Mild prenatal protein malnutrition increases α_{2C}-adrenoceptor expression in the rat cerebral cortex during postnatal life

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Abstract

Mild reduction in the protein content in the diet of pregnant rats from 25 to 8% casein, calorically compensated by carbohydrates, does not alter body and brain weights of rat pups at birth, but results in significant changes of the concentration and release of cortical noradrenaline during postnatal life, together with impaired long-term potentiation and memory formation. Since some central noradrenergic receptors are critically involved in neuroplasticity, the present study evaluated, by utilizing immunohistochemical methods, the effect of mild prenatal protein malnutrition on the α_{2C}-adrenoceptor expression in the frontal and occipital cortices of 8- and 60-day-old rats. At day 8 of postnatal age, prenatally malnourished rats exhibited a three-fold increase of α_{2C}-adrenoceptor expression in both the frontal and the occipital cortices, as compared to well-nourished controls. At 60 days of age, prenatally malnourished rats showed normal expression levels scores of α_{2C}-adrenoceptor in the neocortex. Results suggest that overexpression of neocortical α_{2C}-adrenoceptors during early postnatal life, subsequent to mild prenatal protein malnutrition, could in part be responsible for neural and behavioral disturbances showing prenatally malnourished animals during the postnatal life.

Keywords: Prenatal malnutrition; Adrenoceptor overexpression; Immunohistochemistry

1. Introduction

Mild reduction of the protein content in the diet of pregnant rats, calorically compensated by carbohydrates, results in apparently normal development in utero of fetuses as assessed by normal maternal weight gain during pregnancy and normal body and brain weights of pups at birth [29]. Nevertheless, this insidious form of protein maternal malnutrition in the rat, the so-called "hidden" prenatal malnutrition [29], results in altered noradrenergic function in the neocortex of the offspring, as revealed by increased concentrations and release of cortical noradrenaline during early postnatal life, followed by decreased cortical release of the neurotransmitter during adulthood [37]. Together with decreased cortical noradrenaline release, the neocortex of adult prenatally malnourished rats shows altered electrophysiological indices, including decreased ability of callosal-cortical synapses to perform temporal summation [37] as well as impaired long-term potentiation induced by tetanizing stimulation [38]. Besides, prenatally malnourished animals presented lower visuo-spatial memory performance, as revealed by a greater number of errors and time spent during task solving in an eighth-arm radial maze [38].

Several studies indicate that central nervous system noradrenaline critically influences long-term potentiation (LTP) in cerebral cortex [21,27] and hippocampus [5,20,28], as well as memory formation [10,13,14,40]. For example, it has been reported that activation of α_2-adrenoceptors [7,12,34], specially the α_{2C}-subtype [1–3,16,17], is related to decreased memory formation. In this regard, a recent neurochemical study reported that at day 8 of postnatal age, prenatally malnourished rats showed increased neocortical α_{2C}-adrenoceptor density as revealed by higher [^{3}H]-rauwolscine binding [38]. However, whether the effects of prenatal malnutrition on long-term potentiation and visuo-spatial memory are related to altered

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noradrenergic function observed in the neocortex of malnourished rats is presently unknown.

The α_{2C}-subtype of adrenoceptors is involved in many physiological processes, such as the presynaptic control of noradrenaline release from central neurons, modulation of dopamine and serotonin release, body-temperature regulation, and sensorimotor integration and cognitive functions, including modulation of the acoustic startle reflex and its prepulse inhibition, isolation-induced aggression, development of behavioral despair, and spatial working memory [1–3,6,18,30–33,42]. Since changes in α_{2C}-adrenoceptors after hidden prenatal malnutrition may be of high relevance for the development and function of the mammalian central nervous system, we studied the expression of α_{2C}-adrenoceptors in the frontal and occipital cortex of normal and prenatally malnourished rats by means of immunohistochemical techniques.

2. Materials and methods

2.1. Animals

The experimental protocol and animal management were in accordance with the NIH Guide for the Care and Use of Laboratory Animals [25] and was approved by the Committee for the Ethical Use of Experimental Animals, INTA, University of Chile. The experiments were carried out on male and female Sprague–Dawley rats born from mothers submitted to rearing procedures already described in the literatures [29,37,38]. Briefly, virgin female rats were fed isocaloric purified diets containing either normal (25% casein, providing 22.5% protein) or low (8% casein, providing 7.2% protein) amounts of protein. Components of purified diets were the following: (i) Normal diet: protein, 22.5%; carbohydrate, 50.2%; fat, 15.0%; vitamin mix, 1.0%; salt mix, 4.7%; water, 1.7%; cellulose, 4.2%; L-methionine, 0.4%. (ii) Low protein diet: protein, 7.2%; carbohydrate, 66.5%; fat, 15.0%; vitamin mix, 1.0%; salt mix, 4.7%; water, 1.0%; cellulose, 4.2%; L-methionine, 0.4%. Both diets provide about 4.3 kcal/g. The dietary paradigm was started 5 weeks prior to mating and continued through pregnancy. Body weight gain of pregnant mothers was controlled periodically. At birth all pups were weighed and litters were culled to eight pups (four males, four females). To limit the malnutrition regimen solely to the gestational period, thus, avoiding malnutrition during the lactation period, pups born from mothers fed the 7.2% protein diet were fostered to well-nourished dams (22.5% protein diet) giving birth on that day; pups born from mothers receiving the 22.5% protein diet were nursed by their own mothers who continued to receive the normal diet. After weaning at 22 days of age, all pups were fed on a standard laboratory diet containing 22.5% protein.

2.2. α_{2C}-Adrenoceptor immunohistochemistry

Normal and malnourished rats of 8 and 60 days of age ($N = 4$ each group, two male and two female, taken from two different litters) were deeply anesthetized with urethane (1.5 g/kg i.p.) and perfused transcardially with freshly made fixative [4% paraformaldehyde, 0.2% picric acid in 0.1 M phosphate buffer (PB), pH 6.9]. The brains were removed from the skulls and postfixed for 14 h by immersion in the same fixative. After rinsing in 0.1 M PB, the brains were dehydrated with graded alcohol solutions and xylenes, and embedded in Histowax. Sagital sections, of 5 μm thickness, were cutted with a Reichert–Jung rotary microtome, collected on slides coated with chromalaun–gelatine and stored until immunostaining.

The immunostaining of the α_{2C}-adrenoreceptor was done in humid chambers following antigen retrieval by treatment with a microwave oven (model H2500, Energy Beam Sciences Inc., MA, USA) and using the Tyramide Signal Amplification system (TSA) kit NEL 700 (Perkin-Elmer Life Sciences Inc., MA, USA) to detect the attachment of the antibody. For antigen retrieval, the de-waxed sections were microwave-irradiated exactly as described before [36]. To quench any remaining free aldehyde group, the sections were incubated for 30 min in 0.1 M glycine dissolved in buffer TNT (0.1% Triton X100/150 mM NaCl/50 mM Tris/HCl, pH 7.4). After blocking for 30 min with freshly prepared blocking solution from the TSA-kit, the sections were incubated (4 h RT and overnight at 4 °C) with a guinea pig antibody against the α_{2C}-adrenergic receptor (Prod. GP 10102, Neuromics, MN, USA). The antibody was diluted (1:500) with TNT containing 1% BSA and 1% normal rabbit serum. After washes with TNT containing BSA and normal rabbit serum, the sections were incubated for 1 h at RT with a 1:1000 dilution of a secondary antibody conjugated with peroxidase (rabbit anti-guinea pig, Zymed Laboratories, CA, USA). After washings with TNT, the sections were incubated at RT with biotinylated-tyramine (TSA-kit, NEN, USA) for exactly 8 min, washed with TNT and incubated for 30 min with streptavidine–peroxidase from the TSA-kit. After washings, the peroxidase reaction was developed using buffered 3,3′-diaminobenzidine tablets dissolved in 0.03% H_2O_2 (Rockland, PA, USA).

In the whole immunostaining procedure, the sections were washed three times for 15 min between each step. One set of sections was used for control staining, substituting the primary antibodies with normal guinea pig serum, or with the α_{2C}-adrenoceptor antibody preincubated with a 10-fold excess of the antigen, provided by Neuromics (control peptide). Counterstain was done with Meyer's hematoxylin and Eukitt was used for mounting. Images were acquired with an Olympus CX31 microscope fitted with a Cool Snap-Pro Color Digital camera controlled by Image Pro (Media Cybernetics, MD, USA). The same software was used to calculate the expression level scores (ELS) of the receptor.

2.3. Statistics

All statistical analyses were performed with GraphPad Instat software (GraphPad Software, Inc., San Diego, CA, USA). For the effect of dietary treatments on body and brain weights and for immunohistochemistry, intergroup comparisons were made utilizing two-tailed unpaired Student's *t*-test.

3. Results

3.1. Effect of dietary treatment on body and brain weights

Measurements of body and brain weights revealed that there were no significant differences in body weight gain of pregnant mothers receiving 7.2 or 22.5% protein diet (data not shown). Full data for the effect of this dietary treatment on maternal weight gain during the first, second and third weeks of pregnancy is published elsewhere [37]. At days 1, 8 and 60 of postnatal life, no significant differences in body weight were found between rats born from mothers receiving 7.2 or 22.5% protein diet (Fig. 1). A similar trend was observed for brain weight of normal and malnourished rats at days 1, 8 and 60 of postnatal life (Fig. 1).

3.2. α_{2C}-Adrenoceptor expression in cerebral cortex

The α_{2C}-subtype of noradrenergic receptors was visualized immunohistochemically with a commercially available antibody previously characterized by Western blots and immunoprecipitation of biologically active protein [41]. Preabsorption of the antibody with the control peptide supplied, resulted in no attachment of the secondary, peroxidase-labeled immunoglobulins, demonstrating the specificity of our staining approach (see Section 2). Expression of α_{2C}-adrenoceptors increased slightly in the frontal and occipital cortices of normal rats during postnatal life, as revealed by the higher immunostaining observed at 60 days of age compared to that of 8-day-old pups (Figs. 2 and 3). In the cortex of both, 8- and 60-day old normal

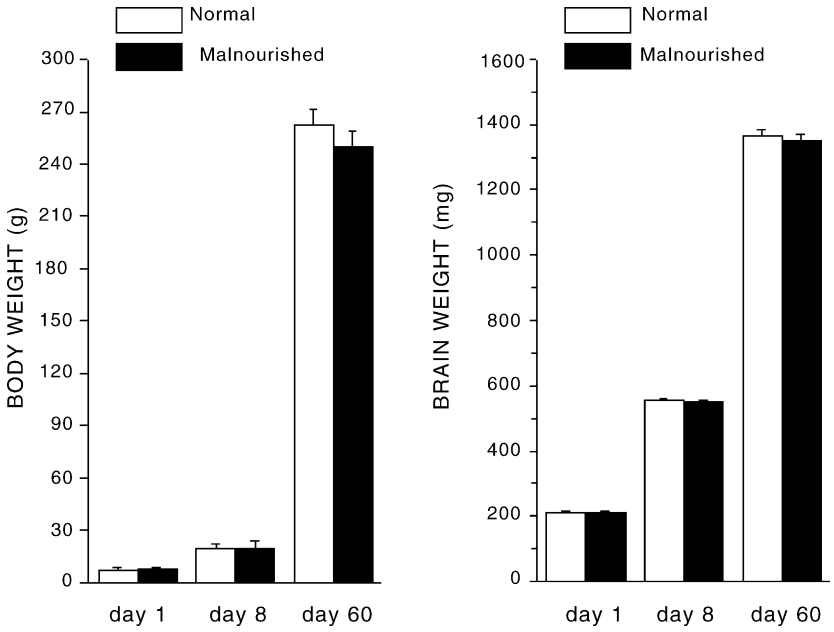

Fig. 1. Body and brain weights of normal and malnourished rats of 1, 8 and 60 days of age. Values are means ± S.E.M. $N = 16$ in each group. No statistically significant differences were found when comparing body and brain weights of normal and malnourished groups of same ages (two-tailed unpaired Student's *t*-test).

rats, α_{2C}-adrenoceptor-immunoreactive neurons were observed in all regions, without significant variation in the distribution of α_{2C}-adrenoceptor-immunoreactivity in the dorsoventral aspects in individual sections; the analysis of serial sections showed no marked mediolateral variation in the distribution. In the 8-day old normal animals, immunoreactivity was concentrated in neuronal perikarya, where they occurred predominantly as puncta. α_{2C}-Adrenoceptor-immunoreactivity was also observed in processes extending into proximal dendrites. In the cortex of 60-day old normal animals, larger and smaller neurons exhibited strong α_{2C}-adrenoceptor-immunoreactivity. Peroxidase labeling for α_{2C}-adrenoceptors was localized in extranuclear regions and extended also into processes between labeled perikarya, especially in normal animals.

Immunostaining showed that at 8 days of age there was a markedly higher expression of α_{2C}-adrenoceptors in both the frontal and the occipital cortices of malnourished animals compared to normals (Figs. 2 and 3). The intensity of staining achieved was similar in the perikarya of both groups. However, the cell density was much higher in the cortex of malnourished animals as compared with the control rats. In fact, in either cortical area the malnourished animals exhibited almost twice the number of cells that express the receptor ($P < 0.05$ and $P < 0.01$ for the frontal and occipital cortices, respectively, two-tailed unpaired Student's *t*-test) and showed a more than three-fold expression levels scores of the receptor/area ($P < 0.01$ for both the frontal and occipital cortices, two-tailed unpaired Student's *t*-test), while the endowment of α_{2C}-adrenoceptors/cell did not change significantly (Fig. 3). At 60 days of age, malnourished rats exhibited normal α_{2C}-adrenoceptor expression levels scores in both the frontal and the occipital cortices, and no statistical significant differences were observed in these parameters when compared normal and malnourished rats (Figs. 2 and 3).

4. Discussion

Mild reduction of the protein content of the maternal diet of pregnant rats did not significantly alter body and brain weights of pups at birth, indicating that protein deficiency in the 7.2% protein group was masked by caloric compensation with carbohydrates, leading to apparently normal fetal development as assessed by body and brain weights at birth. A similar result has been reported elsewhere [24,29]. As discussed by others [23,29], fetal growth retardation and reductions in brain weight after prenatal malnutrition are only produced by severe protein restriction, i.e. reduction of the protein content of the maternal diet to less than 6%.

Immunostaining revealed that α_{2C}-adrenoceptors were already present in the frontal and occipital cortices of 8-day normal rats; after this age the number of binding sites increased slightly, as revealed by the moderately higher immunostaining observed at postnatal day 60. These results are in agreement with previous studies showing moderate increase of both α_{2C}-adrenoceptor mRNA expression and [^{3}H]-rauwolscine binding during postnatal development of the rat cerebral cortex [38,44]. In contrast, prenatally malnourished pups exhibited at postnatal day 8 an approximately three-fold increase of α_{2C}-adrenoceptor expression in both frontal and occipital cortices, as revealed by α_{2C}-receptor immunostaining. This increase in α_{2C}-adrenoceptor expression is closely similar to the overexpression of the α_{2C}-adrenoceptor subtype obtained by mutation of the α_{2C}-gene in mice [19]. To our knowledge, this is the first immunohistochemical demonstration of the enhancing effect of maternal protein malnutrition on the number of α_{2C}-adrenoceptors in the cerebral cortex of pups, which are known to develop postnatally in the rat [26,44]. Changes in adrenoceptor density induced by other forms of malnutrition have already

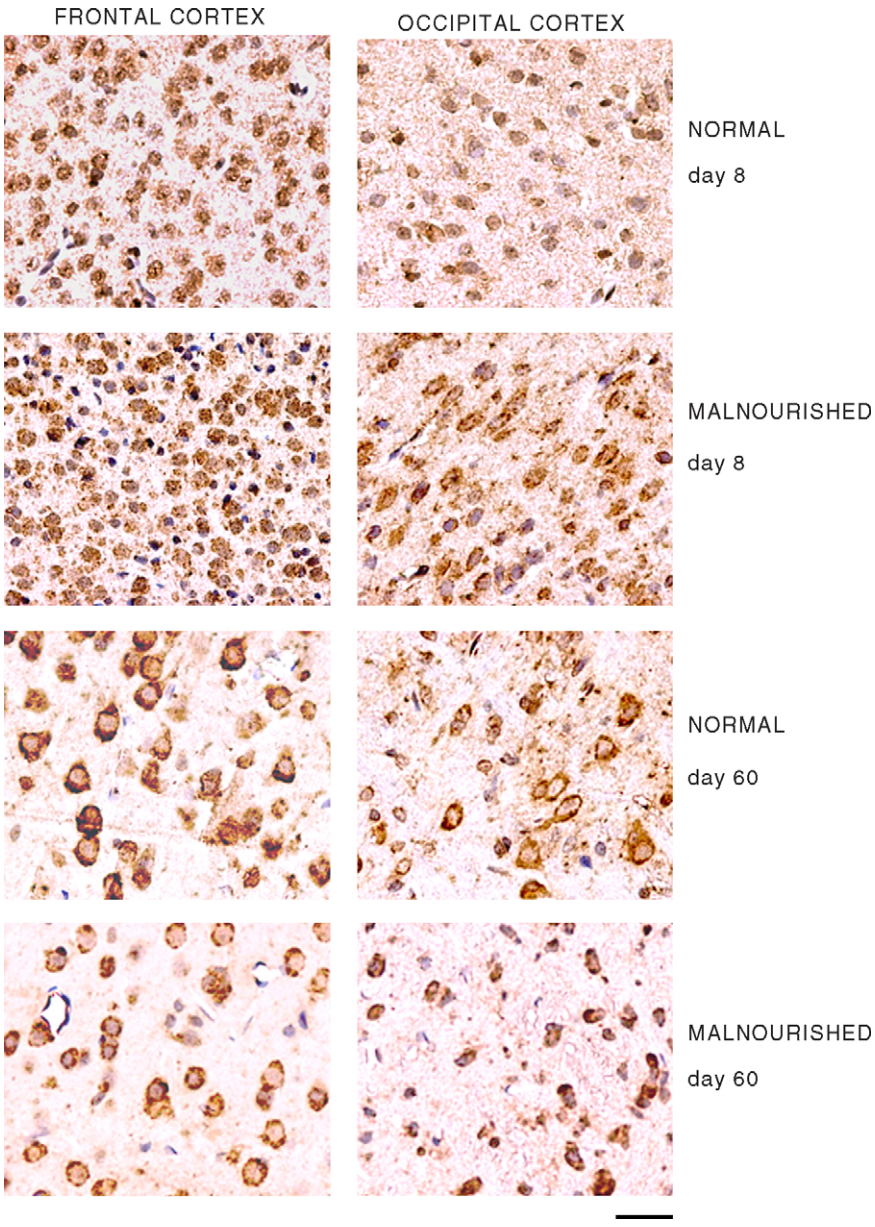

Fig. 2. Expression pattern (50×) of α_{2C}-adrenoceptors by immunohistochemistry (stained with antibody GP 10102), in sections of the frontal and occipital cortices of normal and malnourished rats of 8 and 60 days of age. The α_{2C}-adrenoceptor immunoreactivity appears as small punctate structures concentrated in the perikarya of neurons distributed throughout the cortex of the 8-day-old animals, while in the 60-day-old animals, the α_{2C}-adrenoceptor immunoreactivity was homogeneously distributed throughout the perikarya of small and larger neurons. In addition, immunoreactivity was localized to dendrites. In the 60-day-old animals, the α_{2C}-adrenoceptor immunoreactivity was allocated in dark, diffusely stained neurons intermingled with lighter labeled cells. Scale bar, 50 μm.

been reported using radioligand binding techniques. In fact, rats submitted to a low protein diet between day 14 of fetal life and day 50 of postnatal age, showed a reduced whole brain alpha and beta adrenoceptor binding at adulthood [22]. Nevertheless, it is not possible to compare these data to the present results, due to the different period of development during which nutritional injury was imposed (partial prenatal plus postnatal malnutrition versus purely prenatal malnutrition) and, on the other hand, due to the different type of receptors measured (whole population of α-receptors versus the α_{2C}-adrenoceptor subtype). Increased α_{2C}-adrenoceptor expression in the neocortex of malnourished animals seems to be the result of an increased number of neocortical neurons expressing the receptor, rather than enhanced endowment of α_{2C}-adrenoceptors/cell. The reason by which in malnourished animals some neurons begin to express this receptor subtype is presently unknown. Nevertheless, it has been reported that severe protein restriction during gestation in rats increases the expression of microtubule-associated protein type 1 (MAP 1) which remains elevated until adulthood [15]. As it has been pointed out in the literature, MAP 1B is abundant in the newborn rat brain [35] and is associated to neurite outgrowth [11], while MAP 1A is associated to development and maturation of dendritic processes [35]. During development, neurite transport includes numerous factors or components that are important for axonal function, including G protein-coupled receptors such as α_{2C}-adrenoceptors. For example, it has been

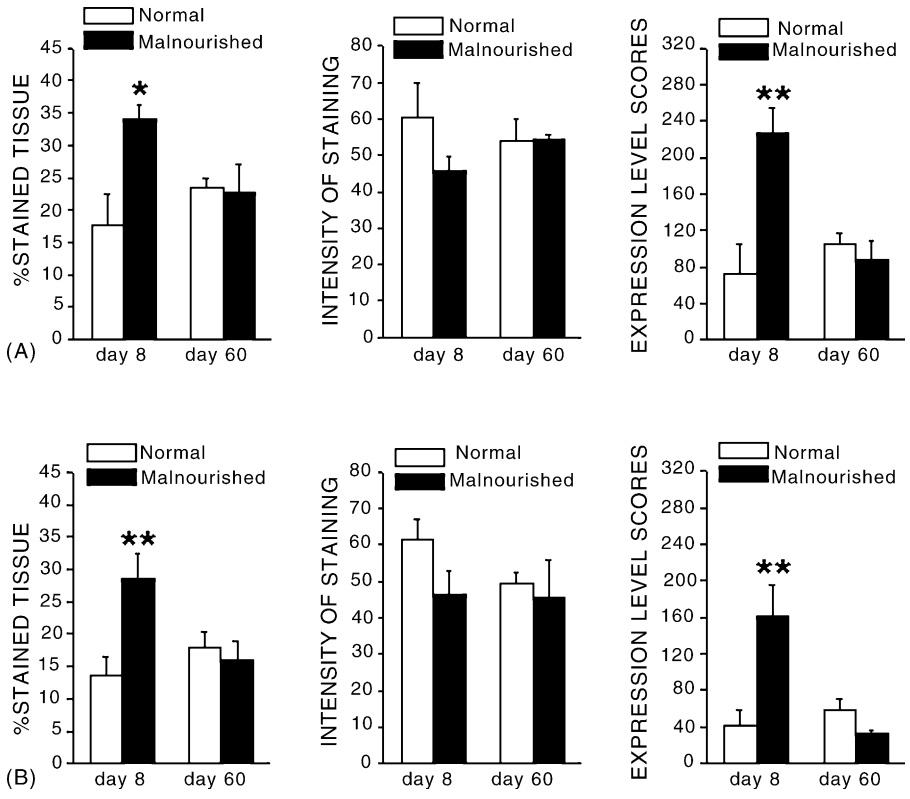

Fig. 3. Expression of α_{2C}-adrenoceptors in the frontal (A) and occipital (B) cortices of normal and malnourished rats of 8 and 60 days of age. Left panels show % stained tissue (percent of cells expressing α_{2C}-adrenoceptors); middle panels show the intensity of staining (number of α_{2C}-adrenoceptors/cell); right panels show the expression levels scores (number of α_{2C}-adrenoceptors/area). Values are means ± S.E.M. For each group $N = 4$ rats. Comparisons between normal and malnourished rats were made using two-tailed unpaired Student's t-test: $^{*}P < 0.05$, $^{**}P < 0.01$.

reported that β-adrenoceptor trafficking, a subclass of G protein-coupled receptors, seems to be microtubule dependent and therefore it could be regulated by microtubule-associated proteins such as MAP 4 [9]. Thus, it is possible to speculate that in prenatally malnourished rats the increased MAP 1 expression could lead to enhanced α_{2C}-adrenoceptor insertion in noradrenergic axon terminals, thereby increasing the density of this adrenoceptor subtype in the neocortex. A similar mechanism, but involving MAP 1A and other microtubule-associated proteins, could be responsible for increased trafficking and insertion of receptors in cell body perikarya and dendrites of neocortical tissue, but the effect of prenatal protein malnutrition in the expression of MAP 1A and other microtubule-associated proteins remains to be determined.

At 60 days of age, prenatally malnourished rats exhibited a reduction of α_{2C}-adrenoceptor expression in the two cortical regions studied, as compared to 8-day-old pups. In fact, at this age the expression levels score of α_{2C}-adrenoceptors in the malnourished group has already regressed to normality. Previous studies using [^{3}H]-rauwolscine binding have also shown a reduction in the number of neocortical α_{2C}-adrenoceptors at adulthood in the rat, but the receptor number still persisted moderately elevated respect to well-nourished controls [38]. This apparent discrepancy could be explained on the basis that [^{3}H]-rauwolscine, in addition to bind to α_{2C}-adrenoceptors, may also detect the presence of some α_{2A}-adrenoceptors in brain regions showing high expression of this receptor subtype, such as the cerebral cortex and hippocampus, where 90% of α_2-adrenoceptors belong to the α_{2A}-subtype and only 10% are of the α_{2C}-subtype, at least in adult mice [6].

The functional consequences of α_{2C}-adrenoceptor overexpression in the neocortex of developing rats could be analyzed on the basis of the reported role of these receptors in the brain. As mentioned above, it has been shown that α_{2C}-adrenoceptors are involved in many physiological processes, i.e. in presynaptic control of noradrenaline release from central neurons, modulation of dopamine and serotonin release, body-temperature regulation, and sensorimotor integration and cognitive functions, including modulation of the acoustic startle reflex and its prepulse inhibition, isolation-induced aggression, development of behavioral despair, and spatial working memory [1–3,6,18,30–33,42]. The fact that α_{2C}-adrenoceptors plays a negative role in long-term memory formation [1–3], suggests that the remarkable increase of this receptor subtype found early in life in the neocortex of malnourished rats could be involved in the decreased neocortical LTP and in the lower visuospatial memory presenting these animals at adulthood [38]. In this regard, it has been reported that noradrenaline is crucially involved in the generation of brain regressive events during development [4,8,43], and that the concentration and release of this neurotransmitter are significantly increased in the brain of neonates born from mothers receiving a 7.2% protein diet [24,37,39]. Thus, it is possible that hyperactive central noradrenergic mechanisms induced by hidden malnutrition operating

upon overexpressed neocortical α_{2C}-adrenoceptors, could lead to disruption of the developmental programming of several processes, including receptor expression of some neurotransmitter systems, axonal growth and synaptic network formation, thereby contributing to impair LTP-dependent plasticity in the neocortex and to depress memory formation a later stages of development.

In conclusion, the present results showed that prenatally malnourished pups exhibited at postnatal day 8 a three-fold increase of α_{2C}-adrenoceptor expression in both frontal and occipital cortices, while at 60 days of age no differences were observed in these parameters when compared normal and malnourished rats. Taking into account the role of α_{2C}-adrenoceptors in memory formation, overexpression of this receptor subtype during early postnatal life subsequent to mild prenatal protein malnutrition could in part be responsible for neural and behavioral disturbances showing prenatally malnourished animals during the postnatal life.

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