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Dear Dr. Gabella,

I am enclosing our response to the reviewer's comments as well as changes made to the manuscript "Purkinje cell loss and motor impairment in rats developing at altered gravity", submission no.: NR-D-05-3787.

Thank you again for giving us the opportunity to clarify the issues addressed below and to revise our manuscript. We hope that our answers will allow you to proceed with the publication of our manuscript in the NeuroReport.

With best regards,
Elizabeth M. Sajdel-Sulkowska

Response to the Section Editor's evaluation and criticism

1. This is a well-written paper that describes a significant reduction of cerebellar Purkinje cell number in male and female rats after fetal and perinatal exposure to hypergravity. There was a correlation between Purkinje cell number and performance on the rotorod test in male rats whether exposed to hypergravity or not, but in females the relationship between Purkinje cell number and rotorod performance was not clear.

Re 1: Thank You.

2. Overall, the results are quite interesting in that they add to a literature on the impact of environmental factors on brain development but the authors do not make explicit why hypergravity is of particular interest. Presumably it is a highly unusual experience. Some discussion would be helpful.

Re 2. Our experiments were part of the attempt to ascertain what happens to the developing CNS in space, when exposed to altered gravity, as part of the collaborative project with NASA. However, by observing that certain environmental impacts can be exerted by physical as well as chemical manipulations we begin to consider other forms of environmental factors not usually considered as developmentally critical. For example, it is possible that frequent air travel or excessive noise during development may be detrimental to the developing CNS. Furthermore, when one considers that the external factors are mediated by the hormonal mechanisms as discussed in the revision, the finding reported here in the context of hypergravity can be extrapolated to other more usual experiences. Pursuing the hypothesis that changes in thyroid hormone levels may be related to an underlying mechanism involved in the effect of hypergravity on the developing cerebellum, one can foresee an applicability of our observations to other thyroid status-disrupting environmental factors, both chemical such as PCB (Nguon et al., 2005) and nonchemical, such as high altitude (Connors and Martin, 2005). We have included the above statement as part of the revised discussion.

3. There was good attention to the subjects feeding and weight control, ensuring that the cerebellar changes are unlikely to be due to nutritional deficiencies. However, it is difficult to know exactly how hypergravity interacts with the developing brain. One important possibility relates to the subjects' general motor behaviour under hypergravity conditions. Were there reduced levels of spontaneous activity and could this have impact on cerebellar development directly because of reduced motor demand or indirectly because of lower levels of sensory feedback?

Re 3. There are no direct data regarding spontaneous activity under hypergravity conditions. We have recorded the behavior of litters on the centrifuge and eventually plan to analyze these data. There are, however, conflicting reports on spontaneous activity upon return from exposure to hypergravity. A decrease in spontaneous activity has been observed in mice at 24 to 48 hours following developmental exposure to 2G (Francia et al., 2004). However, others observed an increased spontaneous activity in rats conceived and reared at 2G for three months (Bouet et al., 2003).

Even if there were reduced levels of spontaneous activity or lower levels of sensory feedback, they would most likely not impact cerebellar development in our experimental paradigm. Such integration is function of Purkinje cells, which do not become physiologically or morphologically fully mature in rats until postnatal day (PD) 18 (McKay and Turner, 2005). With exposure to hypergravity being terminated on PD21 such impact would be negligible.

4. Alternatively, I would be interested to know if there have been measurements of stress-related factors under similar hypergravity conditions. Could stress-related hormonal changes mediate the Purkinje cell changes and might they be related to the sex differences in the rotorod test?

Re 4. Analysis of corticosterone levels in HG rats showed no changes (Moran et al., 2001).

5. As it stands, the paper does not make a clear case as to why hypergravity was used as the environmental factor and does not elaborate on how it may have exerted its effects upon Purkinje cell number. I would like to see some discussion of these two issues in a revised version of the paper.

Re 5. We hope that the sections inserted into the revised discussion adequately addresses these issues. In order to address the Editor's point we have revised the references; however in order to maintain the overall integrity of the original manuscript we have increased the number of references to 28.

Purkinje cell loss accompanies motor impairment in rats developing at altered gravity.

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Abstract

We have previously reported that the developmental exposure of rats to altered gravity (1.65G) from gestational day (GD) 8 to postnatal day (PD) 21 impacts motor functions and cerebellar structure. The present study examined whether the decrease in cerebellar mass accompanied by impaired performance on a rotorod in hypergravity-exposed rats was related to a decrease in Purkinje cell number. The total number of Purkinje cells was determined on PD21 using a stereological analysis applied to paraformaldehyde-fixed cerebellar samples subsequently embedded in celloidin. Total Purkinje cell number was decreased by 17.7-25.3%. These results imply that exposure to altered gravity during Purkinje cell birth may affect their proliferation, resulting in a decrease in Purkinje cell number which in turn leads to motor impairment.

Key words: cerebellum, development, hypergravity, Purkinje cells, rotorod, motor behavior.

Introduction

The cerebellum is crucially linked to motor behavior, although more recent evidence points out cerebellar involvement in learning, motor planning, cognitive flexibility, sensory processing, exploratory activity [1], and other higher cognitive functions [2]. Studies of genetically and non-genetically lesioned rodents suggest cerebellar involvement in spatial learning [3]. Furthermore, selective destruction of Purkinje cells resulted in impaired acquisition and performance of the Morris water maze task [4], supporting a role of the cerebellum in spatial learning.

A relationship between Purkinje cell number and motor behavior is derived mostly from observations of mutant mice. *Nervous* [3], and *lurcher* [5] mutant mice exhibit a relatively selective degeneration of Purkinje cells (3) as does the natural mutation of Purkinje cell degeneration [*pcd*; 6]. All of these mutations are associated with motor impairment as assessed by a rotarod test. Furthermore, bilateral transplants of fetal Purkinje cells to *pcd* mutant mice improve motor performance on the rotarod [7]. In a mouse model of Purkinje cell loss ataxia is dependent upon both the number and distribution of Purkinje cells in the cerebellum [8]. The *Lc/+* mice, which lose Purkinje cells postnatally, show ataxia and impaired rotarod performance [9]. There is some evidence that the Purkinje cell number and function can be affected by nongenetic means, suggesting a potential vulnerability of cerebellar structure and functions to environmental impairments. Purkinje cells appear to be particularly sensitive to developmental exposure to ethanol [10]. In rats modeling fetal alcohol syndrome, depletion of Purkinje cells in the cerebellum results in deficit of motor coordination [11]. Purkinje cells are also sensitive to

brain ischemia [12], to gestational exposure to X-irradiation [13] and low dose ionizing radiation [14]. Data on Purkinje cell loss in cerebella lesioned with intraventricular injections of anti-neuronal immunotoxin OX7-saporin indicates dose- and time-dependent changes in motor performance [15]. Our own work [16] demonstrated sex-dependent motor function impairment following developmental exposure to PCBs that was particularly pronounced in male rats.

In the present study we explored the possibility that the impaired motor function observed in rat neonates developmentally exposed to altered gravity is related to a decrease in Purkinje cell number.

Methods

Timed-pregnant Sprague-Dawley dams were exposed to hypergravity (HG) as described earlier [17]. Pregnant dams were shipped from Taconic Farms (Germantown, NY) to NASA Ames Research Center (ARC) at gestational day (GD) 2 (GD1 defined as the first day after males and females are co-housed 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:12-hr light cycle, with 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 GD7, dams were weight-matched, deselected if not pregnant, and assigned to either hypergravity group (HG, n=6) or stationary control group (SC, n=5). 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 GD8, continuous centrifugation was initiated, with brief daily stops for checks of animal health and data collection both before and after animals' birth. The centrifugation was carried out at 17.6 rpm; the cabs were placed at 136.5 inches from the center, resulting in hypergravity of 1.65 G. The duration of the daily stops ranged from 30 min. during pregnancy to 90 min. after birth.

On postnatal day (PD) 21 neonatal body mass was recorded and each neonate was tested on a rotorod. Following the rotorod test, the neonates were either euthanized by decapitation, cerebella rapidly removed, cerebellar mass recorded and tissue frozen in methyl butane, or the neonates were perfused with formalin and transferred to formalin for storage.

Rotorod tests. Motor behavior was tested on PD21 using a rotorod apparatus as described earlier [16]. The rotorod is a rotating horizontal cylinder. The animals are placed perpendicular to the cylinder with their head facing against the direction of rotation. The animals have to progress forward to maintain equilibrium in order to remain on the cylinder. Each neonate was subjected to one trial on a cylinder rotating at the maximal speed (20 rev/5min). The length of time the animal remained on the rotating cylinder was recorded.

Stereological cerebellar analysis. Rats were deeply anesthetized by inhalation of isoflurane and perfused intracardially with physiological saline, followed by 4% paraformaldehyde, pH 7. The cerebella were removed, weighed, and incubated overnight in the same fixative at 4°C. Subsequently, the tissue was washed with water and dehydrated with 80%, 95% and 100% ethanol and ethyl ether and then infused with 3% celloidin and

then 12% celloidin [18]. Embedded tissue was cut into 30- μ m sections, rehydrated, stained with the cresyl violet stain and dehydrated with ethanol-rosin solution. Every 10th section was used for analysis.

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). Cerebellar volume was estimated from serial sections using point counting and Cavalieri's rule as described elsewhere [19]. The total number of Purkinje cells was estimated using the optical fractionator probe, a 500 x 500 μ m sampling grid and a 30 x 30 x 20 μ m counting box; sections were sampled every 600 μ m.

Results

Motor coordination. Motor coordination was tested on a rotarod on PD21. All hypergravity-exposed PD21 rats showed shorter latency before falling. This latency was reduced by 67.7% in HG males and it was reduced by 61.1% in HG females. ANOVA with hypergravity treatment and sex as between-subjects factors, presented in Table 1, revealed main effect of hypergravity exposure $F(1,108)=8.01$, $p=0.0055$, but no hypergravity by sex interaction. Thus motor coordination is affected in rats developing under hypergravity conditions.

Body mass. On PD21 neither body mass of HG males (SC, 49.43 ± 2.1 ; HG, 50.38 ± 0.93) nor HG females (SC, 48.65 ± 1.69 ; HG, 49.11 ± 1.04) was reduced (Table 1).

Thus it appears that on PD21 impairment of motor coordination is independent of nutritional impact.

Cerebellar mass. Cerebellar mass on PD21 was reduced by 5.0% in HG males and by 6.3% in HG females (Table 1). ANOVA with hypergravity treatment and sex as between-subjects factors, presented in Table 1, revealed main effects of hypergravity exposure, $F(1,91)=15.27$, $p=0.0002$, and sex, $F(1,91)=21.60$, $p<0.0001$, but no hypergravity by sex interaction. Thus cerebellar mass is decreased in rats developing under hypergravity conditions and the decrease is sex-independent.

Total Purkinje cell number. Total Purkinje cell number was determined stereologically in a subset of PD21 rats tested on the rotorod, and was decreased in both HG males and HG females. ANOVA with hypergravity treatment and sex as between-subjects factors, presented in Table 1, revealed a main effect of hypergravity exposure, $F(1,13)=9.26$, $p=0.0094$, but no hypergravity by sex interaction. Thus hypergravity exposure during development results in a decreased number of Purkinje cells.

(Table)

Purkinje cell number and performance on the rotorod. We were interested in whether rotorod performance on PD21 was significantly related to Purkinje cell number when effects of treatment, sex, and mass on rotorod performance were removed. We first calculated a regression model where treatment, sex, their interaction, and mass predicted rotorod performance. The second regression model added total Purkinje cell number as a predictor. The difference in variance accounted for by the two models (ΔR^2) represents the variance in rotorod performance uniquely accounted for by rotorod performance, excluding

effects of treatment, sex, and mass. This value was $\Delta R^2 = 0.276$, $F(1,11) = 8.00$, $p = .0164$. Thus, total Purkinje cell number accounted for a significant proportion of variance in rotorod performance, when effects of treatment, sex, and mass were taken into account. The strength of this relationship is established by the partial correlation between rotorod performance and total Purkinje cell number, partialling out treatment, sex, the treatment by sex interaction, and mass; this value is $\underline{pr} = .649$. This relationship is (approximately) represented graphically in the right panel of Figure 1. This figure plots Purkinje cell number vs. rotorod performance, with values mean-centered by treatment/sex group. (The relationship of mass to rotorod performance is negligible and not significant in the multiple regressions, so it is neglected in this graphical representation.)

(Figure)

Inspection of the scatterplot of the raw data (Figure 1, left) suggested that the relationship between Purkinje cell number and rotorod performance might differ between males and females. Although a clear positive relationship was observed between Purkinje cell number and rotorod performance within each group individually (SC male, SC female, HG male, HG female), this relationship seemed to be consistent across HG and SC males, but not females. That is, there appeared to be a consistent relationship between Purkinje cell number and rotorod performance in male rats regardless of whether they had been exposed to hypergravity, whereas this did not seem to be the case in females. Indeed, the correlation between Purkinje cell number and rotorod performance in males was significant and positive, $\underline{r} = 0.71$ ($\underline{p} = .033$), whereas it was not significant in females, $\underline{r} = -0.14$, $\underline{p} = .75$. However, these correlations did not differ significantly from each other, $\underline{z} =$

1.68, $p = .09$, so the conclusion that the relationship between Purkinje cell number and rotorod performance differs in males and females must remain speculative.

Discussion

Gene-environment interactions are increasingly recognized as powerful determinants during the course of development affecting both physical and psychological outcomes. We have previously reported that developing rats exposed perinatally to hypergravity show a reduction in cerebellar mass. Furthermore, the effect of hypergravity was sex-dependent [17].

In the present study we examined the effect of hypergravity on Purkinje cell number and motor functions. The results presented here indicate that developmental exposure to altered gravity contributes to the deficit in Purkinje cell number and to impaired motor coordination. These data are among the first to show the environmental impact on a specific neuronal population in the developing CNS and the first to report a non-chemical effect. Previously it has been shown that the developing cerebellum is particularly sensitive to the chemical toxins, including ethanol [10, 20] and PCB [16]. We also show for the first time that the decrease in Purkinje cell number due to non-chemical environmental perturbations associated with the impairment of motor functions is similar to the effects of chemical toxins such as ethanol [11].

This effect is most likely not due to under-nutrition since there are no differences in body mass between HG and SC offspring on P21. Others have found that early nutritional deprivation does not affect Purkinje cell number [21]. Furthermore, the effect is most

likely not due to chronic stress since there were no changes in corticosterone levels [17] in HG rats.

However, it is possible that the deficit in Purkinje cell number and impairment in motor behavior may in part be due to a transient hypothyroidism as indicated by increased circulating levels of thyroid stimulating hormone (TSH; 22). Thyroid hormone (TH) is critical for the normal development of brain structure and function [23]. TH deficiency results in impaired motor behavior [24]. Furthermore, TH is involved in the regulation of cell apoptosis and TH deficiency results in decreased expression of antiapoptotic proteins of the Bcl-2 family resulting in enhanced cell apoptosis [25]. Sex-dependent regulation of TSH secretion as well as sex-dependent differences in cerebellar structure may contribute to sex-dependent impairment of cerebellar functions. Furthermore, pursuing the hypothesis that changes in TH levels may be related to an underlying mechanism involved in the effect of hypergravity on the developing cerebellum, one can foresee an applicability of our observations to other thyroid status-disrupting environmental factors -- both chemical, such as PCB exposure [16] and non-chemical, such as high altitude exposure [26].

The relationship between Purkinje cell number and performance on the rotorod appeared to be more consistent in HG and SC males, but not females. However, at this point, the sex-specific nature of the relationship between Purkinje cells and motor coordination is speculative. Our previous data indicated that developmental exposure to PCBs affects cerebellar structure and motor coordination more profoundly in males than in females [16]. Interestingly, both the loss of Purkinje cells with age, only observed in males [27], and rotorod performance in mice [28] are sex dependent. The data presented here

suggest that physical forms of environmental perturbations, just like chemical neurotoxins, may impact developing cerebellar Purkinje cells and motor coordination more significantly in males. Such sex-dependent vulnerability to environmental impacts could contribute to a greater preponderance of neurodevelopmental disorders in males.

Our experiments were part of the attempt to ascertain what happens to the developing CNS in space when exposed to altered gravity, as part of the collaborative project with NASA. However, by observing that certain environmental impacts can be exerted by physical as well as chemical manipulations, we began to consider other forms of environmental factors not usually considered as developmentally critical. For example, it is possible that frequent air travel or excessive noise exposure during development may be detrimental to the developing CNS. Furthermore, when one considers that the response to external factors is mediated by hormonal mechanisms, the finding reported here in the context of hypergravity can be extrapolated to other more usual experiences.

Acknowledgements

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Table 1. Effect of exposure to 1.65G from gestational day GD8 to PD 21 on neonatal rotorod performance, body mass, cerebellar mass and total Purkinje cell number (mean \pm S.E.M.).

	SC		HG	
	MALE	FEMALE	MALE	FEMALE
ROTOROD PERFORMANCE (SEC)	54.25 \pm 13.30	57.03 \pm 16.23	*17.50 \pm 3.11	*22.57 \pm 6.6
NEONATAL BODY MASS (GRAMS)	49.43 \pm 2.1	48.65 \pm 1.69	50.38 \pm 0.93	49.11 \pm 1.04
NEONATAL CEREBELLAR MASS (GRAMS)	0.201 \pm 0.002	0.189 \pm 0.003	*0.191 \pm 0.003	*0.178 \pm 0.003
TOTAL PURKINJE CELL NUMBER	9.53 \pm 0.84 $\times 10^5$	9.53 \pm 0.35 $\times 10^5$	7.85 \pm 0.68 $\times 10^5$	*7.12 \pm 0.56 $\times 10^5$

Rotorod performance (P=0.006), cerebellar mass (P=0.025) and the total Purkinje cell number (P=0.0093) were significantly altered in PD21-HG neonates; body mass on PD21 was not significantly affected.

Figure 1. The relationship between cerebellar Purkinje cell number and performance on the rotorod. Left panel: raw data shows a positive relationship between Purkinje cell number and rotorod performance within each group, but no consistent relationship across groups. Right panel: Mean-centered data removes main effects of sex, hypergravity, and their interaction, revealing a significant correlation between Purkinje cell number and rotorod performance. The strength of this relationship was derived by partial correlation between rotorod performance and total Purkinje cell number, partialling out treatment, sex, the treatment by sex interaction, and mass; this value is 0.649.

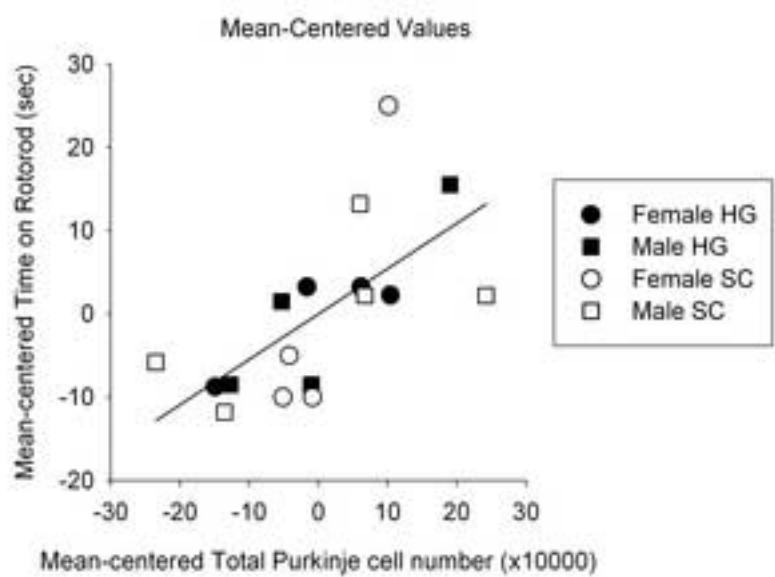
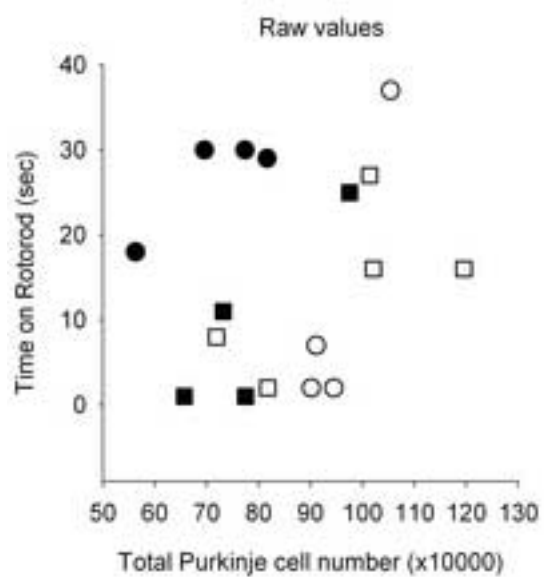


Table 1. Effect of exposure to 1.65G from gestational day GD8 to PD 21 on neonatal rotorod performance, body mass, cerebellar mass and total Purkinje cell number (mean \pm S.E.M.).

	SC		HG	
	MALE	FEMALE	MALE	FEMALE
ROTOROD PERFORMANCE (SEC)	54.25 \pm 13.30	57.03 \pm 16.23	*17.50 \pm 3.11	*22.57 \pm 6.6
NEONATAL BODY MASS (GRAMS)	49.43 \pm 2.1	48.65 \pm 1.69	50.38 \pm 0.93	49.11 \pm 1.04
NEONATAL CEREBELLAR MASS (GRAMS)	0.201 \pm 0.002	0.189 \pm 0.003	*0.191 \pm 0.003	*0.178 \pm 0.003
TOTAL PURKINJE CELL NUMBER	9.53 \pm 0.84X10 ⁵	9.53 \pm 0.35X10 ⁵	7.85 \pm 0.68X10 ⁵	*7.12 \pm 0.56X10 ⁵

Rotorod performance (P=0.006), cerebellar mass (P=0.025) and the total Purkinje cell number (P=0.0093) were significantly altered in PD21-HG neonates; body mass on PD21 was not significantly affected.