Mild prenatal protein malnutrition increases α_{2C}-adrenoceptor density in the cerebral cortex during postnatal life and impairs neocortical long-term potentiation and visuo-spatial performance in rats

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Abstract

Mild reduction in the protein content of the mother's diet from 25 to 8% casein, calorically compensated by carbohydrates, does not alter body and brain weights of rat pups at birth, but leads to significant enhancements in the concentration and release of cortical noradrenaline during early postnatal life. Since central noradrenaline and some of its receptors are critically involved in long-term potentiation (LTP) and memory formation, this study evaluated the effect of mild prenatal protein malnutrition on the α_{2C}-adrenoceptor density in the frontal and occipital cortices, induction of LTP in the same cortical regions and the visuo-spatial memory. Pups born from rats fed a 25% casein diet throughout pregnancy served as controls. At day 8 of postnatal age, prenatally malnourished rats showed a threefold increase in neocortical α_{2C}-adrenoceptor density. At 60 days-of-age, α_{2C}-adrenoceptor density was still elevated in the neocortex, and the animals were unable to maintain neocortical LTP and presented lower visuo-spatial memory performance. Results suggest that overexpression of neocortical α_{2C}-adrenoceptors during postnatal life, subsequent to mild prenatal protein malnutrition, could functionally affect the synaptic networks subserving neocortical LTP and visuo-spatial memory formation.

Keywords: α_{2C} adrenoceptor, long-term potentiation, neocortex, protein malnutrition, visuo-spatial memory.

Central nervous system noradrenaline critically influences long-term potentiation (LTP) in cerebral cortex (Nowicky *et al*. 1992; Kamatsu 1996) and hippocampus (Hopkins and Johnston 1988; Radisavljevic *et al*. 1994; Bramham *et al*. 1997), as well as memory formation (Sternberg *et al*. 1986; Crowe *et al*. 1990; Gibbs 1991), through balanced activation of specific receptors. For instance, animal studies have revealed that β adrenoceptor activation is associated with enhancement of LTP in the hippocampus (Hopkins and Johnston 1988; Radisavljevic *et al*. 1994; Bramham *et al*. 1997) and memory facilitation (Crowe *et al*. 1990; Gibbs 1991; Gibbs and Summers 2000), while activation of α_2 adrenoceptors (Sara and Devauges 1989; Devauges and Sara 1990; Bunsey and Strupp 1995), especially the α_{2C} subtype (Haapalinna *et al*. 1998, 1999; Björklund *et al*. 1998, 1999, 2000), is related to decreased memory formation. This role of β and α_{2C} adrenoceptors is consistent with the widespread distribution of these receptor subtypes in the hippocampus and the cerebral cortex (Lee *et al*. 1998; Gibbs and Summers 2000).

It has been reported that perinatal malnutrition and severe forms of prenatal malnutrition in the rat, in addition to decrease body and brain weights of pups results in functional changes of central noradrenergic systems, including increased activity of brain tyrosine hydroxylase

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Abbreviations used: α_{2C}-AR, α_{2C} adrenoceptor; B_{max}, maximum receptor density; CC, corpus callosum; LTP, long-term potentiation; K_D, dissociation constant.

(Shoemaker and Wurtman 1971; Miller *et al*. 1978; Marichich *et al*. 1979), increased concentration of noradrenaline in the whole brain and neocortex (Stern *et al*. 1975; Morgane *et al*. 1978; Soto-Moyano *et al*. 1995), increased release of noradrenaline in the neocortex (Soto-Moyano *et al*. 1994, 1995, 1998a,b, 1999), and decreased number of α and β adrenoceptors in total brain (Keller *et al*. 1982) and neocortex (Seidler *et al*. 1990). Together with these alterations in central noradrenergic profiles, on reaching adulthood, prenatally-malnourished rats on a 6% prenatal/25% postnatal casein diet exhibit learning disturbances, such as deficits in acquisition of alternation tasks (Tonkiss *et al*. 1990) and impaired visual discrimination learning (Tonkiss *et al*. 1991). Whether altered central noradrenergic function may be partly responsible for the deficits in cognitive processes showing previously malnourished adult animals has remained an elusive question. For example, together with changes in noradrenergic function, perinatally-malnourished animals also exhibit significant elevations of brain serotonin and 5-hydroxyindoleacetic acid, the main metabolite of serotonin, which may be directly correlated with corresponding increases in brain tryptophan (Morgane *et al*. 1978). In addition, 5-HT_{1A} receptors are decreased in the hippocampus of malnourished rats during adulthood (Blatt *et al*. 1994), and dopamine levels have been shown to be diminished in the hippocampus of severely prenatal protein-malnourished rats (Kehoe *et al*. 2001). It is therefore likely that more than one neurotransmitter system could be involved in the learning disturbances appearing in severely malnourished animals.

In contrast to severe forms of maternal malnutrition, mild reduction of the protein content of the mother's diet, calorically compensated for 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 (Resnick *et al*. 1982). However, this insidious form of protein maternal malnutrition, so-called hidden prenatal malnutrition (Resnick *et al*. 1982), 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 (Soto-Moyano *et al*. 1998b). Together with decreased cortical noradrenaline release, the visual cortex of adult prenatally-malnourished animals shows altered electrophysiological indices, including decreased ability of callosal-cortical synapses to perform temporal summation (Soto-Moyano *et al*. 1998b). Changes in central noradrenaline activity after hidden prenatal malnutrition are presumably accompanied by changes in the number of brain adrenoceptors that are relevant for memory processing. However, this question has not yet been addressed in detail. The results presented here provide evidence that hidden prenatal malnutrition in rats results, during adulthood, in increased α_{2C} adrenoceptor (α_{2C}-AR) density in both the frontal and occipital cortices, as well as in impaired neocortical LTP and lower visuo-spatial memory performance.

Materials and methods

Animals and diets

The experimental protocol and animal management were in accordance with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council 1985) and were 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 literature (Resnick *et al*. 1982; Soto-Moyano *et al*. 1998b). 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. The other components of the purified diets were as follows. (i) Normal diet: 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: 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 provided about 4.3 kCal/g. The dietary paradigm was started 5 weeks prior to mating and continued throughout pregnancy. The body weight gain of the pregnant mothers was controlled daily. At birth, all pups were weighed and litters were culled to eight pups (four males, four females). Afterwards, pups born from mothers fed the 7.2% protein diet were fostered by well-nourished dams (22.5% protein diet) giving birth on that day, whereas the 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, all pups were fed a standard laboratory diet providing 22.5% protein.

α_{2C} Adrenoreceptor (α_{2C} -AR) binding assay

Membrane preparation

Normal and malnourished rats aged 8 and 60 days ($n = 8$ each group) were killed by decapitation and their brains rapidly removed, weighed and cooled on ice. The frontal and occipital poles of the brain were dissected and about 30 mg (8-day-old rats) or 80 mg (60-day-old rats) cortex were obtained. Frontal or occipital tissue from different animals was pooled and homogenized, 1 : 8 (wt : vol), in ice-cold buffer (50 mM/L Tris-HCl, pH 7.4, 0.21 M/L sucrose, 1 mM/L MgCl, 0.3 mM/L EGTA, 5 μg/mL leupeptin, 0.1 mM/L benzamidine, and 0.1 mM/L phenylmethylsulfonyl fluoride). Homogenates were then centrifuged at 1000 *g* and the resulting supernatant fluids were centrifuged at 40 000 *g* for 15 min at 15°C. This step was repeated twice and the resulting pellet was then resuspended in a buffer consisting of 50 mM/L Tris-HCl (pH 7.4) and 25 mM/MgCl. Protein was measured using the method of Bradford (1976) with bovine serum albumin as standard.

Adrenoceptor binding assay

α_{2C}-AR binding was tested using [^{3}H]-rauwolscine (Amersham, Chicago, IL, USA) as high affinity ligand because it has been shown that rauwolscine is an α_{2C}-AR-preferring compound in cells trans-

fected with human (Marjamaki *et al*. 1993), rat (Harrison *et al*. 1991) or mouse (Link *et al*. 1992) α_{2C}-AR subtype genes. In rats and mice, rauwolscine has a 20- to 30-fold higher affinity for α_{2C}-AR than α_{2A} adrenoceptor (Harrison *et al*. 1991; Link *et al*. 1992). All saturation assays were performed in triplicate according to the method of Alexander (Colucci *et al*. 1984). Aliquots of membrane suspensions (0.1 mL) were diluted to a final volume of 0.15 mL in the assay buffer (50 mM/L Tris-HCL, pH 7.4, 1 mM/L $MgCl_2$) in the presence of 40 mM/L [^{3}H]-rauwolscine (80 Ci/mM) and then incubated at 25°C for 30 min. The reaction was terminated by the addition of 2 mL ice-cold assay buffer, followed by rapid filtration under reduced pressure through Whatman GF/B glass fiber filters pre-washed with assay buffer. The tubes and filters were rapidly washed with assay buffer (four times with 2 mL) and the radioactivity was counted as automatically quench-corrected disintegrations per min with a Packard 1600TR liquid scintillation counter (Packard Instruments Co., Downers Grove, IL, USA). Specific binding was defined as the amount of total bound radioactivity minus that observed in the presence of a 500-fold molar excess of unlabelled rauwolscine. Saturation binding curves were constructed using increasing concentrations of [^{3}H]-rauwolscine (0.1–45 nM/L). Maximum receptor density (B_{max}) and the dissociation constant (K_D) were calculated from Scatchard-transformed binding data with iterative, mass action, law-based, curve-fitting program LIGAND (GraphPad Software, Inc., San Diego, CA, USA).

Cortical LTP determinations

Experiments were carried out in 22 normal and 22 malnourished 55–60-day-old rats. Rats were weighed, anesthetized with 1.5 g/kg i.p. urethane and placed in a stereotaxic apparatus. A single dose of 1.5 mg/kg of d-tubocurarine was injected i.m. and adequate ventilation was maintained by means of a respirator pump. LTP was induced in the rat frontal and occipital cortices according to the method reported elsewhere (Racine *et al*. 1994; Mondaca *et al*. 2004). For this purpose, field cortical responses evoked by electrical stimulation of the corpus callosum (CC) were recorded by means of an electrode placed on the cortical surface (active electrode) in reference to another electrode located on the excised muscles over the frontal bone (reference electrode). As the responses were led in a differential amplifier, d-tubocurarine was used to avoid muscle responses recorded through the reference electrode. d-Tubocurarine does not penetrate the normal intact brain–blood barrier and is therefore devoid of effects in the central nervous system when administered systemically. Reinforcement of anesthesia during the experiments was not necessary as surgical procedures and recordings lasted no longer than 2 h and, in our experience with non-paralyzed rats, 1.5 g/kg i.p. urethane induces profound anesthesia lasting more than 6 h. Animals never regained consciousness and no changes in heart rate in response to stimulation were detected throughout the experiments.

After exposure of either the frontal or the occipital lobe of both cerebral hemispheres, electrical stimulation of the CC was carried out by means of a bipolar electrode that penetrated through the right frontal cortex at the de Groot co-ordinates A = 6.8 mm, L = 2.0 mm, or the right visual cortex at the de Groot co-ordinates A = 0.0 mm, L = 3.5 mm, according to the atlas of Pellegrino and Cushman (1967). The stimulating electrode consisted of two braided 100 μm diameter wires with a 0.5 mm tip separation; one tip of the electrode was located over the CC and the other tip penetrated the CC at the de Groot co-ordinate V = 2.2 mm (frontal cortex) or V = 2.5 mm (visual cortex). Cortical evoked responses were recorded from either the left frontal or the left visual cortex with a surface monopolar silver ball electrode of 0.5 mm diameter located on the contralateral cortices at similar surface de Groot co-ordinates as those utilized for transcortical stimulation of the CC. Test stimuli consisted of 100 μs duration square-wave pulses generated by means of a Grass S11 stimulator in conjunction with a Grass SIU-5 stimulus isolation unit and a Grass CCU 1 A constant current unit (Astro-Med, Inc., West Warwick, RI, USA). Before beginning each experiment, a full input–output series was performed at a stimulus intensity of 300–1100 μA, and test stimuli with a stimulation intensity yielding responses with peak-to-peak amplitude of 50% of the maximum were used for the remainder of the experiment. After a 30 min stabilization period, a 5 min control period of basal responses (30 averaged responses) was recorded. Thereafter, a tetanizing stimulus consisting of a single train of 100 μs duration square-wave pulses at 312 Hz and 500 ms duration, with intensity 50% higher than the test stimuli, was applied. Recordings were amplified by a Grass P-511 preamplifier (0.8–1000 Hz bandwidth), displayed on a Philips PM 3365 A digital oscilloscope (Fluke Corporation, Netherlands), digitized at a rate of 10 000/s by an A/D converter interfaced to an Acer PC, and stored for retrieval and off-line analysis. In all experiments, body temperature and expired CO_2 were monitored and remained within normal limits. Basal responses evoked in the rat cerebral cortex by contralateral stimulation of the CC begin with an early downward surface positive deflection (P), followed by a late upward surface negative wave (N). P-N latency and P-N peak-to-peak amplitude were measured using time and voltage cursors provided in the digital oscilloscope. Slope was determined as the amplitude/time ratio on the nearest sample to the 10% and the 90% level between cursors set on peaks P and N. The efficacy of the tetanizing train to potentiate cortical-evoked responses was evaluated by measuring both the peak-to-peak amplitude and the maximal slope increases. The results were similar so amplitudes were used for analyses of the experiments according to a procedure reported elsewhere (Racine *et al*. 1994; Mondaca *et al*. 2004). At the end of the electrophysiological experiments, the animals were killed with an overdose of sodium pentobarbital.

Visuo-spatial memory evaluation

Visuo-spatial memory was evaluated in 16 rats (eight normal, eight malnourished) by employing an eight-arm radial Olton maze. It consisted of eight, equally spaced plexiglas arms (70 cm long, 8 cm wide) extending from a central octagonal hub (34 cm across). The maze was placed 92 cm from the floor in a room with white walls. During the adaptation and testing sessions, all the arms of the maze were baited with rice puffs. Spatial cues in the extra-maze environment were provided by the experimenter himself, together with different articles of clothing placed on different hangers fixed on the walls of the room; the position of these articles and the position of the experimenter never changed during the 15 days of maze testing in each group of rats. To test animals in this maze, a strong motivation for food was required; this was induced by keeping them on a restricted diet (8 g/day/rat), starting on day 45 of age and maintained until a 15% body weight deficit was obtained. Thereafter, at 53, 54 and 55 days of age, each animal was submitted

to an adaptation period which consisted of placing the rat in the center of the maze to explore, run to the end of the arms and consume the bait. From 56 to 70 days of age, the animals were submitted daily to the visuo-spatial memory test (one assay daily, 15 days of testing). For this purpose, animals were placed in the central platform where they could freely run the maze until they obtained the food. The time required for solving the task (with a cut-off time of 10 min) and the numbers of errors (entry to already visited arms) were measured as significant parameters for memory evaluation.

Statistical analyses

All statistical analyses were performed with GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). For the effect of dietary treatments on body and brain weights, intergroup comparisons were made using the two-tailed unpaired Student's *t*-test. For binding assays, intergroup comparisons were made using one-way ANOVA followed by the *post hoc* Dunnett multiple comparisons test. For the analysis of the time-course in LTP studies and in visuo-spatial memory evaluation, a two-way ANOVA was performed for both intragroup (time) and intergroup (nutritional condition) comparisons, followed by the Bonferroni multiple comparisons test as *post hoc* test to assess both the effect of time and the effect of the nutritional condition. For comparisons of the total number of errors and the total time spent in visuo-spatial memory evaluation of normal and malnourished animals, the two-tailed unpaired Student's *t*-test was used.

Results

Effect of dietary treatment on body and brain weights

Body and brain weight measurements 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 on the effects of this dietary treatment on maternal weight gain during the first, second and third weeks of pregnancy was published elsewhere (Soto-Moyano *et al.* 1998b). No significant differences in body weights (two-tailed unpaired Student's *t*-test) were observed on days 1, 8 and 55–60 of postnatal life between rats born from mothers receiving 7.2% or 22.5% protein diet. In fact, on day 1 of postnatal life, normal and malnourished rats had mean body weights of 7.2 ± 0.05 g ($n = 56$) and 7.4 ± 0.09 g ($n = 56$), respectively (p = NS); on day 8 of postnatal life, mean body weights of normal and malnourished rats were 19.4 ± 0.30 g ($n = 16$) and 19.7 ± 0.39 g ($n = 16$), respectively (p = NS); on day 60 of postnatal life, mean body weights of normal and malnourished rats were 262.0 ± 9.0 g ($n = 40$) and 250 ± 8.8 g ($n = 38$), respectively (p = NS). A similar trend was observed for brain weights of normal and malnourished rats at days 1, 8 and 60 of postnatal life (two-tailed unpaired Student's *t*-test): on day 1 of postnatal life, normal and malnourished rats had mean brain weights of 211.2 ± 3.9 mg ($n = 8$) and 210.0 ± 5.6 mg ($n = 8$), respectively (p = NS); on day 8 of postnatal life, mean brain weights of normal and malnourished rats were 554.2 ± 8.4 mg ($n = 16$) and 550.6 ± 7.5 mg ($n = 12$), respectively (p = NS); finally, on day 60 of postnatal life, mean brain weights of normal and malnourished rats were 1367.0 ± 19.2 mg ($n = 12$) and 1352.6 ± 17.2 mg ($n = 12$), respectively (p = NS).

α_{2C} -AR binding in cerebral cortex

Density of α_{2C}-ARs increased moderately but significantly in the frontal cortex of normal rats during postnatal life, as revealed by the higher number of [^{3}H]-rauwolscine binding sites observed at 60 days of age ($p < 0.001$, Dunnett multiple comparison test) compared with that of 8-day-old pups (Table 1). A comparable but smaller change ($p < 0.05$, Dunnett multiple comparison test) was observed in the occipital cortex of normal animals of similar ages (Table 1).

On day 8 of postnatal life, malnourished rats exhibited significantly higher binding of [^{3}H]-rauwolscine in both frontal and occipital cortices ($p < 0.001$ for both cortices,

Table 1 Changes in number of binding sites (B_{max}) and in ligand affinity (K_D) in the cerebral cortex of normal and malnourished rats of 8 and 60 days of age

		B_{max} (f_{mol}/mg protein)			K_D (nM)		
Cortex	Age	Normal	Malnourished	p_N	Normal	Malnourished	p_N
Frontal	8 days	23.62 ± 0.72	79.86 ± 4.75	< 0.001	0.25 ± 0.02	0.40 ± 0.09	NS
	60 days	33.29 ± 2.48	60.04 ± 3.05	< 0.001	0.27 ± 0.02	0.37 ± 0.05	NS
	p_A	< 0.001	< 0.001		NS	NS	
Occipital	8 days	31.50 ± 1.59	69.40 ± 7.01	< 0.001	0.19 ± 0.05	0.36 ± 0.08	NS
	60 days	36.40 ± 3.43	51.63 ± 2.51	< 0.05	0.25 ± 0.04	0.42 ± 0.07	NS
	p_A	< 0.05	< 0.01		NS	NS	

Values are means ± SEM. For each group $n = 8$ rats. Comparisons between groups were made using one-way ANOVA followed by the Dunnett multiple comparisons test. For B_{max} values from the frontal cortex, p ANOVA = 0.0001, $F = 67.992$; for B_{max} values of occipital cortex, p ANOVA = 0.0001, $F = 16.742$; for K_D values of frontal cortex,p ANOVA = 0.1520, $F = 1.904$; for K_D values of occipital cortex, p ANOVA = 0.0574, $F = 2.814$. p_N is the probability level for comparisons related to the nutritional condition. p_A is the probability level for comparisons related to age of rats. NS = not significant.

Dunnett multiple comparison test) than that observed in normal animals of the same age (Table 1). At 60 days of age, a reduction in α_{2C}-AR density in both the frontal and the occipital cortices was observed in malnourished rats ($p < 0.001$ and $p < 0.01$, respectively, Dunnett multiple comparison test), as compared with 8-day-old malnourished rats, although α_{2C}-AR density in the frontal and occipital cortices of these animals still remained elevated ($p < 0.001$ and $p < 0.05$, respectively, Dunnett multiple comparison test) when compared with normal rats of the same age (Table 1). In all cases, there were no differences in the dissociation constants for [^{3}H]-rauwolscine binding when comparing normal and malnourished animals (Table 1).

Cortical LTP *in vivo*

Figure 1 A shows typical recordings of basal and post-LTP averaged frontal (left) and visual (right) cortical-evoked responses, which begin with an early downward surface positive deflection (P) followed by a late upward surface negative wave (N). P-N latency was 4.9 ± 0.1 ms in basal frontal responses and 4.7 ± 0.2 ms in potentiated frontal responses, while P-N latency was 8.1 ± 0.3 ms in basal visual responses and 8.0 ± 0.3 ms in potentiated visual responses. For both frontal and visual responses, the differences between P-N latency before and after potentiation was not statistically significant.

In vivo evaluation of cortical LTP revealed that in normal animals, tetanic stimulation of callosal fibers produced a significant increase in peak-to-peak amplitude of transcallosal cortical responses evoked in the frontal and occipital cortices. This increase remained unchanged throughout the recording period (Fig. 1). In the frontal cortex the amplitude increase over the time-course ranged from 32.6 to 39.2% ($p < 0.001$, Bonferroni multiple comparisons test). In the occipital cortex the amplitude increase over the time-course ranged from 36.3 to 44.1% ($p < 0.001$, Bonferroni multiple comparisons test). In contrast, in the frontal cortex of malnourished animals, a lower but significant increase in evoked responses was observed after the tetanic stimulation (about 20% increase, $p < 0.05$ for block 0–5, Bonferroni multiple comparisons test), but the potentiation rapidly declined to become not significant starting in block 5–10. A similar trend was observed in responses evoked in the occipital cortex of malnourished animals, where potentiation

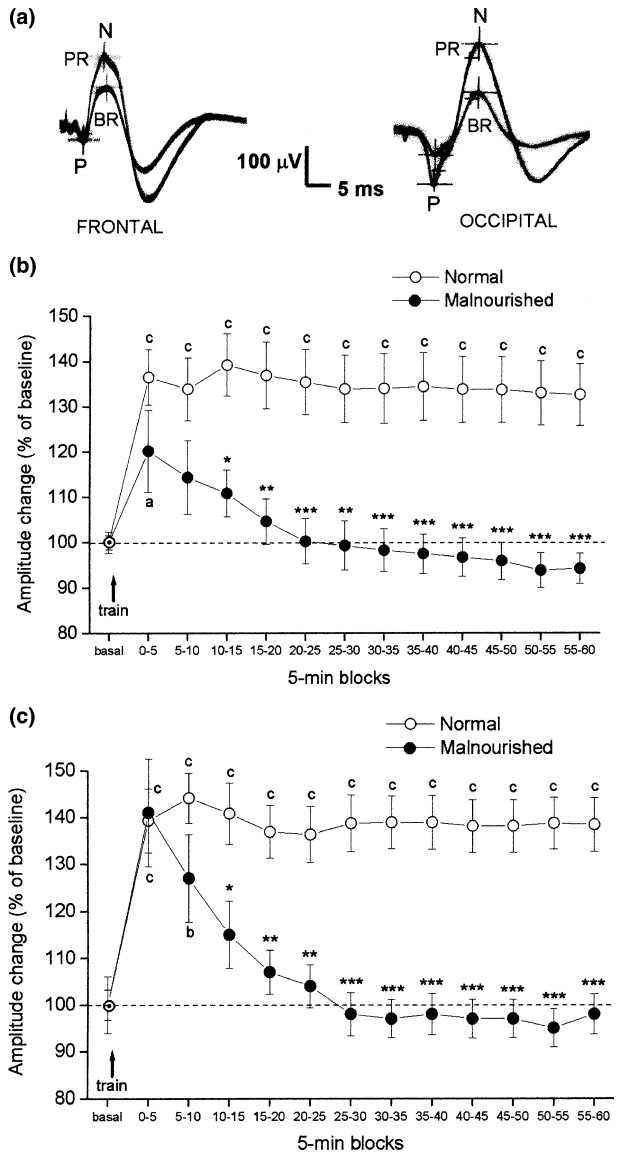

Fig. 1 Representative examples (a) of the average of 12 successive basal responses (BR) and 12 successive potentiated responses (PR) evoked either in the frontal cortex (left) or in the occipital cortex (right) of normal animals by contralateral stimulation of the corpus callosum at 0.1 Hz. P: early downward surface positive deflection; N: late upward surface negative wave. Effect of protein malnutrition during gestation on the time-course of LTP induced in the frontal (b) and the occipital (c) cortices of 55–60-day-old rats. The arrow indicates time of application of the tetanizing train. For the normal group, $n = 22$ (10 rats for frontal LTP, 12 rats for occipital LTP) and $n = 22$ for the malnourished group (10 rats for frontal LTP, 12 rats for occipital LTP). Values are means ± SEM of peak-to-peak amplitudes of 30 cortical-evoked responses per rat. Basal: Average of 30 basal-evoked responses per rat after the 30 min stabilization period. The potentiating effect of the tetanizing train over the time-course (intragroup variable) as well as the effect of the nutritional condition (intergroup variable) on LTP was analyzed using two-way ANOVA followed by the Bonferroni *post hoc* test. For the frontal cortex, the intragroup variable showed a $p_{ANOVA} = 0.0037$, $F = 2.530$, and the intergroup variable showed a $p_{ANOVA} < 0.0001$, $F = 153.6$; the interaction showed a $p_{ANOVA} = 0.0603$, $F = 1.736$. For the occipital cortex, the intragroup variable showed a $p_{ANOVA} < 0.0001$, $F = 5.489$, and the intergroup variable showed a $p_{ANOVA} < 0.0001$, $F = 171.8$; the interaction showed a $p_{ANOVA} < 0.0001$, $F = 3.624$. For intragroup comparisons, $^a p < 0.05$, $^b p < 0.01$, $^c p < 0.001$, according to the Bonferroni multiple comparisons test; for intergroup comparisons, $^* p < 0.05$, $^{**} p < 0.01$, $^{***} p < 0.001$, according to the Bonferroni multiple comparisons test.

was observed only during the first 10 min after application of the tetanizing train (about 40% increase, $p < 0.001$ for block 0–5, $p < 0.01$ for block 5–10, Bonferroni multiple comparisons test) (Fig. 1). Throughout the recording period after block 10–15, malnourished rats showed significantly lower values in peak-to-peak amplitude of cortical responses evoked in both the frontal (Fig. 1b: $p < 0.001$ except for block 10–15 where $p < 0.05$, and blocks 15–20 and 25–30 where $p < 0.01$, Bonferroni multiple comparisons test) and the occipital (Fig. 1c: $p < 0.001$ except for block 10–15 where $p < 0.05$, and blocks 15–20 and 20–25 where $p < 0.01$, Bonferroni multiple comparisons test) cortices as compared with the normal rats.

Visuo-spatial memory

Evaluation of visuo-spatial memory revealed that normal rats committed a number of errors that significantly declined, from a mean of 6.3 for the first 3 day block of assays to a mean of 0.5 for the last 3 day block of assays (Fig. 2a: $p < 0.01$, Bonferroni multiple comparisons test). A similar trend was observed in malnourished rats, but the number of errors performed by these rats declined from a mean of 16.7 for the first 3 day assay block to a mean of 5.9 for the last 3 day assay block (Fig. 2a: $p < 0.05$ for block 4–6, $p < 0.001$ for blocks 7–9, 10–12 annd 13–15, Bonferroni multiple comparisons test). During the 15 days of testing, malnourished rats committed a significantly greater number of errors than normal rats (Fig. 2a: $p < 0.001$ for blocks 1–3, 4–6, 7–9 and 10–12; $p < 0.05$ for block 13–15, Bonferroni multiple comparisons test). Analysis of the total number of errors accumulated during the 15 days of testing revealed that the malnourished group exhibited an increased total number of errors when compared with the control group (Fig. 2b: $p < 0.001$, two-tailed unpaired Student's *t*-test). The time required by normal rats to solve the task decreased progressively from a mean of 305.8 s for the first 3 day block of assays to a mean of 158.2 s for the last 3 day block of assays, showing a significant difference when comparing these two values (Fig. 2c: $p < 0.05$, Bonferroni multiple comparisons test). Malnourished rats also show a declining time required to solve the task, from a mean of 533.7 s for the first 3 day assay block to a mean of 387.9 s for the last 3 day assay block (Fig. 2c: $p < 0.05$, Bonferroni multiple comparisons test). For the 15 days of testing, excepting block 7–9, malnourished rats required significantly more time to solve the task than normal rats (Fig. 2c: $p < 0.05$ for blocks 4–6 and 10–12, $p < 0.001$ for blocks 1–3 and 13–15, Bonferroni multiple comparisons test). Analysis of the total time spent by rats on solving the task, accumulating the data from 15 days of testing, showed that the malnourished group spent significantly more time than the normal group (Fig. 2d: $p < 0.001$, two-tailed unpaired Student's *t*-test).

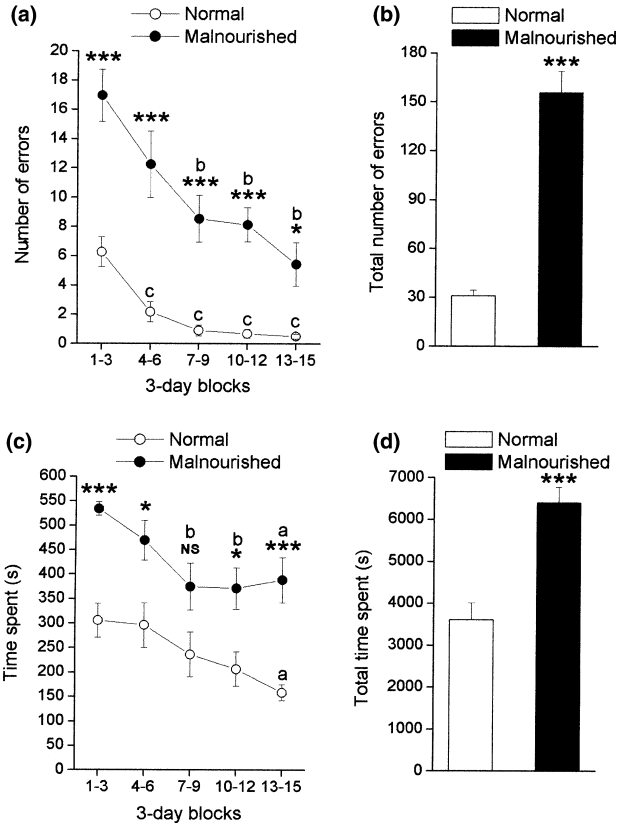

Fig. 2 Effect of protein malnutrition during gestation on visuo-spatial memory of 56–70-day-old rats. (a) The time-course of the number of errors and (b) the total number of errors committed by the animals in solving the task in the radial maze. (c) The time-course of the time spent and (d) the total time spent to solve the task; $n = 8$ rats in each group. Values are the means ± SEM of scores recorded during 15 days of testing (one assay daily) grouped in 3 day blocks. The time-course of the number of errors and of the time spent in solving the task (intragroup variable), as well as the effect of the nutritional condition (intergroup variable) on LTP, was analyzed using two-way ANOVA followed by the Bonferroni *post hoc* test. For the number of errors, the intragroup variable showed a p ANOVA < 0.0001, $F = 14.27$, and the intergroup variable showed a p ANOVA < 0.0001, $F = 102.8$; the interaction showed a p ANOVA $= 0.1733$, $F = 1.642$. For the time spent, the intragroup variable showed a p ANOVA < 0.0007, $F = 5.473$, and the intergroup variable showed a p ANOVA < 0.0001, $F = 58.50$; the interaction showed a p ANOVA < 0.7009, $F = 0.5482$. For intragroup comparisons, $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$, according to the Bonferroni multiple comparisons test; for intergroup comparisons, $^{*}p < 0.05$, $^{***}p < 0.001$, according to the Bonferroni multiple comparisons test. Comparisons of the total number of errors and total time spent between normal and malnourished rats were made using the two-tailed unpaired Student's *t*-test: $^{***}p < 0.001$.

Discussion

A mild reduction in the protein content of the maternal diet did not significantly alter body and brain weights of pups at birth, indicating that the protein diet resulted in apparently

normal fetal development as assessed by birth body and brain weights, similar to reports elsewhere (Morgane *et al*. 1978; Resnick *et al*. 1982). According to Resnick *et al*. (1982) and Morgane *et al*. (1993), fetal growth retardation and reductions in brain weight after prenatal malnutrition occur only after protein restriction, with maternal diets providing less than 6% casein.

[^{3}H]-Rauwolscine binding revealed that α_{2C}-ARs were already present in the frontal and occipital cortices of 8-day-old normal rats; afterwards, the number of binding sites increased slightly, as revealed by [^{3}H]-rauwolscine binding at postnatal day 60. These results are in agreement with studies showing moderate increases in both α_{2C}-AR mRNA expression and [^{3}H]-rauwolscine binding during postnatal development of the rat cerebral cortex, the mRNA for the α_{2C}-AR being already present at birth and [^{3}H]-rauwolscine binding appearing only during the second postnatal week (Winzer-Serhan *et al*. 1997). In contrast, prenatally-malnourished pups at postnatal day 8 exhibited a threefold increase of α_{2C}-AR density in both the frontal and occipital cortices, as revealed by [^{3}H]-rauwolscine binding. This increase in α_{2C}-AR density is similar to the overexpression of the α_{2C}-AR subtype obtained by mutation of the α_{2C}-gene in mice (Holmberg *et al*. 2003). To our knowledge, this is the first demonstration in rat pups of the enhancing effect of maternal protein malnutrition on the number of α_{2C}-ARs in the cerebral cortex, which are known to develop postnatally in this animal species (Nicholas *et al*. 1993; Winzer-Serhan *et al*. 1997). As mentioned previously, it seems likely that [^{3}H]-rauwolscine binding actually reflects α_{2C}-AR density, since rauwolscine has a 20- to 30-fold higher affinity for the α_{2C}-AR compared with the α_{2A} adrenoceptor in rats and mice (Harrison *et al*. 1991; Link *et al*. 1992). Further support for this concept is provided by studies showing that deletion of the gene encoding the α_{2C}-AR results in substantial decreases in [^{3}H]-rauwolscine binding sites in the brain (Link *et al*. 1995). It should be noted, however, that the α_{2C}-AR population could be slightly overestimated when assessed through [^{3}H]-rauwolscine binding as the ligand may also detect some α_{2A}-ARs in brain regions with high expression of this receptor subtype, such as the cerebral cortex and hippocampus, where 90% of α_2-ARs belong to the α_{2A} subtype and only 10% are of the α_{2C} subtype, at least in adult mice (Bücheler *et al*. 2002). Changes in adrenoceptor density induced by other forms of malnutrition have already been reported. In fact, rats submitted to a low protein diet between days 14 of fetal life and 50 of postnatal age showed decreased whole brain α and β adrenoceptor binding at adulthood (Keller *et al*. 1982). Nevertheless, it is not possible to compare these data with those of our study, due to the different periods of development during which the nutritional injury occurred (partial prenatal plus postnatal malnutrition versus purely prenatal malnutrition) as well as to the different type of receptors evaluated (the whole population of α receptors versus the α_{2C}-AR subtype). Other studies have shown that moderate prenatal malnutrition increases hippocampal kainate receptor density in adult rats (Fiacco *et al*. 2003) and striatal NMDA receptor binding in adult female rats (Palmer *et al*. 2004). Furthermore, 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 (Gressens *et al*. 1997). As has been pointed out in the literature, MAP 1B is abundant in the newborn rat brain (Schoenfeld *et al*. 1989) and is associated with neurite outgrowth (Dehmelt and Halpain 2004). Moreover, MAP 1B has been suggested to be important for synapse formation in the rat cerebral cortex (Kawakami *et al*. 2003). In contrast, MAP 1A is very low or absent in developing axonal fibers but increases during development and maturation of dendritic processes (Schoenfeld *et al*. 1989). These results, together with the present observations, support the idea that nutritional insults during fetal life may induce long-term postnatal changes in neurotransmitter receptor expression, and in other proteins that are involved in neuronal growth and development. At 60 days of age, α_{2C}-AR density in the two cortical regions studied had decreased in prenatally-malnourished rats compared with 8-day-old prenatally-malnourished pups, although a 50% increase in [^{3}H]-rauwolscine binding above the control values could still be detected. This suggests that changes induced by prenatal malnutrition in α_{2C}-AR expression persist in the rat long after postnatal nutritional recovery, which could result in altered noradrenergic function in adult age.

Prenatal malnutrition in rats resulted in impaired neocortical LTP and decreased visuo-spatial memory performance in adult animals. The foregoing data suggest that prenatally-malnourished young adult rats were unable to maintain normal LTP in the frontal and occipital cortices *in vivo*, although alternative hypotheses involving increased threshold of neural elements excited in the cortex could also be possible. Also, the progressive decrease in the number of errors and time spent during task execution by malnourished animals indicated that they were able to learn the general strategies of the task. However, their scores were always higher than those of normal animals, indicating that protein malnutrition during fetal life adversely affected their ability to perform in the Olton maze in adulthood. Deficits induced by prenatal malnutrition in task acquisition in adult rats have already been reported but in those studies, moderate protein restriction to pregnant mothers (6% protein diet) had been used as the paradigm (Tonkiss *et al*. 1990). While it is tempting to speculate that the deficits in behavioral performance of rats in the Olton maze reported here are due to visuo-spatial memory deficits, a number of alternative interpretations are possible (for reviews see Cain and Saucier 1996; Cain 1998). For example, it is possible that prenatal protein malnutrition could generate some visual impairment

that interfered with performance in the visuo-spatial memory test. In this regard, it has been reported that visual impairments can interfere with the apparent learning of mazes in rats (O'Steen *et al*. 1995; Spencer *et al*. 1995). However, there are no studies showing that prenatal malnutrition may result in some type of visual impairment at postnatal age. Another possibility is that malnutrition could affect visuo-spatial learning by altering anxiety levels or stress responses to the test situation. In this respect, it has been shown that prenatal malnutrition can affect the exploratory behavior and avoidance of adult rats in the elevated T-maze test, probably through reduction of anxiety (Almeida *et al*. 1996a,b). As another alternative, it is possible that prenatal malnutrition interferes with motor performance at adulthood, rather than producing a true learning impairment. In this regard, it has been shown that motor performance on a revolving drum of adult rats undernourished during gestation plus lactation did not differ from that of well nourished control rats (Tonkiss and Smart 1983). However, other studies suggest that mature rats born from 6 and 8% casein diet-restricted mothers, who continue the protein malnutrition paradigm during the lactation period, were hyperactive in the open field but tended to perform at control levels on the learning measurements in eight-arm radial maze testing (Wolf *et al*. 1986). It is therefore apparent that the question of whether prenatal protein malnutrition altered the performance in the Olton maze at adulthood by affecting visual-spatial memory and/or by generating sensorimotor disturbances remains unresolved, and further research would be necessary to elucidate this aspect.

Whether the effects of prenatal malnutrition on LTP and visuo-spatial memory are related to the marked postnatal increase in α_{2C}-AR density observed in the neocortex of malnourished rats is unknown at present, although reported data have shown a relationship between overexpression of α_{2C}-ARs and poorer navigation capacity in the water maze (Björklund *et al*. 2000). The functional consequences of increased α_{2C}-AR expression in the neocortex of developing rats could be analyzed, keeping in mind the possible role of these receptors in the brain. In this regard, it has been shown that α_{2C}-ARs are involved in many physiological processes, such as body temperature regulation, sensorimotor integration and cognitive functions, including modulation of the acoustic startle reflex and its pre-pulse inhibition, isolation-induced aggression, development of behavioral despair and spatial working memory, as well as modulation of dopamine and serotonin release (Sallinen *et al*. 1997, 1998a,b, 1999; Björklund *et al*. 1998, 1999, 2000; Tanila *et al*. 1999). In addition, α_{2C}-ARs are involved in the presynaptic control of noradrenaline release from peripheral neurons (Hein *et al*. 1999). It has also been reported that this receptor subtype could play a minor role in the release of noradrenaline in the central nervous system (Bücheler *et al*. 2002). α_{2C}-ARs seem to play a negative role in long-term memory formation, since α_{2C}-overexpressing mice performed less well in the water maze (Björklund *et al*. 1998, 1999, 2000). Interestingly, α_{2C}-overexpressing animals also performed less well in a visible platform water maze, suggesting that in addition to memory deficits, other sensorimotor disturbances could be present in these animals. The fact that overexpression of α_{2C}-ARs leads to deficits in water-maze performance suggests that the remarkable increase in this receptor subtype in the neocortex of malnourished rats early in life could be involved in the decreased neocortical LTP and in the lower visuo-spatial memory shown in these animals when they reach adulthood. In this respect, it is worth pointing out that noradrenaline is crucially involved in the generation of brain regressive events during development (Wendlant *et al*. 1977; Blue and Parnavelas 1982; Caviness 1989), and that its concentration and release are significantly increased in the brain of neonates born from dams receiving a 7.2% protein diet (Stern *et al*. 1975; Morgane *et al*. 1978; Soto-Moyano *et al*. 1998b, 1999). Therefore, it can be argued that hyperactive central noradrenergic mechanisms induced by prenatal protein malnutrition, operating upon an unusually high number of neocortical α_{2C}-ARs, could disrupt the developmental programming of several processes, including receptor expression of some neurotransmitter systems, axonal growth and synaptic network formation, thereby contributing to impair plasticity in the neocortex and/or to depress memory formation at later stages of development. The possibility that disturbances in neocortical LTP and visuo-spatial memory are mainly mediated by the increased α_{2C}-AR population persisting until adulthood cannot be discarded, since Björklund *et al*. (1999) showed that the α_2 adrenoceptor antagonist, atipamezole, fully reversed the deficit in platform finding and search strategy in genetically α_{2C}-overexpressing mice submitted to a water maze navigation paradigm. Further studies investigating the effect of specific α_{2C}-AR blocking agents and α_{2C}-AR antisense targetting on neocortical LTP and memory performance in adult prenatally-malnourished adult rats would be helpful in elucidating these aspects.

Abnormalities in hippocampal function of malnourished animals as a factor contributing to the deficits in visuo-spatial learning seem to be less probable, on the basis that early functional alterations induced by prenatal malnutrition on electrophysiological properties of hippocampal neurons seem to disappear on reaching the adult age (Rushmore *et al*. 1998). However, caution must be taken regarding this question, since it is thought that nutritional rehabilitation could contribute to ameliorating long-term alterations in hippocampal plasticity and related learning and memory deficits, though by no means fully reversing them (Morgane *et al*. 2002). In contrast, a bulk of structural changes and functional disturbances are still present in the neocortex long after postnatal nutritional recovery (Morgane *et al*. 1993; Levitsky and Strupp 1995). These are probably related to the limited structural/functional recuperative capacity of the

neocortex due, amongst other factors, to the absence of postnatal neurogenesis. Interestingly, it has been reported that lesions in several neocortical areas, including the prefrontal and occipital cortices, impair the performance in the water maze (Kolb *et al*. 1994; Compton *et al*. 1997; Espinoza *et al*. 1999; Hoh *et al*. 2003). This evidence is consistent with the long held view that the neocortex is an important site for neuronal changes that underlie learning and, in addition, may mechanistically link chemical/functional neocortical disorders induced by prenatal malnutrition in the rat neocortex, such as altered α_{2C}-AR expression and synaptic potentiation, to the impaired visuo-spatial performance observed in these animals.

In summary, the present data show that mild prenatal protein malnutrition results in increased α_{2C}-AR density in the rat cerebral cortex during postnatal life, together with decreased LTP and visuo-spatial performance at adulthood.

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