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Potential Role of Oxidative Stress in Mediating the Effect of Altered Gravity on the Developing Rat Cerebellum

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Abstract

We have previously reported that perinatal exposure to hypergravity affects cerebellar structure and motor coordination in rat neonates. In the present study, we explored the hypothesis that exposure to hypergravity results in oxidative stress that may contribute to the decrease in Purkinje cell number and the impairment of motor coordination in hypergravity-exposed rat neonates. To test this hypothesis we compared cerebellar oxidative stress marker 3-nitrotyrosine (3-NT; an index of oxidative protein modification) and 8-hydroxy-2'-deoxyguanosine (8-OH-dG; an index of oxidative DNA damage) between stationary control (SC) and rat neonates exposed to 1.65 G (HG) on a 24-ft centrifuge from gestational day (G) 8 to P21. The levels of 3-NT and 8-OH-dG were determined by specific ELISAs. We also compared the Purkinje cell number (stereologically) and rotarod performance between the two groups. The levels of 3-NT were increased only in HG females on P6 and on P12 in the cerebellum, and only in HG females on P12 in the extracerebellar tissue. Limited cerebellar data suggests an increase in the levels of 8-OH-dG on P12 only in HG females. In extracerebellar tissue the increase in 8-OH-dG levels was observed in both HG males and HG females except on P6 when it was only observed in HG males. While preliminary, these data suggest that the effect of hypergravity on the developing brain is sex-dependent and may involve oxidative stress. Oxidative stress may, in turn, contribute to the decrease Purkinje cell number and impaired motor behavior observed in hypergravity-exposed rats.

Keywords

Hypergravity; Rat Cerebellum; Purkinje Cells; 3-NT; 8-OH-dG

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1. Introduction

The development of the CNS in organisms on earth is adapted to one unit of gravitational force (1G). Deviations from an environmental paradigm of 1G have been shown to affect CNS structure and function as extensively discussed in a recent review (Sajdel-Sulkowska, 2007). Most notably our recent results (Sajdel-Sulkowska et al., 2005) indicate that the exposure of developing rats to hypergravity results in decreased Purkinje cell number and impairment of motor behavior. Understanding the mechanisms involved in the brain's response to altered gravity at the cellular and molecular level presents a monumental challenge to the space biologist.

The concept of oxidative homeostasis necessary for maintaining normal physiological functions as well as disruption of this homeostasis is gaining increasing recognition. Oxidative stress occurs in the course of normal physiological processes. The damaging action of the reactive oxygen species (ROS) targets key cellular macromolecules, including lipids, carbohydrates, proteins, DNA and RNA. This action is kept in check by the body's natural antioxidant defense system that includes metal binding metalloproteins and other antioxidants. Under the influence of environmental factors, this defense system appears to be overburdened, leading to unchecked oxidative stress damage. Several lines of evidence support the concept that the cellular damage due to oxidative stress plays an important role in gene-environment interplay (Lee and Opanashuk, 2004; Jayaraj et al., 2006). An increase in reactive oxygen species (ROS) and oxidative stress can occur following exposure to various environmental toxins, mostly biological (Nolan et al., 2003) and chemical (Lee and Opanashuk, 2004), especially those that result in hypoxia.

Deviations from an environmental paradigm of 1G have been shown to affect CNS structure and function (Sajdel-Sulkowska, 2007). Can oxidative stress also mediate the effects of altered gravity on the developing CNS? Such a possibility is suggested by observations of changes in organ perfusion and decreased tissue oxygenation under altered gravity (Sanford et al., 1999).

Excessive levels of ROS have been shown to modify lipids proteins and DNA and alter gene expression (Floyd and Carney, 1992; Warner et al., 2004) and can thus affect brain development. The extent of oxidative stress damage to lipids can be assessed by isoprostanes, the damage to proteins by measuring 3-nitrotyrosine (3-NT) and the damage to DNA by measuring the levels of 8-hydroxy-2'-deoxyguanosine (8-OH-dG).

It has been observed that exposure to microgravity during space flights is associated with increased oxidative stress markers reflecting damage to lipids in both humans and rodents (Yang et al., 2003). Repeated exposure of adult rats to short-duration hypergravity (30 sec, 10G) also resulted in lipid peroxidation in the brain (Zhan et al., 1999).

Protein 3-NT is a specific marker of oxidative damage mediated by peroxynitrite, which is formed by the reaction of superoxide with nitric oxide (NO) on tyrosine residues in proteins (Beckman and Koppenol, 1996) or by reaction of nitrite and hydrogen peroxide (Sampson et al., 1998). Immunohistochemical data suggest that increased brain 3-NT occurs during aging (Poon et al., 2006). To date there are no reports of oxidative protein modification in rats exposed to either micro- or hypergravity.

DNA 8-OH-dG is the predominant marker of oxidative damage to DNA. Results of animal studies suggest that the levels of 8-OH-dG are increased in aging (Lopez-Diazguerrero et al., 2005). It has been observed that exposure to microgravity during space flights is associated with increased oxidative stress markers reflecting damage to DNA in both humans and rodents (Yang et al., 2003).

The objective of the present study was to examine the role of oxidative stress in mediating the effect of altered gravity on the developing rat brain and the possible contribution of oxidative stress to decreased cerebellar Purkinje cell number. The study reports for the first time the levels of oxidative stress markers 3-NT and 8-OH-dG in the brain and compares these markers between hypergravity exposed (1.65G) and stationary control (SC) rat neonates. The data presented here, while preliminary, are important to the understanding of the mechanisms involved in the effect of altered gravity on the developing brain.

2. Methods

2.1 Animals and treatment

Timed-pregnant Sprague-Dawley dams were shipped from Taconic Farms (Germantown, NY) to NASA Ames Research Center (ARC) at gestational day (G)2 (G1 defined as the first day after males and females are co-housed and on which the female has either a sperm plug or a sperm-positive vaginal smear). Dams were housed individually under standard vivarium conditions (12-hr light cycle, lights on at 6 AM and off at 6 PM, at 21–24°C). Standard laboratory rat chow and water were available ad libitum.

On G7, dams were weight-matched, deselected if not pregnant, and assigned to either a hypergravity (HG, n=18) group exposed to 1.65G or to stationary controls (SC, n=15). The dams were then transferred to the ARC 24-ft centrifuge rotunda. Both HG and SC dams were placed one per cage, and two or four cages were loaded per cab; all animals were exposed to the same environment. On G8, continuous centrifugation was initiated, with brief daily stops for checks of animal health and data collection both before and after the animals' birth. The centrifugation was carried out at 17.6 rpm and the cabs were placed 136.5 inches from the center, resulting in hypergravity of 1.65 G. The duration of the stops in all experimental groups was 30 minutes before births and 90 min following births.

Selected HG (n=6) and SC (n=5) litters were euthanized on P6, P12 and P21. Their brains were then rapidly removed and the cerebella were dissected from the brain stem, weighed, frozen in methyl butane (Nguon et al., 2004; Sulkowski et al., 2004), and stored at –80°C for further analysis.

2.2 Motor coordination

Motor coordination was measured on P21 by a rotarod test according to the procedure described earlier (Sajdel-Sulkowska et al., 2005). The pups, placed perpendicular to the horizontally rotating cylinder with their heads facing against the direction of rotation, had to move forward to maintain equilibrium and remain on the cylinder. Each neonate was subjected to one trial on a cylinder rotating at 36 rpm. The length of time the animal remained on the rotating cylinder was recorded (in seconds) and the performances were compared among pups in different groups.

2.3. Stereological analysis

On PD21 the neonates were anesthetized by inhalation of isoflurane, perfused with formalin and the cerebella were dissected and transferred to formalin for storage. Subsequently, the tissue was dehydrated with ethanol/ethyl ether and infused with celloidin (3 to 12%). Tissue was cut into 30-µm sections and stained with the cresyl violet stain. All stereological analysis was performed using a Zeiss Axiophot microscope with a Ludl motorized stage and Heidenhein z-axis microcator interfaced to a Dell Precision microcomputer and a Stereo Investigator (MicroBrightField, Colchester, VT). The total number of Purkinje cells was estimated using the optical fractionator probe, a 500 × 500 µm sampling grid and a 30 × 30 × 20 µm counting box; sections were sampled every 600 µm.

2.4. Nitrotyrosine analysis

Cerebellar 3-NT was measured according to the procedure previously described[49]. In brief, cerebellar homogenates were prepared in 2–3 volumes phosphate buffered saline (PBS) containing protease inhibitors (Complete, EDTA-free protease inhibitor cocktail, Boehringer Mannheim, Indianapolis, IN), 0.5% Nonident P40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS; Sigma, St Louis, MO). The homogenates were centrifuged at $10,000 \times g$ for 10 min. The supernatants were assayed for 3-NT content using Nitrotyrosine ELISA Kit (Oxis International Inc., Portland, OR) according to manufacturer's instructions.

2.5. Determination of 8-OH-dG

The DNA content of 8-OH-dG was measured according to the modification of the described procedures (Hellbock et al., 1998). In brief, the DNA was isolated from the tissue using a Qiagen DNA extraction kit based on proteinase K treatment. DNA samples were digested with nuclease P1 followed by treatment with alkaline phosphatase in Tris-HCl pH 8.0. The 8-OH-dG was estimated using a Bioxytech 8-OH-dG –EIA kit (Oxis International Inc., Portland, OR) according to manufacturer's instructions.

2.7. Statistical Analysis

Data was analyzed by ANOVA. All values are reported as Mean $\pm$ SEM. For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

3. Results

Rotarod test of motor behavior

Male and female SC and HG neonates were tested on P21 on a rotarod set at a constant speed of 36 rpm. Both male and female HG pups performed worse than SC pups on the rotarod test as evidenced by a considerably shorter period before falling from the drum (Table 1). ANOVA revealed the main effect of hypergravity exposure $F(5,317)=5.42$, $p<0.001$ and the effect was sex-dependent. In male pups, this latency was decreased by 67.7% ($p<0.050$) in HG as compared with SC pups. In female pups the latency was decreased by 60.4% ($p<0.050$).

Cerebellar Purkinje cell number

Total Purkinje cell number was determined stereologically in cerebellar tissue derived from a subset of P21 rat neonates tested on the rotarod. The number was decreased by 17.6% in HG males and by 25.3% in HG females (Table 1). ANOVA with hypergravity treatment and sex as between-subject factors revealed a main effect of hypergravity exposure $F(1,13)=9.26$, $p=0.0094$.

Cerebellar 3-NT levels

The level of 3-NT in the control rat cerebellum, reported here for the first time, was both age- and sex-dependent (Figure 1A). The highest level of 3-NT was observed on P6 in both the male and female cerebellum. On the other hand, the highest levels in the extracerebellar tissue were observed on P21 (Figure 1B). The data on cerebellar and extracerebellar 3-NT in HG and SC rat neonates is presented in Table 2. As reported by the authors earlier (Sajdel-Sulkowska and Lipinski, 2006), the level of cerebellar 3-NT was increased only in HG females; on P6 the increase was 230% and on P12 the increase was observed to be 1950%. ANOVA revealed a main effect of hypergravity exposure $F(1,68)=5.0$, $p=0.0286$. The level of extracerebellar 3-NT was increased on P6 by 22% only in HG females; there were no changes in extracerebellar 3-NT on P12 or P21.

Cerebellar DNA 8-OH-dG levels

The data on 8-OH-dG is presented in Table 3. The increase in 3-NT concentration in HG female cerebella on P12 was accompanied by a 15.8% increase in 8-OH-dG in cerebellar DNA. The limited amount of cerebellar tissue did not allow for a thorough analysis of cerebellar DNA. The comparison of cerebellar vs. extracerebellar 8-OH-dG on P12 is shown in Table 3. The overall effect of hypergravity on extracerebellar DNA approached significance ($p=0.062$). On P6, while 8-OH-dG showed a 51.4% increase in HG males, it was lower in HG females. The extracerebellar 8-OH-dG was increased in HG males by 51.6% and in females by 69.5%; it was also increased on P21 in HG males by 37.8% and in HG females by 36.1%.

4. Discussion

Markers of oxidative stress in the hypergravity exposed rat cerebellum

This study is the first to report the levels of rat brain 3-NT, which is a marker reflecting oxidative stress-related modification of proteins and 8-OH-dG, a marker reflecting oxidative stress-related modification of DNA. The data derived from our study indicate that the levels of oxidative stress markers vary during the course of normal development, are different in males and females and are further affected by hypergravity exposure in an age- and sex-dependent fashion. Our data also suggests that the levels of 3-NT and 8-OH-dG may be brain region specific.

Our results thus add to the growing evidence supporting the role of oxidative stress in mediating the effect of environmental factors (Lee and Opanashuk, 2004; Jayaraj et al., 2006) including the effect of altered gravity (Yang et al., 2003). Previously, oxidative lipid modification has been reported both in humans and animals exposed to altered gravity (Yang et al., 2003). However, there are no reported quantitative data on the oxidative modification of proteins or DNA in the brains of control rats or rats exposed to altered gravity. Most recent data on 3-NT in rat brain were based on slot blots and reported a relative 3-NT brain content (Butterfield et al., 2007). We have recently reported, also for a first time, human brain levels of 3-NT.

Contrary to our expectations, we did not observe a uniform increase in 3-NT levels, but rather a deviation from the normal levels. These findings could reflect the little known fact that changes in intrinsic antioxidant defense mechanisms accompany normal brain development (Nanda et al., 1996) and may contribute to the age-dependent brain sensitivity to oxidative stress (Driver et al., 2000). While further studies are needed to resolve this ambiguity, it may be speculated that the maturation of the antioxidant system occurs at different discrete points in time in males and females. Additional data on the extracerebellar tissue suggests that hypergravity induces oxidative stress in other parts of brain as well. Most likely hypergravity exerts a global effect in terms of oxidative stress, while the brain region-specific response is defined by regional distribution of specific proteins and other macromolecules that are subjected to oxidative stress. Nitration appears to be a selective process targeting specific proteins. The proteomic approach identified unique target proteins in the aging rat brain (Poon et al., 2006) related to cell apoptosis. It is thus possible that the increased 3-NT levels may be related to increased apoptosis of Purkinje cells resulting in the decreased Purkinje cell number observed in hypergravity-exposed rats.

The data on cerebellar levels of 8-OH-dG is very limited both due to a limited amount of cerebellar tissue and to the large sample requirement for 8-OH-dG analysis. The cerebellar data suggests an increase in the levels of 8-OH-dG on P12 only in females. In extracerebellar tissue, the increase in 8-OH-dG levels was observed in both HG males and HG females on P12 and P21; on P6 it was only observed in males.

Altered gravity and oxidative stress

It has been observed that exposure to microgravity during space flights is associated with increased oxidative stress, assessed in terms of urinary content of 8-isoprostaglandin F₂alpha and 8-oxo-7,8 dihydro-2-deoxyguanosine markers for oxidative damage to lipids and DNA respectively in both humans and rodents (Yang et al., 2003). Exposure of neuron-like PC12 cells, to reduced gravity in clinostat also results in oxidative stress, measured in terms of concentrations of nitric oxide (NO) and nitrotyrosine, which is a marker of oxidative damage to proteins (Qu et al., 2006). It appears that the exposure of rats to hypergravity also results in oxidative stress (Karbachev'ka et al., 2002). Repeated exposure of adult rats to short-duration hypergravity (30 sec, 10G) over a period of four weeks resulted in lipid peroxidation in the brain (Zhan et al., 1999). Our own data indicate that the exposure of developing rats to hypergravity is associated with a reduction in Purkinje cell number (Sajdel-Sulkowska et al., 2005). It is thus of interest that Purkinje cells are sensitive to brain ischemia (Welsh et al., 2002) and consequently may be affected by hypergravity-induced oxidative stress.

Oxidative stress and Purkinje cells

Our data indicate that the increase in oxidative stress observed in the HG females is associated with a decrease in Purkinje cell number in their cerebella. Independent observations indicate both that Purkinje cell loss can be due to oxidative stress in a mouse model of ataxia (Chen et al., 2003) and that Purkinje cells are very sensitive to ischemia (Welsh et al., 2002). Thus it is possible that oxidative stress contributes to the decreased Purkinje cell number in hypergravity-exposed rat neonates. While the Purkinje cell loss was shown on P21 in both male and female cerebella, the increase in the oxidative stress 3-NT preceding this change was only observed in female cerebella and further studies are needed to resolve this ambiguity.

Our data indicating increased oxidative stress markers in hypergravity-exposed rats suggest the possibility of increased cell apoptosis. Such a possibility is supported by the observation of a relationship between oxidative stress and increased apoptosis (Reichenbach et al., 2002). Specifically, Purkinje cell loss in Purkinje cell degeneration (pcd) mice is due to apoptosis that is associated with nitrotyrosine accumulation in the Purkinje cells (Kyuhou et al., 2006). Furthermore, a neurotrophin NGF, when subjected to tyrosine nitration, induces motor neuron apoptosis (Pehar et al., 2006). On the other hand, in the Lurcher mice, while increased oxidative stress and nitrotyrosine are implicated in cell death (McFarland et al., 2007), overexpression of the antiapoptotic protein BCL-2 does not prevent Purkinje cell degeneration, suggesting that apoptosis may not be involved in degeneration (Zanjani et al., 1998). Our studies nevertheless identify an important step in the mediation of the effect of altered gravity on Purkinje cells while future studies will clarify the precise sequence in the pathway. It is possible that while oxidative stress is likely involved in the effect of altered gravity, measures of additional oxidative stress markers may contribute new insight into oxidative stress-induced damage to the Purkinje cells.

At this point neither human nor animal studies consistently indicate whether oxidative stress levels are higher in males or females. However, some studies suggest that males may be more susceptible to oxidative stress (Chakraborti et al., 2007). Thus, while male and female rats subjected to stress of restraint show similar increases in brain superoxide dismutase (SOD), males exhibit more severe behavioral changes. It is possible that while hypergravity-exposed females show a greater increase in 3-NT, male Purkinje cells are more sensitive to other oxidative stress changes. Other studies involving hypertensive rats suggest that gender affects hypertension-induced oxidative stress and oxidative damage to lipids and proteins as well as cell apoptosis in the brain (Ren, 2007). As discussed above, little is known about the

relative degree and sensitivity to oxidative stress in males and females. One may speculate that the sensitivity to oxidative stress is subject to hormonal regulation. For example, progesterone can minimize oxidative insults to the female but not the male (Dhote and Balaraman, 2007).

Mechanisms underlying induction of oxidative stress under altered gravity

Both microgravity encountered by astronauts in space and simulated microgravity on Earth induce a tremendous shift in body fluids, an increase in brain fluid (Pettis et al., 2004; Sarkar et al., 2006) and alterations in tissue perfusion (Sanford et al. 1999). Data derived from experiments simulating microgravity in adult rats have shown that this fluid shift results in altered brain density in females but not in males (Pettis et al., 2004). Hypergravity exposure on the other hand, results in decreased cerebral blood flow (Kawai et al., 1997), changes in lung structure and function (Sanford et al., 1999) and in cerebral hypoperfusion (Serrador, 2005). Changes in fluid distribution within brain tissue affect oxygen supply; it is of interest that in humans exposed to hypergravity, brain oxygen supply is decreased more in males than in females (Tripp et al., 1998). Thus, the effect of the fluid shift is likely to result in hypoxia followed by the oxidative stress. The effect of altered fluid distribution on the CNS, however, appears to be independent of the vestibular response (Fujiki et al., 2000). Independent studies (Fidelini et al., 1999) suggest that oxidative stress may be related to the induction of nitric oxide and stress-related peptides.

The animal studies described here are relevant to understanding the mechanisms responsible for behavioral changes observed in humans exposed to altered gravity. Already some studies report that space flight or rather return from space to earth is associated with oxidative stress damage to lipids and DNA (Yang et al., 2003). Little is known about oxidative protein modification and the sex-, age- and brain region-specificity of the oxidative stress. Some of these questions can be answered more easily in animals and the hypotheses tested further in humans. Furthermore, understanding the role of oxidative stress in mediating the effect of altered gravity is likely to contribute to the identification of potential preventive or therapeutic interventions. For example, the supplementation of diets with antioxidants may counteract post-flight oxidative stress.

In conclusion, this study compared for the first time the levels of oxidative stress markers 3-NT (related to protein modification) and 8-OH-dG (related to DNA modification) between hypergravity-exposed and stationary control rat neonates. Our data indicate a statistically significant increase in 3-NT levels in the hypergravity-exposed rat cerebellum along with an elevation in 8-OH-dG. These levels in control rats are age- and sex-dependent. While preliminary, the data lend further support to the concept of oxidative stress involvement in the effects of hypergravity on the developing rat cerebellum and specifically the decrease in Purkinje cell number that may contribute to a decline in motor functions under altered gravity.

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Figure 1. Developmental profile of cerebellar (1A) and extracerebellar (Figure 1B) 3-NT in male and female rat neonates. Cerebellar 3NT content is expressed in picomoles/gram tissue.

Table 1

Effect of exposure to hypergravity (1.65G) on rotarod performance ($p<0.001$) and cerebellar Purkinje cell number ($p=0.0094$) in male and female rat neonates on P21.

	SC MALE	SC FEMALE	HG MALE	HG FEMALE
Rotarod time (seconds)	47.61±6.73	46.7±7.37	28.75±3.50	33.11±5.23
Purkinje cell number on P21	9.53±0.84×10 ³	9.53±0.35×10 ³	7.85±0.0.68×10 ³	7.12±0.56×10 ³

Table 2
3-NT levels in HG and SC cerebellar and extracerebellar regions

3-NT was measured by ELISA in homogenates prepared from frozen tissue from HG and SC rat neonates. Data are converted to picomole per gram of tissue (pmol/g) and presented as mean±SEM.

TREATMENT	CEREBELLAR TISSUE		EXTRACEREBELLAR TISSUE	
	3-NT (pmol/g tissue)		3-NT (pmol/g tissue)	
	MALE	FEMALE	MALE	FEMALE
SC	1.18±0.21	0.68±0.11	0.74±0.03	0.87±0.10
HG	0.56±0.10	13.26±2.15	0.0.74±0.05	0.74±0.03

Table 3
8-OH-dG levels in HG and SC cerebellar and extracerebellar regions

Cerebellar 8-OH-dG was measured by ELISA in homogenates prepared from frozen postmortem tissue from HG and SC rat neonates. Data are converted to picomole per ug DNA (pmol/ug DNA) and presented as mean $\pm$ SEM.

TREATMENT	CEREBELLAR TISSUE		EXTRACEREBELLAR TISSUE	
	8-OH-dG (pmol/ug DNA $\times 10^{-3}$)		8-OH-dG (pmol/ug DNA $\times 10^{-3}$)	
	MALE	FEMALE	MALE	FEMALE
SC	83	32	14.86 $\pm$ 4.10	10.43 $\pm$ 3.55
HG	10	39	22.53 $\pm$ 7.80	17.68 $\pm$ 3.12