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Early postweaning social isolation but not environmental enrichment modifies vermal Purkinje cell dendritic outgrowth in rats

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In the present study, we analyzed the effects of enriched, social and isolated experiences on vermal Purkinje cell of the rat, together with anxiety-like behavior in the elevated-plus maze. Sprague-Dawley male rats were randomly submitted to either enriched, social, or isolated environments during the early postweaning period (postnatal days 22–32) and were then behaviorally evaluated in the elevated-plus maze and euthanized for histological analysis. Vermal Purkinje cells (sub-lobules VIa and VIb) were sampled, drawn under camera lucida and morphometrically assessed using the Sholl's concentric ring method. Data obtained indicate that environmental enrichment did not significantly modify the Purkinje cell dendritic branching. On the contrary, Purkinje cell of animals reared in social isolation exhibited a significant reduction in dendritic arborization, which was closely associated with anxiety-like behaviors. The data obtained indicate that, although environmental stimulation in normal animals does not produce significant changes in vermal Purkinje cell dendritic arborization, these cells are vulnerable to early stressful experiences, which is in close association with anxiety-like behaviors.

Key words: Purkinje cell dendrites, environmental enrichment, social isolation, anxiety-like behaviors

INTRODUCTION

Numerous studies in animal models have consistently shown that social and sensorimotor experiences can influence brain maturation, most likely to adjust the neural networks to diverse environmental challenges. Most studies related to the impact of early experiences on neural development have been carried out in the cerebral cortex at the macroscopic, microscopic, ultrastructural and electrophysiological levels (Diamond et al. 1972, Devonshire et al. 2010, van Praag et al. 2000, Li et al 2012). Furthermore, the few works that have studied cerebellar plasticity induced by differential experiences revealed a similar structural malleability of Purkinje and stellate cell dendritic trees. For example, rats submitted to motor skill learning have more synapses per Purkinje cell in relation to age-matched exercise and inactive controls (Kleim et al. 1997a, Black et al. 1990, Federmeier et al. 2002). Apparently, these changes in the number of synapses per neuron occur due to an increased number of terminal branches of parallel fibers (axons from granule cells) and terminals from climbing fibers (olivocerebellar projections) (Anderson et al. 1996). Moreover, these plastic changes are accompanied by a greater glia/Purkinje cell ratio, together with a larger volume of the molecular layer (Black et al. 1990, Anderson et al. 1994). Consistent with these data, another study reported that rats subjected to similar motor tasks exhibited significant increases in dendritic density (more intersections according to an overlay of concentric rings; Sholl's method) compared to animals that were subjected to voluntary motor activity (Kleim et al. 1997b). Conversely, the Purkinje cells (PCs) of mice reared under movement restriction had a significant reduction in dendritic complexity and spine density

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compared both to animals subjected to motor activity and to control animals, and this reduced complexity was closely associated with decreased exploratory behavior (Pysh and Weiss 1979, Pascual et al. 1998). Taken together, these data show that cerebellar architecture is highly plastic in response to motor training.

A second strategy, now classic, that demonstrates that experience can modify the neuronal cytoarchitecture is so-called environmental enrichment. In this paradigm, animals voluntarily interact in an environment of social interaction that is coupled with various possibilities for exploration in a complex, more natural physical environment that is enriched with tunnels, mazes, string height, wheels, diversely textured surfaces, and different olfactory stimuli. Under these environmental conditions, cortical or hippocampal neurons exhibit significantly increased dendritic arborization, more synapses per neuron and greater spine density, along with other neurochemical changes, compared to their aged-matched controls (Nithianantharajah and Hannan 2006, Leuner and Gould 2010). Likewise, animals raised in social isolation generally show an opposite effect (Will et al. 2004).

On the other hand, numerous studies performed in animals and humans have provided substantial evidence to support the participation of the cerebellum in several cognitive and emotional dysfunctions. For example, there is a relationship between vermal cerebellar abnormalities and certain psychopathological illnesses, comprising attention deficit hyperactivity disorder, schizophrenia, bipolar disorder, depression, anxiety, and autism (Del Bello et al 1999, Ichimiya et al 2001, Loeber et al 2001, Kaufmann et al 2003, Beversdorf et al 2005, Schutter and Van Honk 2005, Mackie et al 2007, Tavano et al 2007, Kinney et al 2008). The relationship between these psychiatric disorders and cerebellar structures are not surprising when one considers that the cerebellar vermis indirectly establishes a substantial number of connections with the limbic structures, including hypothalamus, amygdala, hippocampus and orbitofrontal cortex (Heath and Harper 1974, Heath et al. 1978, Schutter and van Honk 2005). Since socioemotional and environmental influences can modify brain maturation, it is possible that postweaning experiences may affect the development of cerebellar Purkinje cells, together with changes in the emotional behavior. Thus, in the current study we evaluated whether postweaning enriched or isolated environments change vermal dendritic PC morphology (sub-lobules VIa or VIb) (Larsell 1952), and whether this structural change are accompanied by anxiolytic or anxiogenic behaviors, as evaluated in the elevated-plus maze (EPM) (Pellow and File 1986, Walf and Frye 2007).

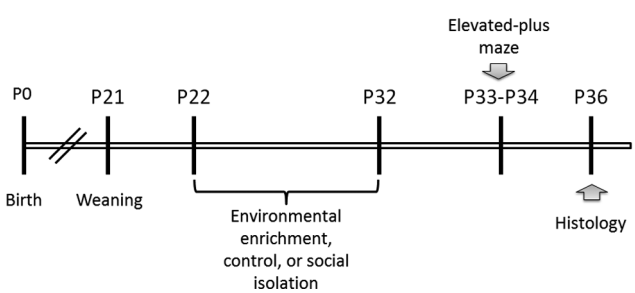

Fig. 1. Timeline showing the experimental design. (P) postnatal age (days).

METHODS

Study design

Pregnant Sprague-Dawley male albino rats were housed individually in laboratory cages (47 × 26 × 15 cm) and maintained under controlled conditions: light (12/12 h), temperature (21 ± 2ºC), and humidity (60 to 70%), with food and water available ad libitum. Once born, pups were cross-fostered and maintained undisturbed during the first 21 postnatal days (breastfeeding period). At 21 postnatal days (P21), the animals were weaned; on the following day (P22), they were randomly assigned to three experimental groups: enriched environment (EE, *n*=14), social condition (SC, *n*=15), and isolation condition (IC, *n*=12). The animals belonging to the EE group were placed in an acrylic chamber (100 × 100 × 100 cm) with various objects inside (ramps, tunnels, wheels, and ropes to climb) three times a day, one hour each (09:00 AM, 01:00 PM and 05:00 PM). Thus, these animals not only interacted with a socially enriched environment but also a physically complex one. SC animals were placed in standard laboratory cages (50 × 30 × 25 cm), 3–4 pups per cage. Finally, IC animals were kept undisturbed in individual cages (30 × 18 × 12 cm, handled only once a week to clean their cages). All three experimental groups were submitted to these different environments in separate rooms for 10 consecutive days (P22–P32) (see timeline in Fig. 1).

Elevated-plus maze

Between P33 and P34, all animals were tested on the EPM. The EPM was constructed of black Plexiglas and consisted of two open arms (60 × 6 cm) and two closed arms (60 × 6 × 14 cm). The device was mounted on a fixed base, 41.5 cm above the floor. Each animal was placed in the center of the EPM and allowed to freely explore the maze for 5 minutes. The number of entries into the open arms were recorded and expressed as a percentage of the total number of entries into any of the four arms. The EPM presents the animal with a choice between the exploration of novel, open environments and the safety of closed spaces. Animals that explored the open arms more frequently were considered less anxious than animals that remained in the enclosed arms (Walf and Frye 2007). Anxiety-like behavior was recorded under dim light by a digital camera (Logitech Quick Cam 9.5.0) situated 50 cm above the EPM. Animals were treated and housed in accordance with the "Principles of Laboratory Animal Care" (NIH publication Nº 86-23, revised 1985), and experimental protocols received approval from the local animal ethics committee.

Purkinje cell morphology

At P36, male rats were weighed and sacrificed under deep ether anesthesia. The cerebella were carefully dissected, fixed and stained in Golgi-Cox-Sholl solution. After 60 days of impregnation, cerebella were dehydrated in 50% alcohol-acetone and 50% alcohol-ether, embedded in celoidin, and hardened with chloroform vapors (Merck). Parasagittal sections (100–120 μm thick) were obtained from the cerebellar vermis, mounted on slides, cleared with α-terpineol, covered with Canadian balsam (Merck), and cover slipped for light microscopic analysis (Olympus CX-31) (Fig. 2). Vermal sections were examined in sequence moving in the rostral to caudal direction. To sample equivalent cerebellar Purkinje cells, in all animals we analyzed sub-lobules VIa or VIb according to Larsell´s terminology (Larsell 1952). A total of 1 033 Purkinje cells were morphometrically analyzed: SC: n=298 (at least 16 per animal), IC: n=325 (at least 24 per animal); EE: n=410 (at least 26 per animal). To assure consistent and reliable neuronal sampling, all of the Purkinje cells that were assessed met the following parameters: homogeneous impregnation, symmetrical dendritic trees, dendritic arbor parallel to the plane of section, and the absence of dendritic breaks. Selected neurons meeting the above criteria were drawn under the Lucida camera (400×) attached to the microscope, and the dendritic tree of each Purkinje cell was

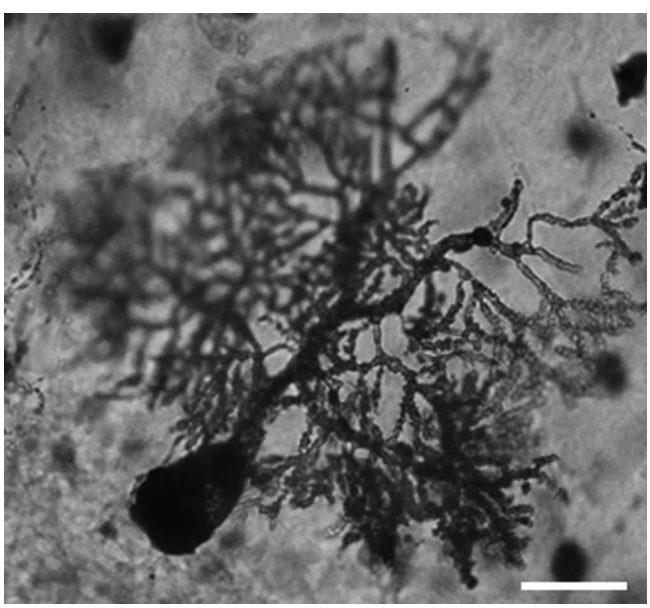

Fig. 2. Golgi-stained cerebellar Purkinje cell typically observed in vermal lobule VI. Scale bar is 30 μm.

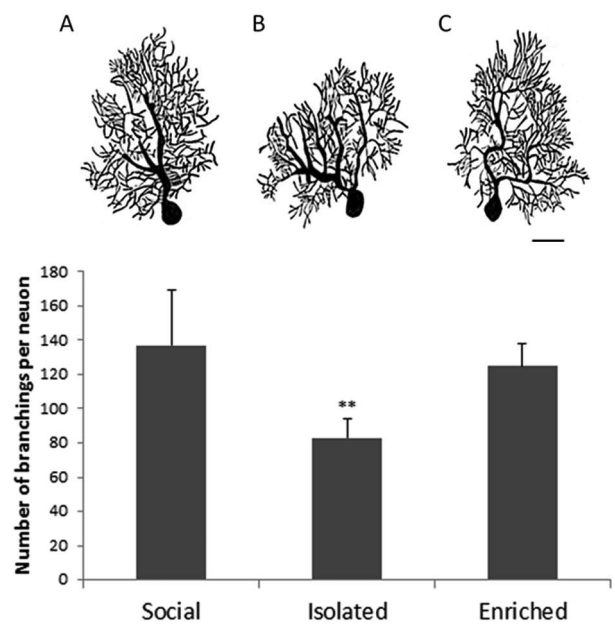

Fig. 3. (A, B, C) representative Purkinje cells drawn under camera lucida of social (control), isolated, and enriched animals, respectively (scale bar is 50 μm). Bottom panel: graphic showing a quantitative analysis of the number of dendritic branches per neuron in social, isolated and enriched animals (means ± SD; $^{**}P$<0.01, ANOVA-test).

drawn and analyzed by counting the number of (1) total dendritic branches (quantified centrifugally from proximal to distal dendrites; considering the large number of dendritic branches Purkinje cells have, we only quantified the total number of branches per neuron), and (2) dendritic intersections per concentric ring, using Sholl's concentric ring analysis (Sholl 1953; see Fig.4).

Statistical analysis

Experimental data were statistically analyzed with a one-way ANOVA test. When significant differences ($P<0.05$) were detected, analyses were complemented *post-hoc* with the Scheffé test (STATA 9.1 software).

RESULTS

As shown in Figure 3, Purkinje cells from animals kept in an isolated environment during the early postweaning period exhibited significantly fewer dendritic branches than their age-matched control (social) (**$P<0.01$, ANOVA test). However, contrary to our expectations, the Purkinje cell dendritic branching of enriched animals showed no significant changes compared to the aged-matched controls. Performing a more detailed morphometric dendritic tree analysis, specifically examining the number of dendritic intersections per concentric ring, social isolation primarily affects the growth in the most distal branches (Fig. 4; *$P<0.05$, ANOVA-test). Again, no changes generated by the enriched environment were detected. Consistent with the morphological data, the percentage of animals that entered the open, "risky" arms of the elevated-plus maze was significantly lower in isolated rats than their age-matched social or enriched animals (Fig. 5; *$P<0.05$, ANOVA-test), suggesting that the isolated animals exhibited anxiety-like behavior.

DISCUSSION

In the present study, it was observed that vermal Purkinje cells of animals reared in a socially isolated environment during the early postweaning period showed significant less dendritic arborization than their age-matched controls. Furthermore, the enriched environment did not produce major changes in this morphological variable. Additionally, consistent with cellular data, only isolated animals exhibited anxious behavior in the elevated-plus maze.

The deleterious impact of social isolation on Purkinje cell development is consistent with other studies, confirming the vulnerability of these cells to stressful postnatal experiences. For example, rats exposed to

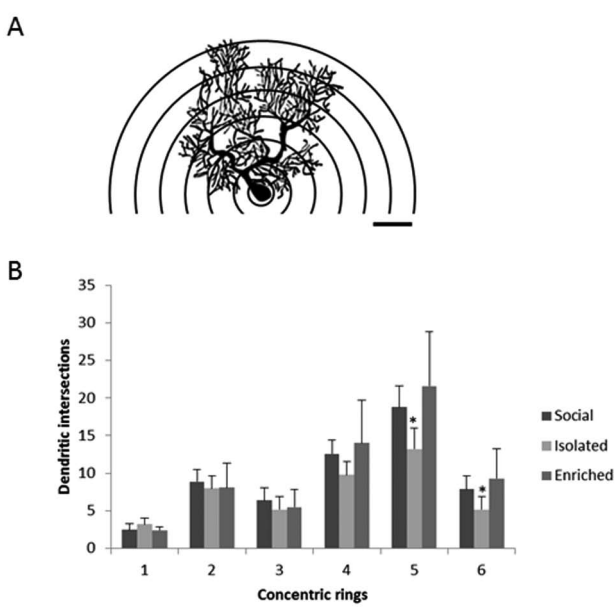

Fig. 4. (A) Sholl's method used to quantify the number of dendritic intersections per concentric ring in social, isolated and enriched animals (scale bar is 50 μm). (B) Dendritic intersections per concentric ring (means ± SD; *$P<0.05$, ANOVA-test).

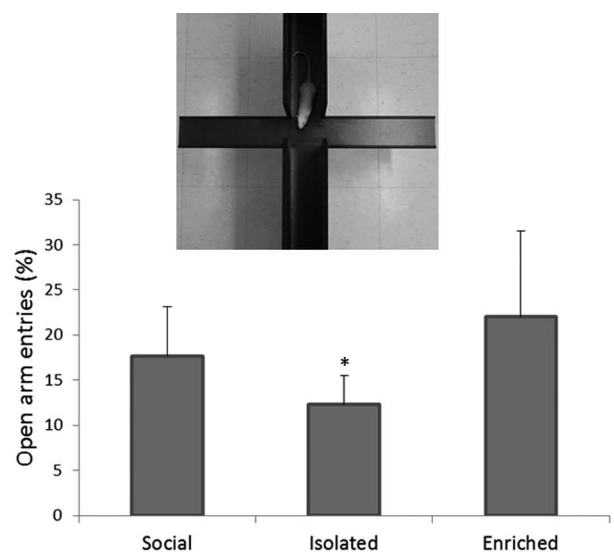

Fig. 5. Anxiety-like behavior in the elevated plus maze (top panel) and the percentage of open arm entries per animal in social, isolated and enriched animals (means ± SD; *$P<0.05$, ANOVA-test).

body movement restriction between postnatal days 18 (P18) and P31 showed a significant developmental delay in Purkinje cell dendritic outgrowth in relation to the aged-matched control animals (Pascual et al. 1998). In this other study, animals were socially deprived slightly earlier (P18–P32) than in the current one (P22–P32), and a different experimental design was used, i.e., restriction of movement into small compartments (10 × 10 × 19 cm) versus social isolation in normal laboratory cages (47 × 26 × 15 cm). Although neither of these studies analyzed plasma corticosterone levels, the experience was likely more stressful in the first study than in the current one and the morphological changes thus appear to be greater than in the present study (~40% and ~25%, respectively). The vulnerability of vermal cerebellar PCs in response to social isolation was also supported by the fact that isolated P18–32 rats exhibit a significant reduction in the expression of the calcium-binding protein calbindin-D28k (Pascual et al. 1999).

Because PCs are the unique projection neurons from the cerebellar cortex and, in this way, modulate all the cerebellar output through deep cerebellar nuclei, it is possible that the deleterious impact of social isolation on dendritic morphology reported in the current work has different functional consequences depending on what other neural networks these cells modulate. In that respect, it is important to note that mammalian vermal cerebellar PCs establish indirect connections with the amygdala, which is the main nuclear complex involved in emotional processing and valence. For example, electrical stimulation of the fastigial nucleus (which receives direct vermal inhibitory Purkinje cell input) evoked strong neuronal responses in both the amygdala and the septo-hippocampal network, and that vermal electrical stimulation modified the activity of amygdalae networks (Snider et al. 1976, Heath et al. 1978). Furthermore, the fact that human cerebellar lesions produce significant functional amygdalae changes, together with the fact that vermal lesions in rats impair the acquisition of classically conditioned bradycardia and fear, support the role of the cerebellar vermis in emotional processing (Supple and Leaton 1990a,b). Consistent with these reports, hotfoot mutant mice, characterized by a deficiency in the synapses made by parallel fibers on the Purkinje cell dendritic tree, exhibit significant impairments in the capacity to learn fear (but nor motor) conditioned responses (Sacchetti et al. 2005). Thus, the current results are consistent with these findings, as the changes in vermal Purkinje cell morphology induced by social isolation are in close association with the anxiety-like behaviors assessed in the elevated-plus maze.

Conversely, animals exposed to an enriched environment showed no significant differences when compared to the age-matched controls. The lack of significant dendritic changes observed in environmentally enriched animals differs from the changes observed by Floeter and Greenough (1979), who reported that monkeys maintained in a "semi-natural" (enriched) environment exhibited more profuse PC dendritic branching and spines in relation to isolated animals. Likewise, Pysh and Weiss (1979) observed that mice subjected to motor training exhibited Purkinje neurons with significantly more dendritic arborization than isolated age-matched controls. In addition, Kleim and coauthors (1997a,b) reported that the dendritic morphology of impregnated cerebellar stellate cells in adult rats submitted to a complex environment, called "acrobatic condition", exhibited greater dendritic arborization than "controls" exposed to motor activity. However, these studies differ markedly from ours regarding the paradigm employed and type of cerebellar cell analyzed (stellate *versus* Purkinje cells). The classic complex environment that we used allows free exploratory activity and social interactions for enriched animals, experiences that are less exigent and more variable than the motor training used by previous authors. Furthermore, in the current study, environmental stimulation was applied during an ontogenetic stage when PC dendritogenesis is nearly over. In fact, as was shown by McKay and Turner (2005), the period of increased speed in rat PC dendritogenesis ranges from postnatal days 6 to 21. Because the animals in the current study were submitted to environmental enrichment between postnatal days 22–32, it is possible that the dendrites at this stage have lower plasticity in response to experiences that involve enriched sensorimotor exploratory activities and social interactions. It is possible that if the rats had undergone more demanding sensorimotor experiences, as in the paradigm of acrobatic condition used by others, structural changes would have detected. Another explanation that would account for the lack of environmental enrichment effects on PC dendritogenesis is that, in the current study, the environmental stimulation was performed in normal animals, whose neurons appeared to be less sensitive to environmental cues. In fact, we

recently showed that PC dendritogenesis stunted by prenatal stress was significantly recovered by an environmental enrichment paradigm similar to that used in the current study (submitted).

CONCLUSION

The present study indicates that vermal Purkinje cell dendritic tree are significantly altered by postweaning social isolation but not by environmental enrichment during the early postweaning period. Furthermore, consistent with other reports, animals exposed to isolation exhibit anxiety-like behaviors in the elevated plus maze, supporting the role of cerebellar vermis in emotional behavior (Supple and Leaton 1990a,b, Sacchetti et al. 2005).

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