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Early hypergravity exposure effects calbindin-D28k and inositol-3-phosphate expression in Purkinje cells

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Abstract

In this study the effects of hypergravity were analyzed on cerebellar Purkinje cells during early development in rats. The cerebellum is a key structure in the control and the adaptation of posture and anti-gravity activities. This holds particularly when external conditions are modified. Three groups of rats were conceived, born and reared in hypergravity (2 g). At postnatal day 5 (P5), P10 or P15, they were exposed to normal gravity and at P40, the cerebella were investigated on the expression of calbindin-D28k and inositol-3-phosphate (IP3) in Purkinje cells. Control animals were bred in the same conditions but at 1 g. Immunoreactivity of Purkinje cells was studied in lobules III and IX of the vermis. Lobule IX of the vermis is one of the targets of primary otolithic vestibular projections, and lobule III served as a control, being much less related with vestibular inputs. The results show that hypergravity induces a decrease in calbindin and IP3 labeling in 20% of Purkinje cells of lobule IX without any change in lobule III. Animals transferred from 2 g to 1 g at P5 or P10 showed the most pronounced effects and much less at P15. This study demonstrates that early development of the cerebellum is highly sensitive to changes in gravity. Ages until P10 are critical for the development of vestibulo-cerebellar connections, and in particularly the calcium signaling in Purkinje cells.

Keywords: Maturation; Calbindin-D28k; Inositol-3-phosphate; Cerebellum; Motor development; Vestibular system

Changes in gravity have strong effects on the maintenance of balance and postural control [6,14]. The sensori-motor functions involved are closely dependent on the cerebellum and imply a proper functioning of the vestibular system. In adult animals, hypergravity exposure followed by a transfer to normal gravity induces postural and locomotor deficits that recover in about 3 weeks [7,8]. In young animals, early hypergravity (HG) exposure induces a delay in the development of behaviors related to the vestibular system, followed by rapid adaptations lasting a few days [9]. Adaptations to changes in gravity are supposed to occur in central regions rather than in peripheral structures, and particularly in the otolith-olivo-cerebellar pathways [11,13]. The cerebellum plays a pivotal role in postural adjustments in relation to ongoing movements (so-called feed forward control of posture) [10]. For these reasons, it is likely that the cerebellum is strongly involved in adaptations to changes in gravity.

The cerebellar circuitry develops largely during the early postnatal period in rats. This development is governed by intrinsic genetic programming, however, external and environmental factors are known to actively modulate the neuronal maturation. During early development, the brain is most plastic and sensitive to external factors [12]. The gravitational vector is such a factor, important for postural control and extremity movements. Several sensory systems are related to gravity, e.g., cutaneous receptors in the plantar soles and extensor muscles are strongly influenced by gravity. The main sensory apparatus which specifically depends on the direction and magnitude of the gravitational vector is the otolithic

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vestibular system. Indeed, it possesses hair cells sensitive to linear acceleration which inform the brain about positions and movements of the head in relation to the vertical gravity. Vestibular afferents reach the vestibular nuclei complex where they are integrated, and in addition, they also directly reach the cerebellum and particularly the vermis [2]. Through its afferent and efferent connections, the cerebellum uses vestibular information to control and adjust body equilibrium and this is essential for motor coordination.

The cerebellum is a highly organized structure in which Purkinje cells are the sole output of the cerebellar cortex. These cells are aligned in a single plane, parallel to the surface of the cortex and they can be specifically stained with antibodies directed against calmodulin, calbindin-D28K, parvalbumin, or inositol-3-phosphate (IP3) [4]. In most species, all Purkinje cells (soma and dendrites) are calbindin immunopositive from an early stage of embryonic development [5]. Calcium-binding proteins are suggested to play a role in the regulation of intracellular calcium signaling cascades, therefore, in the cell firing [4] and they are also related to changes in sensory inputs [19]. IP3 is also powerful in labeling Purkinje cells since it is a second messenger that links the activation of several membrane receptors with the intracellular calcium release. There is strong evidence that its expression is related to growth of Purkinje cells and synaptogenesis [21]. More recently it could be demonstrated that cerebellar motor control is impaired in mice with a selective genetic deletion of the calbindin protein. Indeed, these mutants display marked deficits in motor coordination and behavioral alterations [3]. From this perspective, we have chosen antibodies directed against calcium-binding proteins, like calbindin and IP3 as markers able to identify Purkinje cells and to reflect their activity. Since the cerebellum develops during the first 3 postnatal weeks in rats, we hypothesize that hypergravity from gestation and during postnatal periods could interfere with its development.

Animals were exposed to hypergravity in a centrifuge previously described [23]. The exposure to hypergravity took place in the Vestibular Department of the Academic Medical Center of Amsterdam. In brief, the apparatus consisted of two horizontal arms (length: 1.10 m) equipped of two free-swinging gondolas (length: 1.10 m, width: 0.45 m, height: 0.725 m) situated at the end of the arms. Each gondola contained one to four cages which sizes were adjustable to the number of animals. A camera situated in each gondola allowed observation of the animals. The HG vector was perpendicular to the bottom of the cages and had a magnitude of 2 g in our conditions (angular velocity: 3.18 rad/s; tilt of the gondolas: 57°). Cages were cleaned twice a week and rotation was then alternated from clockwise to anti-clockwise or back to prevent the development of unilateral compensation for Coriolis forces during locomotion. Ambient noise level for HG and control rats was approximately the same (53 dBA) and the main source of this noise was caused by ventilation.

Seven females and four males of the black and white Hooded Lister strain were used for the study. Five females and two males were kept under hypergravity (2 g) while two females and two males were kept under normal gravity (1 g). The females and males kept in hypergravity firstly adapted to the new situation during 1 week and thereafter, one male was housed with two to three females. Rats were weighed twice a week. After a significant increase in female weight, indicating pregnancy, they were separated from the males and kept, one per cage. When parturition was imminent, the centrifuge was stopped daily for inspection. General conditions of breeding were similar in control animals, except the centrifugation. The control rats remained in the experimental room to ensure that they would be exposed to the same environmental conditions, as noise, humidity and temperature.

The day of birth was indicated as P1. Hypergravity was applied from conception until three different ages, leading to three different HG groups and one control group (CONT, $n = 4$): HG-P5, pups remained in the centrifuge from conception until the age of P5 ($n = 4$); HG-P10 until P10 ($n = 4$) and HG-P15 until P15 ($n = 4$). After these periods, they were all kept in 1 g, and their behavior was tested (see [9]) until the age of P40.

At P40, rats were lightly anesthetized with 1% halothane (Fluothane) with O_2/N_2O to minimize pain and discomfort. They were then deeply anesthetized with sodium pentobarbital (Nembutal, i.p., 50 mg/kg) and perfused transcardially. We used first a solution containing 0.8% NaCl, 0.8% sucrose, 0.4% D-glucose in 0.05 M phosphate buffer (pH 7.5), thereafter the perfusion continued with the fixative containing 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.5). The brains were removed and stored overnight in the fixative solution at 4 °C.

For light microscopy, cerebella were stored in sucrose solutions (10% for 1 h, 20% for 1 h, 30% overnight) for cryoprotection. The cerebella were then frozen and cryo-cut (25 μm) in the sagittal plane. Free-floating sections were immersed for 2 h in a pre-incubation medium containing 1% normal rabbit serum and 1% bovine serum albumin in 1.0 M phosphate buffered saline (PBS, pH 7.4). For each vermis, half of the sections was then incubated 17 h at 4 °C with monoclonal antibody anti-calbindin-D28k (Sigma Aldrich, clone CBq55) diluted 1:20,000 and the other half with anti-inositol-3-phosphate diluted 1:2000 in PBS. After washing with PBS, the sections were incubated with biotinylated rabbit anti-mouse (calbindin) and goat anti-rabbit (IP3) IgG diluted in 1:200 for 1.5 h at room temperature. After further washing, the sections were incubated with ABC (avidin–biotin-peroxidase complex, Vectastain Elite, Vector Labs, Burlingame, CA) for 1 h at room temperature. Immunoreactivity was visualized by incubation with 5 mg 3,3-diaminobenzidine HCl (DAB) and 0.03% hydrogen peroxide in 10 ml of 0.1 M PBS (pH 7.4) for 5–7 min at room temperature. The reaction was stopped by washing the sections in cold PBS. In the sections labeled with IP3, reaction was visualized by adding in the solution 8% nickel ($Ni(NH4)_3$) diluted in TBS instead of PBS, the staining was then stopped after 2–5 min.

Light microscopy was used to assess the regional distribution of calbindin and IP3 immunoreactivity along all the different lobuli of the cerebellar sections. Pictures of each section were made with an Olympus digital camera, mounted on an Olympus B50 optical microscope. Images were adjusted to enhance contrast and saved at 300 dpi. Lobules III and IX (Larsell's terminology) were photographed and counted for occurrence of labeled Purkinje cells and non-labeled Purkinje cells. Limits of each lobule were taken at the two gyral fissures. The morphological analysis of the labeled Purkinje cells was performed on serial sagittal sections. By using differential interference contrast microscopy (DIC), Purkinje cells that were not stained for either calbindin or IP3 could be identified. Only those cells were included in the analysis that showed the nucleus in the plane of the sectioning. We calculated the total number of Purkinje cells (labeled and non-labeled) and the percentages of non-labeled cells among the total of counted cells. For each group, the mean and standard deviation was calculated for each of these parameters. Each of the parameters described above was analyzed using a one-way analysis of variance (ANOVA), applied for the number of labeled cells and for the percentages of non-labeled cells. In case of a global effect, multiple comparisons were done with Bonferroni's post hoc tests.

All the procedures involving animals have been reviewed and approved by the Ethics Committee of the Medical Faculty of the University of Amsterdam.

After the transfer to normal gravity, although they displayed a slight decrease in weight, the behavior of the HG rats was basically similar to that of the controls. However, a more extensive analysis of their sensori-motor functions showed a retardation in the development of contact-righting, air-righting, and negative geotaxis while grasp and tail reflexes were normal (results published elsewhere, see [9]).

Calbindin labeling visualizes the soma and the dendrites of Purkinje cells. Fig. 1 shows the typical pattern of calbindin-labeling in the lobule IX in a control (Fig. 1A) and in a HG-P10 cerebellum (Fig. 1B). Purkinje cells in this lobule are not evenly immunoreactive in HG compared to those in controls. It appears that some portions of the Purkinje cell layer show weaker reactivity to the antibody anti-calbindin indicating that they contain less calbindin compared to the controls. The labeling with IP3 also revealed somata and dendrites of Purkinje cells. Fig. 1C and D show the pattern of labeling of the lobule IX with IP3. While in control rats, the Purkinje cell layer was regularly labeled, we found that in HGP5 and in HGP10 rats, the labeled cells were irregularly spaced. The spaces between labeled Purkinje cells contain cells without reaction to the antibody. Fig. 2 shows the aspects of these cells. With the DIC filter, we observed that in between darkly labeled Purkinje cells, some unlabeled cells were embedded.

Fig. 3 shows the proportions of non-labeled Purkinje cells for IP3 in the lobule IX (Fig. 3A) and for calbindin (Fig. 3B). We found a global effect for all experimental groups with respect to the number of labeled cells and for the percentage of non-labeled cells ($p < 0.001$ for both). With IP3, lobule IX of the control cerebella contained on average 205 ± 41.4 labeled Purkinje cells. In HGP5, there were 117 ± 36.8 labeled Purkinje cells ($p < 0.001$), and in HGP10, 126.6 ± 24.1 ($p = 0.003$). No difference was observed with HGP15 rats ($p = 0.143$). The percentage of non-labeled Purkinje cells was of 1.9 (± 2.8) in controls (Fig. 3A). It was

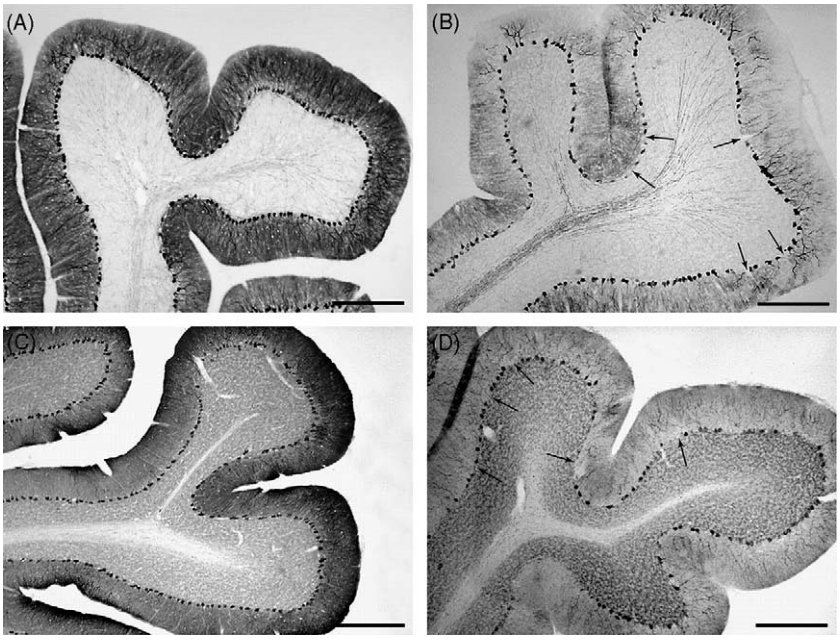

Fig. 1. Immunoreactivity to calbindin (A and B) and IP3 (C and D) in Purkinje cells of cerebellar lobule IX in a control rat (A and C) and in a rat exposed to HG from conception until postnatal day 10 (HGP10), (B and D). In control rats, the lobule IX is evenly labeled while in the HG rat, some portions of the Purkinje cell layer show a weaker reactivity to calbindin (B) and also to IP3 (D). Arrows indicate Purkinje cells, still present, but not stained for calbindin (B) or for IP3 (D) in a hypergravity rat. Scale bar: 600 μm.

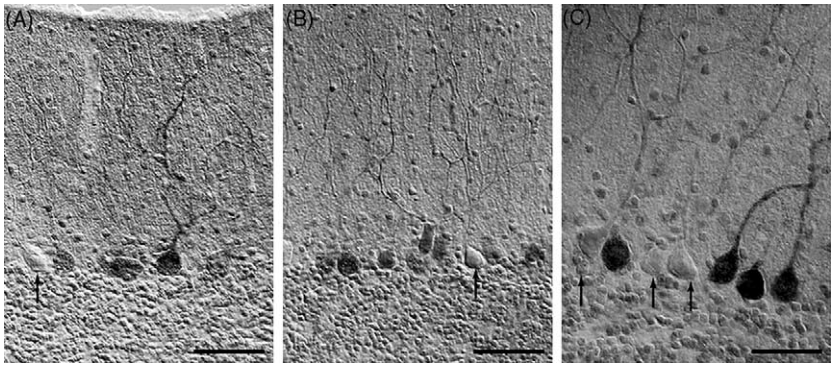

Fig. 2. Examples of Purkinje cell labeling for IP3 in lobule IX, from a HGP10 rat (exposed to HG from conception until postnatal day 10). The figure presents three light microscopic images taken with differential interference contrast optics (DIC) showing a row of Purkinje cells, with their dendrites extending into the molecular layer. Some Purkinje cells somata are stained for IP3, others are devoid of labeling. A-bar indicates 75 μm, B-bar 50 μm, C-bar 100 μm. Arrows indicate the non-labeled Purkinje cells.

significantly increased in HGP5 (19.9 ± 10.9, $p < 0.001$) and in HGP10 groups ($24.2 \pm 4.7\%$, $p < 0.001$). HGP15 group also displayed a higher number of non-labeled cells than in controls ($12.2 \pm 4.1\%$), however, the difference was not significant ($p = 0.12$).

For the calbindin labeling, no significant difference was observed in the number of labeled cells, however, we found that the percentage of non-labeled Purkinje cells (Fig. 3B) was higher in HG than in control rats (global group effect, $p = 0.002$). Post hoc tests revealed, that HGP5 and HGP10 were statistically different from controls. The percentage of non-labeled cells which was of 1.3% (±3.42) in controls increased to 20.2% (±16.6) in HGP5 and to 16.6% (±2.8) for HGP10 ($p = 0.002$ and 0.047, respectively). HGP15 showed on average 5.7% (±3.3) of unlabeled Purkinje cells, the difference with controls being not significant ($p = 0.8$).

Fig. 4 shows the pattern of labeling with calbindin (A in a control, and B in a HGP10 rat) and with IP3 antibodies (C in a control, and D in a HGP10 rat) in lobule III. In control as well as in HG animals, the lobule III was evenly labeled by these two antibodies. We only detected a very small number of "white cells" in this lobule. With calbindin (Fig. 4A), the averaged number of labeled Purkinje cells observed in control animals was of 125 cells (±29) and was not significantly different in HGP5 (116 ± 28), HGP10 (151 ± 13) or HGP15 rats (123 ± 17). For the percentages, HGP5 displayed 1.65% of non-labeled cells, none were observed in the other groups. With IP3 (Fig. 4B), control, HGP5, HGP10 and HGP15 displayed 145.8 (±24), 117.7 (±25), 127.6 (±16.1) and 107.6 (±20) labeled cells, respectively. The percentages of non-labeled cells were 0.17% for controls, 0.5% for HGP5, 3.0% for HGP10 and 0.2% for HGP15. No significant differences were observed between the groups.

Our results show that hypergravity during early development followed by a transfer to normal gravity affects the expression of calcium-binding proteins in Purkinje cells in lobule IX of the cerebellum, whereas in lobule III no differences are observed. In animals transferred to normal gravity at P5 or at P10, about 20% of Purkinje cells lost their reactivity with antibodies directed either against calbindin or IP3, when assessed at P40. When the transfer was performed at P15, Purkinje cells in lobule IX also lost their reactivity, but not significantly. A change in gravity occurring at P5 and P10, therefore, seems to be critical for the development of the cerebellum. Sajdel-Sulkowska et al. [18] demonstrated that hypergravity during gestation until P6 induced a decrease in the mass of the cerebellum of 25.6%, that was not seen in HG rats aged P15. The authors suggested that during neonatal development, the cerebellum is more sensitive to hypergravity exposure than the rest of the brain. The present data

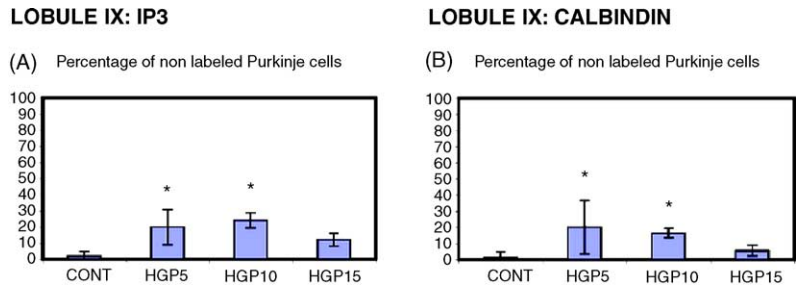

Fig. 3. Quantitative data on IP3 and calbindin immunoreactivity of Purkinje cells in lobule IX of the cerebellar vermis in control (CONT) and hypergravity rats (HG: exposed to hypergravity from conception until postnatal day 5, HGP5, day 10, HGP10 and day 15, HGP15). A. Percentages of non-IP3-labeled Purkinje cells. Vertical bars represent the standard deviation. Asterisks represent a significant difference ($p < 0.05$). B. Percentages of non-calbindin-labeled Purkinje cells. Vertical bars represent the standard deviation. Asterisks represent a significant difference ($p < 0.05$).

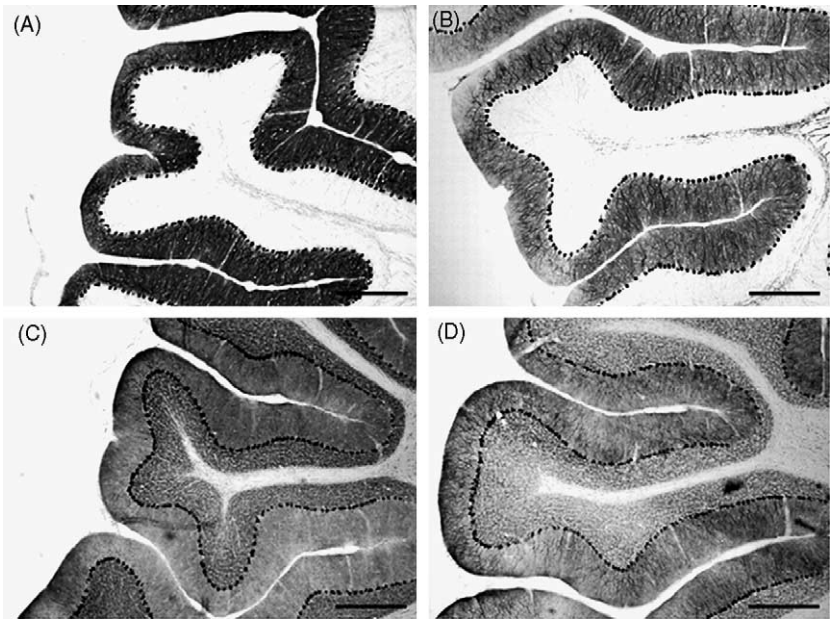

Fig. 4. Immunoreactivity to calbindin (A and B) and IP3 (C and D) in Purkinje cells of cerebellar lobule III in a control (A and C) and a HGP10 (B and D) rats (exposed to HG from conception until postnatal day 10). In control, as well as after HG, lobule III is evenly labeled. Scale bar: 600 μm.

are in full agreement with this conclusion. Furthermore, we observed that only the areas specifically related to vestibular processing are affected. In our experiments lobule III was not affected by hypergravity, all the Purkinje cells being equally stained (Fig. 4), whatever the antibody used. In contrast, lobule IX receiving vestibular afferents, displayed a lack of reactivity meaning that hypergravity selectively modifies the development of lobule IX (Fig. 3) and not the whole cerebellum in a global manner. The vermal lobule IX of the cerebellum, so-called uvula, has been shown to be related with sensory information coming from the saccular and the utricular sensory membranes [2,16]. Possibly, the transfer from 2 g to 1 g (a high gravity level to a lower one), inducing a decrease in otolithic stimulation, has in turn induced a decrease in climbing fiber and mossy fiber activity connected to groups of Purkinje cells. As these cells are still developing at P5 and P10, they could therefore, display their highest sensitivity to environmental factors at these ages. On the contrary, at P15 the cerebellum appears to be less sensitive to gravitational changes than earlier in life, as also suggested by others [18].

Behavioral studies show that hypergravity at early ages induces deficits and alterations in motor control and postural reactions [7–9]. The present experiment suggests that the behavioral deficits observed, could be related to a modified cerebellar development. Indeed, the presence of calbindin in Purkinje cells is necessary for normal motor coordination and sensory integration [1,3]. A decrease in the expression of calbindin in Purkinje cells has also been observed by Pascual et al. [17] after early social isolation in rats. Our situation of hypergravity could be hardly considered as a condition of social isolation, as the pups remained within the litter, but more like a condition of decreased motor activity. Bastianelli [4] suggested that the deleterious effect of social isolation on the reactivity of Purkinje cells to calbindin could be due to the lower activity induced by social isolation. In hypergravity conditions, rats indeed display a lower motor activity than in normal gravity [20]. Calcium-binding proteins regulate intracellular calcium signaling. Several studies have shown that calbindin expression is related with cell activity [2,15,22]. It has also been suggested that these proteins buffer intracellular calcium to modulate the intracellular calcium level and consequently modulate the cell sensitivity to the synaptic signals [2].

In conclusion, the present study demonstrates that animals bred from conception to P5, P10 or P15 in a high gravitational field (2 g) and then transferred to normal terrestrial conditions displayed an altered calcium signaling in lobule IX of the cerebellum. The transfer obviously leads to a decrease in the stimulation of the otolithic vestibular system. This sensory system is closely interconnected with the vestibular nuclear complex and to the cerebellum. Our results suggest that the decreased stimulation of the peripheral vestibular apparatus influences CNS centers processing vestibular information.

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