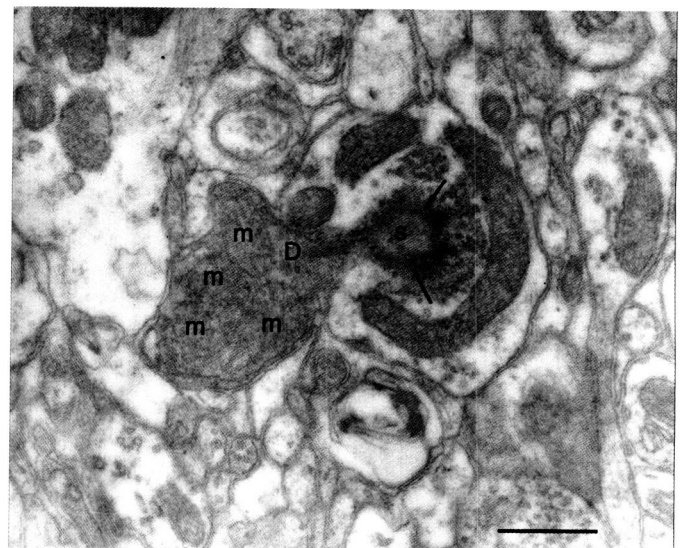
**Figure 5.** An electron micrograph through a Purkinje cell dendrite from an otolith-recipient zone of the nodulus from an FD2 flight rat. The arrows indicate long, stacked lamellar bodies that were observed throughout entire Purkinje cells, including the somata, dendrites, and thorns of flight rats. The lamellar bodies are closely associated with the adjacent mitochondria and nearby plasma membrane. Scale bar: 0.5  $\mu$ m.



**Figure 7.** Electron micrograph of the molecular layer of the nodulus from an FD2 flight rat. The arrow indicates an example of a cellular protrusion between adjacent structures. Both structures are axon terminals, as indicated by the presence of clusters of synaptic vesicles (sv). Scale bar: 0.5  $\mu$ m.



**Figure 6.** Electron micrograph through a Purkinje cell body from an otolith-recipient zone of the nodulus from an FD2 flight rat. An example of an extremely large mitochondrion is indicated, and normal mitochondria in the same cell body are labeled (m) for comparison. Scale bar: 1  $\mu$ m.



**Figure 8.** Electron-dense degeneration is present in a distal Purkinje cell dendrite (D) and spine (s) in the molecular layer of the nodulus from an FD2 flight rat. Degenerated mitochondria (m) are also present in the dendrite. Black arrows indicate synaptic contacts with the degenerated cell. Scale bar: 0.5  $\mu$ m.

## DISCUSSION

Qualitative studies conducted after 24 hours of spaceflight on the cerebellar nodulus show ultrastructural signs of plasticity in the otolith-recipient zones of the nodulus, which are not observed in this region in cage-matched ground-control rats. Such alterations include the formation of lamellar bodies and profoundly enlarged Purkinje cell mitochondria, the presence of inter-neuronal cellular protrusions in the molecular layer, and signs of degeneration in the distal dendrites of the Purkinje cells. Since these morphologic signs are not apparent in the control rats, they are not likely to be due to caging or tissue processing effects.

In the flight tissue, the subsurface cisterns of smooth endoplasmic reticulum, which are common in the cytoplasm of normal Purkinje cells, develop into complex lamellar bodies. These organelles are closely associated with adjacent mitochondria and the nearby plasmalemma. In the initial descriptions of these organelles, the lamellar bodies were shown to consist of stacks of several (between four and 12) flattened, parallel, regularly spaced (300–400 Å) tubular cisterns alternating with plates of dense granular material. The stacks were often observed to be subjacent to the plasma membrane in close proximity to the smooth endoplasmic reticulum, and closely apposed to the outer membranes of mitochondria. They were sometimes, but not always, associated with the rough endoplasmic reticulum as well. More recently, some investigators have suggested that these organelles may be artifacts of fixation or lack of oxygen before death. However, most studies to date view the cisternal stacks as distinct neuronal structures (Yamamoto, 1991). In fact, lamellar bodies have been observed in Purkinje cells using a variety of fixation protocols and embedding and sectioning methods (Yamamoto, 1991).

It has been suggested that markedly increased lamellar body numbers or complexity result from experimental manipulations and/or pathological conditions (Bestetti, 1980; Hansson, 1981). For example, lamellar body formation in Purkinje cell dendrites can be induced by a brief (five-minute) period without oxygen, or by the administration of L-glutamate into the brain. Although L-glutamate is normally found in the brain and participates in the transmission impulses between some neurons, too much glutamate can overstimulate neurons and kill them. This process is called excitotoxicity. In one study, the enhanced formation of cisternal stacks was inhibited by co-administration of glutamate and a glutamate receptor antagonist. Since the antagonist prevents the formation of the stacks, this suggests that glutamate excitotoxicity may play a role in cisternal stack formation. Finding these stacks (lamellae) in the brain (as occurred in our study) may be an indication of excitotoxicity. It is also possible that the entire network of smooth endoplasmic reticulum in Purkinje cells has the relatively unique property to form cisternal aggregates. The number of cisternal stacks present *in situ* under a given set of experimental conditions may reflect a dynamic equilibrium between the tendencies for aggregation and dispersion of the aggregates.

Regarding the other major findings of the present study, the presence of gigantic mitochondria in Purkinje cells has been suggested by other investigators to serve as an ultrastructural sign of early cell degeneration reflecting an underlying process of synaptic remodeling. Moreover, the presence of inter-neuronal cellular protrusions suggests enhanced membrane fluidity, also possibly reflecting an underlying process of neuronal plasticity. Lastly, electron-dense degeneration was apparent in the Purkinje cell dendrites of the flight rats. Such structures contained increased numbers of lysosomes and degenerated mitochondria, but maintained apparently healthy synaptic contacts with normal-appearing axon terminals.

To date, there are few studies on the impact of exposure to altered gravitational environments on the central nervous system structure. In the granular layer of the nodulus of rats raised in a two-G environment for 60 days, 80% of the glomeruli have been reported to show modifications in synaptic morphology. These alterations include changes in the density of pre- and postsynaptic membranes, increased thickness of the postsynaptic density, enlargement of the synaptic cleft, increased packing density of synaptic vesicles, enlarged mitochondria, and an increase in the number of microtubules (Krasnov, 1986; Krasnov, 1991). Two days after such animals return to a one-G environment, the ultrastructure of the nodulus is reported to resemble that of control rats. The synaptic vesicle packing density is diminished, and the number of microtubules is decreased, suggesting that such gravity-induced effects are reversible (Krasnov, 1991). Complementary morphologic changes have been reported to occur in the nodulus of rats following spaceflight (Krasnov, 1986; Krasnov, 1990). In these rats, ultrastructural changes in Purkinje cell dendritic synaptology have been reported.

In the peripheral vestibular system, light microscopic observations of the rat sacculus have been reported following development in 2.3-G and 4.15-G (Lim, 1974). In 60-day-old rats raised pre- and postnatally in a two-G environment induced by continuous centrifugation, the large-sized otoconia (stones in the otoliths) present in normal control rats do not develop. Type I receptor cells exhibit abnormal ultrastructural features, including increased chromatin, increased perinuclear space, and increased intercellular space (Krasnov, 1987; Krasnov, 1991). An increase in otoconial size has been reported in the utricle of rats after seven days of exposure to microgravity (Ross, 1985), suggesting that the otoconia are capable of plastic changes in response to altered gravity conditions. Ultrastructural studies have been conducted on the utricle and sacculus of adult rats after 30 days of exposure to two-G and on the utricular hair cells of adult rats flown on Space Shuttle missions. In these studies, Type II hair cells, and to a lesser extent Type I hair cells, showed evidence of neuronal plasticity. Increases in the mean number of synaptic ribbons were observed in such cells from rats studied on FD13 of a 14-day mission as well as in those sacrificed immediately following Space Shuttle landing, and others sacrificed after a mission-length, one-G re-adaptation period. Taken together, these data support the notion that the adult utricle retains the potential for plastic morphologic reorganization, and that the Type II hair

cells are more directly involved in these adaptations to altered gravity conditions (Ross, 1997).

While there has been evidence for plasticity in the peripheral nervous system, the present results provide ultrastructural evidence for neuronal plasticity in the central nervous system of adult rats after 24 hours of exposure to spaceflight. The particular nature of the structural alterations in the nodulus, including the formation of lamellar bodies and the presence of degeneration, further suggests that excitotoxicity may play a role in the short-term neural response to spaceflight. The specificity of the alterations for particular organelles of the Purkinje cell, the overall acceptability of the ultrastructural tissue preservation, and the paucity of similar observations in control tissues all suggest the soundness of the observations. Nevertheless, it remains critical to demonstrate the extent of these morphological alterations in perfusion-fixed tissue as well.

Our findings suggest a structural basis for the neuronal and synaptic plasticity accompanying the central nervous system response to altered gravity. Specifically, the studies of neuronal degeneration provide new insight into the immediate and short-term cellular responses to varying gravitational fields. Since the short- and long-term changes in neural structure occurring during such periods of adaptation resemble the neuronal alterations that occur in some neurologic disorders such as stroke, these findings may offer guidance in the developing of strategies for rehabilitation and treatment of such disorders. Lastly, since habituation of the time constant of the vestibulo-ocular reflex critically involves the cerebellar nodulus, it seems clear that the vestibular portion of the cerebellar cortex is critical for mediating plastic changes compatible with sensory-motor integration. In that light, the present results help to identify the cellular bases underlying the vestibular abnormalities experienced by astronauts during periods of adaptation and re-adaptation to different gravitational forces.

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