available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Developmental changes in content of glial marker proteins in rats exposed to protein malnutrition

Ana Maria Feoli[a,b], *Marina C. Leite*[a], *Ana Carolina Tramontina*[a], *Francine Tramontina*[a], *Thais Posser*[c], *Letícia Rodrigues*[d], *Alessandra Swarowsky*[d], *André Quincozes-Santos*[a], *Rodrigo B. Leal*[c], *Carmem Gottfried*[a], *Marcos Luiz Perry*[a], *Carlos-Alberto Gonçalves*[a,d,*]

[a]*Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil*
[b]*Faculdade de Enfermagem, Nutrição e Fisioterapia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[c]*Departamento de Bioquímica, CCB, Universidade Federal de Santa Catarina, Florianópolis, Brazil*
[d]*Programa de Pós-Graduação em Neurociências, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil*

ARTICLE INFO

Article history:
Accepted 16 October 2007
Available online 22 October 2007

Keywords:
Astrocyte
GFAP
Protein malnutrition
S100B

ABSTRACT

Pre- and postnatal protein malnutrition (PMN) adversely affects the developing brain in numerous ways, but only a few studies have investigated specific glial parameters. This study aimed to evaluate specific glial changes of rats exposed to pre and postnatal PMN, based on glial fibrillary acidic protein (GFAP) and S100B immunocontents as well as glutamine synthetase (GS), in cerebral cortex, hippocampus, cerebellum and cerebrospinal fluid, on the 2nd, 15th and 60th postnatal days. We found increases in GFAP, S100B and GS in the cerebral cortex at birth, suggesting an astrogliosis. Hippocampus and cerebellum also exhibited this profile at birth. However, a significant interaction between age and diet in postnatal life was observed only in the S100B of the cerebral cortex. No changes in the content of GFAP and S100B and GS activity were found on the 60th postnatal day in malnourished rats. In contrast, following an increase in the levels of S100B in the cerebrospinal fluid, during the early developmental stages, levels remained elevated on the 60th postnatal day. Our data support the concept of astrogliosis at birth, induced by PMN, and involve extracellular-regulated kinase activation. Specific alterations in cerebral cortex emphasize the regional vulnerability of the brain to malnutrition; some alterations were observed only at birth (e.g. GFAP); others were observed on the 2nd and 15th post-natal days (e.g. ERK phosphorylation). Taken together, transient and persistent alterations (e.g. elevated extracellular levels of S100B) suggest some brain damage or a risk of brain diseases in rats exposed to PMN.

1. Introduction

Malnutrition is an important and very common insult to human brain development and function. The global prevalence of stunting in <5-year-old children associated with undernourishment, particularly protein restriction, is increasing despite the decline in childhood mortality (Caballero, 2002). Prenatal and early postnatal protein malnutrition (PMN) adversely affects the developing brain in numerous ways, depending largely on its timing in relation to various develop-

* *Corresponding author*. Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos, 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil.
E-mail address: casg@ufrgs.br (C.-A. Gonçalves).

0006-8993/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2007.10.035

mental events in the brain and, to a lesser extent, on the type and severity of the deprivation (Morgane et al., 1993).

Several studies have shown biochemical changes in the central nervous system (CNS) in experimental models of PMN, particularly involving specific neurotransmitter systems (Morgane et al., 2002; Steiger et al., 2003; Wiggins et al., 1984; Rocha and Souza, 1994). More recently, we have shown changes in the neurotransmission mediated by glutamate, the main excitatory neurotransmitter in the CNS (Rotta et al., 2003). Glial cells, particularly astrocytes, are involved in the metabolic support of neurons, glutamate uptake, synthesis of glutamine, secretion of neurotrophic factors and antioxidant defense (Takuma et al., 2004). Surprisingly, only a few studies have investigated specific glial parameters (Clos et al., 1982; Giuffrida et al., 1980; Gressens et al., 1997), despite growing evidence of the importance of glial cells for neuronal development, survival and plasticity. In fact, during CNS development, the generation of cell types occurs sequentially, and neurons are predominantly generated before glial cells (Sauvageot and Stiles, 2002). Precocious or delayed glial differentiation may cause severe disorganization and dysfunction of the CNS (He et al., 2005).

Glial fibrillary acidic protein (GFAP) is a specific marker of mature astrocytes. CNS injuries are commonly accompanied by astrogliosis, characterized by an increase in GFAP (O'Callaghan, 1991). On the other hand, reduction of GFAP during prenatal development induced by protein restriction has been associated with delayed astrocytogenesis (Giuffrida et al., 1980). S100B protein is another useful marker of brain injury, expressed and secreted by astrocytes (Donato, 2001) and a lower content of S100 protein (possibly S100B) has been found in the cerebellum of malnourished rats (Clos et al., 1982). We have previously demonstrated brain oxidative stress in malnourished rats (Feoli et al., 2006a,b), suggesting a glial alteration in this kind of insult.

The present study aimed to evaluate astrocytic changes in different brain regions during pre- and postnatal developmental periods in response to protein malnourishment. In order to address this issue, specific markers – GFAP and S100B immunocontent and glutamine synthetase activity – were analysed on the 2nd, 15th and 60th postnatal days in the cerebral cortex, hippocampus and cerebellum of normal and protein malnourished rats. S100B content in cerebrospinal fluid was also measured on the 21st and 60th postnatal days.

2. Results

Rats were submitted to prenatal and postnatal PMN. The severity of the PMN model in the postnatal development was evaluated by measuring the body and brain weight on the 2nd, 15th and 60th postnatal days (Fig. 1). There was a significant effect of age ($p<10^{-4}$, $F_{(2,\ 53)}=335.54$) and diet ($p<10^{-4}$, $F_{(1,\ 53)}=46.30$) on brain weight, as well as a significant interaction between age–diet ($p<10^{-4}$, $F_{(2,\ 53)}=23.95$). With regard to body weight, we also observed an effect dependent on age ($p<10^{-4}$, $F_{(2,\ 42)}=693.70$) or diet ($p<10^{-4}$, $F_{(1,\ 42)}=142.96$) and a significant interaction between age and diet ($p<10^{-4}$, $F_{(2,\ 42)}=88.51$).

The brain weight and glial protein parameters on the 2nd postnatal day depict the final representation of the prenatal PMN insult at birth; we found a significant decrease in the brain tissue, accompanied by an increase in glial proteins in all brain structures analysed (Table 2, Student's t test). A qualitative analysis of the cerebral cortex on the 2nd postnatal day, employing immunohistochemical staining for GFAP, confirmed that GFAP-positive cells are apparently more immunostained in malnourished rats (Fig. 2B). However, this scenario changed when mean values from the 15th and 60th postnatal days were added to analyze postnatal development (by two-way ANOVA).

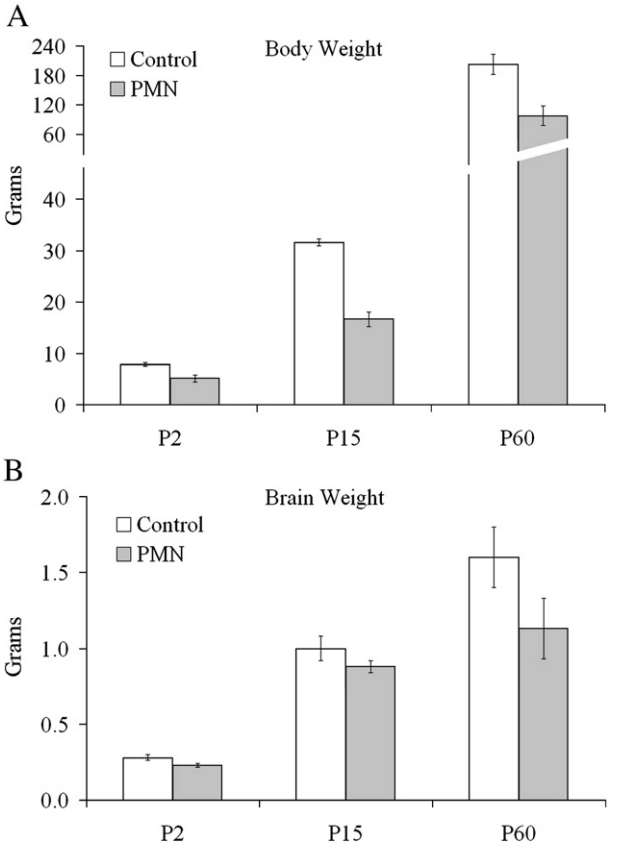

Fig. 1 – Post-natal development profile of body and brain weight of rats exposed to PMN. Rats were exposed to a 7% protein diet during pre- and postnatal life. Weights were measured on the 2nd (P2), 15th (P15) and 60th (P60) postnatal days. Values are mean ± standard error of 6–8 rats in each group. Significant effects, dependent on age or diet, were observed (two-way ANOVA, $p<0.05$).

The effect of age on GFAP content was observed in all brain structures, in both normal and malnourished animals (Figs. 2A, C and D). In cerebral cortex, the effect of age was significant ($p<10^{-4}$, $F_{(2,\ 40)}=37.34$), but no significant effect of diet was observed ($p=0.225$, $F_{(1,\ 40)}=1.52$); no age–diet interaction was found ($p=0.64$, $F_{(2,\ 40)}=0.450$). Similarly, the effect of age was significant ($p<10^{-4}$) on GFAP content of hippocampus and cerebellum ($F_{(2,\ 41)}=25.47$ and $F_{(2,\ 31)}=145.88$, respectively) with no effect of diet ($p=0.334$, $F_{(1,\ 41)}=0.918$ and $p=0.917$, $F_{(1,\ 31)}=0.011$, for hippocampus and cerebellum, respectively). No interaction (age–diet) was observed in the hippocampus and cerebellum ($p=0.283$, $F_{(2,\ 41)}=1.30$ and $p=0.543$, $F_{(2,\ 31)}=0.622$, respectively).

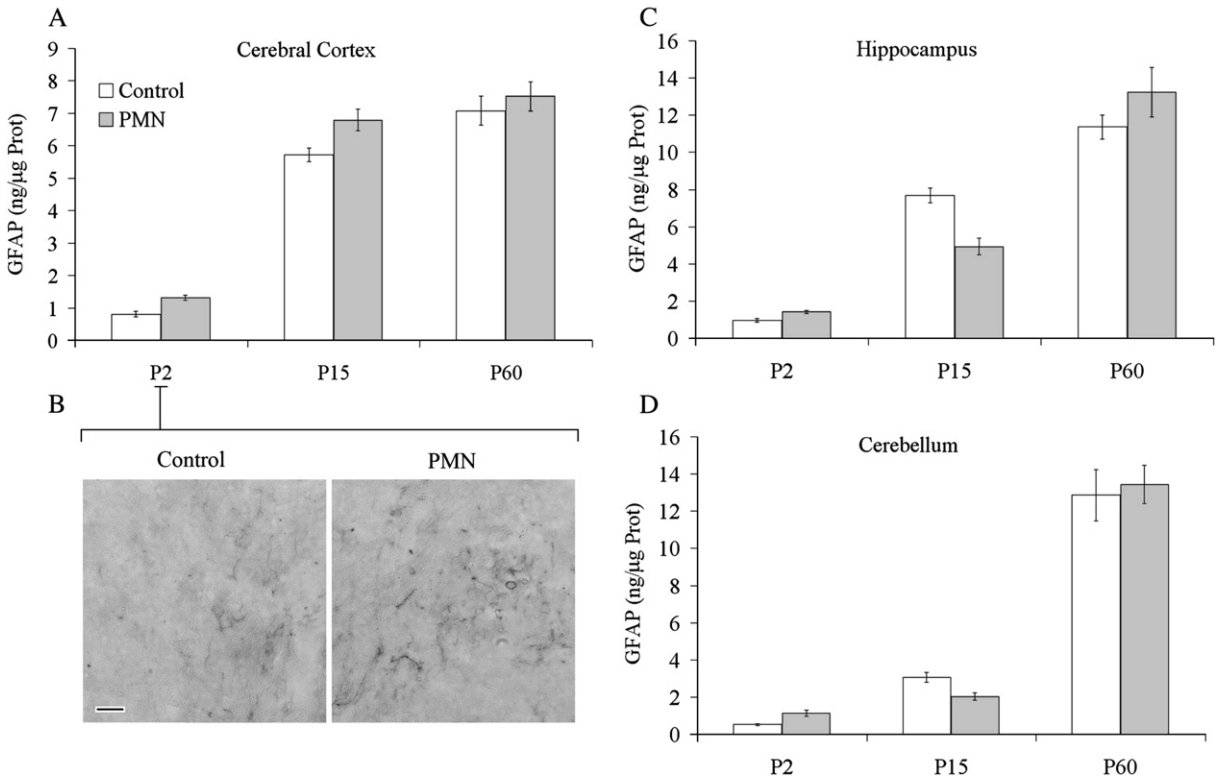

Fig. 2 – Post-natal development profile of GFAP content in brain tissue of rats exposed to PMN. Rats were exposed to a 7% protein diet during pre- and postnatal life. Three brain regions were dissected out (cerebral cortex in panel A, hippocampus in panel C and cerebellum in panel D) and GFAP contents were analyzed by ELISA, on the 2nd, 15th and 60th postnatal days. Values are mean ± standard error of 6–8 rats in each group. Significant effect dependent on age was observed, but not dependent of diet (two-way ANOVA, $p<0.05$). (B) Representative images of immunohistochemistry for GFAP of 3 independent experiments in parietal cerebral cortex from control and PMN rats on 2nd postnatal day. Scale bar = 50 µm.

Similarly to GFAP, an increase in S100B content dependent on age was observed in all brain structures, in both normal and malnourished animals (Fig. 3). It should be noted that two scales of values of S100B (in ng/µg protein) are depicted in the graphs. In cerebral cortex, the effect of age was significant ($p<10^{-4}$, $F_{(2,\ 32)}=391.98$) and the diet effect was almost significant ($p=0.064$, $F_{(1,\ 32)}=3.68$). Interestingly, a significant interaction (age–diet) was observed ($p=0.034$, $F_{(2,\ 32)}=3.75$). Similarly, the effect of age ($p<10^{-4}$) on S100B content of hippocampus and cerebellum was significant ($F_{(2,\ 31)}=170.27$ and $F_{(2,\ 24)}=1745.78$, respectively) and no effect of diet was observed ($p=0.207$, $F_{(1,\ 31)}=1.66$ and $p=0.538$, $F_{(1,\ 24)}=0.390$, for hippocampus and cerebellum, respectively). No interaction (age–diet) was observed in the hippocampus and cerebellum ($p=0.609$, $F_{(2,31)}=0.504$ and $p=0.560$, $F_{(2,24)}=0.594$, respectively).

In cerebrospinal fluid, an increase in S100B content was observed in malnourished rats on the 21st and 60th postnatal days (Fig. 4, $p=0.003$, $F_{(1,\ 32)}=10.55$). A decrease during development was observed in both groups ($p<10^{-4}$, $F_{(1,\ 32)}=6.76$). No interaction (age–diet) was observed in CSF S100B content ($p=0.859$, $F_{(1,\ 32)}=0.32$). It should be mentioned that we were not able to collect CSF without injury (indicated by blood contamination) in malnourished rats before the 21st postnatal day.

An increase in glutamine synthetase activity, dependent on age, was observed between the 2nd and 60th postnatal days, regardless of the nutritional status and the brain region (Fig. 5). In cerebral cortex, the effect of age was significant ($p<10^{-4}$, $F_{(2,\ 47)}=60.11$), but the effect of diet was not significant ($p=0.343$, $F_{(1,\ 47)}=0.919$). No interaction (age–diet) was observed ($p=0.288$, $F_{(2,\ 47)}=1.27$). Similarly, the effect of age ($p<10^{-4}$) on GS activity of hippocampus and cerebellum was significant ($F_{(2,\ 46)}=28.05$ and $F_{(2,\ 48)}=27.81$, respectively) and no effect of diet was observed ($p=0.838$, $F_{(1,46)}=0.42$ and $p=0.987$, $F_{(1,\ 48)}=0.000$, for hippocampus and cerebellum, respectively). No interaction (age–diet) was observed in the hippocampus and cerebellum ($p=0.248$, $F_{(2,46)}=1.43$ and $p=0.740$, $F_{(2,\ 48)}=0.303$, respectively).

In order to investigate the signaling pathway involved in the putative astrogliosis, we evaluated ERK activation in the cerebral cortex on the 2nd, 15th and 60th postnatal days (Fig. 6). We detected an increase in ERK phosphorylation (types 1 and 2) on the 2nd and 15th postnatal days in malnourished rats, but not on the 60th postnatal day. Total ERK 1/2 immunocontents were not different between groups in each age.

3. Discussion

3.1. *Glial alterations in malnourished rats at birth*

Regardless of the progress in reducing global infant mortality, fetal and early postnatal PMN is still a challenge for public health in the developing world; a situation that demands

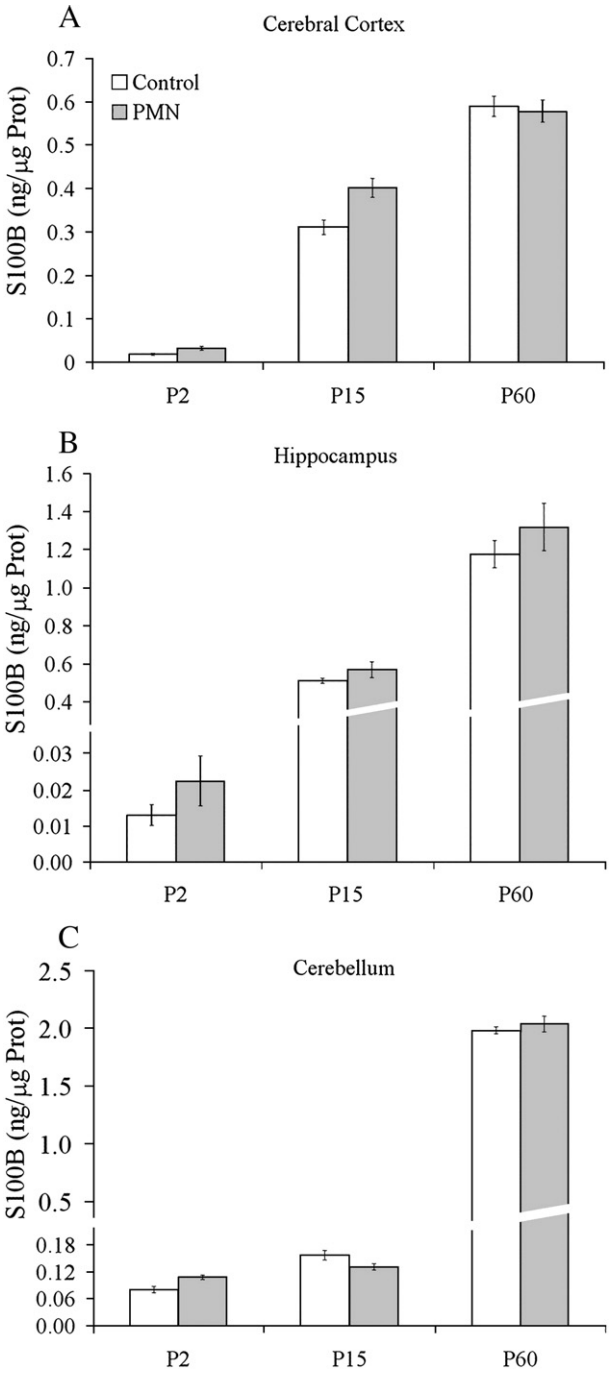

Fig. 3 – Post-natal development profile of S100B content in brain tissue of rats exposed to PMN. Rats were exposed to a 7% protein diet during pre- and postnatal life. Three brain regions were dissected out (cerebral cortex, hippocampus and cerebellum) and S100B contents were analyzed by ELISA on the 2nd, 15th and 60th postnatal days. Values are mean ± standard error from 6 to 8 rats in each group. Notice two scales of S100B values on the Y axis in panels B and C. A significant effect, dependent on age, was observed, but this was not dependent on diet (two-way ANOVA, $p<0.05$). Moreover, a significant interaction (age–diet) was observed in the cerebral cortex.

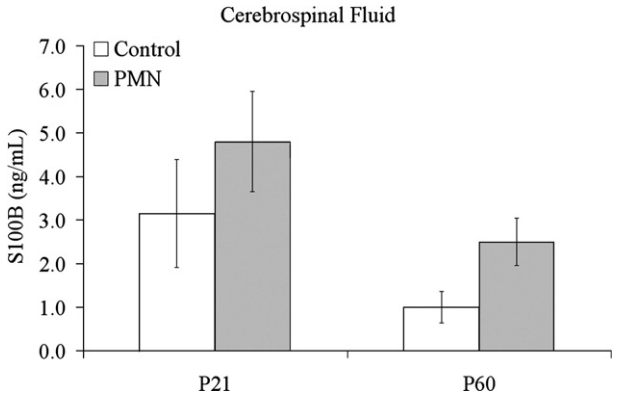

Fig. 4 – Post-natal development profile of S100B in cerebrospinal fluid of rats exposed to PMN. Rats were exposed to 7% protein diet during pre- and postnatal life. Cerebrospinal fluid was collected by cisterna magna puncture under anesthesia with thiopental, on the 21st and 60th postnatal days. S100B content was measured by ELISA. Values are mean ± standard error of 6–8 rats in each group. Significant effects dependent on age or diet were observed (two-way ANOVA, $p<0.05$).

additional efforts to investigate biochemical changes and risks for chronic diseases. Early nutritional deficiencies, even when transient, may affect long-term disease risk, possibly by modifying cellular differentiation and/or function at critical stages of development (Caballero, 2002).

A study carried out 25 years ago showed that pre- and post-natal malnutrition in rats reduced proliferation of neuronal and glial cells (Giuffrida et al., 1980); since this first report, numerous studies have described lower brain weight in malnourished animals, as observed herein. A later study demonstrated a decrease in GFAP-positive cells in the cerebral cortex of rats on the 2nd postnatal day, following exposure to protein restriction during just the first two weeks of gestation (Gressens et al., 1997). We found an augmented GFAP content (as well as S100B content and GS activity) in the cerebral cortex on the 2nd postnatal day. Hippocampus and cerebellum also exhibited this profile at birth.

It is accepted that GS is developmentally regulated and that an increase in its activity is associated with astrocyte differentiation rather than proliferation (Weir et al., 1984). Thus, the increased GS activity observed in malnourished rats at birth is possibly due to a greater amount of the enzyme. It is important to mention that this increment in GS activity could compensate the reduced glutamate uptake activity and glutathione content observed in brain slices of malnourished rats at birth (Feoli et al., 2006b).

GFAP, S100B and GS are markers of astrocyte maturity in brain tissue during development. In fact, all these markers exhibited an increment with post-natal age. PMN did not alter the postnatal development profile of the astrocyte markers. The increase observed at birth (see Table 2) could be the consequence of cell maturation, rather than an increase in astrocyte population during prenatal life. A qualitative analysis of cerebral cortex on the 2nd postnatal day suggests a higher immunoreactivity in GFAP-positive cells in malnourished animals; however, the methodology of this study did not allow

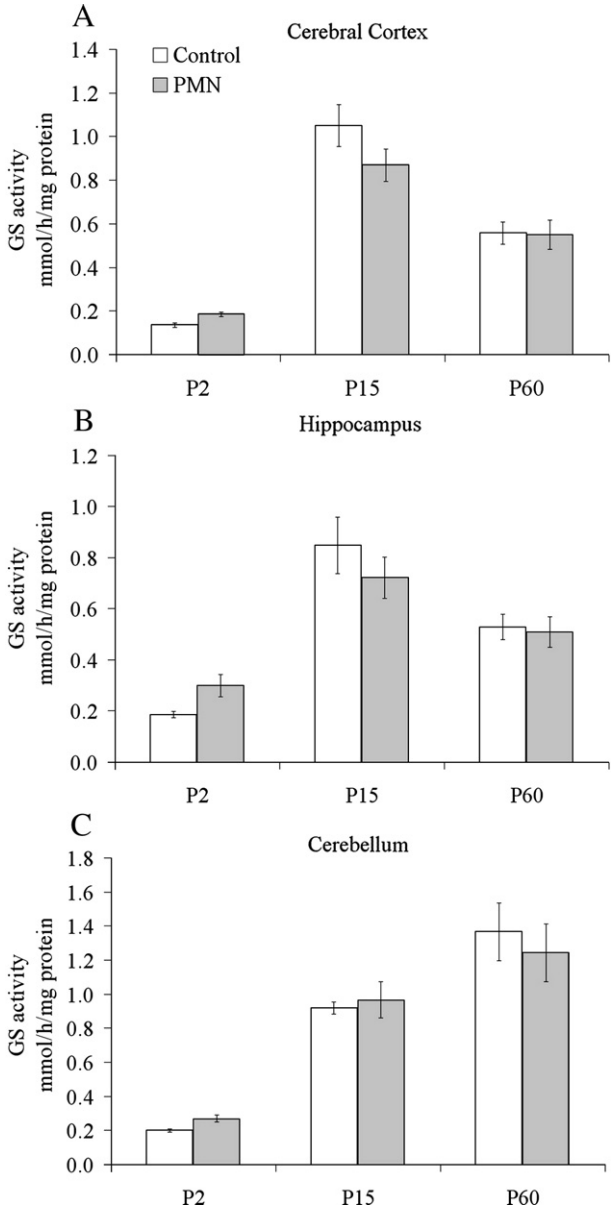

Fig. 5 – Post-natal development profile of GS activity in brain tissue of rats exposed to PMN. Rats were exposed to a 7% protein diet during pre- and postnatal life. Three brain regions were dissected out (cerebral cortex, hippocampus and cerebellum) and GS activity was analyzed by enzymatic colorimetric assay on the 2nd, 15th and 60th postnatal days. Values are mean ± standard error from 6 to 8 rats in each group. Significant effect dependent on age was observed, but not dependent of diet (two-way ANOVA, $p < 0.05$).

the evaluation of astrocyte number and cell density. A more detailed and appropriate densitometry should be carried out in future studies in specific cell layers of cerebral cortex and other brain regions to confirm and extend previous results (Gressens et al., 1997).

As such, prenatal PMN might cause a precocious maturation of astrocytes, which in turn is accompanied by an interruption of neuronal generation (He et al., 2005; Sauvageot and Stiles, 2002). The different developmental profiles of these proteins in malnourished rats, when comparing hippocampus/cerebellum and cerebral cortex, were possibly due to the different profile of glial and neuronal postnatal proliferation and differentiation in these brain regions (Giuffrida et al., 1980; Morgane et al., 2002).

Conversely, after injury of the CNS, either as a result of trauma, disease, genetic disorders or chemical insult, astrocytes become reactive and this reactivity, termed astrogliosis, is characterized by an increase in GFAP (Eng et al., 2000; O'Callaghan, 1991). Therefore, another possible explanation would be to interpret the increase of GFAP, at birth, as an astrogliosis. From this point of view, PMN would cause alterations equivalent to other insults, cited above, that may occur

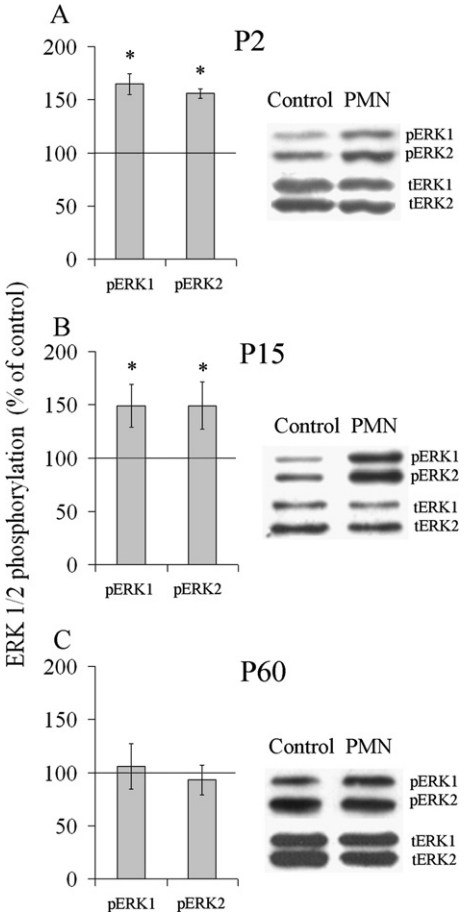

Fig. 6 – Post-natal development profile of ERK 1/2 phosphorylation in cerebral cortex of rats exposed to PMN. Rats were exposed to a 7% protein diet during pre- and postnatal life. Cerebral cortex was dissected out and ERK 1/2 phosphorylation was analyzed by immunoblotting on the 2nd, 15th and 60th postnatal days. Panels A–C show the quantitative analysis of ERK 1/2 phosphorylation on the 2nd, 15th and 60th postnatal days, respectively. Representative immunoblots of phosphorylated ERK 1/2 (pERK 1/2) and total ERK 1/2 (tERK 1/2) are inserted. The data are expressed as percentages of control values (considered as 100%) and represent the mean ± standard error of 3 independent experiments performed in triplicate. *Different from respective control (Student's t test, $p < 0.05$).

during prenatal brain development (see Little and O'Callaghan, 2001, for a review).

In this study, we evaluated the extracellular regulated kinase 1 and 2 (ERK 1/2) signaling pathway system, which is commonly associated with astrogliosis (Mandell and Vanden-Berg, 1999; Webster et al., 2006). We observed increased ERK 1/2 activation in malnourished animals on the 2nd and 15th postnatal days in the cerebral cortex of the brain tissue of malnourished animals, but not on the 60th postnatal day. This elevated basal ERK activation in malnourished neonate rats could participate in the altered cellular response to injury, as observed, for example, in hepatocytes of malnourished rats exposed to interleukin-6 (Ling et al., 2004).

The question remains as to which process, precocious astrocyte maturation or astrogliosis, is more plausibly triggered by PMN during prenatal development. During brain development, neurons and glia are generated in a sequential manner. The Janus kinase (JAK)-signal transducer and activator of the transcription (STAT) 1/3 pathway have been postulated to regulate the later onset of astrogliogenesis (He et al., 2005). Interestingly, in hepatocytes, activation has been observed in the JAK-STAT 3 pathway following induction by PMN (Ling et al., 2004); however, JAK-STAT signaling pathway has also been involved in the induction of astrogliosis (Sriram et al., 2004). Therefore, an increased activation of the JAK-STAT signaling pathway in the brain tissue of malnourished rats could be triggering either earlier glial maturation or astrogliosis. Moreover, the possibility that, during early development, precocious astrocyte maturation and astrogliosis are the same response to PMN should not be ruled out and this issue deserves further investigation.

3.2. *Glial alterations in malnourished rats during the postnatal life*

After birth, an astrocytogenesis peak occurs at around the 15th postnatal day in rodents; this peak is associated with an intense cell granule proliferation, particularly in hippocampus and cerebellum (Morgane et al., 2002). A decrease in the immunohistochemical staining for GFAP on the 30th and 60th postnatal days, particularly in the suprachiasmatic nucleus, induced by a multideficient diet has been reported (Mendonca, 2004).

In the cerebral cortex of rats exposed to PMN, we observed an age–diet interaction effect on S100B content. Such an interaction was not observed in the cerebellum or hippocampus; however, PMN had no effect on GFAP content and GS activity in malnourished rats during postnatal life. Taken together, these data suggest that the glial heterogeneity in the brain should be considered; it is possible that the changes in S100B could be due to differences in the manner in which PMN affects different populations of astrocytes and molecular targets.

Considering the putative role of S100B in synaptogenesis (Donato, 2001; Van Eldik and Wainwright, 2003), we speculate that, in malnourished rats, hippocampal and cerebellar neurons are less affected than cerebral cortex. The elevated content of S100B in cerebral cortex could impair neuronal survival; however, the S100B increase in cerebral cortex does not necessarily correspond to an increase in the extracellular levels of this protein in this tissue. In fact, distinct intra- and extracellular changes in S100B levels have been reported during postnatal development and in astrocyte culture (Eng et al., 2000), indicating that the extracellular S100B level does not necessarily reflect the intracellular S100B content.

3.3. *CSF S100B in malnourished rats*

Several studies have demonstrated the existence of a positive relationship between injury and CNS and S100B levels in CSF (Rothermundt et al., 2003). In agreement with these findings and with the idea of nutritional injury, we found an early and persistent increase in CSF S100B in malnourished rats. The elevation in CSF S100B, observed on the 21st postnatal day, is possibly secondary to the variation of this protein in a determined brain region, such as the cerebral cortex, which exhibited a significant age–diet interaction.

On the other hand, the increase in CSF S100B may occur as a consequence of delayed glial maturation in malnourished rats. In fact, a developmental decrease in CSF S100B between postnatal days 15 and 60 was observed in normal and malnourished rats (Fig. 4; Tramontina et al., 2002). CSF S100B was persistently more elevated in malnourished rats than in control rats. A direct and specific effect of PMN on extracellular S100B cannot be ruled out, as observed in ketogenic fed rats (Ziegler et al., 2004) and astrocyte cultures treated with β-hydroxy-butyrate (Leite et al., 2004).

In summary, these data suggest a significant and transient astrogliosis at birth in rats induced by PMN that could, in turn, affect neuronal development, survival and plasticity. GFAP, S100B, GS and ERK data would support the concept of astrogliosis or precocious astrogliogenesis at birth, which may represent the same response to PMN at this age. Specific postnatal alteration in the S100B content of cerebral cortex emphasizes the heterogeneous response to PMN of brain regions and molecular targets. Another interesting aspect is the recovery (based on contents of GFAP and S100B and GS activity) observed in adult rats, confirming the remarkable plasticity of brain tissue following the prenatal nutritional insult observed by many researchers (e.g. Gressens et al., 1997). However, the persistently elevated extracellular levels of S100B, together with some signals of oxidative damage (Feoli et al., 2006a) as well as the early and transient astrogliosis, reinforce the idea of brain damage or risk of brain diseases in individuals exposed to nutritional insult during development.

4. Experimental procedure

4.1. *Material*

Sodium carbonate, barbital, albumin, Tween-20, EGTA, PMSF, acrylamide, bys-acrylamide, 3,3-diaminobenzidine (DAB), o-phenylenediamine (OPD) and monoclonal anti-S100B antibody were purchased from SIGMA, USA. Anti-S100 antibody conjugated with peroxidase and anti-GFAP antibody were from DAKO, USA. Antibodies against phosphorylated-ERK 1/2 and total ERK 1/2 were purchased from Santa Cruz Biotechnology. Nitrocellulose and peroxidase-conjugated secondary antibodies from Amersham. Maxisorp microtiter plates were from NUNC.

4.2. *Animals and PMN protocol*

Male Wistar rats from our breeding colony were used. Prenatal and lactational malnutrition was induced in pups by restricting the protein content of the mother's diet to 7% (Table 1) during the entire gestation and lactation periods (when the malnutrition was produced by decreasing the global amount of nutrients accessible by the pups), whereas additional malnutrition until 60 days old was induced by maintaining the same 7% protein diet (Feoli et al., 2006b). The litter size was adjusted to eight pups per mother on the first postnatal day. Animals were maintained at 22 °C, on a 12-h light/12-h dark cycle until the experimental age. The protocol concerning this research was used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil (de Souza et al., 2004).

4.3. *Brain tissue and CSF samples*

Rats were anaesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) and positioned in a stereotaxic apparatus. Cerebrospinal fluid was obtained by cisterna magna puncture using a 0.33 mm diameter needle that was inserted no more than 1.5 mm and a maximum volume of 30 μl was collected in a 3 min period to minimize risk of brainstem damage. CSF samples were frozen (−70 °C) until further analysis. After decapitation, the brain was dissected and cut into 0.4 mm slices with a McIlwain chopper. Microslices (1 mm in diameter) were obtained from hippocampus, cerebellum and cerebral cortex with a stainless steel punch. Brain slices were homogenized in PBS, pH 7.4, containing 1 mM EGTA and 1 mM PMSF and stored at −70 °C (Tramontina et al., 2002).

Table 1 – Nutritional composition of the diets

	25% protein diet (g/kg/diet)	7% protein diet (g/kg/diet)
Casein (87% protein)[b]	287	80.50
L-Methionine	1.5	1.5
Fat (soybean oil)	150	150
Carbohydrate (corn starch)	501.5	708
Salt mix[c]	40	40
Vitamin mix[d]	10	10
Non-nutritive fiber	10	10

Energy for both diets: 4.3 kcal/g diet in both diets.
[a] Salt and vitamin compositions are according to Horwitz (1980).
[b] Casein, purity 87% (from Farmaquímica, Porto Alegre, Brazil) supplemented with 0.15% L-methionine (from Merck, Rio de Janeiro, Brazil).
[c] Mineral mixture (from Roche, São Paulo, Brazil) mg/100 g of ration: NaCl, 557; KCl, 3.2; KH_2PO_4, 1556; $MgSO_4$, 229; $CaCO_3$, 1526; $FeSO_4\_7H_2O$, 108; $MnSO_4\_H_2O$, 16; $ZnSO_4\_7H_2O$, 2.2; $CuSO_4\_5H_2O$, 1.9; $CaCl_2\_6H_2O$, 0.09.
[d] Vitamin mixture (from Roche, São Paulo, Brazil), mg/100 g of ration: vitamin A, 4; vitamin D, 0.5; vitamin E, 10; menadione, 0.5; choline, 200; PABA, 10; inositol, 10; niacin, 4; pantothenic acid, 4; riboflavin,0.8; thiamin, 0.5; pyridoxine, 0.5; folic acid, 0.2; biotin, 0.04; vitamin B12, 0.003.

Table 2 – Brain weight and glial proteins at birth of rats exposed to PMN during pre-natal life

	Control (mean ± SE)	PMN (mean ± SE)	p[a]
Brain weight[b]	0.288 ± 0.018	0.232 ± 0.013	$<10^{-4}$
Glial proteins[c]			
Cerebral cortex			
GFAP	0.806 ± 0.085	1.314 ± 0.070	0.006
S100B	0.018 ± 0.001	0.031 ± 0.004	0.028
GS	0.136 ± 0.000	0.185 ± 0.000	$<10^{-4}$
Hippocampus			
GFAP	0.969 ± 0.102	1.416 ± 0.093	0.008
S100B	0.012 ± 0.002	0.022 ± 0.006	0.261
GS	0.187 ± 0.012	0.300 ± 0.043	0.020
Cerebellum			
GFAP	0.521 ± 0.044	1.126 ± 0.168	0.016
S100B	0.080 ± 0.007	0.107 ± 0.005	0.026
GS	0.200 ± 0.010	0.270 ± 0.020	0.030

Rats were exposed to a 7% protein diet (or 25% protein diet, controls) during prenatal life. Brain weights and glial protein were measured on the 2nd postnatal day. Values are mean ± standard error of 8 rats in each group.
[a] Significance evaluated by Student's t test.
[b] Brain weight (in grams).
[c] Glial proteins: GFAP and S100B (in ng/μg of total protein); GS activity (in mmol/h/mg protein).

4.4. *Quantification of S100B and GFAP*

S100B content in CSF and brain tissue was measured by ELISA, as described previously (Tramontina et al., 2000; Vicente et al., 2004). Briefly, 50 μl of sample plus 50 μl of barbital buffer was incubated for 3 h on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1, from Sigma). Peroxidase-conjugated anti-S100 (from DAKO) was then incubated for 1 h. A colorimetric reaction with o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.025 to 2.5 ng/ml. Protein was measured by Lowry's method using bovine serum albumin as a standard. ELISA for GFAP was carried out by coating the microtiter plate with 100 μl samples containing 30 μg of protein for 48 h at 4 °C. Incubation with a polyclonal anti-GFAP from rabbit (DAKO) for 2 h was followed by incubation with a secondary antibody conjugated with peroxidase for 1 h, at room temperature; the standard GFAP curve ranged from 0.1 to 10 ng/ml.

4.5. *Glutamine synthetase activity assay*

The enzymatic assay was performed according to Petito et al. (1992). Briefly, homogenized tissue samples were added to a reaction mixture containing (in mM): 10 $MgCl_2$; 50 L-glutamate; 100 imidazole–HCl buffer (pH 7.4); 10 2-mercaptoethanol; 50 hydroxylamine–HCl; 10 ATP and incubated for 15 min at 37 °C. The reaction was stopped by the addition of 0.4 ml of a solution containing: 370 mM ferric chloride; 670 mM HCl; 200 mM trichloroacetic acid. After centrifugation, the supernatant was measured at 530 nm and compared to the absorbance generated by standard quantities of γ-glutamylhydroxamate (from Sigma) treated with ferric chloride reagent.

4.6. *Detection of ERK activity*

Immunoblotting analysis was performed, as previously described (Leal et al., 2007). Briefly, the brain slices were solubilized with SDS-stopping solution (4% SDS, 2 mM EDTA, 8% β-mercaptoethanol, and 50 mM Tris, pH 6.8). Samples (80 μg of total protein/track) were separated by SDS-PAGE (10% acrylamide/bis-acrylamide). The proteins were transferred to nitrocellulose membrane using a semidry blotting apparatus (1.2 mA/cm^2; 90 min). The membranes were blocked with 5% skimmed milk in TBS (10 mM Tris, 150 mM NaCl, pH 7.5). All steps were followed by washing three times with TBS-T (10 mM Tris, 150 mM NaCl, Tween-20 0.1%, pH 7.5). Phospho-ERK 1/2 and total-ERK 1/2 were detected with anti-phospho-ERK 1/2 (1:10,000) and anti-ERK 1/2 (1:40,000) antibodies both diluted in TBS-T containing 2.5% BSA. The reactions were developed by ECL. ERK 1/2 phosphorylation was measured by scanning the films and the bands were quantified using the Scion Image software.

4.7. *Immunohistochemistry for GFAP in cerebral cortex*

P2 rats were anesthetized using ketamine/xylazine and were perfused through the left cardiac ventricle with 20 ml of saline solution followed by 20 ml of 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. The brains were removed and left for post-fixation in the same fixative solution at 4 °C for 2 h. After this, the material was cryoprotected by immersing the brain in 30% sucrose in phosphate buffer at 4 °C. The brains were sectioned (60 μm) on a cryostat (Leitz) and sections were treated in 10% methanol and 3% H_2O_2 for 30 min. The sections were then preincubated in 2% BSA in PBS containing 0.3% Triton X-100 for 30 min and incubated with polyclonal GFAP antiserum raised in rabbit (from DAKO) diluted 1:50 in 2% BSA in PBS-Triton X-100 for 48 h at 4 °C. After washing several times, tissue sections were incubated in a rabbit PAP-conjugated anti-rabbit IgG diluted 1:50 in PBS at room temperature for 2 h. The immunohistochemical reaction was revealed by incubating the sections in a histochemical medium that contained 0.06% 3,3-diaminobenzidine (DAB) dissolved in PBS for 10 min and then, in the same solution containing 1 μM of 3% H_2O_2 per ml of DAB medium for approximately 10 min. Afterwards, the sections were rinsed in PBS, dehydrated in ethanol, cleared with xylene and covered with Entellan and coverslips. Images were viewed with a Nikon inverted microscope and images transferred to computer with a digital camera (Sound Vision Inc. Wayland, MA).

4.8. *Statistical analysis*

Results are expressed as mean ± standard error of the mean. Two-way ANOVA, assuming significance (p) < 0.05, was used to characterize the postnatal developmental profile of the studied parameters, with the exceptions of ERK 1/2 phosphorylation and the glial parameters of the cerebral cortex at birth (Table 2), which were analyzed statistically using the Student's t test.

Acknowledgments

Brazilian funds from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior (CAPES), FINEP/Rede IBN 01.06.0842-00 and Blanver-Farmoquimica for providing the fiber used in diet composition.

REFERENCES

Caballero, B., 2002. Global patterns of child health: the role of nutrition. Ann. Nutr. Metab. 46, 3–7.

Clos, J., Legrand, C., Legrand, J., Ghandour, M.S., Labourdette, G., Vincendon, G., Gombos, G., 1982. Effects of thyroid state and undernutrition on S100 protein and astroglial development in rat cerebellum. Dev. Neurosci. 5, 285–292.

de Souza, K.B., Feoli, A.M., Kruger, A.H., de Souza, M.R., Perry, C.T., Rotta, L.N., Souza, D.O., Perry, M.L., 2004. Effects of undernutrition on glycine metabolism in the cerebellum of rats. Ann. Nutr. Metab. 48, 246–250.

Donato, R., 2001. S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. Int. J. Biochem. Cell Biol. 33, 637–668.

Eng, L.F., Ghirnikar, R.S., Lee, Y.L., 2000. Glial fibrillary acidic protein: GFAP-thirty-one years (1969–2000). Neurochem. Res. 25, 1439–1451.

Feoli, A.M., Siqueira, I.R., Almeida, L., Tramontina, A.C., Vanzella, C., Sbaraini, S., Schweigert, I.D., Netto, C.A., Perry, M.L.S., Gonçalves, C.A., 2006a. Effects of protein malnutrition on oxidative status in rat brain. Nutrition 22, 160–165.

Feoli, A.M., Siqueira, I., Almeida, L.M., Tramontina, A.C., Battu, C., Wofchuk, S.T., Gottfried, C., Perry, M.L., Goncalves, C.A., 2006b. Brain glutathione content and glutamate uptake are reduced in rats exposed to pre- and postnatal protein malnutrition. J. Nutr. 136, 2357–2361.

Giuffrida, A.M., Hamberger, A., Serra, I., Geremia, E., 1980. Effects of undernutrition on nucleic acid synthesis in neuronal and glial cells from different regions of developing rat brain. Nutr. Metab. 24, 189–198.

Gressens, P., Muaku, S.M., Besse, L., Nsegbe, E., Gallego, J., Delpech, B., Gaultier, C., Evrard, P., Ketelslegers, J.M., Maiter, D., 1997. Maternal protein restriction early in rat pregnancy alters brain development in the progeny. Dev. Brain Res. 103, 21–35.

He, F., Ge, W., Martinowich, K., Becker-Catania, S., Coskun, V., Zhu, W., Wu, H., Castro, D., Guillemot, F., Fan, G., de Vellis, J., Sun, Y.E., 2005. A positive autoregulatory loop of Jak-STAT signaling controls the onset of astrogliogenesis. Nat. Neurosci. 8, 616–625.

Horwitz, W., 1980. Official Methods of Analysis of the Association of Official Analytical Chemists. AOAC, Washington, DC.

Leal, R.B., Posser, T., Rigon, A.P., Oliveira, C.S., Gonçalves, C.A., Gelain, D.P., Dunkley, P.R., 2007. Cadmium stimulates MAPKs and Hsp27 phosphorylation in bovine adrenal chromaffin cells. Toxicology 234, 34–43.

Leite, M., Frizzo, J.K., Nardin, P., de Almeida, L.M., Tramontina, F., Gottfried, C., Goncalves, C.A., 2004. Beta-hydroxy-butyrate alters the extracellular content of S100B in astrocyte cultures. Brain Res. Bull. 64, 139–143.

Ling, P.R., Smith, R.J., Kie, S., Boyce, P., Bistrian, B.R., 2004. Effects of protein malnutrition on IL-6-mediated signaling in the liver and the systemic acute-phase response in rats. Am. J. Physiol., Regul. Integr. Comp. Physiol. 287, 801–808.

Little, A.R., O'Callaghan, J.P., 2001. Astrogliosis in the adult and developing CNS: is there a role for proinflammatory cytokines? Neurotoxicology 22, 607–618.

Mandell, J.W., VandenBerg, S.R., 1999. ERK/MAP kinase is chronically activated in human reactive astrocytes. Neuroreport 10, 3567–3572.

Mendonca, J.E., Vilela, M.C., Bittencourt, H., Lapa, R.M., Oliveira, F.G., Alessio, M.L., Guedes, R.C., De Oliveira Costa, M.S., Da Costa, B.L., 2004. GFAP expression in astrocytes of suprachiasmatic

nucleus and medial preoptic area are differentially affected by malnutrition during rat brain development. Nutr. Neurosci. 7, 223–234.
Morgane, P.J., Austin-LaFrance, R., Bronzino, J., Tonkiss, J., Diaz-Cintra, S., Cintra, L., Kemper, T., Gallerm, J.R., 1993. Prenatal malnutrition and development of the brain. Neurosci. Biobehav. Rev. 17, 91–128.
Morgane, P.J., Mokler, D.J., Galler, J.R., 2002. Effects of prenatal protein malnutrition on the hippocampal formation. Neurosci. Biobehav. Rev. 26, 471–483.
O'Callaghan, J.P., 1991. Assessment of neurotoxicity: use of glial fibrillary acidic protein as a biomarker. Biomed. Environ. Sci. 4, 197–206.
Petito, C.K., Chung, M.C., Verkhovsky, L.M., Cooper, A.J.L., 1992. Brain glutamine synthetase increases following cerebral ischemia in the rat. Brain Res. 569, 275–280.
Rocha, J.B., Souza, D.O., 1994. Effects of undernutrition during suckling and early post-weaning on the inhibition by met-enkephalin of striatal adenylate cyclase activity in adult rats. Pharmacol. Toxicol. 75, 321–323.
Rothermundt, M., Peters, M., Prehn, J.H., Arolt, V., 2003. S100B in brain damage and neurodegeneration. Microsc. Res. Tech. 60, 614–632.
Rotta, L.N., Schmidt, A.P., Mello e Souza, T., Nogueira, C.W., Souza, K.B., Izquierdo, I.A., Perry, M.L., Souza, D.O., 2003. Effects of undernutrition on glutamatergic parameters in rat brain. Neurochem. Res. 28, 1181–1186.
Sauvageot, C.M., Stiles, C.D., 2002. Molecular mechanisms controlling cortical gliogenesis. Curr. Opin. Neurobiol. 12, 244–249.
Sriram, K., Benkovic, S.A., Hebert, M.A., Miller, D.B., O'Callaghan, J.P., 2004. Induction of gp130-related cytokines and activation of JAK2/STAT3 pathway in astrocytes precedes up-regulation of glial fibrillary acidic protein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of neurodegeneration: key signaling pathway for astrogliosis in vivo? J. Biol. Chem. 279, 19936–19947.
Steiger, J.L., Alexander, M.J., Galler, J.R., Farb, D.H., Russek, S.J., 2003. Effects of prenatal malnutrition on GABAA receptor alpha1, alpha3 and beta2 mRNA levels. Neuroreport 14, 1731–1735.
Takuma, K., Baba, A., Matsuda, T., 2004. Astrocyte apoptosis: implications for neuroprotection. Prog. Neurobiol. 72, 111–127.
Tramontina, F., Karl, J., Gottfried, C., Mendez, A., Goncalves, D., Portela, L.V., Gonçalves, C.A., 2000. Digitonin-permeabilization of astrocytes in culture monitored by trypan blue exclusion and loss of S100B by ELISA. Brain Res. Protoc. 6, 686–690.
Tramontina, F., Conte, S., Gonçalves, D., Gottfried, C., Portela, L.V., Vinade, L., Salbego, C., Gonçalves, C.A., 2002. Developmental changes in S100B content in brain tissue, cerebrospinal fluid, and astrocyte cultures of rats. Cell. Mol. Neurobiol. 22, 373–378.
Van Eldik, L.J., Wainwright, M.S., 2003. The Janus face of glial-derived S100B: beneficial and detrimental functions in the brain. Restor. Neurol. Neurosci. 21, 97–108.
Vicente, E., Bôer, M., Leite, M., Silva, M., Tramontina, F., Porciúncula, L., Dalmaz, C., Gonçalves, C.A., 2004. Cerebrospinal fluid S100B increases reversibly in neonates of methylmercury-intoxicated pregnant rats. Neurotoxicology 25, 771–777.
Weir, M.D., Patel, A.J., Hunt, A., Thomas, D.G., 1984. Developmental changes in the amount of glial fibrillary acidic protein in three regions of the rat brain. Brain Res. 317, 147–154.
Webster, B., Hansen, L., Adame, A., Crews, L., Torrance, M., Thal, L., Masliah, E., 2006. Astroglial activation of extracellular-regulated kinase in early stages of Alzheimer disease. J. Neuropathol. Exp. Neurol. 65, 142–151.
Wiggins, R.C., Fuller, G., Enna, S.J., 1984. Malnutrition and the development of brain neurotransmitter systems. Life Sci. 35, 2085–2094.
Ziegler, D.R., Oliveira, D.L., Pires, C., Ribeiro, L., Leite, M., Mendez, A., Gonçalves, D., Tramontina, F., Portela, L.V., Wofchuk, S.T., Perry, M.L., Gonçalves, C.A., 2004. Ketogenic diet fed rats have low levels of S100B in cerebrospinal fluid. Neurosci. Res. 50, 375–379.