Spaceflight Induces Changes in the Synaptic Circuitry of the Postnatal Developing Neocortex

The establishment of the adult pattern of neocortical circuitry depends on various intrinsic and extrinsic factors, whose modification during development can lead to alterations in cortical organization and function. We report the effect of 16 days of spaceflight [Neurolab mission; from postnatal day 14 (P14) to P30] on the neocortical representation of the hindlimb synaptic circuitry in rats. As a result, we show, for the first time, that development in microgravity leads to changes in the number and morphology of cortical synapses in a laminar-specific manner. In the layers II/III and Va, the synaptic cross-sectional lengths were significantly larger in flight animals than in ground control animals. Flight animals also showed significantly lower synaptic densities in layers II/III, IV and Va. The greatest difference was found in layer I/III, where there was a difference of 344 million synapses per mm² (15.6% decrease). Furthermore, after a 4 month period of re-adaptation to terrestrial gravity, some changes disappeared (i.e. the alterations were transient), while conversely, some new differences also appeared. For example, significant differences in synaptic density in layers II/III and Va after re-adaptation were no longer observed, whereas in layer IV the density of synapses increased notably in flight animals (a difference of 185 million synapses per mm² or 13.4%). In addition, all the changes observed only affected asymmetrical synapses, which are known to be excitatory. These results indicate that terrestrial gravity is a necessary environmental parameter for normal cortical synaptogenesis. These findings are fundamental in planning future long-term spaceflights.

Introduction
It has been well established that environmental manipulation may lead to anatomical, chemical and physiological changes in the cerebral cortex subserving sensory and motor function (Van der Loos and Woolsey, 1973; Hubel et al., 1977; Kaas et al., 1983; Donoghue, 1995; Buonomano and Merzenich, 1998; Jones, 2000; Sur and Leamey, 2001). Since the nervous system has evolved over millions of years in the presence of a constant gravitational field, this environmental parameter must influence significantly both posture and motricity. Ground and spaceflight-based studies in adult animals support this hypothesis (Krasnov, 1994; Clarac et al., 1998). Given the changes observed in adult animals and the increased plasticity of neonatal animals (Berardi et al., 2000), one would expect that neonates would be particularly sensitive to the effects of microgravity during spaceflight such as hindlimb unloading.

The results from several studies suggest that a terrestrial gravitational field is necessary for the normal, early postnatal development of the motor system. For example, by simulating weightlessness in rats using a tail-suspension model (a system that allows the animals to move about the cage, extend and flex their hindlimbs), a critical period of development from postnatal day 13 (P13) to P31 has been identified (Walton et al., 1992). During this neonatal period, suspended animals are sensitive to hindlimb unloading and develop marked abnormalities in locomotion that persist into adulthood, particularly in the hindlimbs (Walton et al., 1992). Studies on neonatal hindlimb muscle have shown that the soleus, a prototypical antigravity hindlimb muscle, becomes atrophied after tail suspension from P8 to P24 (Huckstorf et al., 2000), and recent studies have reported changes in the expression of myosin isoforms in neonatal rats after spaceflight (Adams et al., 2000; Ikemoto et al., 2001). At the cortical level, spaceflight studies have revealed increases in the overall density of dendritic spines in the sensorimotor cortex of adult rats after 7 and 14 days in microgravity (Belichenko, 1988; Belichenko and Krasnov, 1991). However, these studies did not examine the tissue at ultrastructural level to determine quantitative changes in synaptic size or connectivity. In addition, the transitory or permanent nature of these changes has not been assessed. Electrophysiological and electron microscope studies have shown that rodent neocortical circuits mature gradually between the first and sixth week postnatal (Blue and Parnavelas, 1983; Bähr and Wolff, 1985; Luhmann and Prince, 1991; Agmon and O’Dowd, 1992; Agmon et al., 1993, 1996; Micheva and Beaulieu, 1996; White et al., 1997; DeFelipe et al., 1997; Wells et al., 2000). Thus, the changes in theafferent information that reaches the sensorimotor cortex induced by selected muscle atrophy and the altered use of hindlimb muscles in microgravity may affect cortical synaptic organization. Therefore, we examined the maturation of synaptic circuits of the neocortex of neonatal rats that developed for 16 days in a low Earth orbit (Neurolab mission, STS-90).

[Neurolab was a NASA research mission dedicated mainly to study how the nervous system responds in microgravity, a fundamental question for future long-duration space flights. Neurolab was born when the US President declared the 1990s the Decade of the Brain, and NASA proposed the Neurolab mission as its contribution to this dictate. Other International Space Agencies also participated in the Neurolab mission. The seven-member crew were not only involved in various experiments with animals (rats, mice, fish, snails and crickets) aboard the Space Shuttle Columbia, but they were also themselves the subjects of a number of sophisticated biomedical examinations. The Shuttle was launched on 17 April and landed on 4 May 1998 at Kennedy Space Center in Cape Canaveral (Florida, USA). The Shuttle reached an altitude of ∼320 km above the planet’s surface and traveled at a speed of ∼7.5 km/s. Since the shuttle orbited the Earth every 92 min, during the 16 day spaceflight there were 16 sunsets and 16 sunrises every 24 h. Therefore, a total of 256 complete orbits around the Earth were undertaken. Crew members were Scott D. Altman, Jay C. Buckey, Richard M. Linnehan, Kathryn P. Hire, James A. Pawelczyk, Richard A. Searfoss and Dafydd Rhys Williams.]  

We focused our ultrastructural analysis on the hindlimb region of the cortex as extensive overlapping of the motor and sensory maps occurs in this region (Hall and Lindholm, 1974;
Jones and Porter, 1980; Donoghue, 1995). We discuss the relationship between the effects observed and the motor strategies adopted by neonates in microgravity as well as the persistence of microgravity-induced alterations in the motor system after returning to terrestrial gravity.

**Materials and Methods**

A total of 22 female Sprague–Dawley rats were used. Eleven were flight (FLT) and 11 were asynchronous ground control (AGC) animals. The AGC animals were housed in cages with dimensions identical to the flight cages in the Life Science Support Facility at the Kennedy Space Center. Their eyes were open and they were able to walk when loaded into either the shuttle (FLT) or the flight-like cages (AGC) on P13. Because of a 24 h launch delay, the animals were P14 at launch (flight-day 1, FD1). The animals were in microgravity for 16 days and were P30 on the day of landing (recovery day 0, R+0). FLT and AGC animals were videotaped on FD 6, FD 11 and R+0, either inside a general-purpose work station in the shuttle laboratory (FLT) or in the Life Science Support Facility (AGC). An ‘animal walking apparatus’ was constructed for these experiments. This consisted of a rotating rectangular platform with a foam surface on one side and a wire grid on the other. There were two metal bars above, and one metal bar below the platform. The animal’s movements were recorded with two video cameras at right angles to each other (TEAC, Sekai, 60 fps). Five FLT and five AGC animals were killed 4–5 h after landing. The remaining animals were utilized for behavioral studies and killed after 4 months after the recovery day having re-adapted to terrestrial gravity (FLT+R+135, P165; AGC+R+129, P159).

**Ultrastructural Analysis**

Since fresh tissue from the different regions of the nervous system or from other organs from these rats was to be used in other studies, animals were anesthetized with Nembutal and decapitated. The brains were removed immediately and three blocks of tissue per hemisphere were obtained from each rat. These blocks were fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4, for 36 h at 4°C. Serial 50 µm Vibratome sections were cut from the blocks containing the hindlimb representation of the somatosensory cortex (between Bregma –0.26 mm and Bregma –2.12 mm; Paxinos and Watson (Paxinos and Watson, 1997)). Some sections were stained with thionin to identify the hindlimb cortex through the presence of a prominent granular cell layer (layer IV) and of large pyramidal cells in layer V, similar in size to those found in the adjacent agranular (motor) cortex (Jones and Porter, 1980) (Fig. 1A, B). Cortical layers were identified as layers I, II/III, IV, Vα, Vβ and VI (Zilles and Wree, 1995). Serial adjacent sections to those Nissl-stained sections including the hindlimb cortex were processed for electron microscopy, as follows. Sections were post-fixed in 2% glutaraldehyde in PB for 1 h and in 1% osmium tetroxide, dehydrated and embedded in Araldite. These plastic-embedded sections were sectioned serially into semithin (2 µm thick) sections (Fig. 1C, D) with a Reichert ultramicrotome. The semithin sections were stained with 1% toluidine blue in 1% borax and examined by light microscopy to identify the cortical layers. Semithin sections containing the hindlimb cortex were photographed and then resectioned into serial ultrathin sections with a silver–gray interference color (DeFelipe and Fairén, 1993). The ultrathin sections were collected on Formvar-coated single-slot grids, stained with uranyl acetate and lead citrate, and examined in a Jel-1200 EX electron microscope.

**Quantification and Statistical Analyses**

Synaptic density per unit area \( (N_s) \) was estimated from 10 electron microscope samples of neuropil from each layer (layers I, II/III, IV, Vα, Vβ and VI) from each animal. These samples were non-overlapping electron micrographs taken at an initial magnification of 10 000 and printed at a final magnification of 50 000 (DeFelipe et al., 1999). All synaptic profiles were counted in each print within an unbiased counting frame (Gundersen, 1977) which represented 35 µm² of tissue. Synaptic profiles that touched the exclusion lines were not counted. These counts were used to determine the numerical density of synapses per unit volume of neuropil, using the formula \( N_s = N_s/d \) (Weibel, 1979), where \( N_s \) is the number of synaptic profiles per unit area and \( d \) the average cross-sectional length of synaptic junctions (see DeFelipe et al. (DeFelipe et al., 1999)). The cross-sectional lengths of synaptic junctions (length of the paired membrane densities at each junction) of all asymmetrical and symmetrical synaptic profiles, as well as the lengths of the postsynaptic densities in the case of oblique and en face synaptic profiles, were measured in the prints using a magnetic tablet (SummaSketch III) and the Scion Image image analysis program (Scion corporation, Frederick, MD). Statistical comparisons of the means were carried out with an unpaired Student’s t-test. All these studies were performed with the aid of the SPSS statistical package (SPSS Science, Chicago, IL).

**Results**

**General Considerations**

We have examined the effects of microgravity on the maturation of synaptic circuits of the neocortex of neonatal rats that developed for 16 days in a low Earth orbit (Neurolab mission, STS-90). After landing, we first analyzed the body weight of the FLT and AGC animals and found no significant difference between the two groups of animals (mean ± SEM (97.9 ± 3.2 and 103.7 ± 3.2 g, respectively). These observations suggest that feeding during the spaceflight was normal and that no apparent nutritional problems might have affected our colony of animals.

**Light Microscopy and Ultrastructural Analysis**

We first analyzed Nissl-stained cortical sections of the hindlimb region of the cortex obtained from the animals using light microscopy. No differences in the cytoarchitectonic (Table 1) or cytological characteristics of neurons were detected between FLT and AGC animals (Fig. 1C, D).

In the absence of gross changes in cytoarchitecture, a more detailed examination of the cortex was carried out. Correlative light microscopy and electron microscopy (DeFelipe and Fairén, 1995) was used to measure synaptic cross-sectional lengths and to estimate the density of synapses in the neuropil of each cortical layer from the left hindlimb area of FLT and AGC animals (Fig. 2). A total of 19 164 synaptic profiles were examined (summarized in Table 2) and the synaptic profiles were classified into three types: asymmetrical, symmetrical and uncharacterized. In the first two types, the synaptic clefts could be visualized and synapses were identified on the basis of the morphology of the postsynaptic density. Essentially, asymmetrical synapses had a prominent postsynaptic density, while symmetrical synapses had a thin postsynaptic density (Gray, 1959; Colonnier, 1968, 1981; Peters, 1987; Peters et al., 1991; Peters and Palay, 1996). In the uncharacterized synapses, the synaptic clefts could not be visualized, due to the oblique plane of section.

The ultrastructure of both the neurons and neuropil in FLT animals was indistinguishable from AGC animals (Fig. 2A, B). Likewise, the proportions of asymmetrical (88%) and symmetrical (12%) synapses, as well as the numbers of perforated synapses (4–7%) [i.e. those with two or more separated postsynaptic densities; Peters and Kaiserman-Abramof (Peters and Kaiserman-Abramof, 1969)] were similar in FLT and AGC animals (Table 2).

**Changes in Synaptic Cross-sectional Lengths in FLT Rats**

To determine whether the synaptic cross-sectional lengths and the densities of synapses were affected by spaceflight, we compared these parameters between FLT and AGC animals on the day of landing (R+0) and after 4 months of re-adaptation to terrestrial gravity (R+129 or R+135). At R+0, the cross-sectional lengths of asymmetrical synapses of FLT rats were longer in all layers except layers I and VI (Fig. 3). The increase in length of asymmetrical synapses was significantly longer in layers II/III and
No significant differences were found in the lengths of symmetrical synapses between the two groups of animals. However, on R+0 the symmetrical synaptic profiles were shorter in FLT animals in all layers, except in layer IV where they were longer than in AGC animals.

After 4 months of re-adaptation, a change in the lengths of the asymmetrical synapses was detected. Asymmetrical synapses were shorter in FLT than in AGC rats in all layers (Fig. 3). This was most pronounced in layer I, where these synapses were significantly shorter in FLT animals. Symmetrical synapses were also smaller in the majority of layers in FLT animals, except for layers I and VI where they were longer. Thus, there was a differential growth of synaptic junctions between FLT and AGC animals that affected asymmetrical synapses in a different manner on R+0 and after a 4 month period of re-adaptation.

Changes in Synaptic Density in FLT Rats
A comparison of the synaptic density for all synapses between FLT and AGC animals on R+0 also revealed differences (Fig. 4). Lower synaptic densities were observed in the cortex of FLT animals except in layers I and VI, although these differences

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness of cortical layers (means ± SEM in µm) in the hindlimb area of AGC and FLT rats sacrificed on the day of landing (P30)</td>
</tr>
<tr>
<td>Cortical layers</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Layer I</td>
</tr>
<tr>
<td>Layer II/III</td>
</tr>
<tr>
<td>Layer IV</td>
</tr>
<tr>
<td>Layer Va</td>
</tr>
<tr>
<td>Layer Vb</td>
</tr>
<tr>
<td>Layer VI</td>
</tr>
<tr>
<td>Layers I–VI</td>
</tr>
</tbody>
</table>

Each value was calculated by measuring the thickness of cortical layers from four Nissl-stained sections per animal, using a video camera attached to the microscope to obtain images at a final magnification of 75. There were no significant differences between AGC and FLT rats.
were only statistically significant in layers II/III, IV and Va. The greatest difference was found in layer II/III, where 344 million fewer synapses per mm$^3$ were found, a decrease of 15.6%. After the period of re-adaptation, however, significant differences in the density of synapses were found in layers IV and Vb.

When asymmetrical and symmetrical synapses were analyzed separately, a significantly lower density was found for asymmetrical synapses in layer II/III of FLT rats (a difference of 161 million synapses per mm$^3$; 18.4% fewer synapses). No statistically significant changes were found in other layers or for symmetrical synapses. After re-adaptation, the difference in layer II/III was no longer significant, whereas in layer IV there was a

Figure 2. Electron micrographs illustrating the neuropil of layer II/III from AGC (A) and FLT (B) rats. Arrows indicate some synapses. Scale bar: 0.5 µm.
significantly higher density of asymmetrical synapses in FLT rats (a difference of 104 million synapses per mm$^3$, a 20% increase in density).

In summary, exposure to microgravity for 16 days produced laminar-specific changes in asymmetrical synapses but not in symmetric synapses. Both the length and density of asymmetric synapses in certain layers was initially affected and while some changes disappeared after 4 months of re-adaptation to terrestrial gravity, new alterations also appeared (late changes).

### Changes in Synaptic Cross-sectional Lengths and Synaptic Density from P30 to Adult in AGC and FLT Rats

From P30 to adult (∼5 months old), AGC animals showed significant increases in synaptic cross-sectional lengths in all cortical layers ($P < 0.001$; Fig. 5). These increases varied from a minimum of 12% in layer VI to a maximum of 22% in layer II/III. In FLT animals there were also significant increases ($P < 0.001$) in synaptic cross-sectional lengths during the recovery period from P30 to adult in all cortical layers except in layer IV, where the synaptic lengths did not change significantly (Fig. 5). The increase in length in FLT animals was less marked than in AGC animals, only varying between 6% (layer Va) and 13% (layer II/III).

Regarding the changes in synaptic density from P30 to adult, a decrease in synaptic density in all cortical layers of AGC animals was seen, which varied between 11% and 33% in layer I and layer II/III, respectively (Fig. 5). This reduction was significant in all layers (layer I, $P < 0.05$; rest of layers, $P < 0.001$). In FLT animals, a significant reduction in the density of synapses also occurred. It ranged from a minimum of 12% in layer IV to 33% in layer VI (layers I and IV, $P < 0.05$; layer II/III, $P < 0.005$; Va, Vb and VI, $P < 0.001$) (Fig. 5). The most marked differences between AGC and FLT rats took place in layer IV (32% decrease in AGC animals against only 12% in FLT rats) and layer II/III (33% decrease in the AGC animals and 18% in the FLT group).

Thus, during normal late development and maturation of...
cortex, there were significant increases in synaptic cross-sectional lengths and reductions in synaptic densities in all layers of AGC animals. The increase in synaptic cross-sectional length, however, was less marked in all layers after spaceflight, and especially in layer IV, where synaptic length did not change at all. The decrease in synaptic density was also less accentuated, especially in layers IV and II/III.

Differences in Locomotion in FLT and AGC Animals
Since changes in cortical circuitry were most probably related to use-dependent changes in locomotion and hindlimb posture in microgravity, we analyzed the videotapes taken on flight day 6 (FD6) and FD11. On both occasions, the animals used their forelimbs, but not their hindlimbs for propulsion. Sometimes the hindlimbs floated behind the animals, but usually the animals grasped the surface with their toes to maintain a normal ‘horizontal’ posture. Another clear difference in locomotion between FLT and AGC animals was the lack of weight-bearing undertaken in microgravity. Thus, both the muscle action and afferent signals from the hindlimbs clearly differed in FLT and AGC animals. One example of the changes was the tactic used to reverse direction while progressing along a rod. FLT animals lifted both hindlimbs at the same time during 26% of the movements, while AGC animals never did this.

Discussion
Our results show that development in microgravity from P14 to P30 selectively affects the morphology and number of certain cortical synapses. At landing, significant differences were found between FLT animals and AGC animals in terms of the

Figure 4. Comparison of the means (± SEM) of synaptic densities between AGC and FLT animals on landing day and after a 4 month period of re-adaptation to terrestrial gravity. 'All synapses' includes asymmetrical, symmetrical and uncharacterized synapses.
Cross-sectional length of synaptic profiles

<table>
<thead>
<tr>
<th>Cortical layers</th>
<th>% of Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
</tr>
<tr>
<td>II-III</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Va</td>
<td></td>
</tr>
<tr>
<td>Vb</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td></td>
</tr>
</tbody>
</table>

Control (AGC layers)  Flight (FLT rats)

Figure 5. Graphics showing the percentage increases in cross-sectional length of synaptic profiles (A) and percentage decrease of synaptic densities (B) that occurs from P30 to adult (~5 months old) in AGC and FLT animals. See text for the statistical significance of these changes.

cross-sectional length and density of asymmetrical synapses. No statistically significant differences were found for symmetrical synapses. After a period of re-adaptation (~4 months), the changes in synaptic length or density were no longer significant. However, in FLT animals new significant changes were observed in asymmetrical but not in symmetrical synapses. Synapses were shorter in layer I and the density of these synapses was higher in layer IV and lower in Vb.

During normal late development and maturation of the cortex in AGC animals (from P30 to 5 months old), synaptic lengths increase and synaptic densities decrease in all layers. Whilst these two phenomena were maintained in the late development and maturation after spaceflight, they were less accentuated, across all cortical layers when considering synaptic cross-sectional lengths and in layers II/III, IV and Va for synaptic density. These results suggest that the development of synaptic circuits in certain layers of the hindlimb neocortex is sensitive to microgravity during the second and third postnatal weeks. The changes that we observed probably occurred during the 16 days of microgravity and not during the relatively short recovery period after landing. In addition, after 4 months of re-adaptation to terrestrial gravity some recovery could be seen, suggesting that some of the changes were transient. Moreover, new changes appeared after re-adaptation, indicating that spaceflight induces more long-term effects on development.

Synaptic size seems to play an important role in the functional properties of synapses (Lisman and Harris, 1993; Nusser et al., 1997; Mackenzie et al., 1999; Takumi et al., 1999; Kubota and Kawaguchi, 2000; Lüscher et al., 2000; Lee and Sheng, 2000). For example, larger synapses appear to express a greater number of postsynaptic receptors (Mackenzie et al., 1999). Thus, the changes in synapse density and size found after spaceflight may reflect functional alterations of the cortical circuits involved. As only asymmetrical synapses were significantly affected by the changes, and this type of synapse is known to be excitatory (Houser et al., 1984; White, 1989; Peters et al., 1991; DeFelipe and Fariñas, 1992; Peters and Palay, 1996; Conti and Weinberg, 1999), we conclude that microgravity affects excitatory synaptic circuits in a laminar-specific manner. However, our data do not permit us to determine which of the possible sources of these synapses were involved. For example, the higher synaptic density found in layer IV of FLT rats after re-adaptation could be due to an increase in the number of axon terminals originating from layer IV spiny stellate cells, from pyramidal cells, from thalamocortical afferents, or from all of these cells [being the major sources of excitatory asymmetrical synapses (White, 1989; DeFelipe and Fariñas, 1992; Jones, 2000; Amitai, 2001)]

The variation in cortical circuitry between FLT and AGC animals is likely to be related to the differences observed in the use of the hindlimbs between these two groups of animals during the 16 day period. Movement in FLT animals may be termed ‘modified quadrupedal’ locomotion (Sulica et al., 1999), in microgravity a unique gait predominates where the major propulsive power is provided by the forelimbs. In this modified quadrupedal locomotion the hindlimbs are sometimes placed anterior to the forelimbs, as in a gallop, to help propel the animal forward. In no case are the hindlimbs used to bear weight as in AGC animals. Thus, both the motor and sensory aspects of locomotion are changed in microgravity. In the presence of terrestrial gravity, extensor ‘antigravity’ muscles play a key role in posture and locomotion. This is not the case in microgravity, where flexor muscle activity predominates. The need for weight-bearing during development is well documented at the muscle level. Indeed, in age-matched animals on the same spaceflight, expression of the type I MHC gene in antigravity skeletal muscles was markedly reduced (Adams et al., 2000; Ikemoto et al., 2001). The impact of these structural changes on sensory–motor control of locomotion and postural reflexes after landing is currently being analyzed. The surface righting reaction does not occur in microgravity (Harding et al., 1999) and preliminary results indicate that surface righting does not mature post-flight. Rather, FLT animals right themselves using the same immature tactics seen at launch. In contrast, righting in AGC animals is typical of adult rats (Harding et al., 1999).

Synaptic plasticity in the cerebral cortex (neof ormation and/ or loss of synapses, and the ultrastructural changes in synapses) has been associated with a number of factors that include learning motor skills, complex environment exposure, and recovery from cortical injury (Cragg, 1974; Greenough and Chang, 1988; Calverley and Jones, 1990; Horner, 1995; Rakic et
Furthermore, modifications in the volume and distribution of extracellular fluids and plasma occur during spaceflight that in turn induce a number of hormonal, physiological and biochemical modifications that are also involved in the regulation of nervous system function. Some of these behavioral alterations may represent an anatomical substrate for some aspects of brain functions, the synaptic plasticity induced by microgravity may be of particular relevance for future prolonged human spaceflights.

**Notes**

We thank Ignacio Busturia for assistance with the statistical analyses. Supported by PNIE grants ESP97-1744-E and ESP98-1310-E, by NASA grants NAG2-951 and NINDS (NS33467), and a Basque country grant to J.I.A.

Address correspondence to Javier DeFelipe, Instituto Cajal (CSIC), Avenida Dr Arce, 37, 28002 Madrid, Spain. Email: defelipe@cajal.csic.es.

**References**


Constantine-Paton M, Cline HT (1998) LTP and activity-dependent synaptogenesis: the more alike they are, the more different they become. Curr Opin Neurobiol 8:139–148.

