
Effects of neonatal maternal deprivation and postweaning environmental complexity on dendritic morphology of prefrontal pyramidal neurons in the rat

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Abstract. It has been reported that periodic maternal separation in rats leads to a variety of endure behavioral, neurochemical and microstructural sequelae associated with the pathophysiology of anxiety disorders. Since it has been proposed that these changes might be permanent, we examined whether environmental complexity aid to recover the structural dendritic impairment induced by neonatal maternal deprivation in the medial prefrontal cortex of the rat. In addition, the anxiety-like behavior was assessed in the elevated plus-maze. Repeated maternal separation between postnatal days 6–21 (3 hours daily) significantly reduced the dendritic material in layer II/III pyramidal neurons and induced anxiety-like behaviors in the elevated plus maze. Furthermore, environmental stimulation (twice a day, 1 h each) during 12 consecutive days (postnatal days 23–35) failed to recover the neuronal and behavioral disorders induced by neonatal maternal separation. The results demonstrated that (i) neonatal maternal separation severely altered pyramidal dendritic outgrowth in close association with anxiety-like behavior assessed in the elevated plus maze, and (ii) postweaning environmental complexity was unable to recover neither the prefrontocortical neuronal impairment nor the novelty-induced anxiety-like behavior triggered by early maternal deprivation.

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Key words: dendritic impairment, environmental complexity, maternal separation, medial prefrontal cortex, elevated-plus maze

INTRODUCTION

Clinical studies have suggested that disruption of the mother-infant socioemotional bond during the first year of life constitutes a severe early life stressful experience that may contribute to the pathophysiology of some psychiatric disorders, such as anxiety substance abuse disorders and antisocial behavior (Foley et al. 2004, Lipman et al. 2001, MacMillan et al. 2001). In addition, several studies performed with laboratory animals have clearly demonstrated that repeated maternal separation (3–6 h) during the first 2–3 weeks of life have long-term consequences on endocrine, behavioral, and brain development later in life. For example, maternal separation (MS) in rats showed stress hyper-reactivity, anxious behavior in the elevated plus-maze test (EPM), anhedonia, increased ethanol consumption, and hyper-reactivity of the hypothalamic-pituitary-adrenal (HPA) axis in response to stress (Caldji et al. 2000, Francis and Meaney 1999, Hofer 1996, Huot et al. 2001, Wigger and Neumann 1999). Most of these behavioral and endocrinal dysfunctions persist throughout adulthood (Daniels et al. 2004). In addition, it has been shown that these long-term effects induced by MS appear to depend upon changes in the structure and function of the medial prefrontal cortex (mPFC) neurons, which are involved in the regulation of the stress response (Spencer et al. 2005, Williams et al. 2006). In fact, mPFC pyramidal neurons of MS rats showed changes in dendritic branching, spine density, and monoaminergic fiber innervation (Bock et al. 2005, Helmeke et al. 2001, Ovtscharoff and Braun 2001, Poeggel et al. 2003), indicating that early adverse socioemotional experiences, such as maternal deprivation, interfere with the development of neuronal and synaptic composition of the mPFC.

On the other hand, several reports in laboratory animals indicated that environmental complexity (EC) enhanced the individual's brain and behavioral development. For instance, rats exposed to EC showed an increase in brain weight, cortical depth (Rosenzweig and Bennett 1996), dendritic outgrowth (Green et al. 1983), synaptogenesis (Comery et al. 1996) and neurogenesis (Olson et al. 2006). Additionally, it has been demonstrated that environmental stimulation during the postweaning period reverses the effects of MS on both HPA axis and behavioral responses to stress (Francis et al. 2002). However, there are no studies examining whether a similar environmental manipula-

tion may compensate neuronal impairments induced by neonatal MS. Thus, the objective of the present study was to determine (i) whether MS alters dendritic length and branching of mPFC pyramidal cells, which are involved in the regulation of complex socio-emotional behaviors, and (ii) if postweaning EC ameliorates the MS-induced neuronal impairments. In addition, we evaluated the effect of both MS and EC on the anxiety-like behavior in the elevated plus-maze, the most widely screening assay employed for anxiolytic and anxiogenic agents in rodents.

METHODS

Animals and experimental design

Adult female Sprague-Dawley rats were mated with colony breeder male rats. One week before birth, dams were individually housed in standard laboratory Plexiglas cages (40 × 30 × 18 cm), with sawdust as bedding in an air-conditioned room under standard laboratory conditions: 12 h light-dark cycle (lights on at 7:00 AM), 22 ± 2°C, and free access to pellets and water. The date of birth was designated as postnatal day 0 (P0). One day after delivery, litters (10–14 pups) were cross-fostered and culled to 10 pups each (5 males and 5 females) and housed together with a mother in standard rat cages (50 × 30 × 20 cm). Half of the litters were randomly assigned to the maternal separation group (MS, *n*=20) and the other half to the mother-reared control group (MR, *n*=20). MS pups were separated every day from their dams for 3 h (1:00–4:00 PM) from P6 until P21. During that time MS pups were kept individually in small aluminium foil nests (12 cm diameter, 15 cm height) filled with clean bedding and placed on a 36°C heating blanket. There was no visual, tactile, olfactory or auditory communication between the pups during the deprivation period. After isolation, the pups were placed back in their home cage. MR pups were left undisturbed, except for cage cleaning 2 times a week. At P21, all animals were weaned and housed in groups of 4 rats per cage under standard laboratory conditions. At P23, half of the MS (*n*=10) and MR (*n*=10) animals were randomly selected (MS-EC and MR-EC groups) and transferred to an enriched environment (EC), twice a day for 1 h each during 12 consecutive days (P23–P35). The EC consist of a large Plexiglas/aluminum cage (100 × 100 × 70 cm) with a variety of

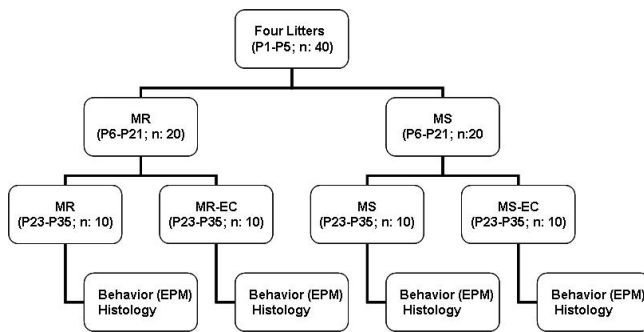


Fig. 1. Experimental design. (P1–P35) postnatal days; (MR) maternally-reared animals; (MS) maternally-separated animals; (MR-EC) maternally-reared animals submitted to environmental complexity; (MS-EC) maternally-separated animals submitted to environmental complexity; (EPM) elevated-plus maze.

objects, such as tunnels, shelves, running wheels, ladders, and different kinds of manipulable objects (e.g., jars, glass balls, wooden objects) that were changed twice a week in order to avoid habituation. At P36, all animals were behaviorally evaluated and, the following day, sacrificed under deep pentobarbital anesthesia (50 mg/kg, i.p.). The experimental design is shown in Fig. 1.

All experimental protocols followed guidelines given at “Principles of laboratory animal care” (NIH publication No. 86-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee at Universidad Católica del Maule.

Elevated plus maze

At P36, all animals were evaluated in the elevated plus maze (EPM). The EPM was made of black Plexiglas and consisted of 2 open arms (50 × 15 cm) and 2 closed arms (50 × 15 × 20 cm). The apparatus was mounted on a fixed base, 50 cm above the floor. Each rat was placed in the center of the EPM facing an open arm, and was allowed to freely explore the EPM for 5 minutes. The number of entries and the time spent in the open arms was recorded. Measures were carried out under dim light condition between 8:00–11:00 PM by 2 observers, who were “blind” to the rearing condition of the pups. The EPM was cleaned between each pup with 5% ethanol to remove odor cues. This test has been validated in rodents to evaluate anxiety related behaviors (Andreatini and Bacellar 2000).

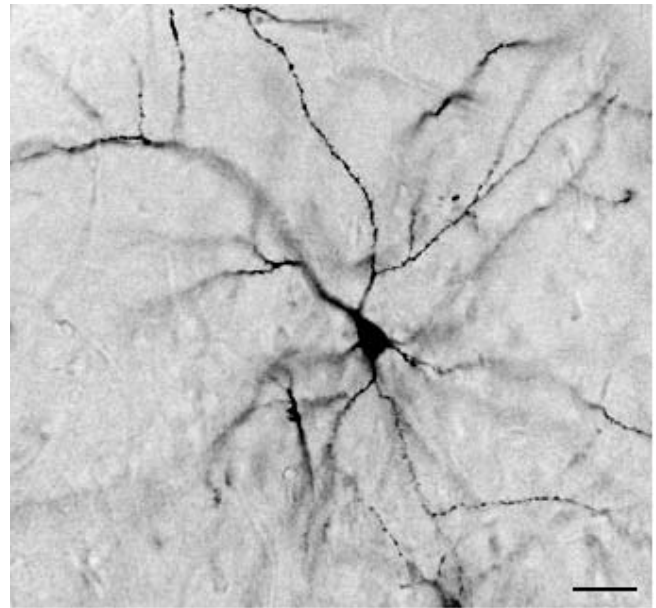


Fig. 2. Representative Golgi-impregnated pyramidal neuron of the rat's mPFC. Scale bar is 45 μ m.

Neuronal evaluations

In order to study the impact of periodic MS on mPFC pyramidal dendritic development, the brains were stained with the Golgi-Cox-Sholl procedure (MR, $n=10$; MS, $n=10$; MR-EC, $n=10$; MS-EC, $n=10$; Sholl 1953; Fig. 2), technique that stains 1–5% of randomly distributed neurons (Pasternak and Woolsey 1975). After 60 days of slow impregnation, brains were dehydrated in ethanol-acetone and ethanol-ether solutions (50%/50%), embedded in celoidin, and hardened in chloroform vapors. Coronal sections (120 μ m thickness) were cut using a sledge microtome and mounted with DPX (Fluka).

In order to qualify for the morphometrical evaluations, pyramidal cells should fulfill the following criteria: (i) have a well-defined pyramidal shape, (ii) show an adequate staining of the soma and dendrites, (iii) have no extensive processes overlapping neighboring neurons, and (iv) be located in a cortical strip between 100 and 350 μ m under the pial surface (2.2 mm and 3.2 mm anterior to bregma; Paxinos and Watson 1998). The first 10–12 cells per brain section meeting the above criteria were traced with the aid of a camera lucida (Olympus, model BH-DA-LB, 400 \times magnification). Two dendritic variables were quantified: (i) the mean basilar dendritic length/neuron by means of an electronic map reader (Pascual et al.

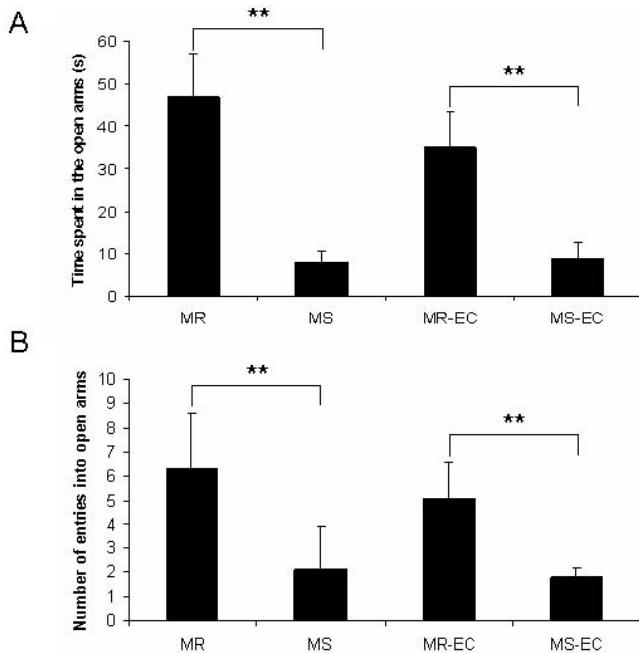


Fig. 3. Effect of maternal separation and environmental complexity on (A) time spent (s) and (B) number of entries into the open arms of the elevated-plus maze. (MR), (MS) maternally-reared and maternally-separated animals, respectively; (MR-EC), (MS-EC) maternally-reared and maternally-separated animals submitted to environmental complexity, respectively. ** $P < 0.01$ (means \pm SD).

1996), and (ii) the mean basilar dendritic ramification/order, determined according to the method of Coleman and Riesen (1968). This method states that dendrites coming out directly from the cell body are considered as first order, direct branches from first order dendrites are considered as second order, and so on. A total of 391 cells were sampled from mPFC (MR: 87; MS: 98; MR-EC: 94; MS-EC: 112). All cells were drawn and analyzed by the same person in a blind manner to maximize reliability.

Statistical analysis

Unpaired two-tailed *t*-test statistical analyses were performed. Results were expressed as means \pm SD and differences were considered significant when $P < 0.05$.

RESULTS

Rats exposed to maternal separation (MS group) did not explore the open arms of the EPM as much as the control rats (MR group). In addition, MS rats spent less

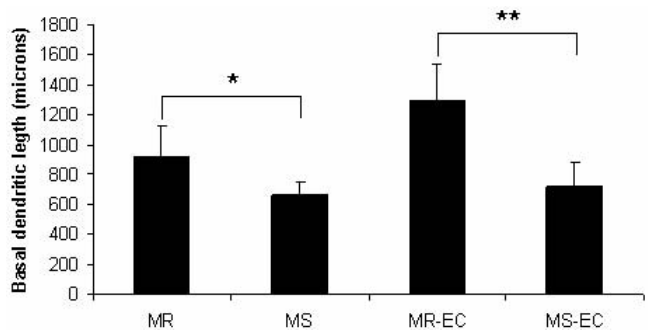


Fig. 4. Effect of maternal separation and environmental complexity on basal dendritic length. (MR), (MS) maternally-reared and maternally-separated animals, respectively; (MR-EC), (MS-EC) maternally-reared and maternally-separated animals submitted to environmental complexity, respectively. * $P < 0.05$, ** $P < 0.01$ (means \pm SD).

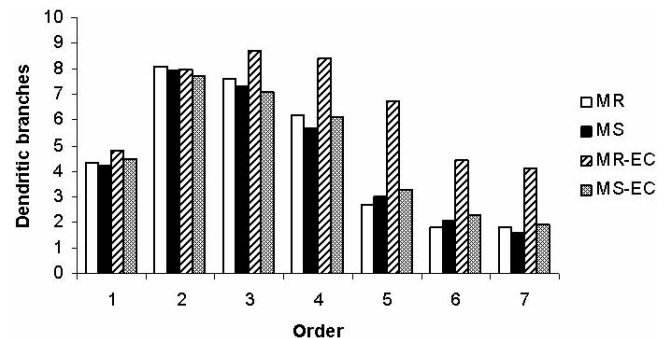


Fig. 5. Effect of maternal separation and environmental complexity on basal dendritic branching. (MR), (MS) maternally-reared and maternally-separated animals, respectively; (MR-EC), (MS-EC) maternally-reared and maternally-separated, animals submitted to environmental complexity, respectively. * $P < 0.05$, ** $P < 0.01$.

time and did not enter the open arms as many times as the MR control rats (Fig. 3A and B, respectively; $P < 0.01$). Unexpectedly, these behavioral alterations appeared to be permanent, since MS environmentally stimulated animals (MS-EC group) did not differ from MS non-stimulated rats (Fig. 3A and B). On the other hand, MS rats displayed a decrease in dendritic length compared to the MR control group ($\sim 28\%$; $P < 0.05$; Fig. 4). Again, the EC condition was unable to ameliorate the dendritic impairment induced by MS ($\sim 44\%$; $P < 0.01$; Fig. 4). Surprisingly, MS did not affect the number of dendritic branches per neuron (Fig. 5). Only control rat pyramidal cells submitted to EC condition (MR-EC group) showed a significant increase in dendritic branching (dendritic orders 4–7; $P < 0.05$, $P < 0.01$; Fig. 5). Those results indicate that post-

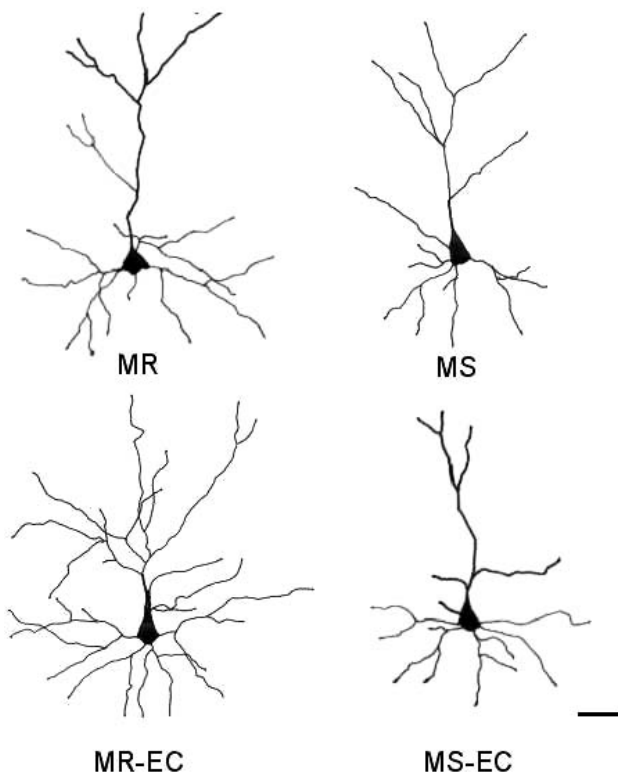


Fig. 6. Representative camera lucida drawings of prefrontocortical layer II/III pyramidal cells. (MR), (MS) maternally-reared and maternally-separated animals, respectively; (MR-EC), (MS-EC) maternally-reared and maternally-separated animals submitted to environmental complexity, respectively. Scale bar is 50 μ m.

weaning environmental complexity appears to be unable to offset the neurobehavioral alterations induced by early maternal separation. Representative camera lucida drawings of mPFC pyramidal cells are shown in Fig. 6.

At weaning, measurements of body weight did not show significant differences between the experimental groups (MR: 40.6 ± 4.2 ; MS: 43.7 ± 3.8 ; n.s.)

DISCUSSION

We demonstrated that repeated maternal separation during the early preweaning period (P6–P21) induced a significant dendritic impairment in mPFC pyramidal neurons and a significant reduction in the rat's exploratory behavior in the EPM, alterations that were not attenuated by postweaning environmental stimulation.

To our knowledge, there is only one report (Bock et al. 2005) analyzing the impact of early maternal deprivation

on mPFC pyramidal cells. Bock and coauthors (2005) examined the effects of brief and repeated maternal deprivation periods (P1–P3, P5–P7, or P14–P16) on dendritic length development in the rat's mPFC. These authors showed, opposite to our results, that repeated maternal deprivation during the ontogenetic stage P5–P7 and P14–P16 induced a significant increase in dendritic length of layer II/III pyramidal neurons. In that report, MS was induced in alternated and shorter periods (1 h); in our study, the pups were submitted to MS during continued and prolonged periods (3 h). This is a critical methodological difference, since it has been demonstrated that short periods of MS actually enhance maternal care and leads to an increased exploratory behavior, less defecation, and a reduced taste neophobia on handled pups (reviewed in Sánchez et al. 2001) and a to a more effective hypothalamic-pituitary-adrenal (HPA) axis regulation (Liu et al. 2000). In addition, short alternating periods of MS are similar to normal wild-type maternal behavior, since dams often leave the nest 15–30 min to forage (Jans and Woodside 1990). Therefore, short periods of MS could enhance dendritic outgrowth (or avoid its damage), as reported in the study of Bock and others (2005). On the contrary, longer periods of MS alter maternal care quality and impair HPA negative feedback (Berger et al. 2000, Brake et al. 2004). These findings suggest that postnatal manipulations performed at different periods can induce opposite effects on neuronal development.

Even though the mechanisms involved in neuronal alterations induced by maternal deprivation are not well known, we speculate that the endocrine response induced by stress, and/or changes in neurotrophin expression, could be involved. One of the most well characterized biological features of maternal deprivation in laboratory animals (McCormick et al. 1998) and human infants (Buss et al. 2003, Gunnar et al. 2001) is the hyper-reactivity of the HPA axis in response to socio-emotional stressful experiences. As a consequence, glucocorticoids (GCs) levels are extremely elevated (Kalinichev et al. 2002), exerting a broad range of deleterious effects on developing GCs receptor expressing neurons, i.e., prefrontal (Meaney and Aitken 1985) or hippocampal pyramidal cells (Meaney et al. 1988). It has been demonstrated that chronic social stress or direct administration of GCs produce dendritic atrophy in prefrontal (Wellman 2001) and hippocampal (McKittrick et al. 2000) neurons.

Alternatively, it has been demonstrated that the expression of brain ornithine decarboxylase (ODC), the first and rate-limiting enzyme in the synthesis of endogenous polyamines (Kuhn et al. 1978), is dramatically decreased in MR rats. Since ODC is involved in neural growth and differentiation (Shimizu et al. 1965, Tabor and Tabor 1984), the reduced ODC activity observed in MS rats could induce dendritic outgrowth abnormalities in specific neuronal groups (Soulet and Rivest 2003). On the other hand, since MS significantly decreases growth hormone (GH) secretion (Kuhn et al. 1978), and GH can regulate neuronal differentiation (Turnley 2005), it is possible that the dendritic outgrowth impairment observed in the current study could be induced by low GH levels in the brain. Finally, since dendritic outgrowth is regulated by the action of brain-derived neurotrophic factor (BDNF; McAllister 1999), and MS animals express lower levels of BDNF on both prefrontal and hippocampal neurons (Roceri et al. 2002, 2004; but see Greisen et al. 2005), we cannot discard that the downregulation of BDNF expression could induce neuronal atrophy in MS rats. Further preclinical studies, at the molecular and cellular levels, are required in order to prove these pathophysiological relationships.

Environmental complexity (EC) was not effective recovering both dendritic impairment and exploratory behavior in the EPM. Since EC is a condition of inanimate and social over-stimulation, it is possible that the social challenge and the novel environment could act as a stressful condition in vulnerable animals, such as MS pups. As a consequence, the “therapeutic” effects of the EC on dendritic and behavioral parameters described in other studies (Green et al. 1983, Rosenzweig and Bennett 1996) could not be effective in previously deprived animals. This suggestion is consistent with the fact that EC experienced by control rats (MR) significantly promoted mPFC dendritic development (see Figs 4 and 5). However, the dramatic neuronal plasticity observed in mPFC neurons of MR animals contrasted with previous data reported by Kolb and colleagues (2003), study that failed to demonstrate any significant change in mPFC dendritic outgrowth of rats submitted to EC. The most critical methodological difference between both studies was the age of the animals at the time of the EC stimulation. We submitted the rats to EC during the early postweaning period (P23–P35), when mPFC neurons are highly plastic and modifiable (Wedzony et al. 2005); in contrast, Kolb and others (2003) performed the EC manipulation in

adult rats (P90), when the critical dendritic developmental period is currently over (Petit et al. 1988).

It is possible that EC, in combination with antidepressant drugs, may be effective in promoting dendritic outgrowth. This hypothesis is supported by at least 2 evidences. First, paroxetine or reboxetine, administered during P21–P28, reversed most of the emotional abnormalities detected in MS rats (Huot et al. 2001, Ladd et al. 1999). Second, BDNF expression, the major neurotrophin involved in dendritic outgrowth and plasticity (McAllister 1999), is increased in response to EC (Spires et al. 2004) and antidepressant drugs (such as desipramine or phenelzine) (Dias et al. 2003). Accordingly, it is possible that the administration of antidepressant drugs combined with EC could attenuate the dendritic impairment induced by neonatal MS. Further preclinical studies will be performed in order to prove these hypotheses.

The EPM test is widely used to assess anxiety-like behaviors and is based on unconditioned responses to a potentially dangerous environment, i.e., the avoidance of open and novel spaces. We observed, consistent with previous reports, that MS rats entered the EPM open arms less times compared to MR controls (Fig. 3; Daniels et al. 2004, Boccia and Pedersen 2001, McIntosh et al. 1999, Romeo et al. 2003). The fact that the anxiety-like behavior continued even after 30-days of resocialization or EC, suggests that early neonatal MS produced endured behavioral sequelae. Opposite to our results, Hellemans and coauthors (2004) showed that EC significantly ameliorated the anxiety-like behavior induced by postweaning social isolation. In that report, social deprivation was imposed later in development (postweaning period) when the environmental hostile experiences may be less harmful. In our study, maternal deprivation was imposed during preweaning, a more vulnerable period. These opposite results suggest how important and critical is the time when the deprivation period begins.

CONCLUSION

The present data demonstrated that neonatal maternal deprivation impaired postnatal mPFC neuronal development and caused long-term anxiety disorders. In addition, postweaning environmental enrichment was unable to ameliorate any of the neurobehavioral variables studied, suggesting that MS induces permanent neurobehavioral sequelae.

ACKNOWLEDGEMENT

This research was supported by Grant FONDECYT 1020114

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Received 19 January 2007, accepted 4 July 2007

