UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PPG-NEUROCIÊNCIAS

IMUNOCONTEÚDO DA PROTEÍNA S100B E COMPORTAMENTO COGNITIVO EM RATOS EXPOSTOS AO METIL-MERCÚRIO DURANTE A GESTAÇÃO

DISSERTAÇÃO DE MESTRADO

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PORTO ALEGRE

2004

Resumo

Metilmercúrio (MeHg), a forma orgânica do mercúrio, é um dos poluentes de maior risco ao ambiente. MeHg é uma potente neurotoxina, principalmente durante o desenvolvimento do sistema nervoso central. A neurotoxicidade induzida pelo MeHg no período pré-natal pode causar desordens mentais, paralisia cerebral e convulsões. Nós investigamos o imunoconteúdo de S100B no fluído cerebroespinal (FCE) e no tecido encefálico, uma proteína ligante de cálcio produzida e secretada pelos astrócitos, na qual tem uma atividade trófica e tóxica, dependendo da sua concentração. Ratas grávidas foram expostas ao MeHg (5 mg/kg/dia) no 12°, 13° e 14° dias de gestação. O fluído cerebroespinal e o tecido encefálico (mais especificamente hipocampo, córtex cerebral e cerebelo) foram obtidos dos neonatos no 1°, 15° e 30° dia pós-natal. O acúmulo de MeHg foi medido do tecido encefálico após o nascimento e aos 30 dias de vida. Um aumento da S100B no FCE foi observado aos 15 dias de vida pós-natal, mas desapareceu aos 30 dias. No tecido hipocampal mostrou um aumento da S100B (e redução da proteína ácida fibrilar glial) imediatamente após nascimento, mas não posteriormente. Nenhuma mudança foi observada no teste cognitivo (labirinto aquático) desses ratos em idade adulta. Nossos resultados reforçam o envolvimento glial na neurotoxicidade induzida pelo MeHg. As mudanças no hipocampo ao nascimento, poderiam estar relacionadas com as desordens cognitivas e epilépticas atribuídas ao MeHg. O aumento da S100B no FCE reforça a hipótese de que o aumento da S100B está relacionado a danos no sistema nervoso central. Embora, o mecanismo celular envolvido no aumento do conteúdo da S100B no FCE seja desconhecido, os resultados sugerem que a S100B possa ser usada como um marcador periférico na injúria induzida pelo MeHg.

Índice

1. Introdução

1.1 Neurotoxicidade do MeHg

O sistema nervoso central (SNC) concentra metais para uso metabólico, tendo as células neurais propriedades fisiológicas para se proteger da toxicidade dos metais (Tiffany- Castiglioni & Qian., 2001). Metais como cálcio, zinco, ferro, selênio, potássio e sódio, são nutrientes requeridos por muitos organismos vivos, mas estes metais podem apresentar efeitos tóxicos quando estiverem presentes em concentrações elevadas em um determinado organismo (Kataba-Pendias & Pendias, 1993). Outros metais, como alumínio, prata, ouro, chumbo, cádmio, arsênio e o mercúrio não são essenciais para os seres vivos, podendo causar efeitos tóxicos nos organismos expostos a tais elementos (Bruins et al., 2000). Mercúrio está presente no meio ambiente em eventos naturais (erupção vulcânica), como também por fontes antropogênicas (queima de carvão, processos químicos industriais). Na natureza, o mercúrio existe na forma inorgânica e orgânica, como por exemplo, o metilmercúrio (MeHg). A conversão das espécies de mercúrio orgânico para inorgânico, e vice-versa, ocorre no ambiente e *in vivo* (Allen et al., 2002).

Os riscos à saúde pela exposição à esse metal passou a chamar atenção após o trágico acidente em Minamata e Niigata, no Japão. Na década de 50, houve um grande derramamento de mercúrio na baía de Minamata expondo a população ao metal durante anos. As manifestações clínicas em adultos foram perda da função sensorial, visual, auditiva, fraqueza muscular, tremores, enquanto que paralisia cerebral, caracterizada de leve à grave disfunção motora e retardo mental, foi o principal sintoma da exposição pré-natal (Sanfeliu et al., 2001; Kakita et al., 2000; Gressens et al., 2001).

A população em geral expõe-se ao mercúrio a partir de inúmeras fontes, incluindo o ar, o solo, a água e os alimentos (Miller,1998). As amálgamas dentárias, ainda utilizadas, contribuem para a intoxicação, principalmente dos dentistas e seus auxiliares. Entretanto, a maior fonte de contaminação ao MeHg atualmente vem do consumo de peixes de águas contaminadas, onde o mercúrio inorgânico é metilado por bactérias presentes no ecossistema, entrando assim na cadeia alimentar (Bergdahl et al., 1998; Kakita et al., 2000; Giménez-Llort et al., 2001; Tiffany-Castiglioni & Qian., 2001). A concentração de MeHg nos músculos de peixes predadores pode chegar à ordem de mg/Kg (WHO, 1990). Nestes peixes, o mercúrio é acumulado e pode ser transferido para aves e mamíferos, incluindo a população humana. O acúmulo de metais pesados no organismo humano representa um risco significativo para a saúde, levando a uma variedade muito grande de sintomas, como anemia, déficits cognitivos, tremores, gengivite, hipertensão, irritabilidade, câncer, depressão, perda de memória, fadiga, cefaléia, hiperuricemia, gota, insuficiência renal crônica, infertilidade masculina, esclerose e doença de Alzheimer (Miller,1998).

Assim como em humanos, modelos experimentais em roedores mostram que o MeHg atravessa a barreira placentária e tem uma alta afinidade ao sistema nervoso central (SNC) fetal (Haykal-Coates et al., 1998). No SNC os astrócitos desempenham um papel importante na toxicidade do MeHg. O MeHg inibe a captação de glutamato na fenda sináptica, resultando em um aumento do neurotransmissor no meio extracelular (Aschner et al., 2000). Neste contexto, um recente estudo *in vivo* mostrou que o MeHg aumentou os níveis extracelulares de glutamato no córtex frontal de ratos (Juárez et al., 2002).

In vitro, tem sido demonstrado que MeHg pode afetar a função neuronal e astrocítica de

diferentes formas. Os principais mecanismos envolvidos na neurotoxicidade do MeHg são a geração de stress oxidativo, alteração da homeostase do cálcio intracelular e interferência com o transporte de membranas, especialmente a inibição da captação de glutamato pelos astrócitos. A excitotoxicidade neuronal seguida da alteração da captação de glutamato pelos astrócitos é uma hipótese do dano neuronal induzida pelo mercúrio baseada em diferentes experimentos, isso porque ele aumenta a liberação de cálcio das organelas intracelulares e bloqueia o influxo de cálcio através de múltiplos subtipos de canais, modificando assim a concentração intracelular do cálcio e a liberação de neurotransmissores. Além disso, o MeHg tem alta afinidade por grupamentos sulfidrilas (Trombeta & Kromidas., 1992), o qual resulta na depleção intracelular da glutationa levando a um acúmulo das espécies reativas de oxigênio. O próprio stress oxidativo, inibe o mecanismo de captação do glutamato astrocítico através de uma ação direta nos transportadores de proteína (Shanker et al., 2001; Juárez et al., 2002).

Embora, os mecanismos bioquímicos e moleculares da ação do MeHg que levam a uma disfunção e degeneração neuronal não sejam ainda bem compreendidos (Yee & Choi., 1996; Faro et al., 2002; Shanker et al., 2002), os astrócitos apresentam um papel chave na neurotoxicidade induzida por esse metal. Isso devido a propensão do MeHg em se acumular nessas células gliais, um processo que não está ainda bem entendido (Aschner et al., 2000). Na tentativa de entender os mecanismos da toxicidade do MeHg, estudos têm se voltado para as células astrocíticas, que possuem várias características que lhe permitiriam servir como depósitos para metais no SNC. Primeiro, processos citoplasmáticos da astroglia conhecidos como pés terminais envolvem a superfície vascular no SNC e da pia mater, formando duas camadas morfológicas permeáveis conhecidas como membrana glial perivascular e membrana limitante pia-glial, respectivamente.

Além disso, os corpos celulares da astroglia estão posicionados entre os corpos celulares neuronal e o endotélio capilar que formam a barreira hematoencefálica. Portanto, a astroglia está citoarquiteturalmente posicionada para ser a primeira célula do parênquima cerebral a ser exposta aos elementos transportados pelo sangue, incluindo metais. A segunda propriedade é a metalotioneína – MT (Tiffany-Castiglioni & Qian., 2001), que são proteínas caracterizadas em conter muitas cisteínas (que possuem afinidade a metais divalentes), e agem como potentes "scavengers" do mercúrio cerebral. No tecido dos mamíferos, são encontradas duas isoformas MT-I e MT-II. No SNC, a MT-I e a MT-II são quase que ausentes em neurônios, mas são abundantes em astrócitos (Aschner et al., 2000; Tiffany-Castiglioni & Qian., 2001).

Estudos feitos em ratos mostram que exposição pré-natal ao MeHg leva a disfunção motora, no entanto as informações sobre disfunções cognitivas são controversas (Doré et al., 2001; Giménez-Llort et al., 2001).

1.2 Proteína S100B

S100B é uma proteína ligante de cálcio de 21 kDa, do tipo "EF-hand", produzida e secretada pelos astrócitos, sendo considerada uma proteína marcadora destas células. Também é expressa por melanócitos, condrócitos e adipócitos (Donato, 1999). Esta proteína pode ser encontrada solúvel no citoplasma, associada à membrana plasmática, a outras membranas intracelulares e ao citoesqueleto, o que sugere seu papel em diversos processos celulares (Sorci et al., 1998). A literatura pouco nos informa sobre sua distribuição no SNC durante o desenvolvimento, embora a ela tenha sido postulada importante função na maturação astrocítica e neuronal (Tiu et al., 2000).

Em ratos, os estudos datam de 1972. Nesse caso, foi possível medir a proteína logo após o nascimento e seu acúmulo iniciou a partir dos 14 dias aumentando rapidamente até os 6 meses,

mostrando que a sua expressão é regulada pelo desenvolvimento (Cícero et al., 1972).

Muitos estudos sugerem um papel intra e extracelular para essa proteína. Dentre as suas ações intracelulares, ela atua regulando a fosforilação de proteínas, de atividade enzimática, da homeostase do cálcio e da dinâmica do citoesqueleto. Com relação as suas funções extracelulares, a S100B parece estar envolvida na sobrevivência neuronal, extensão neurítica e proliferação glial. Foi observado que culturas primárias de astrócitos tem a secreção estimulada na ausência de soro (Tramontina et al., 2000; Pinto et al., 2000). Entretanto, pouco se conhece sobre o mecanismo pelo qual a secreção ocorre. A ação extracelular dessa proteína em cultura depende da sua concentração, ela pode exercer efeitos tróficos ou tóxicos. Em concentração nanomolar ela exerce efeitos neurotróficos estimulando a extensão de neuritos (Kligman & Marshak., 1985), facilitando a sobrevivência dos neurônios durante o desenvolvimento, além de estimular a proliferação de astrócitos (Van Eldik et al., 1991; Selinfreud et al., 1991). Em concentrações micromolares podem exercer efeitos neurotóxicos. Algumas doenças neurodegenerativas apresentam elevados níveis de S100B, como a Síndrome de Down e Doença de Alzheimer (Griffin et al., 1989). Camundongos transgênicos que expressam elevadas concentrações de S100B apresentam alterações comportamentais, além de apresentar uma alta densidade de dendritos no hipocampo durante períodos precoces de desenvolvimento pós-natal e uma significativa perda de dendritos quando apresentam um ano de idade, sugerindo o efeito deletério da proteína em elevadas concentrações (Whitaker-Azmitia et al., 1997).

1.3 Proteína GFAP

Sabe-se que a proteína glial fibrilar ácida - GFAP é a principal proteína de filamento intermediário em astrócitos maduros do SNC. A GFAP é uma importante moduladora da

estabilidade estrutural dos processos astrocíticos (Eng et al., 2000). Essa é uma proteína marcadora de astrócitos presente em filamentos intermediários do grupo III. Exibe uma atividade dinâmica modulada por fosforilação e desfosforilação, efetuando um papel fundamental na plasticidade astrocítica (Rodnight et al., 1997). O seu estado de fosforilação é regulado, dentre outros fatores, pela proteína S100B (Ziegler et al., 1998). No SNC de vertebrados superiores, seguidos de injúria por trauma, distúrbios genéticos, desordens, ou insultos químicos , astrócitos tornam- se reativos e respondem de uma maneira típica, chamado de astrogliose ou gliose reativa (Eng et al., 2000), à qual é caracterizada pela proliferação e/ou hipertrofia glial (Wishcamper et al., 2003).

As proteínas marcadoras astrocíticas GFAP e S100B podem ser usadas como marcadores de comprometimento glial frente à exposição à metais pesados (Huang et al., 1993; Noack et al., 1996). Além disso, o conteúdo de S100B no FCE poderia dar uma idéia de extensão de dano neural. Dessa forma, o presente estudo pretende avaliar o imunoconteúdo da S100B no tecido nervoso, particularmente hipocampo, cerebelo e córtex cerebral e no FCE frente a exposição ao MeHg.

A exposição ao metal foi feita no 12°, 13° e 14° dia de gestação (5 mg/kg/dia) (Watanabe et al., 1999). Esta escolha deve-se ao fato de que a partir do 12° dia de gestação até o nascimento, a proliferação celular é marcada por uma explosão da atividade no cerebelo, tálamo, estriado, estruturas límbicas e córtex cerebral. Antes desse período, a interferência do MeHg com a proliferação celular resulta frequentemente em malformações e aborto. Além disso, um estudo feito em neonatos de camundongos expostas ao MeHg nesses períodos de gestação com uma dose similar, mostrou alterações neuroquímicas (Watanabe et al., 1999) e motoras (Doré et al., 2001).

2. Objetivos

2.1 Objetivo Geral:

O presente estudo pretende avaliar os possíveis efeitos do metilmercúrio no conteúdo de S100B e o comportamento cognitivo em ratos expostos ao MeHg durante o período pré-natal.

2.2 Objetivos Específicos:

1- Quantificar o imunoconteúdo de S100B presente no tecido nervoso (cerebelo, hipocampo, e córtex cerebral);

2- Quantificar o imunoconteúdo de S100B no líquido cerebroespinal dos neonatos de ratas intoxicadas por MeHg;

3- Avaliar o comportamento cognitivo nos ratos de 60 dias intoxicados durante a gestação.

3. Publicação

Manuscrito submetido ao Neurotoxicology em 12 de janeiro de 2004:

“ Cerebrospinal fluid S100B increases reversibly in neonates of methyl mercury-intoxicated rats”

Cerebrospinal fluid S100B increases reversibly in neonates of methyl mercury-intoxicated pregnant rats

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Abstract

Methylmercury (MeHg), an organic methylated form of mercury, is one of the most hazardous environmental pollutants. MeHg is a potent neurotoxin, particularly during brain development. Neurotoxicity-induced by MeHg in prenatal age can cause mental disorders, cerebral palsy and seizures. We investigated cerebrospinal fluid (CSF) and brain tissue contents of S100B, a calcium binding protein produced and secreted by astrocytes, which has trophic and toxic activity on neurons depending on concentration. Pregnant rats were exposed to MeHg (5mg/kg/day, on the 12^{th}, 13^{th} and 14^{th} days of pregnancy). CSF and brain tissue (hippocampus, cerebral cortex and cerebellum) were obtained from neonate rats on 1, 15 and 30 days postnatal. MeHg accumulation was measured in brain tissue after birth and on the 30^{th} postnatal day. An increase of CSF S100B was observed on the 15^{th}, but not on the 30^{th} postnatal day. Hippocampal tissue demonstrated increased S100B (and reduction in glial fibrillary acidic protein) immediately after birth, but not later. No changes in the S100B content were observed in cerebellum and cerebral cortex. No changes were observed in the spatial learning of these rats at adult age. These specific and reversible changes in the hippocampus could be related to the cognitive and epileptic disorders attributed to MeHg. Our results further indicate the glial involvement in the MeHg-induced neurotoxicity. The increment of CSF S100B in neonates exposed to MeHg reinforces the view that increased S100B is related to damage in the nervous system and that S100B could be a marker for MeHg-neurotoxicity. Although the cellular mechanism related to MeHg-induced increase in S100B content in CSF remains unknown, our results suggest the use of S100B as a peripheral marker of brain damage induced by MeHg.

Key words: S100B, astrocyte, methylmercury, GFAP, brain development

Introduction

Methylmercury (MeHg) is a potent neurotoxin, particularly during fetal nervous system development, and high exposure to this compound can cause mental disorders, cerebral palsy and seizures (Myers and Davidson, 2000). Astrocytes accumulate MeHg, thus constituting brain depots for this well-known toxicant (Tiffany-Castiglioni and Qian, 2001). Whilst the neurotoxic effects of MeHg are well reported, the mechanisms underlying its toxicity are not fully understood.

MeHg toxicity impairs astrocytic glutamate uptake (Aschner, 2000), thus affecting the major excitatory mechanism of neurotransmission. Either death or proliferation of astrocytes have been reported after Hg accumulation in these cells, in turn influencing neuronal survival (Tiffany-Castiglioni and Qian, 2001). Distinct regional patterns of neuronal degeneration were found in neonate rats after MeHg administration during pregnancy, revealing degenerative neurons in the brain stem and the limbic system, including the hippocampus (Kakita et al., 2000).

Neurobehavioral parameters have been studied in intrauterine MeHg intoxication, including epileptogenesis (Szasz et al., 1999) and cognitive disorders (Kakita et al., 2000). MeHg-induced decrease of glutathione peroxidase was reported in fetal mouse brain (Watanabe et al., 1999), reinforcing the theory of the involvement of astrocytes in the neurotoxicity of this metal (Allen et al., 2002).

Two astrocytic markers, glial fibrillary acidic protein (GFAP) and S100B, have been used as indicators of glial response in brain injury, including metal-induced neurotoxicity (O'Callaghan, 1991; Huang et al., 1993). S100B is a 21 kDa calcium binding protein, involved in cytoskeletal modulation and extracellular activity, which is trophic or toxic depending on its concentration. Studies in cell cultures indicate that at nanomolar levels S100B stimulate neuronal survival and glial proliferation.

By contrast, micromolar levels of extracellular S100B can induce apoptosis (Donato, 2001). Increased cerebrospinal fluid (CSF) has been reported in several acute and chronic injuries, including traumatic brain injury, stroke and Alzheimer disease (Rothermundt et al., 2003).

In this study, we investigated S100B levels in CSF and in different brain regions (hippocampus, cerebral cortex and cerebellum) of neonates from pregnant rats exposed to methylmercury. Neonate rats were studied immediately after birth, on the 15th and 30th postnatal days. GFAP content was investigated in hippocampus. Spatial learning of these rats was evaluated later, on the 60th postnatal day.

Material and methods

Material. Primary antibodies against S100B (SH-B1) and GFAP were obtained from Sigma and DAKO, respectively. Chemicals for electrophoresis were purchased from Sigma and immunoblotting reagents were from Amersham Biosciences. Methylmercury (II) chloride was obtained from Sigma. All other chemicals were of analytical reagent grade and purchased from Merck.

Animals and MeHg exposure. Adult female Wistar rats, from the department of Biochemistry of the Universidade Federal do Rio Grande do Sul, were housed with food and water ad libitum under a 12-h light/dark cycle at a temperature of 25°C. These rats were mated for 24 h. The day when sperm were observed in the vaginal smear was taken as the first day of pregnancy. The pregnant females were divided into two groups, namely MeHg group and control group. On the 12th, 13th and 14th days of pregnancy, the MeHg group was injected s.c. with MeHg, at a dose of 5 mg MeHg/Kg/day (Watanabe et al.,1999), while the control group was injected with 25 mM sodium bicarbonate.

CSF and brain tissue samples. Offspring (on postnatal days 1, 15 and 30) were anaesthetized by intraperitonial injection of pentobarbital sodium (50mg/Kg-body weight) and positioned in a stereotactic holder. Cerebrospinal fluid was obtained by cisterna magna puncture using an insulin syringe (27 gauge x 1/2" length). A maximum volume of 30 μl was collected in a 3 min period to minimize risk of brainstem damage. CSF samples were frozen (-20 ^{o}C) until further analysis. After decapitation, brain microslices (0.4 x 1.0 mm) from hippocampus, cerebral cortex and cerebellum were obtained in a McIlwain chopper, homogenized in phosphate buffer saline (PBS) and stored at –20 ^{o}C (Tramontina et al., 2002). Slices for GFAP immunoblotting were dissolved in electrophoresis sample buffer (Pereira et al., 1994).

ELISA for S100B. CSF (diluted 4x in PBS) or brain slice homogenate (diluted 100x in PBS) were used in an ELISA for S100B, as described previously (Tramontina et al., 2000). Briefly, 50 microliters of sample plus 50 microliters of barbital buffer (pH 8.6) were applied to microtiter plates previously coated with monoclonal anti-S100B in carbonate buffer and blocked with 1% bovine serum albumin. The plate was incubated for 3 h at 37º C. After washing, peroxidase-conjugated anti-S100 diluted 1:1000 was added, and incubation continued for 1 h. The absorbance of the oxidized substrate (OPD) was measured at 492 nm. Total protein was measured by Lowry's method using bovine serum albumin as a standard.

Immunoblotting for GFAP. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane by semi-dry blotting (Pereira et al, 1994). Immunodetection of GFAP was carried out using a polyclonal anti-GFAP and a peroxidase-conjugated anti-rabbit antibody. The blots were developed by ECL luminol method.

Spatial learning. To access spatial learning, a Morris-type water maze task was performed (Morris, 1984; Vasconcellos et al., 2003) using a circular pool (180 cm diameter, 60 cm high), filled with water (depth 30 cm; 24 $\pm$ 1 ^{o}C), placed in a room that was rich in consistently located spatial cues (including a large wood door, two prominent posters on one wall, and the experimenter). An escape platform (10 cm diameter) was placed in the middle of one of the quadrants, 1.5 cm below the water surface, equidistant from the sidewall and the middle of the pool. The platform provided the only escape from the water and was located in the same quadrant on every trial. The position of the animal in the pool was recorded during the entire experiment. Four different starting positions were equally spaced around the perimeter of the pool. On each of the training days, all four start positions were used once in a random sequence, i.e., four training trials per day. A trial began by placing the animal in the water facing the wall of the pool at one of the starting points. If the animal failed to escape within 60 s it was gently conducted to the platform by the experimenter. The rat was allowed to stay there for 20 s. The inter-trial interval was 10 min. After each trial the rats were dried, and returned to their cages at the end of the session. Animals were trained for 5 days. Twenty-four h after the last training session, the rats were submitted to a test session. Before this session, the submerged platform was removed. The retention test consisted of placing the animals in the water for 1 min. The latency in reaching the original position of the platform, the number of crossings in that place and the time spent in the target quadrant compared to the opposite quadrant were measured.

Statistical analysis. Parametric data are reported as mean ± SEM and were analyzed by Student's t test (when two groups were considered), two-way ANOVA, or by repeated measures ANOVA.

Determination of mercury. Total mercury was determined in brain tissue by cold vapor atomic absorption spectrometry, according to Bergdahl et al., 1998.

Results

Table 1 shows the levels of mercury in the brain regions of the neonate rats. High mercury contents (expressed in pg/μg of protein) were found immediately after birth. Hippocampus and cerebral cortex demonstrated lower contents compared to whole brain. A strong decrease was found on the 30^{th} postnatal day. No mercury was detected in the hippocampus at this time. It is important to mention that no differences were observed in body or brain weights at birth.

A significant increase in CSF S100B was observed in 15 day-old rats born from mercury-intoxicated pregnant rats (Fig 1), which was not found in 30 day-old rats. Unfortunately, we were unable to obtain a sufficient quantity of CSF S100B without inflicting brain damage in rats before 15 days of age. A developmental decrease in CSF S100B between postnatal days 15 and 30 confirmed previous studies (Tramontina et al, 2002).

Fig 2 shows developmental levels of S100B in different brain regions: hippocampus (A), cerebellum (B) and cerebral cortex (C). S100B was increased in the hippocampus of mercury-exposed rats on the first postnatal day. This difference disappeared on postnatal days 15 and 30. No changes in the S100B immunocontent were observed in cerebral cortex and cerebellum. A strong positive correlation ($r > 0.98$) was observed between age and S100B content in all examined brain regions.

Interestingly, the effect on GFAP contrasted with that observed on S100B content. Immunoblotting for GFAP in hippocampus showed a decrease in mercury-exposed rats on the first postnatal day (Fig 3A). Similar to the results with S100B, no significant changes in GFAP content were observed in 30 day-old rats (Fig 3B).

In order to investigate a possible cognitive damage associated with hippocampal changes observed on the first postnatal day, 60- day-old rats were submitted to a water maze task (Fig 4). In the retention test, no effect of MeHg on memory was found. A two-way ANOVA showed that MeHg-treated and control rats presented the same latency in reaching the original position of the platform (Figure 4A), with no significant differences between sexes ($p > 0.05$ in both cases). No significant interaction was observed between these two variables ($p > 0.05$). The number of times that the animals crossed the platform location was also analyzed (Figure 4B). Two-way ANOVA showed that MeHg-treated and control rats presented similar performances in this parameter ($p > 0.05$ for both cases). Again, no significant interaction was observed between these two treatments. When analyzing the time spent in the target quadrant and the time spent in the opposite quadrant (Figure 2C), a repeated measure ANOVA showed no effect of methyl mercury treatment. There were no interactions between treatment and sex. The animals spent more time in the target quadrant, compared to the opposite quadrant ($p < 0.001$), and no interactions were observed between the time spent in both quadrants and methylmercury treatment, sex, or time in both quadrants versus sex versus treatment.

Discussion

MeHg-intoxicated adult rats show focal cell degeneration in selected brain regions (Kakita et al., 2000). MeHg can be transferred to the fetus through the placenta and to the neonate through breast milk. It remains unclear as to why the fetus displays a higher sensitivity to MeHg in comparison with the adult and there is no sensitive biochemical marker available to demonstrate the exact degree of neurological injury caused by this environmental toxicant (Castoldi et al., 2003). We found lower levels of brain tissue mercury in 30-old-day rats than immediately after birth, in agreement with Sakamoto and co-workers (2002). These authors demonstrated that Hg transference through milk is fairly limited compared to that through the placenta. This limited transference together with intense postnatal changes in brain volume may explain the decrease in brain Hg after birth.

Measurements of the mercury levels in body fluids, such as blood, which have been used as biological indicators for MeHg contamination, do not always relate directly to the brain levels of this compound and do not allow a reliable evaluation of the extent of neurological damage (Apostoli et al., 2003). In this regard, several studies have demonstrated the existence of a positive relationship between injury to the CNS and S100B levels in CSF (Rothermundt et al., 2003). In agreement with these findings, we have recently observed a correlation between peripheral S100B levels and MeHg-induced neurotoxicity in adult rats (M Farina, unpublished results). Here, we demonstrated a reversible increase in CSF S100B content after prenatal exposure to MeHg. Elevation in CSF S100B is possibly secondary to the increment of this protein in determined brain regions, such as the hippocampus. However, a direct effect of MeHg on S100B secretion cannot be ruled out. In fact, in 15-day-old rats, changes in CSF S100B were observed, but at this time hippocampal alterations were not more observed.

Moreover, distinct intra and extracellular changes in S100B levels have been reported during postnatal development (Tramontina et al., 2002), suggesting that the extracellular level does not necessarily reflect the intracellular content.

Hippocampal S100B and GFAP contents were altered immediately after birth in MeHg-exposed rats. An increase of 200% was observed in S100B content, suggesting a strong glial activation in this region, in contrast to the cerebral cortex and cerebellum. A significant decrease of 15% was also observed in GFAP in the hippocampus. Although an increase of GFAP is expected in a reactive gliose (O'Callaghan. 1991), a reduction of GFAP content in astrocyte injury is not uncommon (Pereira et al., 1994; Guo-Ross et al., 1999). However, a reduction of the GFAP content during development could suggest a delay in astroglial maturation, considering that GFAP is a characteristic intermediate filament protein of mature astrocytes (Laping et al, 1994). In contrast to our results, an increase of GFAP was found in hippocampus of Evan-Long rats exposed prenatally to MeHg for a longer time (from 6 to 15 days of pregnancy; Goldey et al, 1994). In addition, we did not observe a decrease in body weight at birth in MeHg-exposed rats, as these authors found.

Based on different profiles of changes that we found in the hippocampus in S100B and GFAP contents of MeHg-exposed rats at birth, it would be possible to speculate that this could be due to differences in the manner in which MeHg acts in different populations of astrocytes and molecular targets. In fact, although GFAP and S100B are astrocyte markers they have different brain distributions and distinct putative roles. One of the putative roles for S100B is the modulation of glial intermediate filaments. However, S100B has many other intracellular targets and it also plays an extracellular role as a cytokine (Donato, 2001; Rothermundt et al, 2003).

The cerebellum is one of the areas characteristically altered by methylmercury intoxication, both in the adult and during development. However, rat cerebellar neurons exposed in culture to MeHg showed specific changes in protein 440-kDa ankyrinB, whereas other the neuronal proteins, MAP-2 and GFAP, coexisting in the culture, remained unchanged (Sakaue et al., 2003). Our results reinforce the theory of the distinct sensitivities of molecular targets, cells and brain regions to MeHg.

Since specific and reversible changes were observed in the hippocampus, we decided to evaluate spatial learning behavior in these animals later on, using the Morris water maze. Performance in this task is strongly linked to hippocampal function. No alterations were observed in the water maze task in 60-day-old rats exposed to MeHg during development. These results are in agreement with those of other reports conducted in models of prenatal MeHg intoxication (Sakamoto et al, 2002; Fredriksson et al., 1996), suggesting that the alterations observed in hippocampus during development of these animals after MeHg treatment are not enough to alter spatial memory.

In summary, our results reinforce the glial involvement in MeHg-induced neurotoxicity. We observed specific and reversible changes in the hippocampus, which could be related to some disorders attributed to MeHg. The increase of CSF S100B in neonates exposed to MeHg during pregnancy reinforces the view that increased S100B is related to damage in the nervous system and further suggests that S100B could be a useful marker in MeHg-neurotoxicity. Although the cellular mechanism related to MeHg-induced increase in S100B content in CSF remains unknown, our results suggest the use of S100B as a peripheral marker of brain damage induced by MeHg.

Table 1. *Hg levels in brain tissue of neonates from MeHg-intoxicated pregnant rats*

	Day 1	Day 30
Whole brain	32.46	0.27
Hippocampus	17.72	ND
Cerebral cortex	6.41	0.11

Hg content in brain tissue from offspring was measured by atomic absorption spectrometry. Values are means of 4 independent experiments and they are expressed in pg of Hg/ μg of protein. ND means not detected (less than 0.10). Values from control rats were not detected.

Figure legends

Figure 1. *Cerebrospinal fluid S100B in neonates from MeHg-intoxicated rats during pregnancy*. Neonates were anaesthetized and CSF was collected by cisterna magna puncture. S100B was measured by ELISA. Each value is a mean of 10-12 animals from different experiments ± SEM. *a*, *b* and *c* indicate different groups by statistical analysis (ANOVA, followed by Tukey's test, $p < 0.05$).

Figure 2. *Immunocontent of S100B in different brain regions in MeHg-intoxicated offspring.* Brain S100B content was measured in 1, 15 and 30-day-old rats intoxicated with MeHg during pregnancy. Three regions were analysed: hippocampus (A), cerebellum (B) and cerebral cortex (C). Each value is a mean of 8-10 animals from different experiments ± SEM. *a*, *b* and *c* indicate different groups by statistical analysis (ANOVA, followed by Tukey's test, $p < 0.05$).

Figure 3. *Hippocampal GFAP content in the offspring from MeHg-intoxicated rats during pregnancy.* Hippocampal content of GFAP was measured by immunoblotting in 1 and 30- day-old rats. Values of the MeHg group were expressed in percentage assuming control samples as being 100% in each experiment. Each value is a mean of 6 animals from different experiments ± SEM. A representative immunoblot from 1-day-old rats is shown.

* Statistically significant (Student t test, $p < 0.05$).

Figure 4. *Cognitive behaviour in water maze task of rats prenatally intoxicated with MeHg.* Spatial learning of 60-day-old control and MeHg prenatally exposed rats were evaluated by measurement of the latency to find the platform (A), crossings (B) and time spent in target (indicate by T) and opposite (indicated by O) quadrants (C). Each group contained 10-12 animals. No statistical differences were found (Two-way ANOVA).

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Figure 1

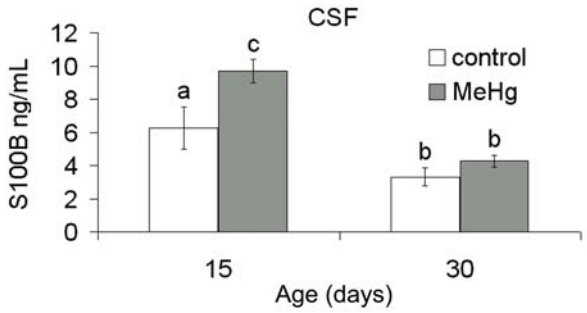


Figure 2

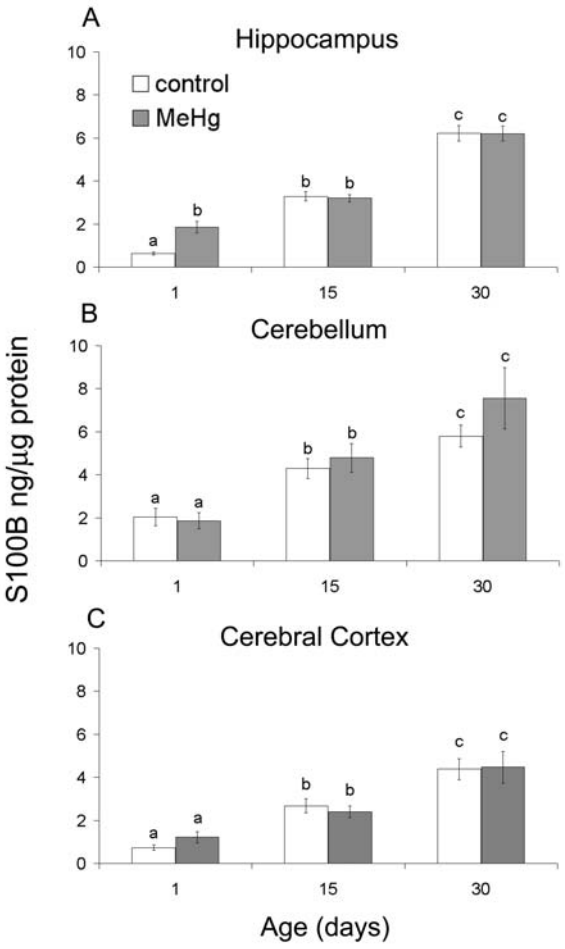

A
Hippocampus
control
MeHg
B
Cerebellum
C
Cerebral Cortex
S100B ng/µg protein
Age (days)
1
15
30

Figure 3

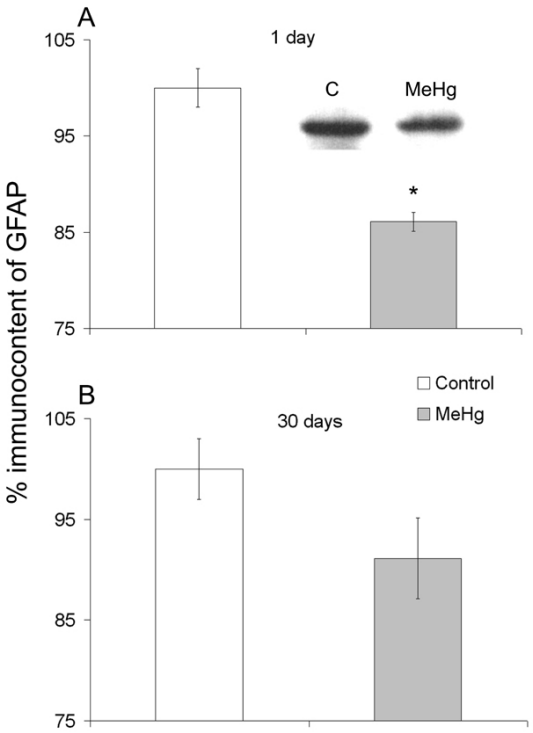
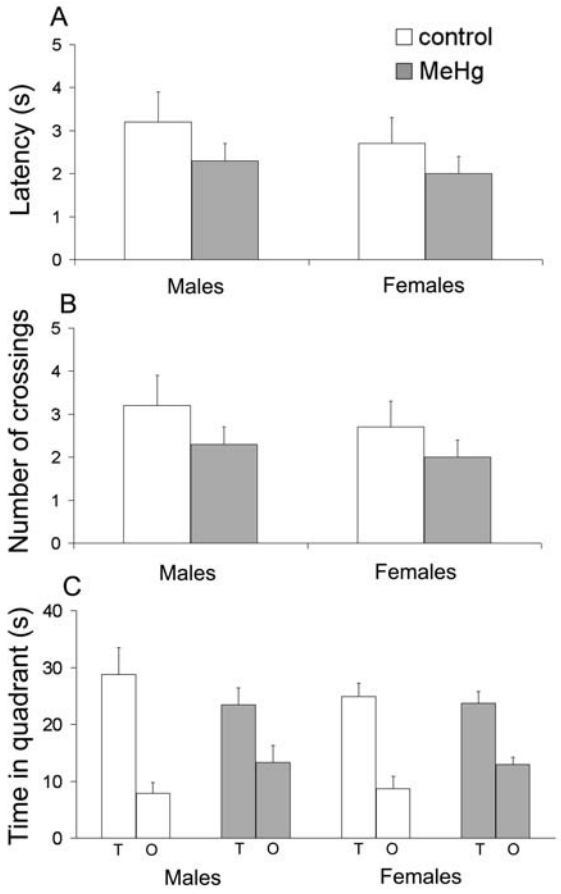

A
1 day
C
MeHg
105
95
85
75
*
% immunocontent of GFAP
B
30 days
Control
MeHg
105
95
85
75

Figure 4


A
control
MeHg
Latency (s)
0
1
2
3
4
5
Males
Females
B
Number of crossings
0
1
2
3
4
5
Males
Females
C
Time in quadrant (s)
0
10
20
30
40
T
O
T
O
T
O
T
O
Males
Females

4. Discussão

Os efeitos da exposição pré-natal ao MeHg tanto em humanos como em animais têm sido bem documentado por décadas (Doré et al., 2001). O MeHg pode ser transferido ao feto atravessando a barreira placentária e ao neonato através do leite, atingindo diversos tecidos incluindo o SNC. Contudo, a maior afinidade do MeHg ao SNC em desenvolvimento com relação ao adulto ainda é desconhecida (Castoldi et al., 2003). Tem sido proposto que o MeHg causa alteração no desenvolvimento cerebral normal através de diversos mecanismos patogênicos incluindo interferência mitótica, defeito cromossomal e inibição de enzimas. Uma vez que esse metal tem sido envolvido na interrupção de um grande número de funções biológicas, é provável que os mesmos mecanismos patogênicos não ocorram nos mesmos estágios do desenvolvimento (Haykal-Coates et al., 1998). Em nosso estudo encontramos baixas concentrações de mercúrio aos 30 dias de vida o que difere dos níveis encontrados ao nascimento de acordo com Sakamoto e colaboradores (2002). Eles demonstraram que a transferência do mercúrio através do leite é muito menor se comparada com a placentária. Essa menor transferência junto com a intensa mudança pós-natal no volume cerebral pode explicar o decréscimo do Hg no cérebro após o nascimento.

As medidas do mercúrio no fluído corporal, assim como o sangue, no qual tem sido usado como um indicador biológico na contaminação ao MeHg, nem sempre reproduz os níveis cerebrais desse metal e não mostra a extensão do dano neurológico (Apostoli et al., 2003). Muitos estudos tem demonstrado a existência de uma relação positiva entre danos no SNC e níveis de S100B no FCE (Rothermundt et al., 2003). Recentemente tem sido mostrada uma correlação entre níveis de S100B periférica e a neurotoxicidade induzida ao MeHg em ratos adultos (M Farina, dados não publicados).

No presente estudo mostramos um aumento reversível do conteúdo de S100B no FCE. Esse aumento é possivelmente secundário ao acréscimo da proteína S100B em determinadas regiões cerebrais, assim como no hipocampo. Entretanto, um efeito direto do MeHg sobre a secreção de S100B não pode ser excluída. De fato, os ratos aos 15 dias de vida apresentaram mudanças no conteúdo de S100B no FCE, mas nesse período no hipocampo nenhuma alteração foi observada. Além disso, distintas mudanças dos níveis intra e extra de S100B foram observadas durante o desenvolvimento pós-natal (Tramontina et al., 2002), sugerindo que os níveis extracelulares de S100B necessariamente não refletem o conteúdo intracelular.

O conteúdo de S100B e GFAP em hipocampo estavam alterados nos ratos expostos ao MeHg imediatamente pós o nascimento. Um aumento de 200% foi observado no conteúdo de S100B sugerindo uma forte ativação glial nesta região em contraste com o córtex cerebral e cerebelo. Sabe-se que o MeHg causa danos ao citoesqueleto (Trombeta & Kromidas., 1992; Szász et al., 1999), em nossos estudos houve um significativo decréscimo de 15% na proteína de filamento intermediário, a GFAP, no hipocampo. Embora, o aumento da GFAP fosse esperado na gliose reativa (O'Callaghan., 1991), não é incomum uma redução do conteúdo de GFAP nas injúrias astrocíticas (Pereira et al., 1994; Guo-Ross et al., 1999). Baseado nas diferentes mudanças no conteúdo de S100B e GFAP poderia ser possível especular que o MeHg age em diferentes populações de astrócitos.

Cerebelo é uma das áreas caracteristicamente alteradas pela intoxicação ao MeHg tanto em adultos como durante o desenvolvimento. Contudo, neurônios cerebelares de ratos expostos ao MeHg em cultura mostraram mudanças específicas na proteína 440-kDa ankirina B, enquanto que outras proteínas neuronais MAP-2 e GFAP, coexistentes na cultura permaneceram sem alterações (Sakaue et al., 2003). Nossos resultados reforçam a idéia sobre a distinta afinidade de alvos moleculares, células

e regiões cerebrais ao MeHg.

Considerando a alteração hipocampal observada ao nascimento poderia haver um dano cognitivo, particularmente no aprendizado espacial. Entretanto, nenhuma alteração foi observada no labirinto aquático nos ratos, expostos ao MeHg durante a gestação, aos 60 dias de vida. Estes resultados estão de acordo com outros modelos de intoxicação pré-natal (Sakamoto et al., 2002; Fredriksson et al., 1996; Kakita et al., 2000).

Concluindo, nossos resultados reforçam o envolvimento glial na neurotoxicidade induzida pelo MeHg. Observamos específicas e reversíveis mudanças em hipocampo, na qual poderia estar relacionada à desordens cognitivas e epilépticas atribuídas ao MeHg. O aumento do conteúdo de S100B no FCE em neonatos expostos ao MeHg reforçam a idéia de que a S100B está relacionada ao dano no sistema nervoso e de que a S100B poderia ser um útil marcador da neurotoxicidade à esse metal. Embora, o mecanismo celular envolvido no aumento da S100B no FCE induzido pelo MeHg permaneça desconhecido nossos resultados sugerem a possibilidade de usar a S100B como um marcador periférico de dano cerebral induzido pelo MeHg.

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