UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

NEUROINFLAMAÇÃO E ESQUIZOFRENIA: AVALIAÇÃO DE PARÂMETROS ASTROGLIAIS *IN VITRO* E *IN VIVO*

Daniela Fraga de Souza

Orientador

Prof. Carlos Alberto Saraiva Gonçalves

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Tese apresentada ao curso de Pós-Graduação em Ciências Biológicas – Bioquímica, da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do grau de Doutor em Bioquímica.

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RESUMO

Embora a inflamação seja um processo de defesa fisiológica, neuroinflamação desequilibrada tem sido associada com a fisiopatologia de doenças do SNC. Além disso, a exposição pré-natal à infecção bacteriana e/ou viral tem sido implicada em vários estudos como um importante fator ambiental capaz de afetar prejudicialmente o neurodesenvolvimento, aumentando assim o risco de esquizofrenia. Resposta imune materna, em vez de infecção direta do feto, poderia ser responsável pelo desenvolvimento da doença. Glia ativada libera uma variedade de citocinas pró-inflamatórias que contribuem para a disfunção neuronal. Além disso, as alterações de marcadores astrogliais parecem estar intimamente relacionadas à patologia da esquizofrenia, por exemplo, níveis elevados de S100B, uma proteína derivada da glia, foram observados no soro e LCR de pacientes com esquizofrenia. Nesta tese avaliou-se se a secreção de S100B (em células de glioma C6 e fatias de hipocampo de ratos Wistar) poderia ser diretamente modulada por citocinas inflamatórias alteradas na esquizofrenia, assim como o possível envolvimento da via da proteína cinase ativada por mítógeno (MAPK) nestas respostas. Os efeitos dos antipsicóticos típicos e atípicos na secreção de S100B induzida por citocinas foram analisadas. Nós também avaliamos se a administração de LPS em ratas prenhas no final da gestação afeta alguns aspectos comportamentais da prole em sua vida adulta, e investigamos os efeitos deste tratamento sobre parâmetros gliais usando preparação *ex vivo* dos descendentes, além de observar se o tratamento pré-natal afeta marcadores gliais (S100B e GFAP) da prole adulta. Usamos a prole aos 30 e 60 dias para avaliar as diferenças entre os ratos jovens e adultos e também investigar a existência de diferenças entre os descendentes do sexo masculino e feminino. Avaliamos também os parâmetros de estresse oxidativo NO e GSH. Nós observamos que secreção de S100B foi aumentada pelas citocinas pró-inflamatórias (IL-1β, TNF-α, IL-6 e IL-8) em glioma C6 e em fatias hipocampais através da via MAPK, e estresse oxidativo pode ser um componente desta modulação. Além disso, IL-6 foi capaz de induzir um aumento no conteúdo de S100B e GFAP em glioma C6 e os antipsicóticos, haloperidol e risperidona, foram capazes de inibir a secreção de S100B estimulada por IL-6. Observamos também que o modelo de alteração imune maternal provocou alterações comportamentais na prole adulta, especialmente no comportamento social dos ratos afetados, além de modificar o padrão de secreção da proteína S100B e da captação de glutamato em fatias *ex vivo* da prole adulta. Constatamos que conteúdo de glutationa e NO são modificados de forma dependente de idade/gênero. Foi observada também uma pronunciada astrogliose particularmente hipocampal, com GFAP apresentando-se aumentada em ratos juvenis e adultos enquanto que conteúdo de S100B apresentou diminuição na prole juvenil e aumento em ratos adultos em resposta a exposição imune maternal. Aparentemente, infecção pré-natal parece levar a anormalidades comportamentais e neuroquímicas, incluindo modificação de marcadores gliais, na vida pós-natal, possivelmente via citocinas.

ABSTRACT

Although inflammation may be a physiological defense process, imbalanced neuroinflammation has been associated with the pathophysiology of brain disorders. Furthermore, prenatal exposure to bacterial and viral infection has been implicated by several studies indicating that such exposure is an important environmental factor out that may detrimentally affect neurodevelopment, increasing the risk of schizophrenia. Maternal immune response, rather than direct infection of the fetus, could be responsible for disease development. Activated glia releases a variety of pro-inflammatory cytokines that contribute to neuronal dysfunction. Moreover, changes in astrogliais markers seem to be closely related pathology of schizophrenia, for example, elevated levels of S100B, a glia derived protein, have been observed in the serum and CSF of schizophrenic patients. We evaluated whether S100B secretion (in C6 glioma cells and hippocampal slices in Wistar rats) could be directly modulated by the main inflammatory cytokines altered in schizophrenia, as well as the possible involvement of mitogen-activated protein kinase (MAPK) pathways in these responses. The effects of typical and atypical antipsychotic drugs on glial cytokine-induced S100B release were analyzing. We also evaluated whether administration of LPS to rats dams in late gestation affects some aspects of the offspring behavior in their adult life, and investigated the effects of this treatment on glial parameters using *ex vivo* preparation of offsprings, and whether it affects astroglial markers (S100B and GFAP) of the offspring in later life. We used the offspring at 30 and 60 days to evaluate the differences between juvenile and adult rats and also investigate the existence of differences between male and female offspring. We also investigated the oxidative stress parameters NO and GSH. We observed that S100B secretion was increased by the pro-inflammatory cytokines (IL-1 β, TNF-α, IL-6 and IL-8) in C6 glioma and in hippocampal slices via the MAPK, and oxidative stress may be a component this modulation. Furthermore, IL-6 was capable of inducing an increase in content GFAP and S100B in C6 glioma and antipsychotic, haloperidol and risperidone have been capable of inhibiting S100B secretion stimulated by IL-6. We also note that the model of maternal immune changes caused behavioral changes in the adult offspring, especially in the social behavior of rat affected and modified the pattern of secretion of S100B protein and glutamate uptake in *ex vivo* slices of adult offspring. We found that content of glutathione and NO are modified so age / gender dependent. There was also a pronounced hippocampal astrogliosis, with GFAP presenting increased in juvenile and adult rats while S100B content was reduced in juvenile and increased in adult offspring rats in response to exposure to maternal immune. Apparently, prenatal infection appears to lead to behavioral and neurochemical abnormalities, including modification of glial markers in the postnatal life, possibly via cytokines.

LISTA DE ABREVEATURAS

5HT: 5-hidroxitriptamina;

DCF: 2′–7′-dichlorofluoresceína;

DPN: dia pós-natal;

ELISA: "enzyme-linked immunosorbent assay" teste imunoenzimático de detecção;

ERK: Cinase regulada por sinal extra-celular

EROS: espécies reativas ao oxigênio;

GFAP proteína glial fibrilar ácida;

GSH: glutationa reduzida;

IL-1β: interleucina-1β**;**

IL-6: interleucina -6;

IL-8: interleucina -8;

JNK: c-Jun N-terminal;

LCR: líquido cefalorraquidiano;

L-DOPA: L-3,4-dihidroxifenilalanina;

LPS: lipopolissacarídeo;

MAPK: proteína cinase ativada por mitógeno

NeuN: antígeno nuclear neuronal

NF-kB: fator nuclear kB;

NMDA: N-metil D-aspartato;

SNC: Sistema nervoso central;

TH: tirosina hidroxilase;

TNF-α: fator de necrose tumoral- α;

TRL4: receptor toll-like 4**;**

INTRODUÇÃO

1. Doenças Neuropsiquiátricas

Desde a época do antigo Egito, as sociedades têm se esforçado para entender a doença mental e para cuidar daqueles afetados por ela. Mas, ao longo dos milênios, a ideia de que a doença mental pode ter uma causa biológica surgiu apenas de forma intermitente, e os tratamentos vão do inofensivo (exercício, humor e música) ao bárbaro (prisão, exorcismo e lobotomia) (Jablensky *et al.* , 1992). Em meados do século XX, no entanto, vários avanços foram feitos: os profissionais de saúde finalmente compreenderam que doenças mentais são doenças encefálicas e não somente psicológicas. Adicionalmente, um conjunto de critérios sistemáticos para o diagnóstico foi desenvolvido em conjunto com terapias farmacêuticas e psicológicas, que ainda são centrais para a psiquiatria moderna (Chou e Chouard, 2008).

Doenças neuropsiquiátricas são um fardo enorme para sociedade, pois essas doenças afetam não só a saúde das pessoas atingidas, como também sua capacidade de aprender, trabalhar e se relacionar (Monji *et al.* , 2009). Além disso, uma falta de marcadores biológicos confiáveis para a caracterização destas doenças acarreta, talvez sem surpresa, em opções de tratamento longe de ser ótimas em termos de eficácia e especificidade. Mesmo hoje, décadas de pesquisas depois, a prevalência de doenças neuropsiquiátricas não diminuiu (Bromet e Fennig, 1999) e nossa compreensão dos mecanismos biológicos de doenças como transtornos de humor, esquizofrenia e autismo é ainda frustrantemente limitada.

Contudo, pesquisadores estão fazendo progressos no sentido de definir a base biológica destas doenças. Avanços tecnológicos nas áreas da genômica e crescente quantidade de estudos na área, bem como o desenvolvimento de novos modelos

animais, estão colaborando com a nossa compreensão a cerca destas doenças e oferecendo perspectivas de diferentes opções de tratamento.

1.1. Doenças Neuropsiquiátricas e Neuroinflamação

O sistema nervoso central (SNC) é tradicionalmente conhecido como um órgão "imunologicamente privilegiado", ou seja, não suscetível à resposta imune e inflamatória sistêmica. Contudo atualmente, sabe-se que ele possui um sistema imune endógeno coordenado por células imunocompetentes, como a microglia e os astrócitos (Dong e Benveniste, 2001, Lucas *et al.* , 2006). A resposta à infecção ou inflamação gerada no cérebro difere daquelas desencadeadas na periferia, sintomas clássicos, como rubor, tumor, calor e dor não são tipicamente observados no SNC. Além disso, o recrutamento de leucócitos, que é rápido nos órgãos periféricos, apresenta-se modesto e tardio no tecido nervoso. Essas diferenças podem ser atribuídas, em parte, à presença da barreira sangue-cérebro, que obstrui a entrada de células inflamatórias, patógenos e macromoléculas, protegendo assim o sensível sistema neuronal de danos tipicamente associados à inflamação (Tuppo e Arias, 2005).

Neuroinflamação desempenha um papel crítico em diferentes doenças neurológicas e psiquiátricas (Jones e Thomsen, 2012). Embora a inflamação possa ser um processo de defesa fisiológica, benéfico para a reparação e recuperação do sistema nervoso central (SNC) (Wee Yong, 2010), inflamação desbalanceada tem sido associada com a patofisiologia de distúrbios cerebrais diversos, incluindo a doença de Alzheimer, depressão e esquizofrenia (Monji, 2012). Resposta inflamatória no SNC inclui um amplo espectro de complexas e integradas respostas celulares, tais como a ativação da glia. A glia, quando ativada, libera uma variedade de mediadores pró-inflamatórios

(citocinas, por exemplo), que podem potencialmente contribuir para a disfunção neuronal e progressão de patologias do SNC (Khansari *et al.* , 2009).

As citocinas estão envolvidas na regulação da comunicação entre as células do sistema imunológico, por conseguinte, elevados níveis periféricos e no sistema nervoso central de citocinas indicam ativação da resposta inflamatória. No entanto, estas citocinas não modulam somente a função SNC normal: sinalização anormal da citocina pode contribuir para as principais doenças agudas e crônicas do sistema nervoso central (Tansey, 2010). Os níveis aumentados de citocinas pró-inflamatórias, tais como IL-1β, TNF-α e IL-6, são encontrados em indivíduos com epilepsia, esquizofrenia e autismo (Miller *et al.* , 2011, Potvin *et al.* , 2008). Além disso, modelos animais utilizando lipopolissacarídeo (LPS), endotoxina indutora de resposta imune componente da membrana externa de bactérias gram-negativas, marcados com iodo^{125} em ratas prenhas indicam que o desenvolvimento de patologia tipo-esquizofrenia manifestadas nos ratos descendentes ocorre, muito provavelmente, devido ao efeito das citocinas, uma vez que as citocinas podem atravessar a placenta e LPS, não (Ashdown *et al.* , 2006).

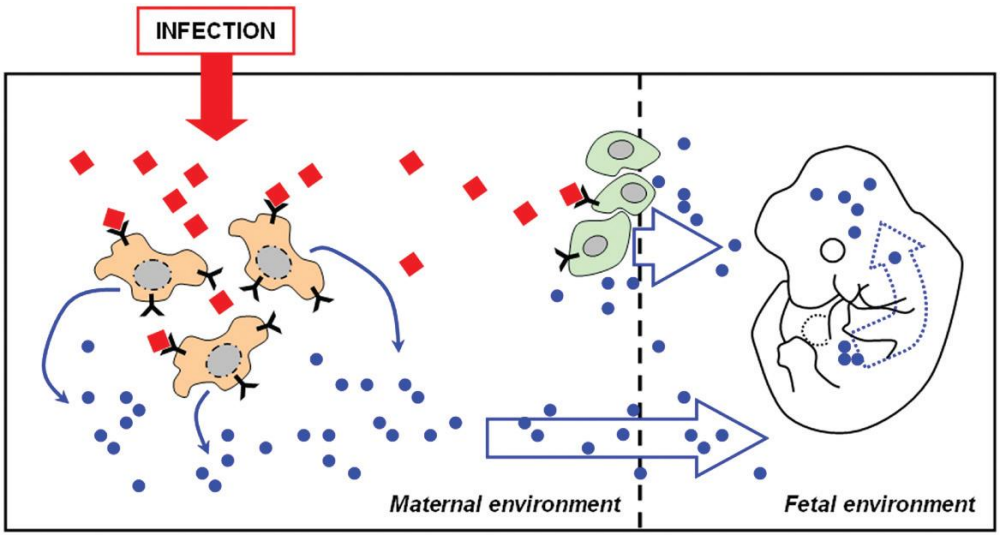

Figura 1 Resposta das citocinas nos compartimentos materno e fetal após a infecção materna durante a gravidez. Adaptado de (Meyer *et al.* , 2007).

1.2. Esquizofrenia

A Esquizofrenia é um distúrbio neuropsíquico complexo que afeta cerca de 1% da população mundial. Trata-se de uma doença psiquiátrica crônica, severa e provavelmente a mais angustiante e incapacitante (McGrath *et al.* , 2008). Os sintomas desta patologia podem ser divididos em três categorias principais: sintomas positivos (delírios e alucinações), sintomas negativos (falta de motivação, apatia, embotamento afetivo e isolamento social) e déficit cognitivo (Carpenter and Buchanan, 1994).

A etiologia da esquizofrenia permanece imprecisa, contudo existem diversas hipóteses a respeito. Diferentes estudos sugerem a desregulação de neurotransmissores na gênese da patologia (Carlsson, 1988). A teoria dopaminérgica, por exemplo, é a mais antiga e mais amplamente estudada na fisiopatologia da esquizofrenia. Essa teoria provém da observação do bloqueio do receptor de dopamina D2 como o mecanismo de ação dos antipsicóticos. Achados como hiperfunção no sistema límbico e hipofunção dopaminérgica no córtex pré-frontal de pacientes esquizofrênicos reforçam a ideia de que o sistema dopaminérgico seja responsável pelos sintomas "positivos" e "negativos" vistos na esquizofrenia (Meltzer, 1989). Hipofunção glutamatérgico, caracterizada por uma perda da neurotransmissão excitatória mediada pelo receptor NMDA, também parece desempenhar papel importante na patofisiologia desta desordem (Lieberman, 1999, Meltzer, 1989, Stahl, 2007, Tamminga, 1998), teoria essa que baseia-se na capacidade de antagonistas de receptor NMDA de induzir sintomas semelhantes aos da esquizofrenia (Javitt, 2010) e replicar até melhor os sintomas negativos e déficits cognitivos do que os agonistas dopaminérgicos (por exemplo, anfetaminas) (Steinpreis, 1996). Além disso, desregulação de serotonina, GABA e acetilcolina também têm sido implicados na patogênese da esquizofrenia (Lieberman, 1999, Stahl, 2007).

Adicionalmente, existe evidência de déficit em metabolismo celular, estresse oxidativo no córtex pré-frontal, disfunção mitocondrial, mobilização aumentada de glucose e / ou hipóxia celular, presentes na esquizofrenia (Bitanihirwe and Woo, 2011, Prabakaran *et al.* , 2004).

Todavia, várias linhas de evidência indicam fortemente que a esquizofrenia pode ter origem no desenvolvimento neurológico envolvendo componentes genéticos e ambientais (Lewis and Levitt, 2002). Diversos estudos epidemiológicos têm demonstrado que a exposição pré-natal de grávidas a fatores ambientais, como complicações obstétricas e infecções materna, virais ou bacterianas, promove o aumento da incidência de esquizofrenia na prole quando em idade adulta (Shi *et al.* , 2009). Isto sugere que a infecção materna durante a gravidez pode contribuir para a origem da esquizofrenia através de seus efeitos no desenvolvimento do cérebro fetal (Patterson, 2009). Além disso, estudos recentes mostraram a presença de desregulação de genes relacionados à imunidade, com forte ativação microglial e astrocítica, bem como regulação positiva de citocinas no sistema nervoso central de esquizofrênicos (Potvin, Stip, 2008).

2. Infecções Pré-natais e Esquizofrenia

Devido à observação da aumentada incidência de esquizofrenia em indivíduos nascidos no final do inverno/início da primavera (Tochigi *et al.* , 2004) e após grandes epidemias virais ou bacterianas, acredita-se hoje que infecção pré-natal seja um fator ambiental relevante no desenvolvimento de esquizofrenia nos descendentes adultos. (Brown, 2011, Meyer *et al.* , 2009, Smith *et al.* , 2007). Estima-se que 38-46% dos casos de esquizofrenia tenham associação com infecção pré-natal (Brown and Derkits, 2010).

Diversos estudos sugerem uma relação causal entre infecções virais ou bacterianas durante a gravidez e comportamento alterado na prole adulta (Borish and Steinke, 2003), no entanto, os fatores específicos que medeiam os efeitos da infecção materna no desenvolvimento do cérebro fetal permanecem incertos. Sabe-se que bactérias e vírus são potentes ativadores de citocinas pró-inflamatórias, tais como o fator de necrose tumoral (TNF-α), interleucina 1-beta (IL-1β) e interleucina-6 (IL-6), que são os principais efetores da resposta imune periférica e os principais mediadores de doenças neurais em resposta à infecção e inflamação (Dantzer and Wollman, 2003, Mansur *et al.* , 2012).

Na periferia, essas citocinas atuam em uma cascata que envolve geralmente a indução sequencial de TNF-α, IL-1β e IL-6 (Luheshi, 1998). Contudo, do mesmo modo que as citocinas têm um papel-chave no desenvolvimento normal do encéfalo, elas também estão implicadas na morte e disfunção neuronal após lesão ou doença crônica do SNC (Allan and Rothwell, 2003), além de serem elevadas em hipóxia, que por sua vez também está associada com um risco aumentado de esquizofrenia (Marx *et al.* , 2001). Sendo assim, as citocinas são candidatas lógicas a mediadoras dos efeitos da infecção materna sobre o feto, é possível que respostas anormais de TNF-α, IL-1β e IL-6 durante a gestação, como resultado de uma infecção, possam afetar o desenvolvimento do SNC fetal no útero (Dammann and Leviton, 1998, Zhao and Schwartz, 1998). Entretanto, existem relatos contraditórios de tanto expressão aumentada, quanto diminuída de citocinas no SNC fetal frente à exposição de ratas prenhas a agentes que mimetizam infecções virais e bacterianas, como Poly I:C, um polímero sintético imunoestimulante que se assemelha a RNA de vírus patogênico, e LPS, por exemplo (Shi, Smith, 2009).

Sinalização do LPS no SNC ocorre através do receptor Toll-like 4 (TLR4) (Poltorak *et al.* , 1998) e a ligação do LPS a este receptor de membrana resulta na ativação do fator nuclear NF-kB (Chow *et al.* , 1999), com posterior transcrição de genes que codificam mediadores inflamatórios, incluindo o TNF- α, IL-1β, IL-6 e IL-8 (Rivest, 2003). A expressão de TLR4 tem sido descrita em tecido cerebral de rato neonatos e adultos, LPS também induz a expressão de citocinas em culturas gliais e neuronais humanas e de ratos (Eklind *et al.* , 2001, Guerra *et al.* , 2011, Hua and Lee, 2000, Laflamme and Rivest, 2001).

Sendo assim, a ativação imune maternal é muito útil como modelo para estudar os mecanismos pelos quais insultos maternais são capazes de afetar os fetos levando a distúrbios bioquímicos e comportamentais na vida adulta (Oskvig *et al.* , 2012).

3. Astrócitos

O SNC é constituído basicamente por dois tipos celulares, células neuronais e células gliais. As células gliais são subdivididas em microglia, células fagocíticas envolvidas na resposta inflamatória, e macroglia – composta por oligodendrócitos, formadores da mielina; células ependimais, que revestem os ventrículos cerebrais, e astrócitos (Jessen, 2004, Perea and Araque, 2005).

Os astrócitos podem ser classificados em dois subtipos principais: astrócitos protoplasmáticos, encontrados em maior concentração na substância cinzenta, e astrócitos fibrosos, localizados principalmente na substância branca cerebral, que correspondem, respectivamente, ao tipo 1 e tipo 2, em cultura (Kimelberg and Norenberg, 1989). Existem ainda astrócitos especializados, encontrados em estruturas

específicas, como a glia de Bergmann no cerebelo, as células de Müller na retina e os pituícitos na neurohipófise (Porter and McCarthy, 1996).

Apesar de serem as células mais abundantes do SNC, somente nas últimas décadas os astrócitos deixaram de ser considerados apenas suporte estrutural para os neurônios e passaram a ser reconhecidos por sua capacidade dinâmica e diversidade funcional (Shao and McCarthy, 1994). Hoje se sabe que os astrócitos não somente estabelecem a arquitetura estrutural do cérebro, mas também organizam e participam de suas vias de comunicação e plasticidade (Blasko *et al.* , 2004).

Além de modular a intensidade e atividade sináptica, os astrócitos também podem regular a neurogênese e gliogênese, assim como estabelecer interações recíprocas com outros tipos celulares do SNC, como neurônios, células endoteliais, oligodendrócitos, microglia e células ependimais (Horner and Palmer, 2003).

Dentre as inúmeras funções exercidas pelos astrócitos podemos também citar a formação da barreira hematoencefálica (Abbott *et al.* , 2006); tamponamento de níveis extracelulares dos íons (Walz, 1989); produção de fatores tróficos (Eriksen and Druse, 2001, Schwartz and Mishler, 1990); metabolismo de neurotransmissores (Kimelberg and Katz, 1985) e suporte energético para os neurônios (Pellerin, 2005).

Além disso, os astrócitos, juntamente com a microglia, são as principais células efetoras de resposta imune inata no SNC, exibindo um fenótipo ativado em doenças neurodegenerativas, produzindo diversos mediadores inflamatórios e exercendo atividade fagocítica (figura 2) (Hauwel *et al.* , 2005, Wyss-Coray and Mucke, 2002). No processo de ativação glial, observa-se superexpressão de citocinas neurorotróficas como interleucina 1- β (IL-1β) e S100B (de Souza *et al.* , 2009, Donato, 2001, Griffin *et al.* , 2000, Liu *et al.* , 2005, Sheng *et al.* , 2000). Recentes trabalhos têm sugerido que a expressão aumentada dessas citocinas exerce papel-chave na progressão da

neuroinflamação, disparando e sustentando uma cascata de eventos que resultam em progressiva morte neuronal (Allan and Rothwell, 2003, Griffin, 2006).

3.1. Proteína S100B

A S100B é uma proteína ligante de cálcio, zinco e cobre pertencente à família das proteínas S100, assim denominadas por serem solúveis em solução 100% de sulfato de amônia (Moore, 1965).

Estruturalmente, a proteína S100B apresenta-se na forma de homodímeros constituídos de duas subunidades, unidas por pontes de dissulfeto, sendo uma proteína do tipo EF-hand (hélice-loop-hélice) de 21 kDa com dois sítios ligantes de Ca2+ (Marenholz *et al.* , 2004). No SNC, a S100B é predominantemente expressa e secretada por astrócitos (Donato, 2001), exercendo efeitos autócrinos e parácrinos sobre outras células gliais e neurônios.

Essa proteína pode atravessar a barreira hematoencefálica, sendo que concentrações basais e aumentadas, devido à lesão cerebral, podem ser determinadas no soro (Marchi *et al.* , 2004). Entretanto, sua concentração sérica não pode ser considerada como exclusivamente proveniente de fonte cerebral (Netto *et al.* , 2006), visto que outras fontes periféricas, como o tecido adiposo, também existem (Haimoto *et al.* , 1987, Marshak *et al.* , 1985).

3.1.1. Papéis Intracelulares

Intracelularmente, a S100B é capaz de se ligar a diversas proteínas-alvo, tais como GAP-43, PKC, fator anti-apoptótico Bcl-2 ou à proteína supressora tumoral P53 (Donato, 2001). S100B também pode regular a ubiquitinação de proteínas, via interação com Sgt1 (Bianchi *et al.* , 1993), e a polimerização do citoesqueleto, inibindo a fosforilação de GFAP e vimentina, dessa forma, possivelmente modulando plasticidade,

metabolismo energético e proliferação celular (Donato, 2003, Van Eldik and Wainwright, 2003).

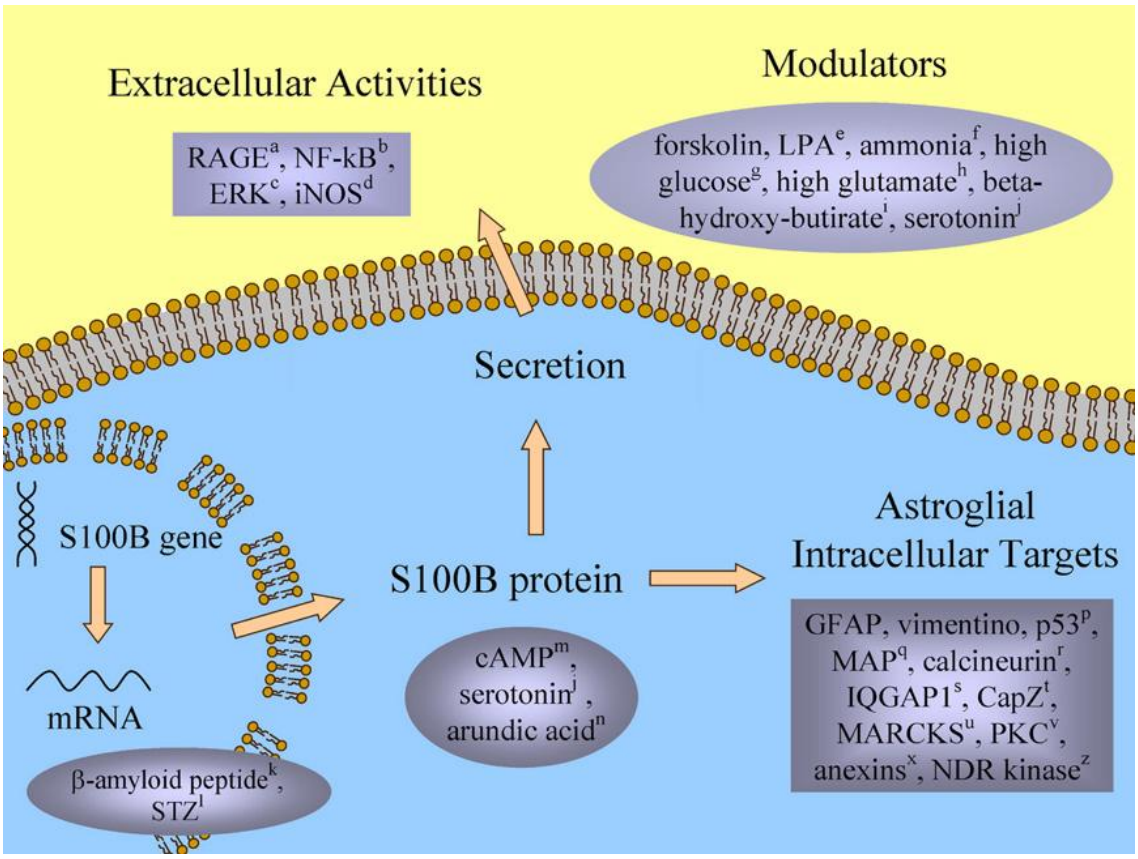

Figura 2. Representação esquemática da expressão e secreção de S100B astroglial. Adaptado de Goncalves *et al.* , 2008.

3.1.2. Papéis Extracelulares

Os efeitos extracelulares da proteína S100B em cultura estão intimamente relacionados com a sua concentração no meio extracelular. Em baixas concentrações, variando entre picomolar e valores baixos de nanomolar, essa proteína é trófica, promovendo crescimento de neuritos, modulação sináptica e sobrevivência neuronal (Van Eldik and Wainwright, 2003).

Já concentrações mais elevadas de S100B, variando entre altos níveis de nanomolar até molar, resultam em efeitos opostos. Altos níveis estimulam a produção e secreção de citocinas pró-inflamatórias IL-6, IL-1β, TNF-α, e enzimas relacionadas ao estresse oxidativo, óxido nítrico sintase induzível (iNOS), podendo até levar à apoptose neuronal (Ahlemeyer *et al.* , 2000, Schmitt *et al.* , 2007, Steiner *et al.* , 2007).

Esses efeitos são mediados, em parte, pela interação da S100B com o RAGE, um receptor multiligante, envolvido na transdução de estímulos inflamatórios e de diversos fatores neurotróficos e neurotóxicos (Donato, 2001).

3.1.3 Expressão e Secreção

Apesar de já identificados diversos papéis intra e extracelulares da S100B, o mecanismo através do qual é secretada ainda não está elucidado (Rothermundt *et al.* , 2001). No entanto, já se tem conhecimento de diversos moduladores da secreção dessa proteína, que é estimulada em estresse metabólico (privação de oxigênio, soro e/ou glicose) e diminuída por altas concentrações de glutamato extracelular, por exemplo (Gerlach *et al.* , 2006, Tramontina *et al.* , 2006a).

Contudo, um aumento da expressão de S100B não está diretamente relacionado com um aumento de sua secreção, pois nem toda S100B produzida é necessariamente exportada (Tramontina *et al.* , 2002, Tramontina *et al.* , 2006b), assim como, alterações morfológicas em astrócitos também não se correlacionam diretamente com a secreção de S100B (Pinto *et al.* , 2000).

3.1.4. Envolvimento em Doenças Neuropsiquiátricas

Vários estudos têm demonstrado uma correlação significativa entre S100B e moléculas pró-inflamatórias em distúrbios neurológicos e psiquiátricos (Ashraf *et al.* ,

1999, Steiner *et al.* , 2009). No entanto, a ideia de que S100B possa ser considerada uma citocina permanece controversa.

Nossos trabalhos anteriores sugerem que S100B pode ser um componente importante na neuroinflamação. Temos demonstrado que secreção de S100B é induzida por IL-1β, mediada por sinalização MAPK-ERK, em culturas de astrócitos, de células de glioma C6 e em fatias hipocampais agudas (de Souza, Leite, 2009). Recentemente, mostramos que secreção de S100B é estimulada pela administração intracerebroventricular (ICV) de LPS e que o LPS estimula diretamente a secreção de S100B em astrócitos e fatias hipocampais (Guerra, Tortorelli, 2011).

A presença de níveis elevados de S100B, observada no soro e CSF de pacientes esquizofrênicos (Lara *et al.* , 2001, Rothermundt *et al.* , 2004a, Steiner *et al.* , 2006), bem como a associação de esquizofrenia com certos haplótipos do gene S100B envolvidos na expressão aumentada de S100B (Liu, Shi, 2005), fornece evidências adicionais de que uma disfunção glial pode representar um fator patogênico na esquizofrenia.

OBJETIVOS

Objetivo Geral

O objetivo deste trabalho foi estudar a participação de fatores astrogliais em modelos de neuroinflamação e esquizofrenia.

Objetivos Específicos

Modelo *in vitro* (células de glioma C6 e fatias hipocampais):

- Investigar o efeito de citocinas envolvidas na patologia da esquizofrenia sobre a secreção de S100B, bem como a possível participação da via MAPK nessa resposta.
- Analisar o efeito das citocinas pró-inflamatórias sobre a proteína GFAP e estresse oxidativo.
- Verificar o efeito de antipsicóticos (típico e atípicos) na secreção de S100B induzida por citocinas.

Modelo *in vivo* (ratas Wistar prenhas):

- Avaliar o impacto da administração de LPS no final da gestação, sobre o comportamento da prole adulta.
- Avaliar parâmetros astrogliais (secreção de S100B e captação de glutamato) em fatias *ex vivo* de animais adultos expostos a LPS pré-natalmente.

- Investigar possíveis alterações na quantificação da tirosina hidroxilase nesse modelo.
- Analisar os marcadores astrogliais S100B e GFAP em tecido de animais juvenis (30 dias) e adultos (60 dias), machos e fêmeas, cujas progenitoras foram expostas a LPS no final da gestação.
- Avaliar possíveis alterações idade e/ou gênero diferente em parâmetros de estresse oxidativo de ratos com exposição pré-natal ao LPS.

Decision Letter

Data: 5 Dec 2012 12:52:23 +0000
De: Progress in NP & BP <pnpbp@neurosciences.ulaval.ca>
Assunto: Your Submission
Para: casg@ufrgs.br

Ms. Ref. No.: PNP-D-12-00419R1
Title: Interleukin-6-induced S100B secretion is inhibited by haloperidol and risperidone
Progress in Neuro-Psychopharmacology & Biological Psychiatry

Dear CA,

A final disposition of "Accept" has been registered for the above-mentioned manuscript.

Kind regards,

Progress in Neuro-Psychopharmacology & Biological Psychiatry

Comments from the Editors and Reviewers:

Reviewer #1: The authors have answered most of the queries. One typo in the highlights section (Neuroinflammation potentially contributeS) needs to be corrected. Interestingly, the new results with DCF ROS measurement does not only not explain the discrepancies in S100B response to antipsychotics, but raises new questions because of opposite effects of first and second generation antipsychotics ; while this does not deter acceptance of the manuscript in its present form, there is obviously some basis for further investigation

Reviewer #2: This revised manuscript has been well revised based on our suggestions.

Final suggestion in abstract is the following:
How about changing the abstract part from ".cytokines altered in schizophrenia, ." to ".cytokines (IL-1beta, TNF-alfa, IL-6, IL-8, etc), ."?

****

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CAPÍTULO I

Interleukin-6-induced S100B secretion is inhibited by haloperidol and risperidone

Daniela Fraga de Souza, Krista Wartchow, Fernanda Hansen, Paula Lunardi, Maria Cristina Guerra, Patrícia Nardin and Carlos-Alberto Gonçalves*

Artigo aceito para publicação no periódico Progress in Neuro-Psychopharmachology & Biological Psychiatry

Interleukin-6-induced S100B secretion is inhibited by haloperidol and risperidone

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Abbreviations: **CSF**, cerebrospinal fluid; **DCF,** 2′–7′-dichlorofluorescein; **ELISA**, enzyme-linked immunosorbent assay; **ERK,** extracellular signal-regulated kinase, one of three members of the MAPK; **GSH,** glutathione; **GFAP**, glial fibrillary acidic protein; **IL-1β**, interleukin-1β**; IL-2**,interleukin-2; **IL-6,** interleukin-6; **IL-8**, interleukin-8; **JNK**, c-Jun N-terminal kinase, another of three members of the MAPK; **LDH,** lactate dehydrogenase**; LPS**, Lipopolysaccharides; **MAPK**, mitogen-activated protein kinase; **MEK**, MAPK/ERK kinase; **MTT**,3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **NF-κB**, nuclear factor kappa B; **p38**, third member of the MAPK; **TNF-α**, tumor necrosis factor- α; **TRL4**, toll-like receptor 4**.**

Abstract

Although inflammation may be a physiological defense process, imbalanced neuroinflammation has been associated with the pathophysiology of brain disorders, including major depression and schizophrenia. Activated glia releases a variety of pro-inflammatory cytokines that contribute to neuronal dysfunction. Elevated levels of S100B, a glia derived protein, have been observed in the serum and CSF of schizophrenic patients suggesting a glial role in the disease. We evaluated whether S100B secretion (in C6 glioma cells and hippocampal slices in Wistar rats) could be directly modulated by the main inflammatory cytokines altered in schizophrenia, as well as the possible involvement of mitogen-activated protein kinase (MAPK) pathways in these responses. We also investigated the effects of typical and atypical antipsychotic drugs on glial cytokine-induced S100B release. Our results suggest that S100B secretion is increased by pro-inflammatory cytokines via MAPK and that oxidative stress may be a component of this modulation. These results reinforce the idea that the S100B protein is involved in the inflammatory response observed in many brain diseases, including schizophrenia. Moreover the antipsychotics, haloperidol and risperidone, were able to inhibit the secretion of S100B following IL-6 stimulation in C6 glioma cells.

Key words: antipsychotics; cytokines; glia; schizophrenia; S100B

1. Introduction

Neuroinflammation plays a critical role in different neurological and psychiatric diseases. Although inflammation may be a physiological defense process, beneficial for repair and recovery of the central nervous system (CNS)(Wee Yong, 2010), imbalanced inflammation has been associated with the pathophysiology of several brain disorders, including Alzheimer's disease, major depression and schizophrenia (Akiyama *et al.* , 2000, Garate *et al.* , 2011, Monji *et al.* , 2009). The inflammatory response in the CNS includes a wide spectrum of complex and integrated cellular responses, such as microglial and astroglial activation. These glial cells, when activated, releases a variety of pro-inflammatory mediators (e.g. cytokines) which can potentially contribute to neuronal dysfunction and result in the progression of the CNS pathology (Khansari *et al.* , 2009).

Cytokines are involved in regulating the communication between immune cells, thus, high peripheral and CNS cytokine levels indicate activation of the inflammatory response. However, these cytokines modulate not only normal CNS function; abnormal cytokine signaling may contribute to major acute and chronic CNS diseases(Tansey, 2010). Increased levels of pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6, have been found in individuals with epilepsy and autism (Mansur *et al.* , 2012) and levels of IL-2 andIL-8 may also be elevated in schizophrenia (Zhang *et al.* , 2002). In fact, meta-analyses have shown consistent alterations of increased IL-1β, IL6, and TNF-α levels in the serum of schizophrenic patients (Miller *et al.* , 2011, Potvin *et al.* , 2008). *et alet al*Furthermore, animals models using iodinated LPS in pregnant rats show that the development of schizophrenia-like manifestations in offspring rats occurs, most probably, due to the effect of cytokines, since cytokines and not LPS can cross the placenta (Ashdown *et al.* , 2006).

S100B, a calcium-binding protein belonging to the S100 family of proteins in the CNS is predominantly expressed and secreted by astrocytes (Donato, 2001, Donato *et al.* , 2009), exerting paracrine and autocrine effects on neurons and glial cells. This protein may cross the blood-brain barrier and concentrations of S100B determined in serum and/or cerebrospinal fluid (CSF) appear to reflect the degree of injury in brain disorders (Pleines *et al.* , 2001).As such, S100B has been proposed as a marker of brain damage (Goncalves *et al.* , 2008, Marchi *et al.* , 2004). S100B seems to be able to modulate cytokine secretion and may also be modulated by pro-inflammatory cytokines(Edwards and Robinson, 2006, Schmitt *et al.* , 2007). Moreover, some studies have demonstrated a significant correlation between S100B and pro-inflammatory molecules in neurological and psychiatric disorders (Ashraf *et al.* , 1999, Steiner *et al.* , 2009).However, the idea that S100B may be considered to be a cytokine remains controversial.

Our previous reports have suggested that S100B may be an important component in neuroinflammation. We have demonstrated that S100B secretion is induced by IL-1β, mediated by MAPK-ERK signaling, in astrocyte cultures, C6 glioma cells and acute hippocampal slices(de Souza *et al.* , 2009). More recently, we showed that S100B secretion is stimulated by intracerebroventricular lipopolysaccharide (LPS) administration and that LPS directly stimulates the secretion of S100B from astrocytes and hippocampal slices (Guerra *et al.* , 2011).

The presence of elevated S100B levels, observed in the serum and CSF of schizophrenic patients (Lara *et al.* , 2001, Rothermundt *et al.* , 2004, Steiner *et al.* , 2006), as well as the association of the schizophrenia with certain haplotypes of the S100B gene involved in increased S100B expression (Liu *et al.* , 2005), provides further evidence that a dysfunction of glia cells might present a pathogenic factor in schizophrenia. For this

reason, C6 glioma cells have been investigated as targets of antipsychotic medication (Nardin *et al.* , 2011, Steiner *et al.* , 2010b). We herein evaluate whether S100B secretion could be directly modulated by the main inflammatory cytokines associated with the pathophysiology of schizophrenia; IL-1β, TNF-α, IL-6 and IL-8 in C6 glioma cells and acute hippocampal slices, as well as the possible involvement of MAPK pathways in these responses. We also investigate the effects of these cytokines on GFAP and oxidative stress. Finally, we explore the effects of typical and atypical antipsychotic drugs on glial cytokine-induced S100B release.

2.Material and Methods

2.1 Animals

For the preparation of hippocampal slices, twelve male 30-day old Wistar rats were obtained from our breeding colony (Department of Biochemistry, UFRGS, Porto Alegre, Brazil), were maintained under controlled light and environmental conditions (12 hour light/12 hour dark cycle at a constant temperature of 22 ± 1°C), and had free access to commercial chow and water. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996 and followed the regulations of the local animal housing authorities**.**

2.2 Materials

Dulbecco's modified Eagle's medium (DMEM) and other materials for cell culture were purchased from Gibco (Belo Horizonte, Brazil). Interleukin-1beta (IL-1β), tumor

necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), S100B protein, anti-S100B antibody (SH-B1), o-phenylenediamine (OPD), Triton X-100, methylthiazolyldiphenyl-tetrazolium bromide (MTT), Griess reagent (modified), standard glutathione, o-phthaldialdehyde and dichlorofluoresceindiacetate (DCF-DA) were purchased from Sigma (St. Louis, MO, USA). Polyclonal anti-S100B was purchased from DAKO (São Paulo, Brazil), anti-GFAP from rabbit and anti-rabbit linked to peroxidase were purchased from GE (Little Chalfont, United Kingdom). Other reagents were purchased from local commercial suppliers (Sulquímica, Labsul or Biogen; Porto Alegre, Brazil).

2.3 Preparation and incubation of brain slices

Rats were decapitated, their hippocampi were quickly dissected out and transverse sections (300 μm) were rapidly obtained using a McIlwain tissue chopper. One slice was placed into each well of a 24-well culture plate. Slices were incubated in oxygenated physiological medium containing, in mM, 120 NaCl, 2.0 KCl, 1.0 $CaCl_2$, 1.0 MgSO4, 25.0 Hepes, 1.0 KH_2PO_4 and 10.0 glucose, pH 7.4, at room temperature. The medium was changed every 15 min for fresh medium. Following a 120-min equilibration period, slices were incubated in medium plus IL-1β, TNF-α, IL-6 or IL-8 (0.1, 1 or 10 ng/mL) for 1 h at 30°C.

2.4 C6 glioma cell culture

A rat glioblastoma cell line (C6 cells) was obtained from the American Type Culture Collection (Rockville, Maryland, USA). Late passage cells (i.e. after at least 100

passages) were seeded in 24-well plates at densities of 104 cells/well, and cultured in DMEM (pH 7.4) supplemented with 5% fetal bovine serum, 2.5mg/mL Fungizone® and 100U/L gentamicin in 5% CO_2/95% air at 37 °C. After cells had reached confluence, the culture medium was replaced by DMEM without serum in the absence or presence of IL-1β, TNF-α, IL-6 or IL-8 (0.1, 1 or 10 ng/mL). The inhibitors, PD98059 (10 μM, MEK inhibitor), SB203580 (10 μM, p38 inhibitor), SP600125 (10 μM, JNK inhibitor) or SN-50 (50 μg/mL, NF-κB inhibitor), as well as the antipsychotics, haloperidol (0.3 μM), clozapine (3 μM) or risperidone (70 nM), were added 15 min before cytokine exposure.

2.5 ELISA for S100B

The S100B concentration was determined in the culture medium at 15 min, 1, 6, and 24 h; and in the incubation medium of slices at 1 h. Cells were washed and scraped at 24 h for measurement of intracellular S100B content. S100B levels were determined by ELISA, as described previously (Leite *et al.* , 2008). Briefly, 50 μL of sample plus 50 μL of Tris buffer were incubated for 2 h on a microtiter plate, previously coated with anti-S100B monoclonal antibody (SH-B1, from Sigma). Anti-S100 polyclonal antibody (from DAKO) was incubated for 30 min and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. The color reaction with o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/mL.

2.6 ELISA for GFAP

ELISA for GFAP was carried out by coating the microtiter plate with 100 µL samples containing 20 ng of protein for 24 h at 4 °C. Incubation with a polyclonal anti-GFAP from rabbit for 1 h was followed by incubation with a secondary antibody conjugated with peroxidase for 1 h, at room temperature. A colorimetric reaction with o-phenylenediamine was measured at 492 nm. The standard human GFAP (from Calbiochem) curve ranged from 0.1 to 5 ng/mL.

2.7 Immunocytochemistry and cell morphology

C6 glioma cells were cultured on circular glass cover slips. After 1 h of IL-6 exposure, cells were fixed for 20 min with 4% paraformaldehyde in phosphate buffer (PBS, mM): 2.9 KH2PO4; 38 Na2HPO47H2O; 130 NaCl; 1.2 KCl, rinsed with PBS and permeabilized for 20 min in PBS containing 0.2% Triton X-100. Fixed cells were then blocked for 60 min with PBS containing 5% bovine serum albumin and incubated overnight with anti-S100B monoclonal antibody from mouse (SH-B1), 1:500 or polyclonal anti-GFAP from rabbit, 1:500. Following incubation with primary antibodies, the cultures were washed in PBS/triton 0,2% (3×5 min) and incubated for 2 h with the respective secondary antibody at a 1:1000 dilution: Alexa Fluor 528 (goat anti-mouse-IgG; red fluorescence) and Alexa 488 (goat anti-rabbit-IgG; green fluorescence).

2.8 MTT reduction assay

Cells were treated with 50 µg/mL Methylthiazolyldiphenyl-tetrazolium bromide (MTT) for 30 min in 5% CO_2 / 95% air at 37°C. Subsequently, the media was removed and

MTT crystals were dissolved in DMSO. Absorbance values were measured at 560 and 650 nm. The reduction in MTT was calculated as (absorbance at 560 nm) - (absorbance at 650 nm).

2.9 Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase assay was carried out in 50 μL of extracellular medium, using a commercial colorimetric assay from Doles (Goiânia, Brazil).

2.10 Evaluation of Intracellular Reactive Oxygen Species (ROS) Production

Intracellular ROS production was detected using the non-fluorescent cell permeating compound, 2′–7′-dichlorofluorescin diacetate (DCF-DA). DCF-DA is hydrolyzed by intracellular esterases and then oxidized by ROS to a fluorescent compound, 2′–7′-dichlorofluorescein. C6 cells were treated with DCF-DA (10 μM) for 30 min at 37°C and rinsed with DMEM without serum. Following DCF-DA exposure, the cells were rinsed and then scraped into PBS with 0.2% Triton X-100. Fluorescence was measured with a spectrophotometer plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm.

2.11 Glutathione (GSH) Content Assay

GSH levels (nmol/mg protein) were measured, as described previously (Browne and Armstrong, 1998). C6 glioma cell homogenates were diluted in 10 volumes of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA and protein was precipitated

with 1.7% meta-phosphoric acid. Supernatant was assayed with o-phthaldialdeyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0–500 μM).

2.12 Nitric Oxide (NO) Production

NO was determined by measurement of nitrite (a stable oxidation product of NO), based on the Griess reaction. The Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 0.5 N HCl and 0.1% N-(1-naphthyl) ethylenediamine in deionized water. The assay was performed as described (Hu *et al.* , 1996) with modifications. Briefly, cells were cultured on 96-well plates and, after treatment, the Griess reagent was added directly to the cell culture and the incubation was maintained under reduced light conditions at room temperature for 15 min. Samples were analyzed at 550 nm on a microplate spectrophotometer. Controls and blanks were run simultaneously. Nitrite concentrations were calculated using a standard curve prepared with sodium nitrite (0–50 μM).

2.13 Protein Determination

Protein content was measured by Lowry's method using bovine serum albumin as standard (Peterson, 1977).

2.14 Statistical analysis

Parametric data are reported as means ± standard error and were analyzed by one-way ANOVA (followed by Tukey's or Dunnett's test). Values of $p < 0.05$ were considered to be significant.

3. Results

Cytokines increase S100B secretion in acute hippocampal slice preparations. Different concentrations of IL-1β, TNF-α, IL-6 and IL-8 (from 0.01 to 10 ng/mL) were added to hippocampal slices and extracellular S100B was measured at 1 h (Figure 1A-1D). Both IL-6 and TNF-α were able to increase S100B secretion at concentrations of between 0.1 to 10 ng/mL ($p < 0.01$, ANOVA). However, IL-8 and IL-1β increased S100B secretion only at concentrations of 1 and 10 ng / mL ($p < 0.05$, ANOVA). No effects were observed at cytokine concentrations of 0.01 ng/mL (data not shown). To make sure that we were determining S100B secretion, we evaluated cell integrity and viability in our preparations by measuring LDH release and capacity for MTT reduction, respectively. No changes in the MTT reduction assay (Figure 1E, $p = 0.58$) or LDH release were observed (Figure 1F, $p = 0.39$).

S100B secretion is also acutely modulated by cytokines in C6 glioma cells. In order to investigate a direct effect of cytokines on glial cells, we added different concentrations of cytokines (from 0.1 to 10 ng/mL) to C6 glioma cell cultures and extracellular S100B was measured at 1, 6 and 24 h (Figure 2). All cytokines, at higher concentrations (10 ng/mL), increased S100B secretion at 1h, but not at 6 h or 24 h afterwards. This acute effect also observed with the cytokine concentration of 1 ng/mL in C6 glioma cells (except with TNF-α) and no effect was observed at the cytokine

concentration of 0.1 ng/mL (except with TNF-α). Similar assays were also carried out in all C6 glioma preparations, confirming cell viability and integrity (data not shown).

Cytokine-stimulated S100B secretion occurs via the MAPK pathway. To assess the role of the MAPK signaling pathway in the cytokine-stimulated secretion of S100B, specific inhibitors of this pathway were added to the C6 glioma culture. The ERK pathway inhibitor, PD98059 (Figure 3) ($p < 0.0001$, ANOVA), the p38 pathway inhibitor, SB203580 (Figure 4) ($p < 0.001$, ANOVA), and the JNK pathway inhibitor, SP600125 (Figure 5) ($p < 0.001$, ANOVA), were all able to prevent cytokine-stimulated S100B secretion, without affecting basal S100B secretion. A possible involvement of NF-κB mediating S100B secretion was investigated using a specific inhibitor of NF-κB, SN-50 peptide (Figure 6). SN-50 was unable to prevent cytokine-stimulated S100B secretion. Importantly, this inhibitor *per se* increased S100B secretion ($p < 0.05$, ANOVA) and therefore it is difficult to affirm whether this inhibitor prevented, or not, the effect induced by cytokines.

IL-6 increases S100B and GFAP content at 24 h in glioma cells. S100B and GFAP content in C6 glioma cells were measured after 24 h of exposure to cytokines (IL-1β, TNF-α, IL-6 and IL-8; 10 ng/mL) (Figure 7A and 7B, respectively). S100B intracellular content was increased only by IL-6 ($p < 0.05$, ANOVA). On the other hand, an increase in GFAP content was observed with all cytokines, except TNF-α ($p < 0.01$, ANOVA). The increment in both proteins induced by IL-6 was confirmed by immunocytochemistry, without changes in cell morphology (Figure 7C).

Signals of oxidative stress are observed in C6 glioma cells exposed to cytokines for 1h or 24 h. Acute exposure of cytokines (IL-1β, TNF-α, IL-6 and IL-8; 10 ng/mL for 1 h) increased DCF levels (Figure 8A, $p < 0.005$), with the exception of IL-8 ($p = 0.077$) and

did not change NO levels (Figure 8B, $p = 0.63$). After cytokine exposure for 24 h, we observed a decrease in intracellular content of glutathione in cell cultures treated with IL-1β and IL-8 (Figure 8C, $p < 0.005$).

The increase in S100B secretion, induced by IL-6 in C6 glioma cells, is prevented by antipsychotics. In order to evaluate a protective effect of antipsychotics on S100B secretion induced by IL-6, we added haloperidol (typical antipsychotic), clozapine and risperidone (atypical antipsychotics). Both haloperidol and risperidone abolished IL-6-induced S100B secretion ($p < 0.0001$). Clozapine did not change the effect of IL-6 on S100B secretion ($p = 0.474$). Interestingly, risperidone per se increased basal S100B secretion. Moreover, risperidone prevented the IL-6 induced S100B secretion, resulting in secretion to levels that were lower than those at basal conditions.

Acute ROS production induced by IL-6 is prevented by haloperidol. To assess the effect of antipsychotic drugs in intracellular ROS production on C6 glioma cells exposed to IL-6, we added haloperidol, risperidone and clozapine. Haloperidol abolished IL-6-induced ROS production ($p < 0.05$). Neither clozapine nor risperidone were able to prevent the effect of IL-6 on DCF levels ($p = 0.59$ and $p = 0.32$, respectively). Moreover, risperidone and clozapine per se acutely increased basal DCF levels ($p < 0.001$ and $p < 0.05$, respectively).

4. Discussion

Several lines of evidence indicate that glial dysfunction could be a pathogenic factor in schizophrenia. Levels of S100B, a protein expressed and secreted by glial cells, astrocytes (mainly) and oligondrocytes (Donato, Sorci, 2009, Steiner *et al.* , 2008), are altered in the serum and CSF of schizophrenic patients, reinforcing this idea (Schroeter and Steiner, 2009). The mechanism of S100B secretion is unknown, but

neurotransmitters including glutamate (Buyukuysal, 2005, Goncalves *et al.* , 2000, Tramontina *et al.* , 2006), serotonin (Tramontina *et al.* , 2008), dopamine (Nardin, Tramontina, 2011) and acetylcholine (P. Lunardi, unpublished data) are able to modulate its secretion in cell cultures and acute brain slices.

However, increments in serum and CSF S100B in schizophrenic patients cannot be explained exclusively by changes in the profile of S100B secretion, caused by altered neurotransmission. For example, assuming that D2 receptors in astrocytes of these preparations are negatively coupled to adenylcyclase, a decrease in S100B secretion would be expected (Nardin, Tramontina, 2011). Recent findings, in animal models and human studies, have provided cumulative evidence for the cytokine hypothesis of schizophrenia (Watanabe *et al.* , 2010). In addition, a correlation between S100B and pro-inflammatory molecules has been demonstrated in neurological and psychiatric disorders (Ashraf, Bhattacharya, 1999, Pleines, Morganti-Kossmann, 2001, Potvin, Stip, 2008). Supporting an inflammatory role for S100B, we have observed changes in S100B secretion in response to interleukin-1β (de Souza, Leite, 2009) and activation of TLR4 (Guerra, Tortorelli, 2011). Furthermore, inflammatory cytokines were found to stimulate S100B secretion in hippocampal slices and this effect was confirmed in C6 glioma cells. The effect on S100B secretion was acute, since no effect was observed at 6 or 24 h after cytokine exposure in cell cultures. We previously demonstrated cytokine-mediated modulation of S100B secretion in primary astrocytes and C6 glioma cells (de Souza, Leite, 2009). Griffin *et al.* previously reported an increase in S100B secretion in C6 glioma cells, induced by IL-1beta, as shown by the increase in the intracellular content of this protein (Griffin *et al.* , 1998). Nonetheless, we know now that an increase in cell content of this protein is not necessarily accompanied by its secretion (de Souza, Leite, 2009). An increase in extracellular S100B was also observed in cell

cultures exposed to TNF-α (Edwards and Robinson, 2006). However, these authors also observed an increase in extracellular GFAP, suggesting that, under the conditions used, TNF-α exposure affected cell integrity. Conversely, S100B appears to modulate expression (and release) of TNF-α and IL-6 (Ponath *et al.* , 2007); suggesting a new role for IL-6 in stimulating S100B release. Our data clearly indicate that S100B secretion is stimulated by the inflammatory cytokines, IL-1β, TNF-α, IL-6 and IL-8, suggesting that the increase in S100B observed in schizophrenic patients could, in part, be explained by cytokine stimulation.

Our next step was to investigate the signaling pathway involved in this mechanism, in particular the MAPK pathway, which is implicated in gene expression as well as in the molecular mechanisms of protein secretion, in response to a diverse range of extracellular stimuli. The MAPK pathway, in fact, encompasses three distinct pathways; the ERK, p38 and JNK pathways. Specific inhibitors of these pathways were able to block the S100B secretion induced by inflammatory cytokines. It is important to mention that these signaling kinases are susceptible to oxidative stress (Gaitanaki *et al.* , 2003). Therefore, the oxidative stress observed in schizophrenic patients and other brain disorders could modulate S100B secretion through MAPK. Moreover, under the conditions used, cytokines were able to induce oxidative stress, as shown by measurements of DCF oxidation and reduced glutathione content. In general, cytokines induced oxidative stress, but not nitrosative stress (based on measurement of nitrate content); however, IL-6 was not able to modify glutathione content and IL-8 did not affect DCF oxidation. These findings may help to explain the presence of markers of oxidative stress and the impaired antioxidant defense system previously found in patients with schizophrenia (Martinez-Cengotitabengoa *et al.* , 2012).

The NF-κB pathway was also investigated using SN-50, an inhibitor for the nuclear migration of this transcription factor. No effect was observed on IL-6-induced S100B secretion. Moreover, SN-50 *per se* caused an increase in S100B secretion. The increase in S100B secretion that we observed, induced by SN-50, could be interpreted as interruption of the tonic inhibition of NF-κB-mediated S100B synthesis. In fact, NF-κB-mediated activation of transcription has been widely characterized, but the mechanisms of NF-κB-mediated repression have not (Tchivileva *et al.* , 2009). *et al* However, to our knowledge, a direct involvement of NF-kappaB in S100B expression has not been reported. Moreover, the effects of MAPK and NF-kappa B inhibitors on S100B secretion (measured in 1 h) possibly do not involve protein synthesis. Therefore, the effect of SN-50 may involve target(s) other than NF-κB (Torgerson *et al.* , 1998)*et al.*

IL-6 was able to induce an increase in GFAP and S100B 24 h afterwards. In fact, IL-6 is an important regulator of neuroinflammation and contributes to astrocytic differentiation of C6 glioma cells (Takanaga *et al.* , 2004). Increased blood levels of IL-6 cytokine in schizophrenia have been described over 20 years (Shintani *et al.* , 1991) and this cytokine has been used in schizophrenia models (Behrens *et al.* , 2008). However, postmortem human brain studies indicate a lack of gliosis in most schizophrenic brains (Arnold *et al.* , 1996, Damadzic *et al.* , 2001). On the other hand, it is possible that cytokine-induced gliosis could be transitory, as observed in animal models (Fatemi and Folsom, 2009). Moreover, it is necessary to consider the effect *per se* on GFAP of chronic antipsychotic administration, which in rats was dependent on type of antipsychotic and brain region (Blazquez Arroyo *et al.* , 2010).

We have chosen IL-6-induced S100B secretion to evaluate the effects of the antipsychotics, haloperidol, clozapine and risperidone. Exposure of C6 and OLN-93 cells to haloperidol and clozapine caused a decrease in basal S100B release (Steiner,

Bernstein, 2008); the authors of this report suggested that serum S100B elevation in schizophrenic patients is normalized rather than increased by the effects of antipsychotic drugs on glial cells. More recently, we showed a decrease in S100B secretion in hippocampal slices and C6 cells, induced by apomorphine (Nardin, Tramontina, 2011); in contrast, antipsychotics (haloperidol and risperidone) were not able to alter this change. In the present study, we demonstrated that S100B secretion, induced by IL-6, is prevented by haloperidol and risperidone, but not clozapine. Only risperidone was able to change basal S100B secretion, confirming a previous study (Quincozes-Santos *et al.* , 2008). Together these data reinforce the idea that antipsychotics help to normalize the elevated serum S100B levels in schizophrenic patients, during acute active phases and that cytokines are underlying these S100B elevations. It should be noted that adipocytes are another important source of serum S100B (Goncalves *et al.* , 2010) and that major metabolic changes in schizophrenic patients are observed in these cells (Steiner *et al.* , 2010a).

However, the effect of antipsychotic drugs on IL-6-induced S100B secretion in C6 glioma cells indicates a modulation via D_2 receptors. It is worth mentioning that atypical antipsychotic drugs, such as clozapine and risperidone, involve more $5HT_{2A}$ than D_2*et al* (Kuroki *et al.* , 2008). Moreover, other receptors such as $5HT_7$, found in astrocytes, are targets of antipsychotic drugs and putatively could underlie the different modulation observed with clozapine and risperidone (Smith *et al.* , 2011)*et al*. Therefore, these and other receptors are modulating basal and/or cytokine-induced S100B secretion in brain tissue, making it difficult to describe a simple scenario to explain the extracellular changes of this protein. Moreover, the effects of antipsychotic drugs on S100B secretion could be due to changes in cell oxidative status*et al* (Donato, Sorci, 2009). However, for haloperidol, in contrast to its *in vivo* long term administration *et al*(Pillai *et al.* , 2007),

we observed a decrease in ROS levels in glioma C6 cells at 1 h after administration. Moreover, we know that haloperidol, at high concentrations (but not risperidone), is able to induce ROS production after 6 h *et al*(Quincozes-Santos *et al.* , 2010) in C6 glioma cells. Therefore, it was not possible to correlate S100B secretion and ROS levels in C6 glioma cells during acute exposure to antipsychotic drugs.

Some limitations of this study should be noted. Firstly, this is an in vitro study, useful for studying responses and mechanisms in an isolated manner, but unable to demonstrate the entire complexity of a system in vivo. Secondly, levels of S100B secretion found are sub-nanomolar levels compatible with the neurotrophic activity of this protein observed in culture; however, the specific effects of the increased secretion of this protein following the stimulation of neurons and other neuronal cells with pro-inflammatory cytokines should be studied. Finally, it is worth mentioning that although the astrocytes are the main source of extracellular S100B, the contribution of other cells should not be excluded, particularly in slice preparations. However, our ability to reproduce previous results validates our preparations for comparative approaches.

Conclusions. Our data provides evidence that S100B secretion is increased by pro-inflammatory cytokines in C6 glioma of cells and hippocampal slices of rats via MAPK, and that oxidative stress may be a component of this modulation. IL-6 induced an increase of S100B and GFAP. These results reinforce the idea that the S100B protein is involved in the inflammatory response observed in many brain diseases including schizophrenia. Moreover the antipsychotics, haloperidol and risperidone, were able to inhibit the secretion of S100B following IL-6 stimulation.

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Figures

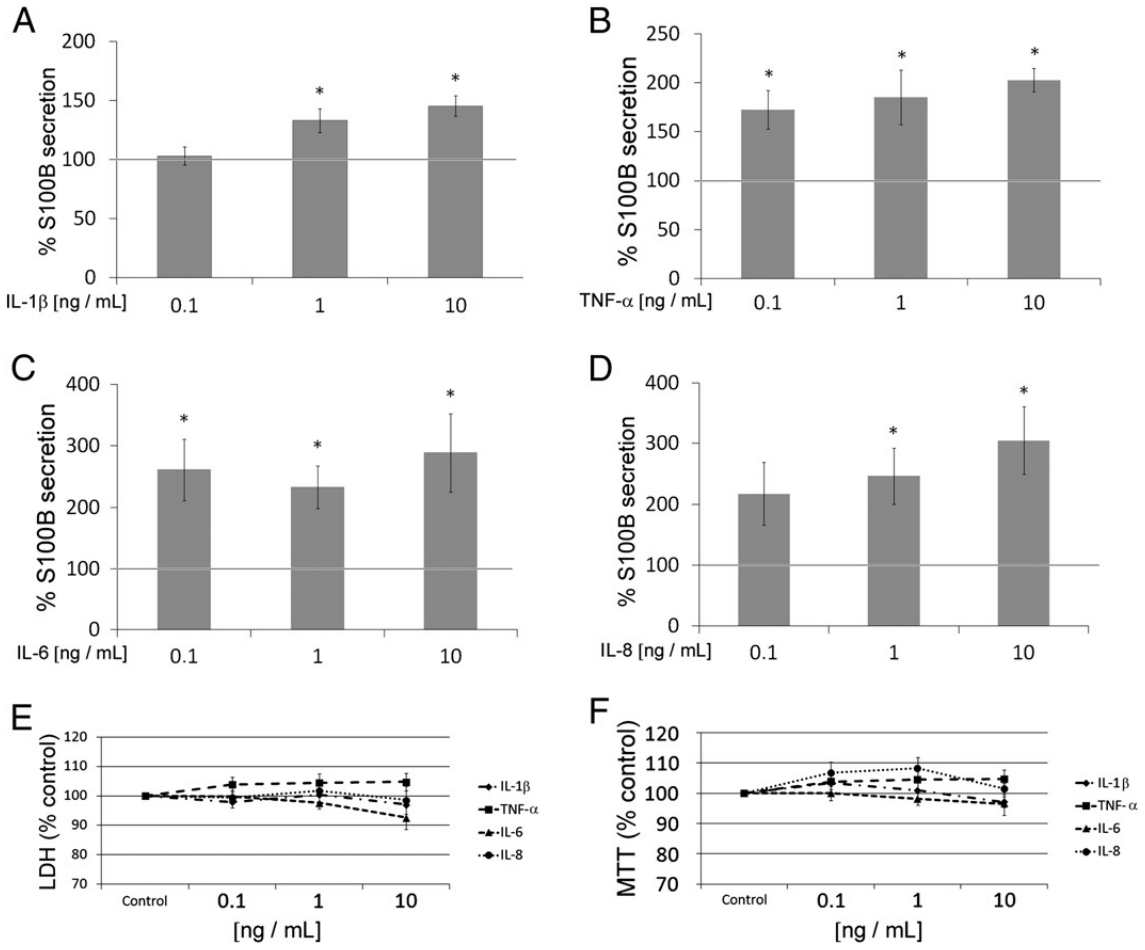

Figure 1. Cytokines increase S100B secretion in acute hippocampal slice preparations. Hippocampal slices from 30-day old Wistar rats were exposed to IL-1β (A), TNF-α (B), IL-6 (C) or IL-8 (D) at concentrations ranging from 0 to 10 ng/mL for 1 h; S100B was measured by ELISA. The line indicates basal secretion, assumed as 100% in each experiment. The basal levels of S100B ranged from 0.1 to 0.24 ng/mg total protein. Cell integrity and viability, LDH release (E) and capacity for MTT reduction (F) were analyzed, respectively. Each value represents the mean (± standard error) of 6 independent experiments performed in triplicate. * Significantly different from basal secretion (one way ANOVA followed by Dunnett's test, with a significance level of $p < 0.05$).

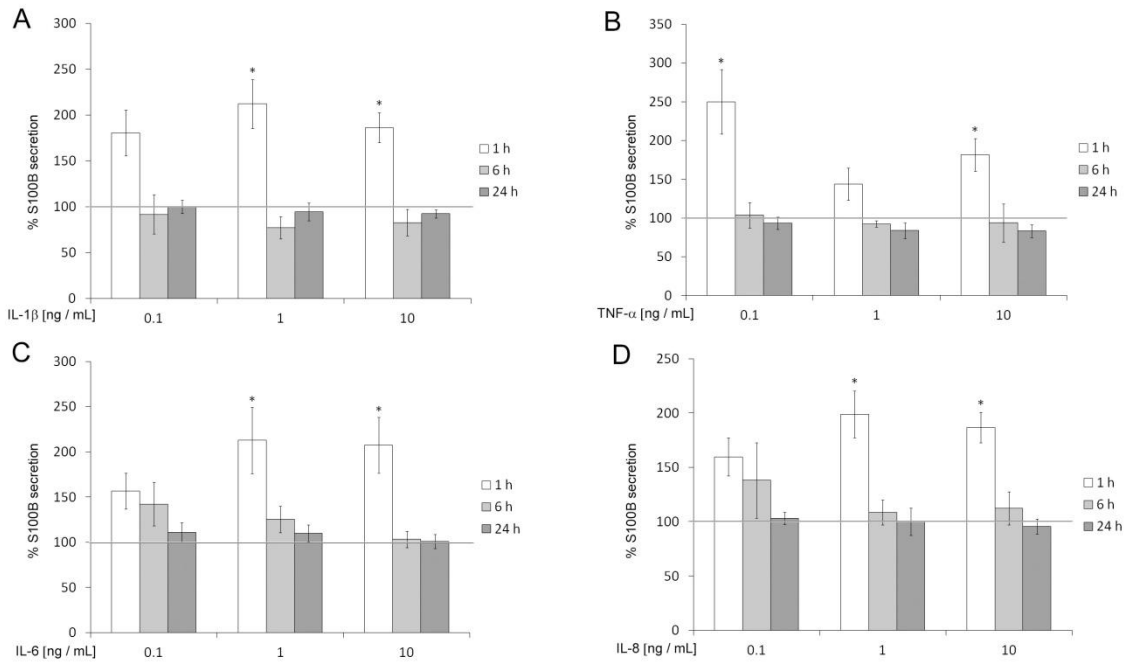

Figure 2. S100B secretion is also acutely modulated by cytokines in C6 glioma cells. S100B secretion at 1, 6 and 24 h in C6 glioma cells exposed to IL-1β, TNF-α, IL-6 or IL-8 (in A, B, C and D, respectively). The line indicates basal secretion, assumed as 100% in each experiment. The basal levels of S100B ranged from 0.08 to 0.12 ng/mg total protein. Each value is the mean (± standard error) of 6 independent experiments performed in triplicate. * Significantly different from basal secretion (one way ANOVA followed by Dunnett's test, with a significance level of $p < 0.05$).

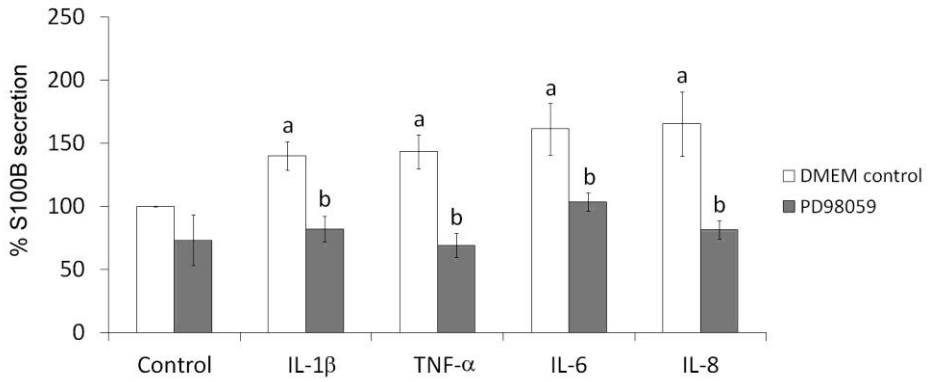

Figure 3. Effect of a MAPK/ERK pathway inhibitor on cytokine-induced S100B secretion. C6 glioma culture cells were exposed to 10 ng/mL IL-1β, TNF-α, IL-6 or IL-8for 1 h, in the presence (or not) of 10 μM PD98059 (MAPK/ERK inhibitor), added 15 min before cytokine exposure. S100B content in the medium was measured by ELISA. Each value is the mean (± standard error) of 6 independent experiments performed in triplicate. Means indicated by different letters are significantly different. [a] Significantly different from basal secretion. [b] Significantly different from cytokine-induced secretion (one way ANOVA followed by Tukey's test, with a significance level of $p < 0.05$).

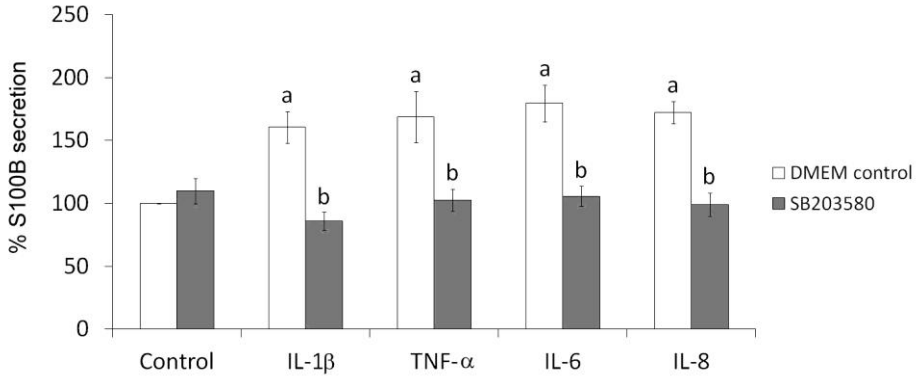

Figure 4. Effect of a MAPK/p38 pathway inhibitor on cytokine-induced S100B secretion. C6 glioma culture cells were exposed to 10 ng/mL IL-1β, TNF-α, IL-6 or IL-8for 1 h, in the presence (or not) of 10 μM SB203580 (MAPK/p38 inhibitor), added 15 min before cytokine exposure. S100B content in the medium was measured by ELISA. Each value is the mean (± standard error) of 6 independent experiments performed in triplicate. Means indicated by different letters are significantly different. [a] Significantly different from basal secretion. [b] Significantly different from cytokine induced secretion (one way ANOVA followed by Tukey's test, with a significance level of $p < 0.05$).

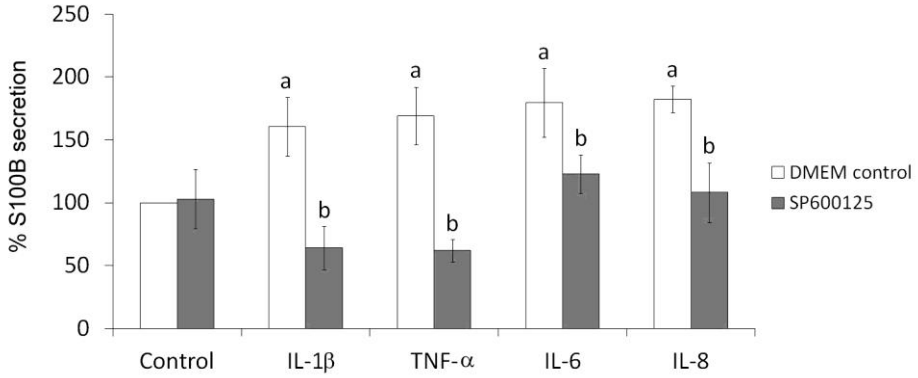

Figure 5. Effect MAPK/JNK pathway inhibitor on cytokine-induced S100B secretion. C6 glioma culture cells were exposed to 10 ng/mL IL-1β, TNF-α, IL-6 or IL-8for 1 h, in the presence (or not) of 10 μM SP600125 (MAPK/JNK inhibitor), added 15 min before cytokine exposure. S100B content in the medium was measured by ELISA. Each value is the mean (± standard error) of 6 independent experiments performed in triplicate. Means indicated by different letters are significantly different. [a] Significantly different from basal secretion. [b] Significantly different from cytokine induced secretion (one way ANOVA followed by Tukey's test, with a significance level of $p < 0.05$).

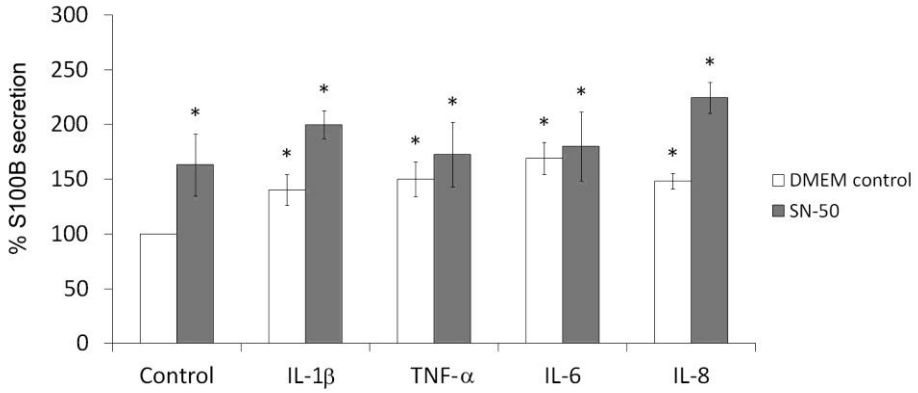

Figure 6. EffectNF-κB inhibitor on cytokine-induced S100B secretion. C6 glioma

culture cells were exposed to 10 ng/mL IL-1β, TNF-α, IL-6 or IL-8for 1 h, in the presence (or not) of 50 μg/mL SN-50 (NF-κB inhibitor), added 15 min before cytokine exposure. S100B content in the medium was measured by ELISA. Each value is the mean (± standard error) of 6 independent experiments performed in triplicate. Means indicated by different letters are significantly different. * Significantly different from basal secretion (one way ANOVA followed by Tukey's test, with a significance level of $p < 0.05$).

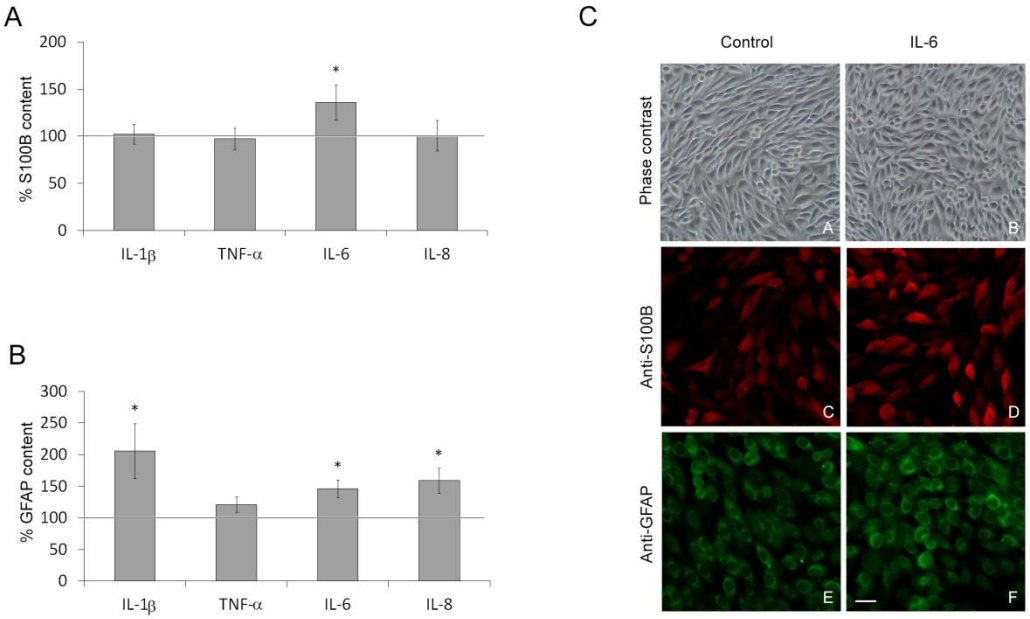

Figure 7. Intracellular GFAP and S100B content are modified by cytokines in C6 glioma cells.C6 glioma cells exposed to 10 ng/mL IL-1β, TNF-α, IL-6 or IL-8for 24 h were lysed and intracellular contents of S100B (A) and GFAP (B) were measured by ELISA. The line indicates basal secretion, assumed as 100% in each experiment. In C, C6 glioma cells were exposed to 10 ng/mL IL-6 for 24 h, phase-contrast images from control and IL-6-exposed cells are shown in panels A and B, respectively; immunocytochemistry for S100B from control and IL-6-exposed cells are shown in panels C and D, respectively and immunocytochemistry for GFAP in control and IL-6-exposed cells are shown in panels E and F, respectively. Each value is the mean (± standard error) of at least 5 independent experiments performed in triplicate.*Significantly different from basal secretion (one way ANOVA followed by Tukey's test, with a significance level of $p < 0.05$). Scale bar = 20 □m.

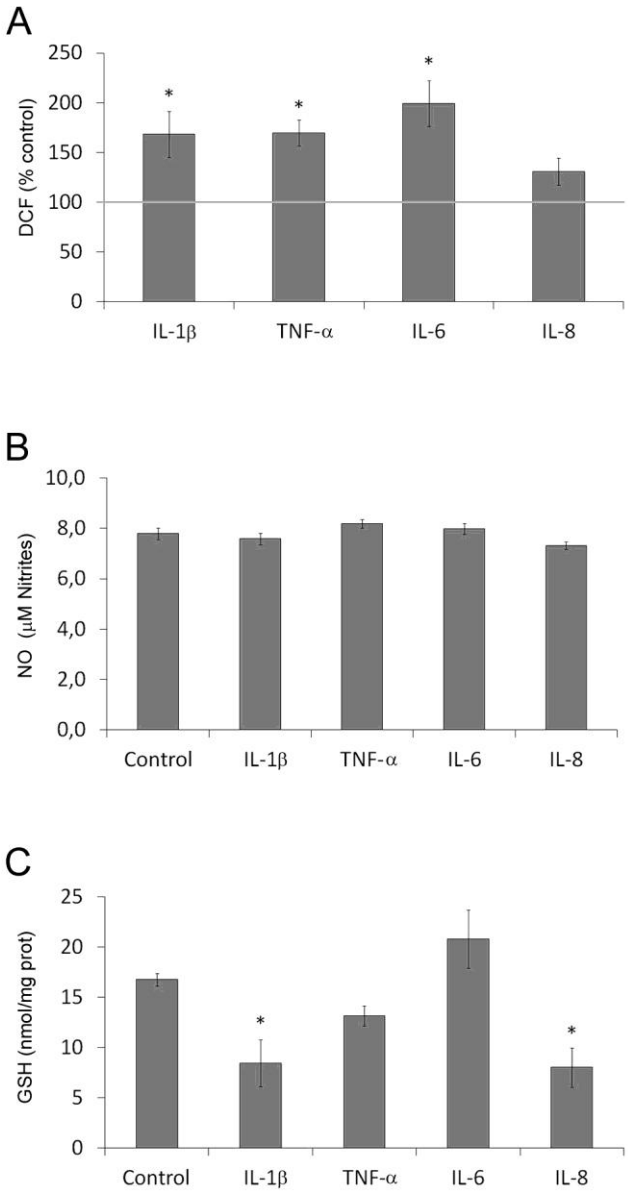

Figure 8. Signals of oxidative stress are observed in C6 glioma cells exposed to cytokines for 1h or 24 h. Intracellular ROS production (A) and nitric oxide production (B) were measured in the C6 cell line in the absence and in the presence of 10 ng/mL IL-1β, TNF-α, IL-6 or IL-8 after 1h of treatment. Glutathione amount (C) was quantified at 24h. In A, the line indicates basal secretion, assumed as 100%. Each value is the mean (± standard error) of at least 5 independent experiments performed in

triplicate.* Significantly different from control (one way ANOVA followed by Tukey's test, with a significance level of $p < 0.05$).

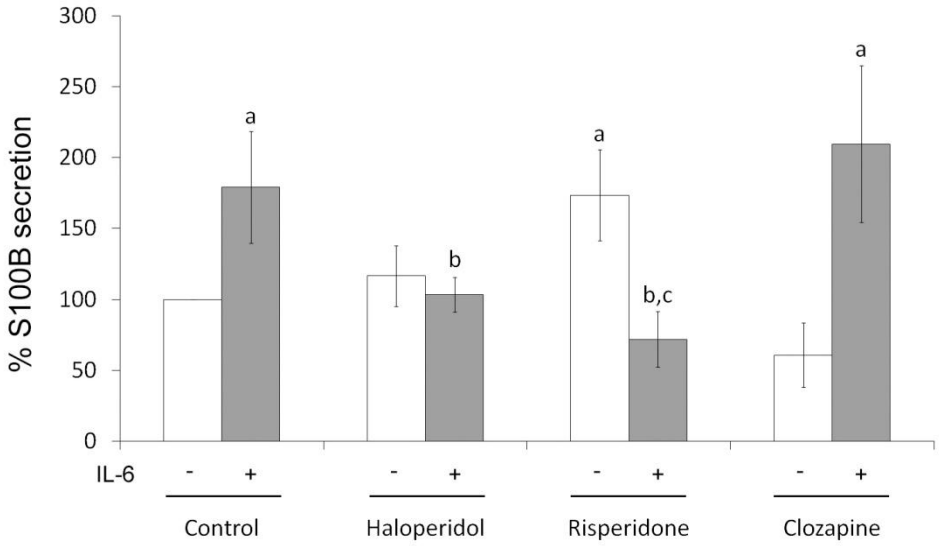

Figure 9. Increase of S100B secretion induced by IL-6 in C6 glioma cells is prevented by antipsychotics. C6 glioma culture was exposed to 10 ng/mL IL-6 for 1 h, in the presence or in the absence of haloperidol (0.3 µM) or risperidone (70 nM) or clozapine (3 µM), added 15 min before cytokine exposure. S100B content in the medium was measured by ELISA. The line indicates basal secretion, assumed as 100% in each experiment. Each value is the mean (± standard error) of 6 independent experiments performed in triplicate. [a] Significantly different from basal secretion. [b] Significantly different from cytokine induced secretion. [c] Significantly different from risperidone induced secretion (one way ANOVA followed by Tukey's test, with a significance level of $p < 0.05$).

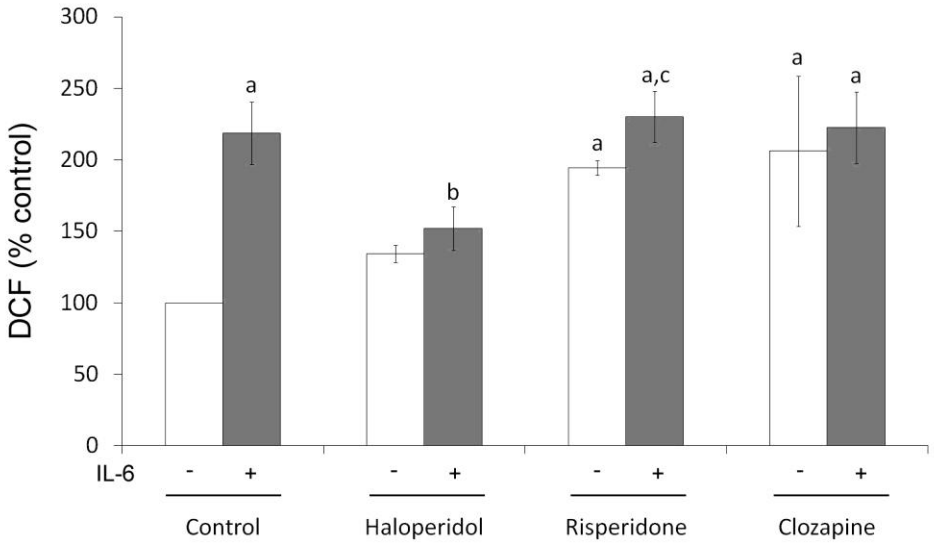

Figure 10. IL-6-induced intracellular ROS production in C6 glioma cells is prevented by haloperidol. Intracellular ROS production was measured in C6 glioma cultures exposed to 10 ng/mL IL-6 for 1 h, in the presence or in the absence of haloperidol (0.3 μM), risperidone (70 nM) or clozapine (3 μM), added 15 min before cytokine exposure. Each value is the mean (± standard error) of 4 independent experiments performed in triplicate. [a] Significantly different from basal secretion. [b] Significantly different from cytokine-induced secretion. [c] Significantly different from risperidone-induced secretion (one way ANOVA followed by Tukey's test, with a significance level of $p < 0.05$).

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CAPÍTULO II

Astroglial parameters in model of schizophrenia induced by LPS during pregnancy of Wistar rats

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Abbreviations: CNS, central nervous system; **ELISA**, enzyme-linked immunosorbent assay; **IL-1β**, interleukin-1β**; IL-6,** interleukin-6; **LPS**, lipopolysaccharide; **NMDA**, N-metil D-Aspartato; **TH**, tyrosine hydroxylase; **TNF-α**, tumor necrosis factor- α.

Abstract

Maternal infection during pregnancy might increase the incidence of the schizophrenia in offspring adulthood. Maternal immune response, rather than direct infection of the fetus, could be responsible for disease development. Various animal models have been used in recent years to investigate this correlation, for example the model of maternal exposure to LPS, moreover there is increasing evidence of structural and functional alterations in glial cells of schizophrenic patients. We evaluated whether administration of LPS to rats dams in late gestation affects some aspects of the offspring behavior in their adult life. We also investigate the effects of this treatment on glial parameters using *ex vivo* preparation of offsprings from pregnant rats exposed to LPS. Hippocampal slices were used to evaluate S100B secretion and glutamate uptake in basal condition and facing a challenge with apomorphine. Moreover we measured tyrosine hydroxylase (TH) to evaluate the effect on catecholaminergic neurons. Our data suggest that this model could mimic schizophrenia, since the finds are consistent with molecular and behavioral patterns seen in schizophrenic patients. This result reinforces the idea that prenatal infection may be an important environmental factor predictive of the development of schizophrenia and that dysfunction of glial parameters in adult offspring may contribute to the pathophysiology of this disease.

Key words: animal model; glia; lipopolysaccharide; schizophrenia; S100B

1. Introduction

Several lines of evidence strongly indicate that schizophrenia is a complex neuropsychiatric disorder of neurodevelopmental origin which involves components genetic and environmental [1]. Substantial epidemiologic studies have demonstrated that prenatal exposure of pregnant women to environmental challenges, such as obstetric complications and maternal infection with either viral or bacterial pathogens, increase the incidence of the schizophrenia in offspring adulthood [2, 3]. This suggests that maternal infection during pregnancy might contribute to the pathophysiology of schizophrenia through effects on fetal brain development [4]. Moreover, recent studies have shown dysregulation of immune-related genes in brain, striking microglial and astrocyte activation, as well as cytokine up-regulation in schizophrenic brain [5].

The variety of infectious agents associated with increased risk of schizophrenia suggests that mechanisms common to several prenatal infections may affect fetal development. Various animal models have been used in recent years to investigate this correlation, for example the model of maternal exposure to bacterial endotoxin, lipopolysaccharide (LPS), which is known not pass the placental barrier, however produces a range neurochemical and behavioral injuries in the offspring [6-8]. Several lines of evidence indicate that the maternal immune response, rather than direct infection of the fetus, is responsible for the increased incidence of schizophrenia [9, 10]. Animal models have indicated that the maternal immune response can influence fetal brain development, possibly via circulating cytokines [11]. LPS administered to pregnant rats changes IL-1β, IL-6, and TNF-α in the maternal circulation , placenta, amniotic fluid and in fetus [12, 13], an altered cytokine profile very similar to that found in schizophrenic patients. These immune-associated changes in the fetus may lead to behavioral modifications or

disorders in adult animals. It has been reported that spatial learning and memory were impaired in adult rats that were prenatally exposed to LPS [14].

The inflammatory response in the brain is coordinated primarily by glial cells, major secreting cytokines and other pro-inflammatory factors. Recently we showed that pro-inflammatory cytokines increased S100B concentration *in vitro* and this increase is prevented by antipsychotic (de Souza *et al,* 2012). S100B, protein predominantly expressed and secreted by astrocytes in central nervous system (CNS), has been proposed as a marker of brain damage [15, 16], as well as elevated S100B levels are observed in schizophrenic patients [17-19]. Furthermore, there is increasing evidence of structural and functional alterations in glial cells of schizophrenic patients [20-22].

Impairment in glutamatergic signaling is another parameter that has been suggested to contribute to the development of schizophrenic pathophysiology. Phencyclidine and ketamine, antagonists of glutamate receptors NMDA, induce neurocognitive disorders and psychotic symptoms similar to schizophrenia, unlike dopaminergic agonists, these drugs mimic all aspects of the disease symptoms [23]. This led to the development of a glutamate hypothesis of schizophrenia postulating that schizophrenic symptoms are caused by glutamatergic hypofunction. Currently it is usually accepted that glutamate hypothesis can be integrated with the dopamine hypothesis, dopaminergic system changes typically associated with schizophrenia, may actually be secondary to an underlying deficit in glutamatergic transmission. In principle glutamatergic hypofunction can be due to hyperactive glutamate transporters, defective receptors, lack of receptors, inadequate glutamate release or lack of glutamatergic nerve terminals. In spite of the large number of studies on glutamatergic transmission in psychiatry disorders, only a handful has been performed on glutamate uptake [24, 25].

We herein evaluate whether administration of LPS to rats dams in late gestation affects some aspects of the offspring behavior in their adult life. We also investigate the effects of this treatment on glial parameters using *ex vivo* preparation of offsprings from pregnant rats exposed to LPS. Hippocampal slices were used to evaluate S100B secretion and glutamate uptake in basal condition and facing a challenge with apomorphine. Moreover we measured tyrosine hydroxylase (TH) to evaluate the effect on catecholaminergic neurons.

2. Materials and Methods

2.1 Animals

Female Wistar rats from our breeding colony (Department of Biochemistry, UFRGS, Porto Alegre, Brazil), weighing 216–263 g each, were used, were maintained under controlled light and environmental conditions (12 hour light/12 hour dark cycle at a constant temperature of 22 ± 1°C), and had free access to commercial chow and water. They had their fertility cycle controlled, and, when on proestrus, mated overnight. In the morning, vaginal secretion was collected to be analyzed . If spermatozoa were found in the morning, it was designated as first day of pregnancy. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996 and followed the regulations of the local animal housing authorities.

2.2 Treatment

For gestational LPS treatment, timed pregnant Wistar rats were injected on day 18 and 19 of pregnancy as follows: 6 pregnant rats were injected intraperitoneally (ip) with 500 μg/kg LPS (from Escherichia coli, serotype 055:B5, Sigma) and 5 were injected with

corresponding volume of sterile saline (control), on once daily. Females were kept separate and with free access to their own litters. Rats from both groups control and LPS were born healthy and the number of offspring was normal. The offspring rats were weaned at 21 days old and were housed separately by sex. The experiments were performed using males rats from each litter. Rats had free access to food and water. All the experiments were performed between 12:00 and 17:00. Behavioral tests were performed at postnatal day 60, considered adult rats. At 65 days old, rats were sacrificed.

2.3 Behavioral test

2.3.1 Open Field Test

The general activity of male pups was tested on postnatal day 60. The open field consisted of an open wooden arena with dimensions (length/width/height in cm) 60/40/50 with 12 equally divided squares. The animals were observed directly and continuously for 5 min. The following behavioral components were measured: number of squares crossed (with four paws), rearing, latency for the crossing of first square (as measures of exploratory activity), and numbers of grooming and fecal bolus (as measures of emotionality) [26]. These measurements were made on two consecutive days (day 1 and day 2).

2.3.2 Three-Chamber Sociability Test

The social test was performed in a three chambered apparatus as described previously in a mice task, with modifications required to perform it in rats [27]. It is a wooden box no painted with partitions separating the box into three chambers with dimensions (length/width/height in cm) 120/40/50, being 60 cm length to the central chamber and

30 cm each side. The openings between compartments allowed free exploration to the different chambers. Time spent in each chamber; as well as the time spent exploring the stranger rat or an object in the chamber, was analyzed by two observers. The object was an empty identical cage used to enclose the stranger rat. Chambers were cleaned with 70% ethanol and water between tests. Animals used as "strangers" were Wistar males with the same age and no previous contact with the test rats. Animals were individually acclimated for 5 min into the apparatus on the day before the experiment. On the sociability test, rats were allowed to expend 5 min in the central chamber, and then the stranger rat was introduced into one of the side chambers. The experiment was performed for up to 10 min, with the stranger rat and an object on each side. A 10-minute test to quantify preference for social novelty began immediately after the 10-minute test for sociability. In this test, the original stranger rat (stranger 1) remained in its wire cage on one side of the apparatus and a new unfamiliar rat (stranger 2) was placed in the wire cage on the opposite side, which was previously empty during the sociability test. The score was evaluated by the time spent in each chamber and by the time spent sniffing each wire cage. Stranger 1 and stranger 2 animals originated from different home cages had never been in physical contact with the subject rat. The three chambered apparatus was centered onto a lab bench to minimize light gradients in, temperature, sound and other environmental conditions that could produce a side preference.

2.4 Hipocampal Slices

Offsprings were killed by decapitation. Hippocampi were removed and sliced in transverse sections of 0.3 mm using a McIlwain tissue chopper. Slices were transferred to 24-well plates containing 0.3 mL of Hepes-buffered saline solution (120 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgSO4, 1 mM KH2PO4, 10 mM glucose and 25 mM

Hepes, pH 7.4) per well. The medium was changed every 15 min for 2 h at room temperature. After this stabilization period, apomorphine (100 μM) was added and the slices incubated for 1 h at 30 °C. Drug concentration was chosen based on pilot experiments and previous reports [28]. All experiments were performed in triplicate (three slices from the same animal, pup of dams treated with LPS or saline, were used for each treatment condition).

2.5 Glutamate Uptake

After the incubation period, the medium was replaced by Hank's balanced salt solution (HBSS) containing 137 mM NaCl, 0.63 mM Na2HPO4, 4.17 mM NaOHCO3, 5.36 mM KCl, 0.44 mM KH2PO4, 1.26 mM CaCl2, 0.41 mM MgSO4, 0.49 mM MgCl2 and 5.55 mM glucose, pH 7.4. The slices were maintained at 35 °C, and the assay was started by adding 0.1 mM l-glutamate and 0.66 μCi/mL l-[2,3-3H]-glutamate; this high unlabeled glutamate concentration renders the assay insensitive to changes in glutamate release. Incubation was stopped after 5 min by removing the medium and rinsing the slices three times with ice-cold HBSS; it has been shown that up to 5 min the glutamate uptake is linear in this slice preparation [29]. Slices were then lysed in a solution containing 0.5 N NaOH. Sodium-independent (nonspecific) uptake was determined using a solution with N-methyl-d-glutamine instead of NaCl. Sodium-dependent glutamate uptake was obtained by subtracting nonspecific uptake. Radioactivity was measured with a scintillation counter (2800TR TriCarb Liquid Scintillation Analyzer, Perkin-Elmer, Waltham, MA, USA). Final glutamate uptake was expressed as nmol/mg protein/min.

2.6 Extracellular S100B Content

Immediately after the incubation period, 10 μL of medium were collected and kept in −20 °C until S100B levels were determined by ELISA, as previously described [30].

Briefly, 50 µL of Tris buffer were added to the samples and incubated for 2 h in a microtiter plate previously coated with monoclonal anti-S100B (SH-B1, from Sigma). Polyclonal anti-S100 (from Dako) was incubated for 30 min; peroxidase-conjugated anti-rabbit antibody (GE healthcare) was added for additional 30-min incubation. The color reaction with o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/mL. Results were expressed at ng/mL.

2.7 Western Blot Analysis of Tyrosine Hydroxylase

Equal amounts (20 µg) of proteins from each sample were boiled in sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) bromphenol blue) and electrophoresed in 10% (w/v) SDS-polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane. Equal loading of each sample was confirmed with Ponceau S staining (Sigma). Anti –TH (Sigma) was used at a dilution of 1:1.000. After incubating with the primary antibody for 1 h at room temperature, filters were washed and incubated with peroxidase-conjugated anti-rabbit immunoglobulin (IgG) at a dilution of 1:2.000. The chemiluminescence signal was detected using an ECL kit from GE Healthcare.

2.8 Statistical analysis

Parametric data are reported as means ± standard error and were analyzed by Student's t-test (when two groups were considered), one-way ANOVA (followed by Tukey's or Dunnett's test) or two-way ANOVA (followed by Bonferroni's test). Values of $p < 0.05$ were considered to be significant.

3. Results

3.1 Behavioral test

3.1.1 Open Field Test

Adult male offspring from dams who received LPS (500 μg/kg) or saline (control) were tested in open field test (Figure 1). Two-way ANOVA (prenatal treatment X time) of the LPS and control data indicated significant interaction ($p = 0.002$) and significant effect of time ($p = 0.048$), but no significant effect of treatment ($p = 0.06$) in latency for the crossing of first squares. Post-hoc analysis indicated that exposure to LPS decreased the latency for the crossing of first squares on day 2 ($P < 0.001$), when compared with the control group. Furthermore, we observed an increased in latency in controls ($p <$ 0,001) in day 2, which was not repeated with the LPS group (Figure 1A). In number of fecal bolus was showed significant main effects of prenatal treatment ($P = 0.042$), but no effects of time ($p = 0.931$) or interaction between the two factors ($p = 0.655$), post hoc reveal that LPS group increased significantly the number of fecal bolus in day 1 ($p < 0.05$) (Figure 1B). No change was observed in the numbers of rearing, grooming or crossing ($p > 0.05$) when compared with the control group (Figure 1C).

3.1.2 Three-Chamber Sociability Test

In order to evaluate the social behavior of the offspring, was performed the three chambers test (Figure 2). In phase 1, the control group spent more time in chamber 1 than in chamber 2 ($p < 0.05$) and the LPS group did not show this difference ($p = 0.583$). A significant effect was observed in the control group: more time spent exploring the stranger mouse ($p <0.005$) relative to the object, but this effect was not replicated in offspring of LPS treated rats ($p = 0.067$) (Figure 2A). In phase 2, there was no difference between groups in the time spent in the following chambers 1(stranger rat) and chamber 2 (object), but a difference was found in time exploration in empty central

chamber between control e LPS group ($p < 0.01$). In addition, both groups spent less time in the central chamber than in other chambers (Figure 2B).

3.2 Glutamate Uptake

To assess the role of prenatal LPS-treatment and of the non-selective dopamine agonist apomorphine in glutamate uptake were used hipocampal slices ex vivo (Figure 3). Two-way ANOVA of the LPS and saline data indicated a significant effect of prenatal treatment ($p = 0.021$) and ex vivo treatment ($p = 0.047$), and no significant interaction. Post hoc analysis revealed that glutamate uptake was significantly less in LPS group ($p < 0.05$) and apomorphine was able to decrease the glutamate uptake only in control group ($p < 0.05$).

3.3 S100B secretion

The effects of apomorphine and LPS on offspring S100B secretion were evaluated in acute hippocampal slices (Figure 4). Two-way ANOVA of the LPS and saline data indicated a significant effect of prenatal treatment ($p = 0.0005$), ex vivo treatment ($p < 0.0001$) and interaction between this factors ($p = 0.024$). Post hoc analysis revealed that apomorphine was able to decrease the glutamate uptake in both control and LPS groups ($p < 0.0001$) and that LPS prenatal treatment was able to increased S100B secretion in ex vivo preparation ($p < 0.05$).

3.4 TH content

TH content was evaluated in hippocampus and frontal cortex samples (Figure 5). Student's t-test indicated that TH content is significantly increased in frontal cortex ($p = 0.04$), but no changes in TH levels were observed in hippocampus.

4. Discussion

The present experimental investigation in rats shows an evaluation of long-term changes in anxiety-like behavior, emotional reactivity, social interaction and social memory. We found that exposure to a bacterial-like response by LPS treatment in late gestation leads to changes in the pattern of response to the open field test: no habituation latency to leave the first square in contrast to controls, in addition the amount of fecal bolus was increased in the offspring of dams treated with LPS, being these two measurements traditionally related with emotionality and /or anxiety, it is clear that the model results in behavioral disturbances, although not modify the ambulation (crossings), rearings or grooming. Another important behavioral parameter impaired in this model is social interaction, since the mice LPS exhibited no preference for chamber two (rat) on the chamber one (object) as with the control rats, as well as time expend exploring the strange rat significantly longer than time expend exploring object present in the controls was not repeated LPS-offspring. However, there was no impairment in social memory of pups of rats treated with LPS.

Several types of animal models have been developed to investigate schizophrenia, including the LPS-induced model in pregnant rats. This model has a significant number of characteristics observed in humans with schizophrenia, for example fear or anxiety (fecal bolus increased) and social dysfunction (no preference for social chamber) found in this work. Prenatal brain inflammation in late gestation especially interferes with cell migration, organization, and synapse maturation [31, 32], the consequences of which may be more restricted at both the functional and structural levels and long-term pharmacological and cognitive consequences of prenatal immune activation in late gestation may thus be more closely related to negative symptoms of schizophrenia (social interaction deficits, presence of repetitive stereotyped behaviors, anhedonia, and

behavioral and/or cognitive inflexibility, among other) [33, 34]. Social cognitive impairments and negative symptoms are core features of schizophrenia directly associated with impaired community functioning [35].

Glutamate neurotransmission plays a critical role in synaptic activity and plasticity throughout the brain, including cognition-, emotion- and reward-related circuits [36]. In schizophrenic patients, different evidence of abnormal glutamatergic transmission has been found, such as disturbances of cortical glutamate release [37, 38], lower glutamate levels in the prefrontal cortex [39] and increased prefrontal and hippocampal glutamate concentration [40]. We found a decrease in glutamate uptake in LPS-offspring, partly contradicting to classical glutamatergic hypothesis that postulates that the symptoms found in schizophrenic patients could arise from glutamatergic hypofunction [41, 42]. However, most of studies are conducted in cortex and our observation was made in hippocampus, moreover our study evaluates glutamate uptake itself while the vast majority uses quantification of glutamate transporters for assessing uptake [25, 43]. Our observations are consistent with findings showing that cytokines are able of attenuating glutamatergic uptake manner dependent [44, 45], as this helps to reinforce the idea that cytokines could be the protagonists of the changes caused in the offspring of mothers exposed to LPS. Interestingly, apomorphine - a non-selective agonist which activates both dopamine D1- and D2-like receptors, with a preference for the latter subtype - significantly decreased glutamate uptake in control animals, but in LPS group this capacity was lost. This shows that a dysfunction in the physiological integration of glutamatergic and dopaminergic systems may occur as a result of prenatal exposure to LPS.

Recent evidences, in animal models and human studies, have provided cumulative evidence for the cytokine hypothesis of schizophrenia [50]. In addition, a correlation

between S100B and pro-inflammatory molecules has been demonstrated in neurological and psychiatric disorders [5, 51, 52]. We found that the apomorphine at 100 uM was able to reduce the secretion of S100B in hippocampal slices of both controls and LPS-offspring. These results are consistent with our previous findings that demonstrated a decrease of S100B in three different preparations *in vitro* used (hippocampal slices, primary cortical astrocytes and C6 glioma cells) [28] when treated with apomorphine. Assuming that D2 receptors negatively coupled to adenyl cyclase are activated in astrocytes by apomorphine, a decrease in the secretion of S100B was an expected result. Other results with forskolin, fluoxetine and serotonin also suggest a link between cAMP and S100B secretion [53, 54].

Our next step was to measure the tyrosine hydroxylase, enzyme responsible for catalyzing the conversion of the amino acid L-tyrosine to L-DOPA, which is the precursor of dopamine, a key neurotransmitter involved in schizophrenia. The evaluation of tyrosine hydroxylase is quite controversial, it is possible to find any studies showing a decreased in its activity / amount in schizophrenic post-mortem patients [46] and in response to cytokines [47], while there are also studies showing an increased in inflammation models [48] and psychiatric diseases [49]. In present study found increased specific region (only cortex) of this protein, this finding is in agreement with studies that show a dopaminergic hyperactivity disorder in the pathology of schizophrenia.

In schizophrenic patients dopaminergic hyperactivation has been proposed for base aggravation of positive symptoms, that is where typical antipsychotics are the most effective. Moreover, an increase in serum S100B has been observed in schizophrenic patients in various studies [19, 55, 56]. However, it is unclear whether elevated serum S100B specifically reflects astrocytic activation. In fact, sources outside the brain may

contribute to serum S100B, particularly adipocytes [57]. Moreover, recent findings suggest that S100B levels in the blood correlates with the fatty tissue in healthy individuals [58], and insulin resistance in patients with schizophrenia [59]. We also found a significant increase in S100B secretion in rat pups whose mothers were exposed to LPS, agreeing with previous results, that S100B secretion was increased by proinflammatory cytokines (DF de Souza, unpublished).

Some limitations of this study should be noted. This is a an animal model study, useful for studying responses and mechanisms, but takes into account only one of many environmental factors that may be related to the development of psychiatric disease. Moreover, in order to perform a direct measurement of levels of S100B secretion and glutamate uptake was used an *ex vivo* preparation, however, we know that cannot be replicated, nor accurately observe all the complex variables could be present an *in vivo* system.

Our data provides evidence that this model could mimic schizophrenia, since the finds are consistent with molecular and behavioral patterns seen in schizophrenic patients, for example impaired social interaction and increased secretion of S100B. This result reinforces the idea that prenatal infection may be an important environmental factor predictive of the development of schizophrenia and that dysfunction of glial parameters, such as S100B and glutamate uptake, in adult offspring may contribute to the pathophysiology of this disease.

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Figures

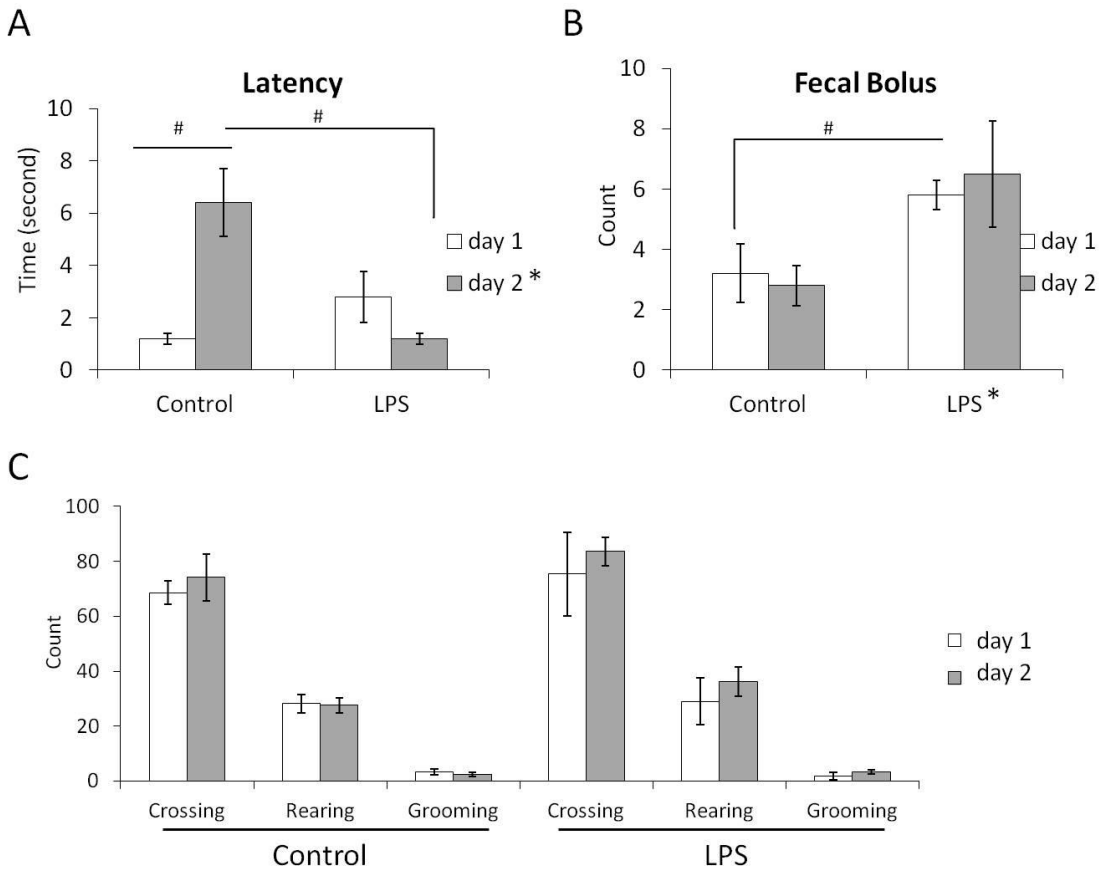

Figure 1. Open field test in rats prenatally exposed to LPS. Was measured latency, defined as time for the crossing of first squares (A), and number of: fecal bolus (B), crossing, rearing and grooming (C). Data are expressed as mean ± standard error (LPS group N=5, control group N=5). *Significant effect of the time (in A) and of the prenatal treatment (in B) (Two-way ANOVA $p < 0.05$). # Significant difference from control group (A and B) and difference within control group (between day 1 and day 2), which don't occurs with LPS group (A) (Bonferroni's Post hoc, $p < 0.05$).

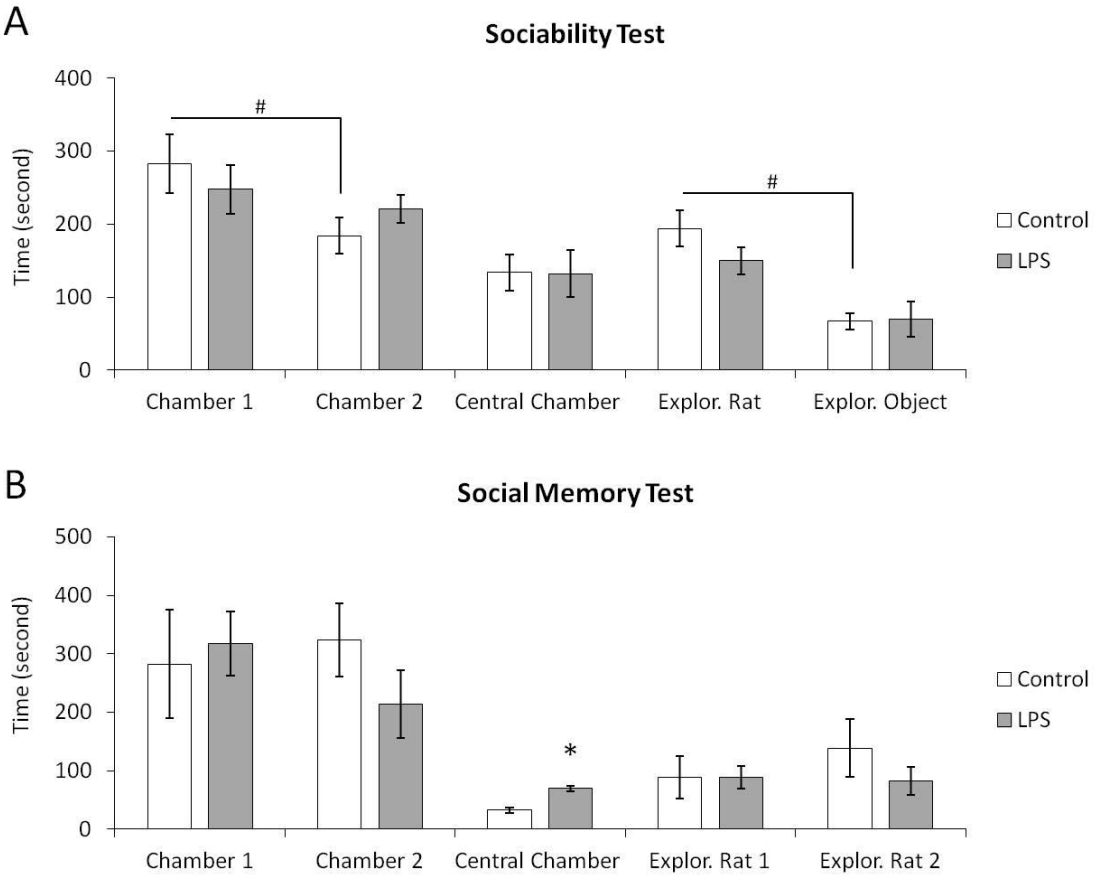

Figure 2. Three-chamber sociability test in rats prenatally exposed to LPS. Phase 1: sociability measured after 5 min to acclimation and10 min of test (A). Phase 2: social memory after 10min of test (B). The score was evaluated by the time spent in each chamber and by the time spent sniffing each wire cage. Data are expressed as mean ± standard error (LPS group N=5, control group N=5). *Significantly different from control group, $p< 0.05$. # Significant difference within groups, $p < 0.05$.

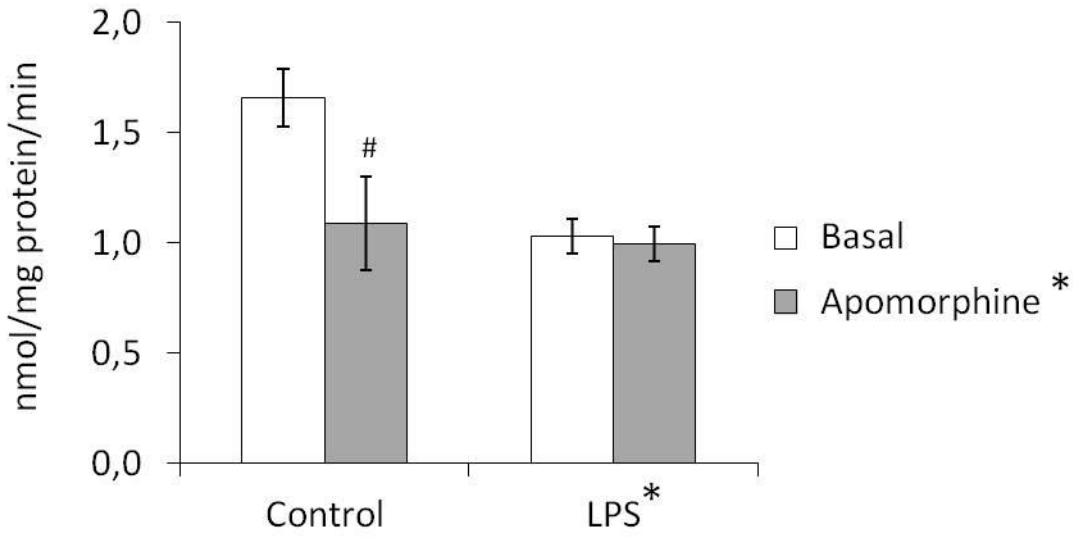

Figure 3. Glutamate Uptake in hippocampal slices *ex vivo* from rats prenatally exposed to LPS. Was measured glutamate uptake of hippocampal slices pretreated or not with apomorphine. Data are expressed as mean ± standard error (LPS group N=5, control group N=5), the experiments were performed in triplicate. *Significant effect of prenatal and post natal treatment (Two-way ANOVA $p < 0.05$). $^{\#}$ Significant difference in control samples pretreated with apomorphine (Bonferroni's Post hoc, $p < 0.05$).

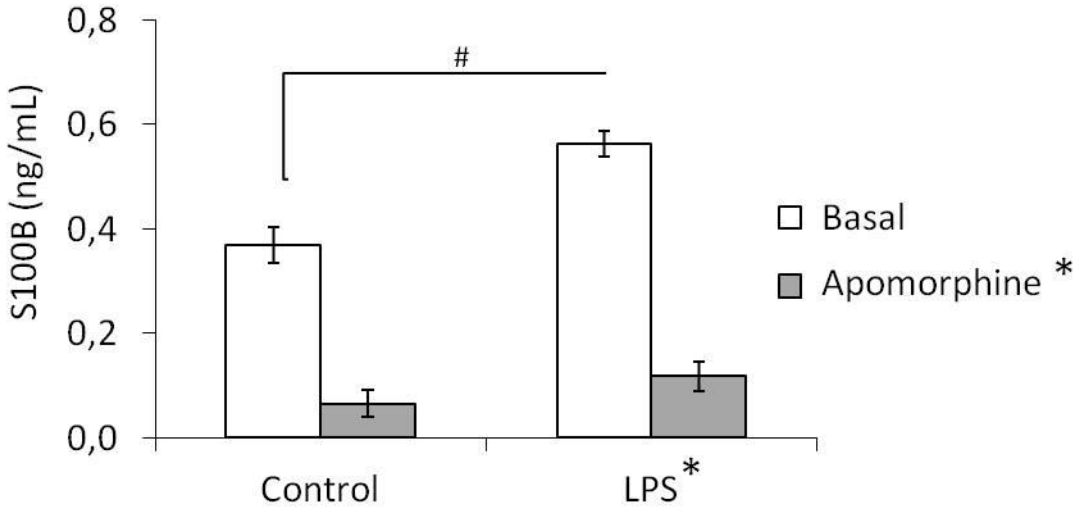

Figure 4. S100B secretion of *ex vivo* preparation from rats prenatally exposed to LPS. Was measured secretion of S100B by ELISA, of hippocampal slices pretreated or not with apomorphine at 100 μM for 1 h. Data are expressed as mean ± standard error (LPS group N=5, control group N=5), the experiments were performed in triplicate *Significant effect of prenatal and post natal treatment (Two-way ANOVA $p < 0.05$). #Significant difference of control and LPS prenatal treatment in basal samples (Bonferroni's Post hoc, $p < 0.05$).

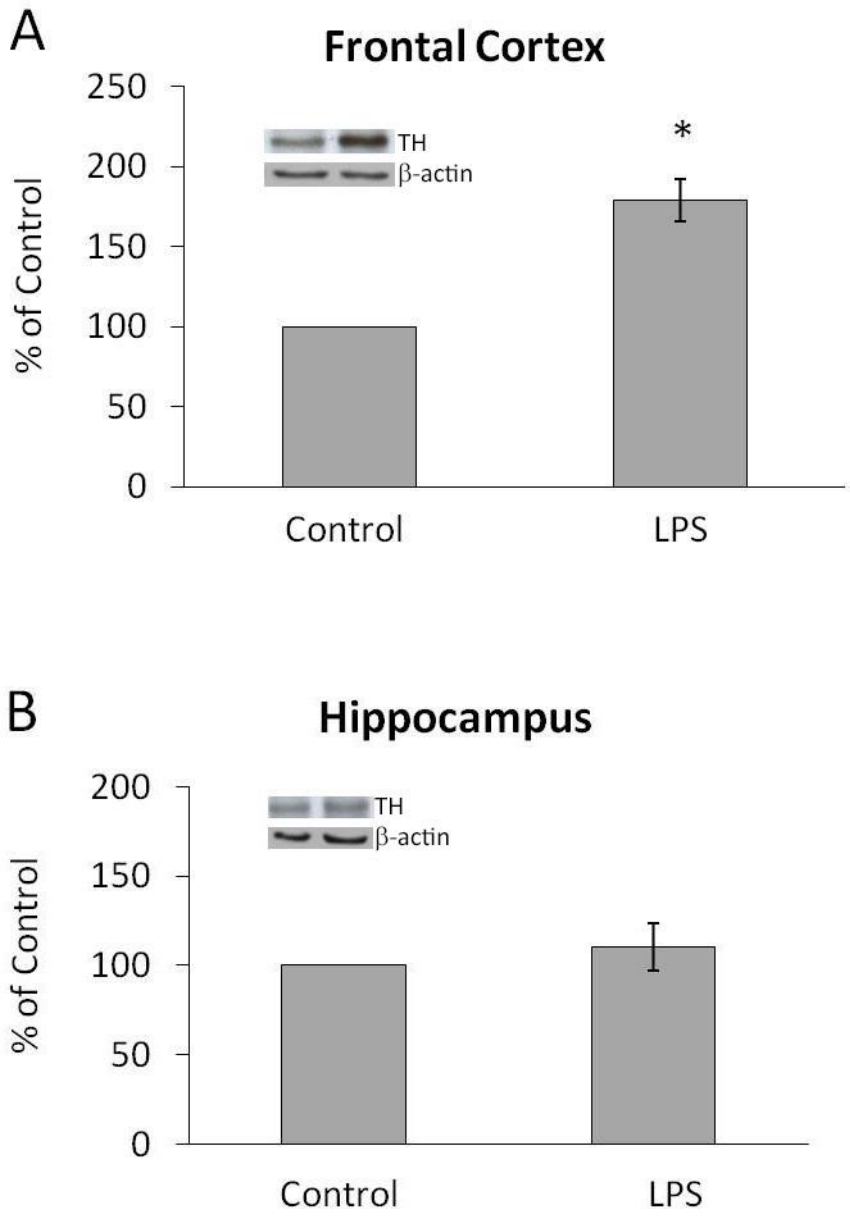

Figure 5. Tyrosine Hydroxylase of rats prenatally exposed to LPS. TH value was measured in slices of frontal cortex and hippocampus. Data are expressed as mean ± standard error (LPS group N=5, control group N=5), the experiments were performed in triplicate * Significantly different from control (Student's t-test), $p < 0.05$.

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CAPÍTULO III

Changes in astrogliosis markers (GFAP and S100B) in a maternal immune activation model of schizophrenia in Wistar rats

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Abbreviations: CNS, central nervous system; **ELISA**, enzyme-linked immunosorbent assay; **GFAP**, glial fibrillary acidic protein; **GSH**, glutathione; **IL-6,** interleukin-6; **LPS**, lipopolysaccharide; **MIA**, maternal immune activation; **NeuN** neuronal nuclear antigen; **PND**, postnatal day; **ROS**, reactive oxygen species.

Abstract

Prenatal exposure to bacterial and viral infection has been implicated by several studies indicating that such exposure is an important environmental factor out that may detrimentally affect neurodevelopment, increasing the risk of schizophrenia. The maternal immune activation animal model is used to study how an insult directed at the maternal host can have adverse effects on the fetus leading to behavioral and neurochemistry changes later in life. Moreover, changes in astrogliosis markers seem to be closely related pathology of schizophrenia. We evaluate whether the administration of LPS in rat dams in late pregnancy affects astroglial markers (S100B and GFAP) of the offspring in later life. We used the offspring at 30 and 60 days to evaluate the differences between juvenile and adult rats and also investigate the existence of differences between male and female offspring. We also investigated the oxidative stress parameters NO and GSH. Our results show that prenatal LPS challenge leads to neurochemical abnormalities in glial marker in postnatal life, manifesting some even earlier, such as glutathione, or in adulthood, such as content NO. Moreover our model led to a particularly hippocampal astrogliosis, an increase of GFAP in juvenile rats already persisting through adulthood, whereas S100B appears to have a time-dependent expression: decreased in rats to PND30 and PND60 increased.

Key words: animal model; astrogliosis; GFAP; lipopolysaccharide; schizophrenia; S100B

1. Introduction

Schizophrenia is a chronic and debilitating illness which affects about 1% of the world population and the onset of manifestation is typically in late adolescence or in early adulthood (Monji *et al.*, 2009). However, recent analysis provides evidence for a sex difference in the development of schizophrenia, in both first-episode and chronic schizophrenia patients at a significantly earlier age in male than female patients (Angermeyer and Kuhn, 1988; Zhang *et al.*, 2012), moreover incidence of schizophrenia is significantly higher in men than in women (male: female ratio = 1.4) (Aleman *et al.*, 2003; McGrath, 2006).

Nevertheless, etiology of schizophrenia remains unclear, although a variety of epidemiological evidence indicates that neurodevelopmental factors contribute to the pathophysiology (Fatemi and Folsom, 2009). Prenatal exposure to bacterial and viral infection has been implicated by several large epidemiological studies indicating that such exposure is an important environmental factor out that may detrimentally affect neurodevelopment, increasing the risk of neuropsychiatric disorders (Zuckerman and Weiner, 2005).

The maternal immune activation (MIA) animal model is used to study how an insult directed at the maternal host can have adverse effects on the fetus leading to behavioral and neurochemistry changes later in life, specifically within abnormal exploration and social behaviors, cytokines levels and gene regulation (Ashdown *et al.*, 2006; Beloosesky *et al.*, 2012; Fatemi *et al.*, 2008; Meyer *et al.*, 2009) (D. de Souza, unpublished data). Systemic administration of the bacterial endotoxin lipopolysaccharide (LPS) is a widely used and accepted MIA model to emulate immune activation and subsequent release of immunoregulatory, cytotoxic and

inflammatory cytokines secondary to gram-negative bacterial infections (Borrell *et al*., 2002).

Changes in glial cells seem to be closely related pathology of schizophrenia (Bernstein *et al*., 2009; Cotter *et al*., 2001; Steiner *et al*., 2011). S100B, a protein mainly expressed and secreted by astrocytes in central nervous system (CNS), has been proposed as a marker of brain damage (Goncalves *et al*., 2008; Marchi *et al*., 2004) and some studies have demonstrated that S100B is increased in neurological and psychiatric disorders (Ashraf *et al*., 1999; Lara *et al*., 2001; Steiner *et al*., 2008; Steiner *et al*., 2009). Recently we showed that S100B protein is increased by intracerebroventricular (LPS) administration and that LPS directly modulate the secretion of S100B from astrocytes and hippocampal slices (Guerra *et al*., 2011), furthermore we observed that S100B secretion cytokine-stimulated is prevent by antipsychotics *in vitro* (de Souza, *et al*., 2012).

Brain inflammation, as defined by astrogliosis (enhanced glial fibrillary acidic protein (GFAP) staining) has been observed in the offspring of models of MIA, as IL-6 or LPS-treated mothers (Hao *et al*., 2010; Samuelsson *et al*., 2006). In general, it is quite controversial role of GFAP in neurological diseases, some studies found no changes or decreased GFAP content in the cortex and cerebellum of schizophrenic patients, (Falkai *et al*., 1999; Fatemi *et al*., 2004; Rajkowska *et al*., 2002), however, there is evidence that it might be significantly greater in demented schizophrenic patients when compared to non-demented (Arnold *et al*., 1996).

Analyses of the molecular mechanisms underlying oxidative stress suggest that cognitive dysfunction may be associated with an imbalance in the generation and clearance of reactive oxygen species (ROS) (Bitanihirwe and Woo, 2011). Additionally, studies suggest that altered regulation of fundamental mechanisms of oxidative stress

may contribute to the pathogenesis of schizophrenia and related disorders (Boskovic *et al*., 2011; Chauhan and Chauhan, 2006; Do *et al*., 2009; Floyd, 1999). In this paper we evaluate whether the administration of LPS in rat dams in late pregnancy affects astroglial markers (S100B and GFAP) of the offspring in later life. We used the offspring at 30 and 60 days to evaluate the possible differences between juvenile and adult rats and also investigate the existence of differences between male and female offspring. We also investigated the oxidative stress parameters NO and GSH in this model.

2. Material and Methods

2.1. Animals

Female Wistar rats from our breeding colony (Department of Biochemistry, UFRGS, Porto Alegre, Brazil), weighing 216–263 g each, were used, were maintained under controlled light and environmental conditions (12 hour light/12 hour dark cycle at a constant temperature of 22 ± 1°C), and had free access to commercial chow and water. They had their fertility cycle controlled, and, when on proestrus, mated overnight. In the morning, vaginal secretion was collected to be analyzed. If spermatozoa were found in the morning, it was designated as first day of pregnancy. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996 and followed the regulations of the local animal housing authorities.

2.2. Treatment

For gestational LPS treatment, timed pregnant Wistar rats were injected on day 18 and 19 of pregnancy as follows: 6 pregnant rats were injected intraperitoneally with 500

μg/kg LPS (from Escherichia coli, serotype 055:B5, Sigma) and 5 were injected with corresponding volume of sterile saline (control), on once daily. Females were kept separate and with free access to their own litters. Rats from both groups control and LPS were born healthy and the number of offspring was normal. The offspring rats were weaned at 21 days old and were housed separately by sex. The experiments were performed using males and females rats from each litter. Rats had free access to food and water. All the experiments were performed between 12:00 and 17:00. In order to analyze the differences between young and adult rats, experiments were performed at postnatal day (PND) 30 and PND 60.

2.3. Obtaining CSF, serum and hippocampal samples

Animals were anesthetized with ketamine/xylazine (75 and 10 mg/kg, respectively, i.p.) and then positioned in a stereotaxic holder and CSF was obtained by cisterna magna puncture using an insulin syringe (27 gauge × 1/2 in. length). Animals were anesthetized as described above and blood was obtained by cardiac puncture. CSF and serum was frozen (−20 °C) until further analysis, at most for two weeks. The animals were killed by decapitation, and the brains were removed and placed in cold saline medium with the following composition (in mM): 120 NaCl; 2 KCl; 1 CaCl2; 1 MgSO4; 25 HEPES; 1 KH2PO4 and 10 glucose, adjusted to pH 7.4. The hippocampi and frontal cortex were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper. Slices were then frozen at −20 °C (for measurement of GFAP and S100B) or −80 °C (for measurement GSH and NO), at most for two weeks.

2.4. ELISA for S100B

The S100B concentration was determined in the hippocampal and cortical samples, in addition to serum and CSF from offsprings PND 30 and PND 60. S100B levels were

determined by ELISA, as described previously (Leite *et al.*, 2008). Briefly, 50 μL of sample plus 50 μL of Tris buffer were incubated for 2 h on a microtiter plate, previously coated with anti-S100B monoclonal antibody (SH-B1, from Sigma). Anti-S100 polyclonal antibody (from DAKO) was incubated for 30 min and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. The color reaction with o-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/mL.

2.5. ELISA for GFAP

ELISA for GFAP was carried out by coating the microtiter plate with 100 μL samples containing 20 ng of protein for 24 h at 4 °C. Incubation with a polyclonal anti-GFAP from rabbit (GE Healthcare) for 1 h was followed by incubation with a secondary antibody conjugated with peroxidase for 1 h, at room temperature. A colorimetric reaction with o-phenylenediamine was measured at 492 nm. The standard human GFAP (from Calbiochem) curve ranged from 0.1 to 5 ng/mL.

2.6. Immunohistochemistry for GFAP and NeuN

Rats were anesthetized using ketamine/xylazine and were perfused through the left cardiac ventricle with 200 mL of saline solution, followed by 200 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 24 h. Subsequently, the material was cryoprotected by immersing the brain in 30% sucrose in phosphate buffer at 4 °C [19]. The brains were sectioned (50 μm) on a cryostat (Leitz). The sections were then preincubated in 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.4% Triton X-100 for 30 min and incubated with polyclonal anti-GFAP from rabbit or -NeuN from mouse, diluted 1:500 in 0.4% BSA in PBS-Triton X-

100, for 48 h at 40 °C. After washing several times, tissue sections were incubated with a secondary antibody Alexa Fluor 488 (goat anti-rabbit-IgG; green fluorescence) and Alexa Fluor 568 (goat anti-mouse-IgG; red fluorescence) diluted 1:500 in PBS, at room temperature for 2 h. Afterwards, the sections were mounted on slides with Fluor Save® and covered with coverslips. Images were viewed with an Olympus microscope and images transferred to a computer with digital camera. Samples were quantified according with (Centenaro *et al.*, 2011).

2.7. Glutathione (GSH) Content Assay

GSH levels (nmol/mg protein) were measured, as described previously (Browne and Armstrong, 1998). Slices were homogenized and assayed in 10 volumes of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA and protein was precipitated with 1.7% meta-phosphoric acid. Supernatant was assayed with o-phthaldialdeyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0–500 μM).

2.8. Nitric Oxide (NO) Production

NO metabolites, NO_3^- (nitrate) and NO_2^- (nitrite) were determined according to (Hu *et al.*, 1996) [24]. Briefly, homogenates from one hippocampus were mixed with 25% trichloroacetic and centrifuged at 1,800 × g for 10 min. The supernatant was immediately neutralized with 2 M potassium bicarbonate. NO_3^- was reduced to NO_2^- by nitrate reductase. The total NO_2^- in the supernatant was measured by a colorimetric assay at 540 nm, based on the Griess reaction. A standard curve was performed using sodium nitrate (0–50 μM).

2.9. Protein Determination

Protein content was measured by Lowry's method using bovine serum albumin as standard (Peterson, 1977).

2.10. Statistical analysis

Parametric data are reported as means ± standard error and were analyzed by two-way ANOVA (followed by Bonferroni's test). Values of $p < 0.05$ were considered to be significant.

3. Results

3.1. Maternal LPS treatment increase S100B in adult and decrease in juvenile offspring rats.

LPS did not significantly change S100B levels in frontal cortex of juvenile rats (Figure 1A), but two-way ANOVA for S100B shows significant effect of treatment ($p = 0.005$), significantly decreased in both male and female hippocampus (Figure 1B), as evidenced by post-hoc ($p < 0.05$). Was observed a significant effect of treatment in S100B immunocontent in the frontal cortex ($p = 0.005$) and hippocampus ($p = 0.040$) of adult rats (Figure 1C and 1D, respectively), but not of gender ($p = 0.08$, $p = 0.07$). Post-hoc test reveal a significant increase in both male- and female-LPS in frontal cortex ($p < 0.05$) and in male-LPS ($p < 0.005$).

3.2. Maternal LPS treatment decreased S100B levels in CSF of young offspring females.

A 2(treatment × gender) between subjects two-way ANOVA revealed a significant interaction in CSF of juvenile rats ($p = 0.003$). Post hoc analysis revealed that S100B

levels were significantly less in female of LPS group ($p < 0.05$) when compared with control group (Figure 2A). This effect in juvenile offspring was not found in serum (Figure 2B). LPS did not significantly change S100B levels in CSF or serum of adult offspring (Figure 2C and 2D).

3.3. GFAP content is altered in offspring born to LPS-treated dams.

A significant effect of gender ($p = 0.02$) and treatment ($p = 0.04$) in GFAP immunocontent of frontal cortex was observed in juvenile offspring rats, post hoc analysis revealed that LPS was able to decrease the GFAP in both male and female PND 30 ($p < 0.05$) and that female have a smaller amount basal of GFAP ($p < 0.05$) (Figure 3A). In hippocampus of rats PND 30, two-way ANOVA of the LPS and control data indicated only a significant effect of treatment ($p = 0.003$), a significant increase was attested by post test in male and female treated when compared to control (p , 0.05) (Figure 3B). No changes were found in frontal cortex of adult rats (Figure 3C). however in hippocampus of PND 60 was found significant effect of treatment ($p < 0.009$) and gender ($p = 0.02$) (Figure 3D), post hoc indicated that male-LPS is significantly different to control male rats.

3.4 Immunohistochemistry confirms the increment of GFAP in hippocampus of rats LPS-offspring and shows a difference of gender in NeuN.

To assess whether the result obtained with ELISA of GFAP in whole hippocampus would be specifically reproduced in the CA1 region and also to evaluate neuronal changes, we performed the immunohistochemistry of GFAP and NeuN (Figure 4 A). After quantifying, was observed a significant effect of gender ($p = 0.002$) and treatment ($p = 0.0002$) in GFAP immunocontent of hippocampus was observed in offspring rats PND 30 . There was also an interaction effect between treatment and gender ($P = 0.001$)

(Figure 4B). In PND 60 two-way ANOVA shows significant effect of gender ($p = 0.0007$), but there was no effect of treatment or interaction between prenatal treatment and gender ($p = 0.23$ and 0.33, respectively) (Figure 4C).

LPS did not significantly change NeuN levels in hippocampus of juvenile or adult rats (Figure 4D and 4E respectively), but two-way ANOVA shows significant effect of gender in both PND 30 ($p < 0.0001$) and PND 60 ($p < 0.0001$). However, there was no interaction effect between prenatal treatment and gender ($p = 0.19$ and 0.12, respectively).

3.5 Rats offspring from LPS-treated dams have alteration in stress oxidative.

Glutathione content and NO production were used as parameters to evaluate possible oxidative stress caused by LPS prenatal exposure. Two-way ANOVA (treatment X gender) indicated significant interaction effect of gender and treatment ($p = 0.017$) in frontal cortex PND 30, but no significant effect of treatment ($p = 0.35$) (Figure 5A). On the other hand, hippocampus of juvenile rats presented a mean effect of treatment ($p = 0.03$), GSH content is increased in PND 30 male, according to post-hoc ($p < 0.05$) (Figure 5B). Reduced glutathione content was modified frontal cortex of PND 60, significant effect of treatment was found ($p = 0.04$) (Figure 5C), no effects were found in hippocampus of PND 60 (Figure 5D).

Two-way ANOVA (treatment X gender) showed no change in the content of NO in PND 30 (Figure 6A), but indicated significant effect of gender ($p = 0.04$) in PND 60, the post-hoc that followed indicates that there was an increase in NO from hippocampus of adult females (Figure 6B).

4. Discussion

Schizophrenia is believed to involve neurochemical, metabolic activities and connectivity impairment between several brain regions as nucleus accubens, prefrontal cortex and hippocampus (Cui *et al*., 2009; Lisman *et al*., 2008). Increasingly it is observed that the imbalance of neurodegenerative and neuroprotective factors may play a key role in this brain disorders. Among the factors that modulate the subtle balance between cell death and survival, the role of cytokines have been consistently reported in schizophrenic (Mansur *et al*., 2012). S100B is a calcium-binding protein secreted by astrocytes into the synapse, where it is thought to participate in synaptic plasticity (Nishiyama *et al*., 2002), being neurotrophic at nanomolar and apoptotic at micromolar levels (Donato *et al*., 2009). Here, we observed that S100B has a pattern of expression age dependent, since it is reduced in the hippocampus of offspring LPS PND 30 and increased in cortex and hippocampus of LPS group PND 60 when compared to control rats. In a recent study, we found that the MIA model used in this work causes behavioral changes in rats offspring adulthood, furthermore, leads to an increased secretion of the S100B protein in slices *ex vivo* of offspring from LPS-treated dams in late pregnancy (DF de Souza, unpublished data), these results support the idea that the signaling machinery of S100B (secretion and expression) can be modified after prenatal exposure to infectious agents. Another aspect is that the expression of S100B is decreased in juvenile offspring LPS and increased in adulthood; these data are particularly interesting when one takes into account that the first signs of schizophrenia occur at the beginning adulthood (Monji *et al.*, 2009) and has been observed an elevation of S100B in patients during the first onset of schizophrenia (Steiner *et al.*, 2006).

Several lines of evidence indicate that glial dysfunction could be an important factor in schizophrenia pathology. Studies reveal that GFAP and S100B are modulated by pro-inflammatory cytokines (de Souza *et al*., 2012) (Edwards and Robinson, 2006) and play

a critical role in neuroinflammation and in neurodegenerative diseases (Maragakis and Rothstein, 2006; Sheng *et al*., 2003). Nevertheless, while increased S100B levels in patients with schizophrenia have been interpreted as a marker of structural damage or, alternatively, as a sign of increased astrocyte activation (Wiesmann *et al*., 1999), the role in psychiatry disease of GFAP, a classical marker of astrogliosis, remains controversial (Hao *et al*., 2010; Samuelsson *et al*., 2006). We found a modulation of GFAP dependent on age, gender and brain region: we observed a GFAP decreased in frontal cortex of juvenile LPS-offsprings and increased in hippocampus in both PND 30 and PND 60, and a significant reduction in the amount of GFAP in juvenile female compared to males of the same age. These findings may help explain the discrepancies found in studies regarding the quantification of GFAP in neurological diseases, since it is not always possible to control these variables, especially in human studies.

In schizophrenia, several studies have demonstrated increased S100B CSF and serum concentrations in the acute stage of disease (Rothermundt *et al*., 2004; Schroeter and Steiner, 2009). Still, surprisingly we found only a decrease of S100B levels in CSF of female offspring prenatally exposure to LPS, however although PND 60 match age media of the apparition of the beginning of the first manifestations of schizophrenia, it is not possible to determine whether S100B increased lead to or result from episode schizophrenia. Moreover, in order to evaluate parallel neuronal changes, NeuN, a neuronal nuclear antigen, was measured in hippocampus, no changes between treated and no treated rats was observed. However it was found an interesting ontogenetic difference of this protein of this content between males and females.

Despite of findings shows an elevation of oxidative stress and a decrease of antioxidant defense in schizophrenia pathology and model (Dadheech *et al*., 2008; Do *et al*., 2009), our data show an increment of NO only in adult rats females prenatally exposure to

LPS, and a surprising elevation of GSH in hippocampus PND 30 and in frontal cortex of PND 60. Thus showing that there is probably a regulatory mechanism of antioxidant defenses and oxidative stress depends on different factors with gender, location and age, further studies are needed with a wider range of free radicals and / or ROS for better preparation of a representative panel.Some limitations of this study should be noted. We used an animal model that although containing some aspects of this pathology, does not address the wide range of environmental, pharmacological and social present in development of schizophrenia in humans. Furthermore we analyze only a few among many neurobiological aspects that may be affected in response to inflammatory prenatal insult.

In conclusion, our results show that prenatal LPS challenge leads to neurochemical abnormalities in glial marker in postnatal life, manifesting some even earlier, such as glutathione, or in adulthood, such as content NO. Moreover our model led to a particularly hippocampal astrogliosis, an increase of GFAP in juvenile rats already persisting through adulthood, whereas S100B appears to have a time-dependent expression: decreased in rats to PND30 and PND60 increased.

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Figures

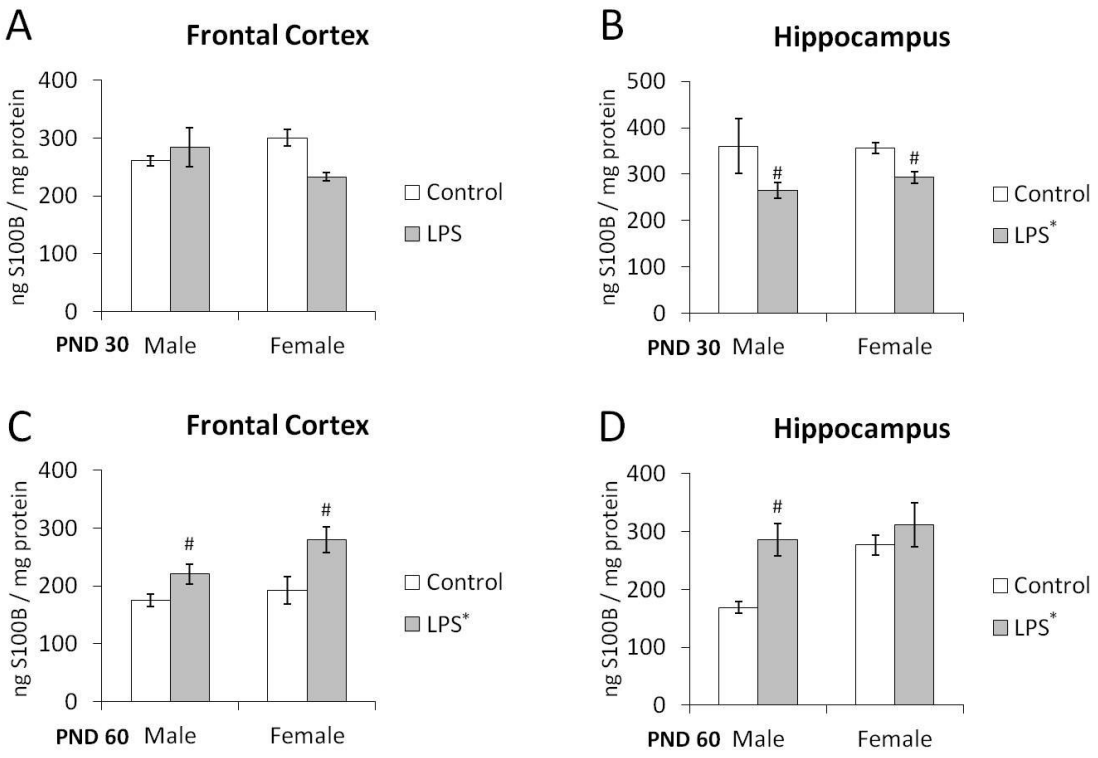

Figure 1. Maternal LPS treatment increase S100B in adult and decrease in juvenile offspring rats. Cortical or hippocampal slices from PND 30 (A and B, respectively) and PND 60 Wistar rats prenatally exposed to LPS (C and D, respectively); S100B was measured by ELISA. Data are expressed as mean ± standard error (LPS group N=5, control group N=5), the experiments were performed in triplicate *Significant effect of prenatal treatment (Two-way ANOVA $p < 0.05$). #Significantly different from control (Bonferroni's post hoc, $p < 0.05$).

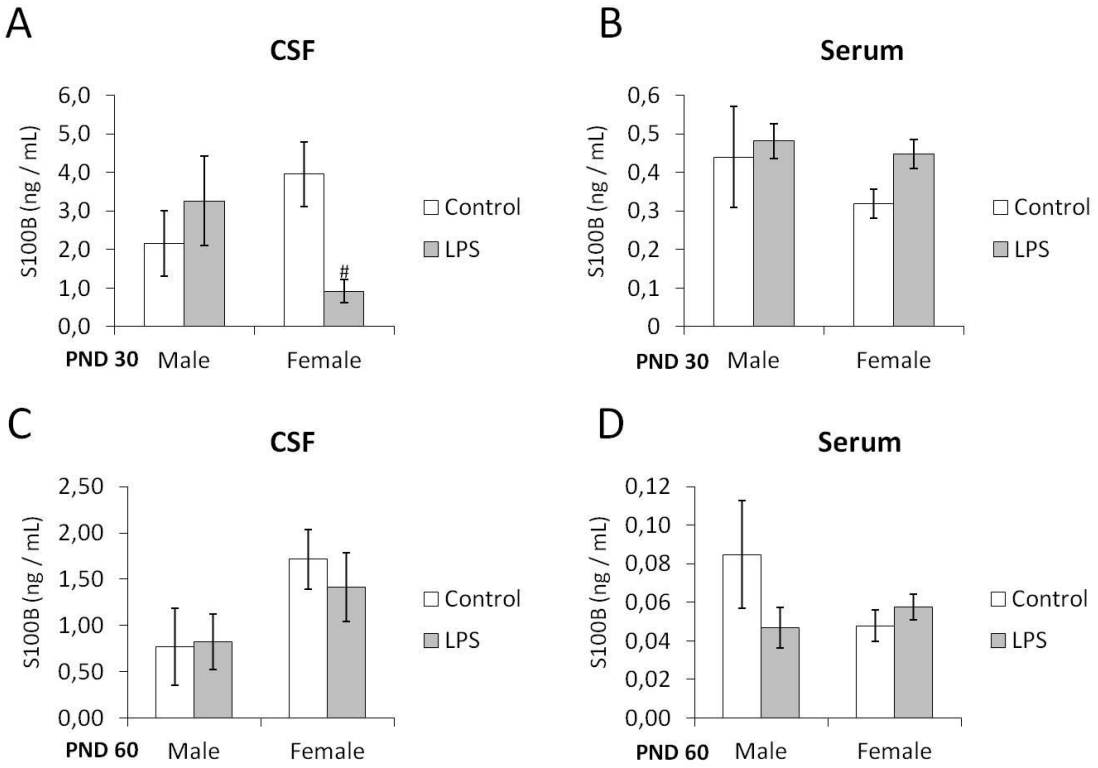

Figure 2. Maternal LPS treatment decreased S100B levels in CSF of young offspring females. CSF and Serum from PND 30 (A and B, respectively) and PND 60 Wistar rats prenatally exposed to LPS (C and D, respectively); S100B was measured by ELISA. Data are expressed as mean ± standard error (LPS group N=5, control group N=5), the experiments were performed in triplicate *Significant effect of prenatal treatment ($p < 0.05$). #Significant interaction Gender X prenatal treatment (Two-way ANOVA followed by Bonferroni's post hoc, $p < 0.05$).

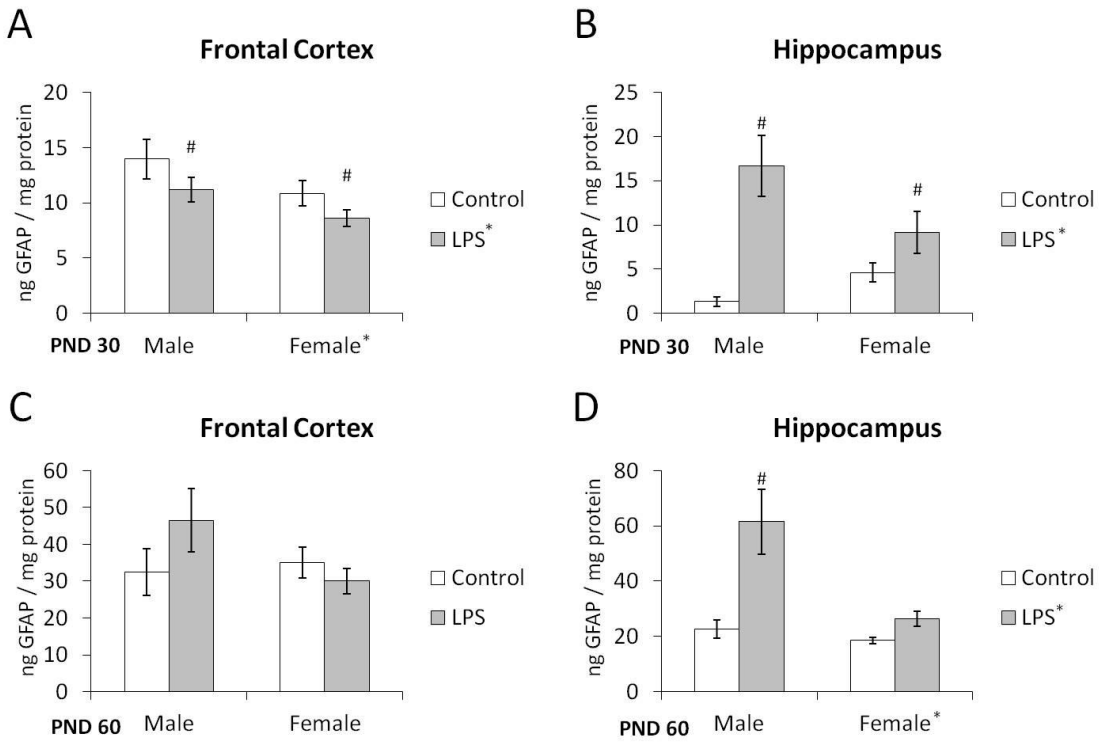

Figure 3. GFAP content is altered in offspring born to LPS-treated dams. Cortical or hippocampal slices from PND 30 (A and B, respectively) and PND 60 Wistar rats prenatally exposed to LPS (C and D, respectively); GFAP was measured by ELISA. Data are expressed as mean ± standard error (LPS group N=5, control group N=5), the experiments were performed in triplicate *Significant effect of prenatal treatment and/or gender (Two-way ANOVA $p < 0.05$). #Significantly different from control (Bonferroni's post hoc, $p < 0.05$).

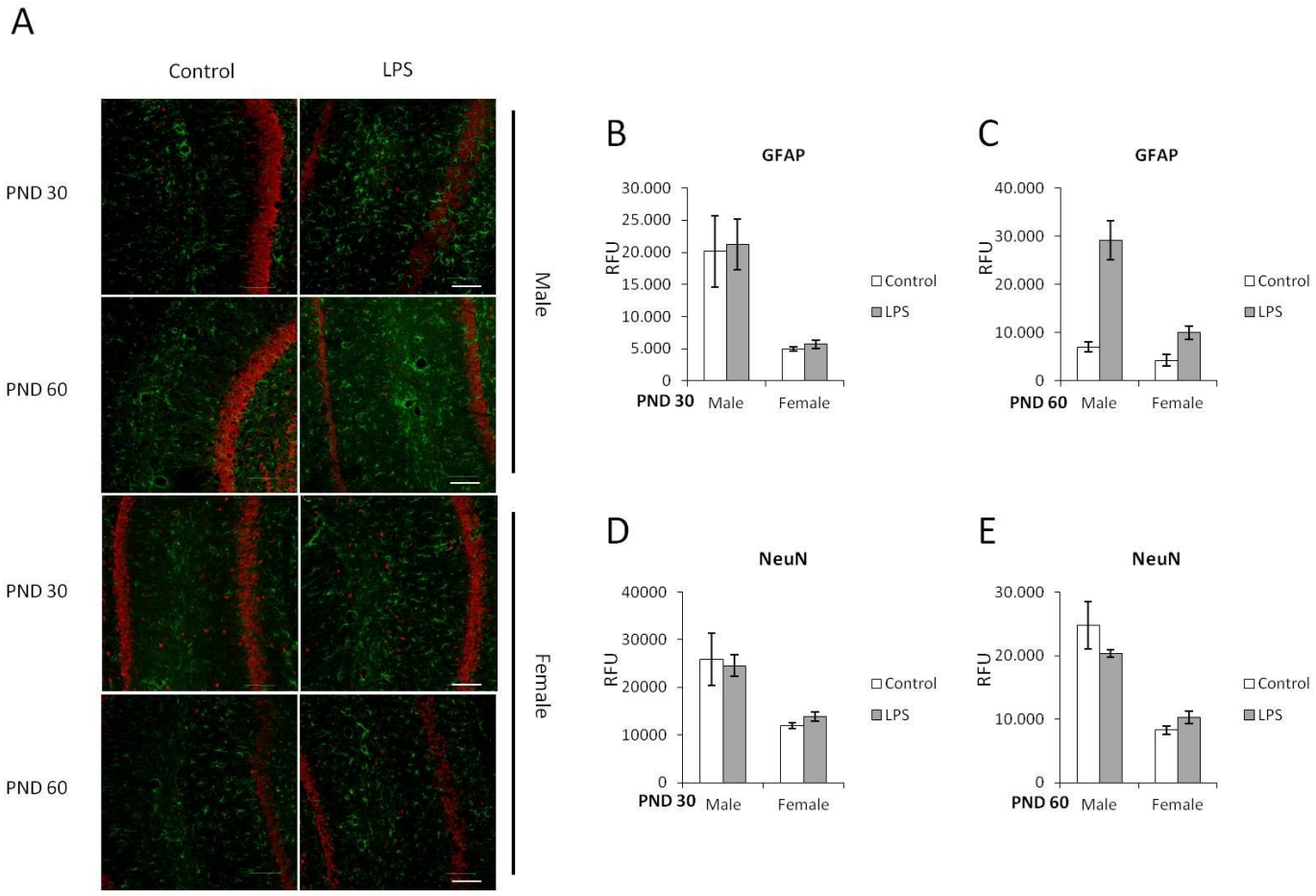

Figure 4. Immunohistochemistry confirms the increment of GFAP in hippocampus of rats LPS-offspring and shows a difference of gender in NeuN. In A, shows immunohistochemistry for GFAP (green) and NeuN (red) from hipocampal slices of 30- and 60-day old Wistar rats prenatally exposed to LPS (male and female). After GFAP and NeuN were quantify in the hippocampal slices from PND 30 (B and D, respectively) and PND 60 Wistar rats prenatally exposed to LPS (C and E, respectively). Data are expressed as mean ± standard error (LPS group N=5, control group N=5), the experiments were performed in triplicate *Significant effect of prenatal treatment and/or gender (Two-way ANOVA $p < 0.05$). $^{\#}$Significantly different from control (Bonferroni's post hoc, $p < 0.05$). Scale bar = 10 mm. RFU: relative fluorescence units.

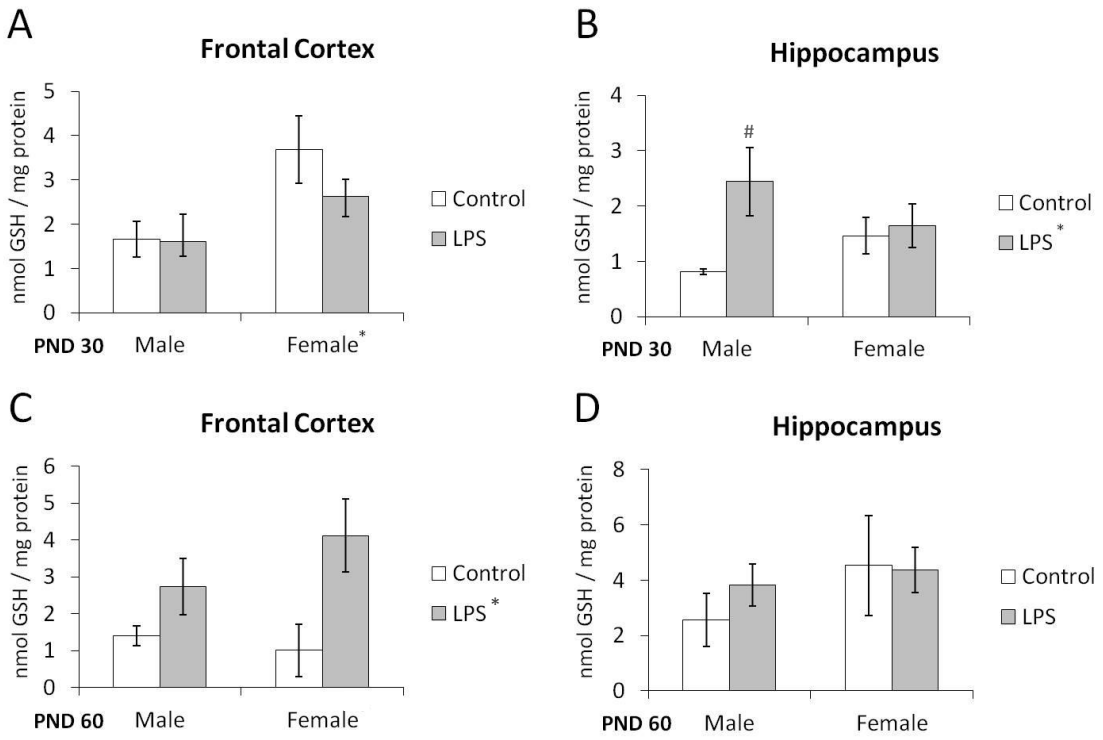

Figure 5. Glutathione content can be dependent of gender or modulated by prenatal treatment with LPS. Cortical or hippocampal slices from PND 30 (A and B, respectively) and PND 60 Wistar rats prenatally exposed to LPS (C and D, respectively). Data are expressed as mean ± standard error (LPS group N=5, control group N=5), the experiments were performed in triplicate *Significant interaction prenatal treatment x gender (A) or effect of prenatal treatment (B,C) (Two-way ANOVA $p < 0.05$). #Significantly different from control (Bonferroni's post hoc, $p < 0.05$).

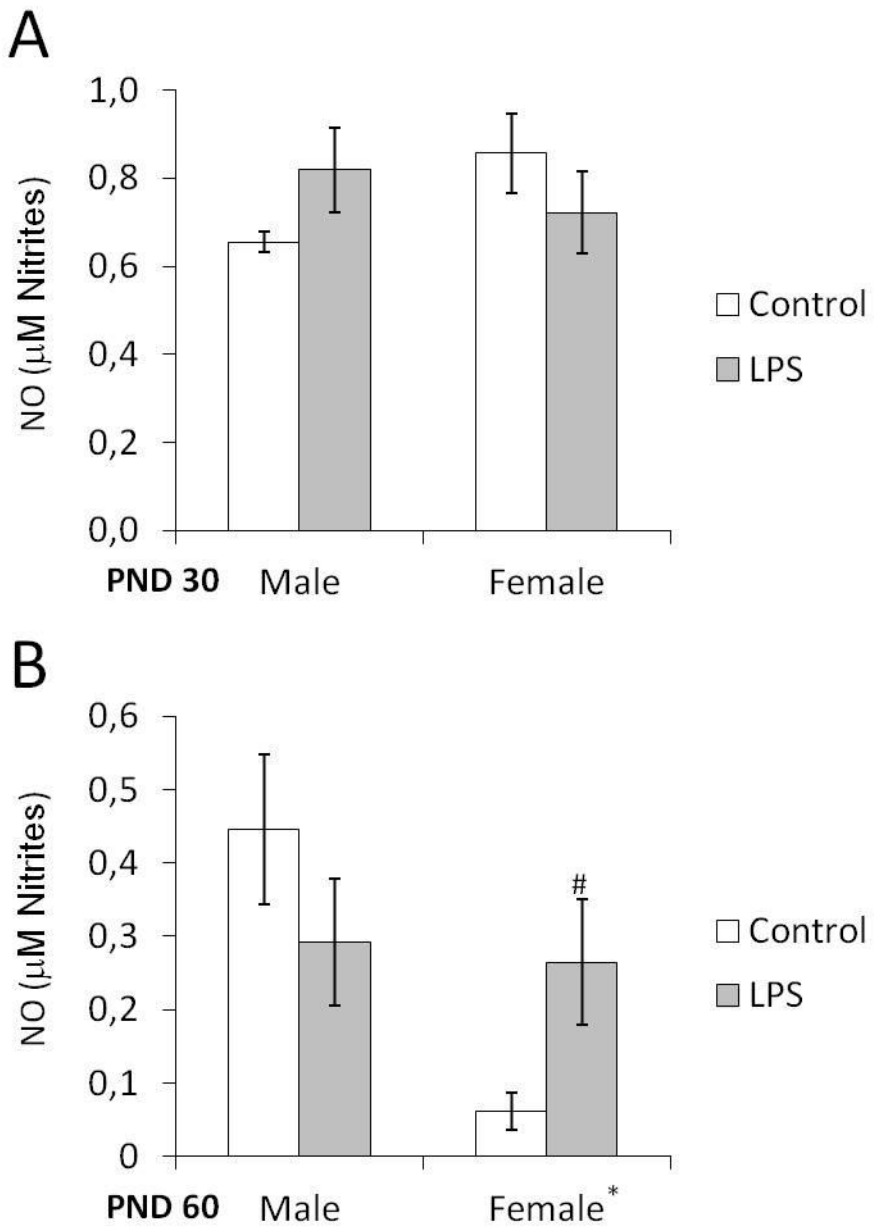

Figure 6. Nitric oxide content can be dependent of gender. Hippocampal slices from PND 30 (A) and PND 60 (B) Wistar rats were prenatally exposed to LPS. Data are expressed as mean ± standard error (LPS group N=5, control group N=5), the experiments were performed in triplicate *Significant effect of gender (Two-way ANOVA $p < 0.05$). #Significantly different from control (Bonferroni's post hoc, $p < 0.05$).

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DISCUSSÃO

Várias evidências apontam que disfunção glial possa ser um fator importante na patogenia da esquizofrenia. Níveis elevados de S100B, proteína expressa e secretada principalmente por astrócitos no SNC, são encontrados no soro e LCR de pacientes esquizofrênicos (Donato *et al.* , 2009, Steiner *et al.* , 2008). Somando-se a isso, descobertas recentes, tanto em modelos animais, quanto em estudos com humanos, têm fornecido evidências para a hipótese da participação de citocinas na gênese da esquizofrenia (Watanabe *et al.* , 2010). Além disso, a correlação entre S100B e citocinas pró-inflamatórias já foi encontrada em distúrbios neurológicos e psiquiátricos (Ashraf, Bhattacharya, 1999, Pleines *et al.* , 2001, Potvin, Stip, 2008) e reforçando essa visão, temos observado mudanças na secreção de S100B em resposta a interleucina-1β (de Souza, Leite, 2009) e à ativação de TLR4 (Guerra, Tortorelli, 2011).

Neste estudo, vimos que IL-6, IL-8 e TNF-α são capazes de estimular a secreção de S100B em fatias hipocampais e em células de glioma C6 e esse efeito encontrado parece ser agudo, uma vez que nenhuma mudança foi observada após 6 ou 24 h de exposição às citocinas nas culturas celulares, contudo nossa avaliação foi realizada após uma única exposição a citocinas, não podemos afirmar portanto se em exposições crônicas essa resposta não seria mais prolongada, uma vez que já foi mostrada a existência de um ciclo de citocinas que através de retroalimentação e superregulação dos seus membros é capaz de perpetuar o sinal de inflamação, podendo levar em última instância a distúrbios neurais (Griffin, 2006).

Um estudo recente também constatou elevação da S100B extracelular em culturas de células expostas a TNF-α, porém estes autores observaram concomitantemente um aumento de liberação de GFAP (Edwards and Robinson, 2006),

o que sugere que sob as condições utilizadas, a integridade celular poderia estar sendo afetada. Por outro lado já foi visto que S100B é capaz de modular a expressão e liberação de TNF-α e IL-6 (Ponath *et al.* , 2007), evidenciando assim uma conversa cruzada dessas moléculas. Corroborando com esses achados, nossos dados indicam claramente que a secreção de S100B é estimulada pelas citocinas inflamatórias, IL-1β, TNF-α, IL-6 e IL-8, o que sugere que o aumento da S100B observado em pacientes com esquizofrenia possa, em parte, ser explicados pela estimulação por citocinas.

Com o intuito de investigar as vias de sinalização envolvidas nestes mecanismos, analisamos a via MAPK, que por ser responsável por dirigir respostas celulares a um conjunto diversificado de estímulos extracelulares, tais como mitógenos e citocinas pró-inflamatórias, além de regular proliferação, expressão gênica, diferenciação, mitose, sobrevivência celular, apoptose entre outros, era uma candidata natural para estar regulando também essa modulação. Inibidores específicos das proteínas MAPK (ERK, p38 e JNK), foram capazes de bloquear a secreção de S100B induzida por citocinas. É importante mencionar que estas cinases de sinalização são ainda sensíveis ao stress oxidativo (Gaitanaki *et al*. , 2003). Portanto, estresse oxidativo observado em pacientes com esquizofrenia e outros transtornos psíquicos pode estar modulando a secreção de S100B através de MAPK. Mais que isso, sob as condições utilizadas, as citocinas foram capazes de induzir estresse oxidativo, como atestado através de medidas de oxidação de DCF e diminuição os níveis de glutationa reduzida, sugerindo uma íntima ligação entre esses fatores. De um modo geral, as citocinas parecem induzir estresse oxidativo, mas não estresse nitrosativo (com base na quantificaçãol de nitritos). Estes resultados podem ajudar a explicar a presença de marcadores de estresse oxidativo e do prejuízo do sistema de defesa antioxidante encontrados em pacientes com esquizofrenia (Martinez-Cengotitabengoa *et al.* , 2012).

A via de NF-kB também foi investigada, utilizamos SN-50, um inibidor da migração nuclear.O fator de transcrição NF-κB é um mediador chave nas vias de transdução do sinal para a atividade das citocinas durante injúria cerebral, em células gliais e neuronais. Quando ativado, o NF-κB transloca-se para o núcleo, promovendo a transcrição de diversos mediadores pró-inflamatórios, como IL-1, IL-6, TNF-α, metaloproteinases, iNOS, e outros, podendo assim, estar envolvido no desenvolvimento de neuropatologias (Memet, 2006, Moynagh, 2005). Contudo não observamos efeito sobre a secreção de S100B induzida por citocina. Além disso, SN-50, por si só provocou um aumento na secreção de S100B. De fato, a ativação de transcrição mediada por NF-kB vem sendo amplamente caracterizada, mas os mecanismos de repressão mediados por NF-kB não (Tchivileva *et al.* , 2009). No entanto, de nosso conhecimento, um envolvimento direto da NF-kappaB na expressão de S100B ainda não havia sido relatado. Além disso, os efeitos dos inibidores da MAPK e NF-kappa B sobre a secreção de S100B (medido em 1 h), possivelmente não envolvam a síntese de proteínas. Portanto, o efeito de SN-50, pode envolver um diferente alvo de NF-kB (Torgerson *et al.* , 1998).

A IL-6 foi capaz de induzir um aumento de GFAP e de S100B em glioma C6, 24 h após a exposição. De fato, a IL-6 é um importante regulador da neuroinflamação, assim como, está envolvida na diferenciação astrocítica das células de glioma C6 (Takanaga *et al.* , 2004). Níveis séricos aumentados de IL-6 na esquizofrenia têm sido descrito a mais de 20 anos (Shintani *et al.* , 1991), além disso, esta citocina tem sido frequentemente utilizada em modelos da doença (Behrens *et al.* , 2008). Porém, estudos post-mortem em humanos, indicam uma ausência de gliose na maioria dos cérebros esquizofrênicos analisados (Arnold *et al.* , 1996, Damadzic *et al.* , 2001).

Recentemente, nosso grupo mostrou uma diminuição da secreção de S100B em fatias hipocampais e em células C6, induzida por apomorfina (Nardin *et al.* , 2011), antipsicóticos (haloperidol e risperidona) não foram capazes de reverter esse aumento. Todavia no presente estudo, mostramos que a secreção de S100B, induzida pela IL-6, em contraste, é prevenida por haloperidol e risperidona, mas não por clozapina. Observamos ainda que apenas a risperidona foi capaz de modular a secreção basal de S100B, acordando com um estudo recente de nosso grupo (Quincozes-Santos *et al.* , 2008). Juntos, esses dados reforçam a ideia de que os antipsicóticos possam ajudar a normalizar os níveis elevados de S100B em pacientes com esquizofrenia, especialmente durante a fase aguda da doença, e que as citocinas sejam subjacentes a estas elevações. Deve-se notar que os adipócitos são outra importante fonte de S100B sérica (Goncalves *et al.* , 2010) e que as principais alterações metabólicas em pacientes com esquizofrenia são observadas nestas células (Steiner *et al.* , 2010a).

O efeito das drogas anti-psicóticas na secreção de S100B induzida por IL-6 em células de glioma C6 indicam uma modulação através de receptores D2. Vale ressaltar que os medicamentos antipsicóticos atípicos, como a clozapina e risperidona, envolvem também $5HT_{2A,}$ não só D_2 (Kuroki *et al.* , 2008). Além disso, outros receptores tais como $5HT_7$, encontrados em astrócitos, são alvos de drogas anti-psicóticas e poderiam estar envolvidos na diferença entre a modulação da clozapina e da risperidona (Smith *et al.* , 2011). Assim, estes e outros receptores podem estar modulando a secreção de S100B basal e / ou a induzida por citocina no tecido cerebral, o que torna difícil descrever um cenário simples para explicar as alterações extracelulares da proteína. Além disso, os efeitos das drogas anti-psicóticas na secreção de S100B podem ser devido a alterações no estado oxidativo da célula (Donato, Sorci, 2009). Contudo, em contraste com administração de longo prazo *in vivo* (Pillai *et al.* , 2007), foi observada

uma redução nos níveis de EROs nas células de glioma C6 1 h após a administração de haloperidol,. Sabe-se que o haloperidol, em concentrações elevadas (mas não risperidona), é capaz de induzir a produção de EROs, em células de glioma C6 (Quincozes-Santos *et al.* , 2010). Por isso, não foi possível correlacionar a secreção de S100B e níveis de EROs nas células de glioma C6, durante a exposição aguda a drogas anti-psicóticas.

Diversos tipos de modelos animais têm sido desenvolvidos para estudar a esquizofrenia, incluindo o modelo induzido por injeção intraperitoneal de LPS em ratas fêmeas prenhas. Este modelo apresenta um número significativo de características relacionadas com os principais fenótipos apresentados por humanos esquizofrênicos, como por exemplo, deficiência sensório-motora, anormalidades de aprendizado, comprometimento da memória de trabalho, sensibilidade aumentada a drogas psicoestimulantes, comprometimento do comportamento social, entre outras (Meyer, Feldon, 2009). Inflamação do SNC pré-natal especialmente no final da gestação interfere com a migração celular, organização e maturação de sinapses (Gilmore *et al.* , 2004, Meyer, Yee, 2007), cujas consequências em níveis funcional e estrutural são mais restritas. Implicações farmacológicas e cognitivas de longo prazo decorrentes de ativação imune pré-natal no final da gestação podem, assim, estar mais relacionado a sintomas negativos da esquizofrenia (déficits de interação social, presença de comportamentos repetitivos estereotipados, anedonia, e inflexibilidade comportamental e / ou cognitiva, entre outras) (Bitanihirwe *et al.* , 2010, Sullivan *et al.* , 2006). Deficiências cognitivas, sociais e os sintomas negativos são as principais características da esquizofrenia diretamente associados com o prejuízo do convívio em sociedade (Bell *et al.* , 2011).

Em nosso desenho experimental os ratos mostram uma resposta de longo prazo, apresentando mudanças em parâmetros comportamentais como ansiedade, reatividade emocional e interação social. Nós constatamos que a resposta a uma exposição do tipo bacteriana, como tratamento com LPS, no final da gestação gera alterações no padrão das respostas no teste campo aberto: perda da habituação da latência (tempo gasto para deixar o primeiro quadrado), em contraste com os controles, e quantidade de bolos fecais, aumentada na prole de mães tratadas com LPS; sendo estas duas medidas tradicionalmente relacionadas com emotividade e / ou ansiedade, fica claro que o modelo resulta em distúrbios comportamentais, embora não modifique a ambulação (crossings), rearings ou grooming. Outro importante parâmetro comportamental que se encontra prejudicado nesse modelo é a interação social, uma vez que os ratos LPS não exibiram preferência pela câmara 2 (rato) sobre a 1 (objeto) como acontece com os ratos controle, assim como o tempo maior de exploração do rato estranho em relação ao objeto presente nos controles não se repetiu com prole-LPS. Contudo não houve prejuízo na memória social dos ratos filhotes de mães tratadas com LPS.

Neurotransmissão glutamatérgica desempenha um papel crítico na atividade sináptica e plasticidade em todo o SNC, incluindo circuitos relacionados com cognição, emoção e recompensa (Moghaddam, 2003). Em pacientes esquizofrênicos, diferentes e conflitantes evidências de transmissão glutamatérgica anormal vem sendo encontradas, tais como distúrbios de liberação cortical de glutamato (Frankle *et al.* , 2003, Lewis *et al.* , 2003),níveis diminuídos de glutamato no córtex pré-frontal (Tsai *et al.* , 1995)e aumento da concentração de glutamato pré-frontal e hipocampal (van Elst *et al.* , 2005). Nós encontramos uma diminuição na captação de glutamato na prole-LPS, contrariando a clássica hipótese glutamatérgica que postula que os sintomas encontrados em pacientes esquizofrênicos poderiam advir de hipofunção glutamatérgica (Coyle, 2006).

Por outro lado, a maior parte dos estudos é realizada em córtex, e a nossa observação foi feita em hipocampo, além disso, nosso trabalho avalia captação de glutamato propriamente dita enquanto que a imensa maioria usa quantificação dos transportadores de glutamato para avaliar a captação (Danbolt, 2001, Spangaro *et al.* , 2012). Nossas observações estão de acordo com achados que mostram que citocinas são capazes de atenuar a captação glutamatérgica de maneira dependente, este dado ajuda a reforçar a ideia de que as citocinas poderiam ser as protagonistas das mudanças causadas nos filhotes de mães expostas ao LPS. Interessantemente, apomorfina - um agonista não-seletivo da dopamina que ativa ambos os receptores D1 e D2, com alguma preferência pelo último subtipo (Koller and Herbster, 1988) - diminuiu significativamente a captação de glutamato nos animais controles, mas no grupo-LPS essa capacidade foi perdida. Isto mostra que uma disfunção na integração fisiológica dos sistemas dopaminérgicos e glutamatérgicos possa ocorrer em decorrência da exposição pré-natal ao LPS.

O mecanismo de secreção de S100B permanece desconhecido, mas sabe-se que neurotransmissores como glutamato (Buyukuysal, 2005, Goncalves *et al.* , 2000, Tramontina, Leite, 2006a), serotonina (Tramontina *et al.* , 2008), dopamina (Nardin, Tramontina, 2011) e acetilcolina (P. Lunardi, dados não publicados), são capazes de modular a sua secreção em culturas de células e em fatias hipocampais agudas. Nós verificamos que a apomorfina foi capaz de reduzir a secreção de S100B em fatias hipocampais tanto dos controles quanto do grupo LPS. Esses resultados estão de acordo com nossos achados prévios que mostravam uma diminuição de S100B nas três diferentes preparações *in vitro* utilizadas (fatias hipocampais, astrócitos corticais e células de glioma C6) quando tratadas com apomorfina (Nardin, Tramontina, 2011).

Supondo-se que os receptores D2 negativamente acoplados a adenilato-ciclase são ativados nos astrócitos pela apomorfina, uma diminuição na secreção de S100B seria um resultado esperado. Outros resultados com forskolina, fluoxetina e serotonina também sugerem uma ligação entre cAMP e secreção de S100B (Goncalves *et al.* , 2002, Tramontina, Tramontina, 2008). Em doentes esquizofrênicos, hiperativação dopaminérgica tem sido proposta para a base da exacerbação dos sintomas positivos, que é onde antipsicóticos típicos são mais eficazes. Por outro lado, um aumento da S100B sérica tem sido observado em vários estudos com pacientes psiquiátricos (Lara, Gama, 2001, Rothermundt *et al.* , 2004b, Schroeter and Steiner, 2009). No entanto, ainda não está claro se S100B sérica elevada reflete especificamente ativação astrocítica. De fato, fontes extra-cerebrais podem contribuir para o aumento de S100B no soro, particularmente adipócitos (Gonçalves *et al.*, 2010). Além disso, descobertas recentes sugerem que os níveis de S100B no sangue correlacionam-se com o tecido adiposo em indivíduos normais (Steiner *et al.* , 2010b) e com a resistência à insulina em pacientes com esquizofrenia (Steiner *et al.* , 2010c). Nós também encontramos um aumento significativo de secreção de S100B em fatias hipocampais de ratos descendentes de mães expostas ao LPS, acordando com os resultados de secreção de S100B aumentada por citocinas pró-inflamatórias.

Nosso passo seguinte foi medir a enzima tirosina hidroxilase, responsável por catalisar a conversão do aminoácido L-tirosina em L-DOPA, que é o precursor da dopamina, um dos principais neurotransmissor envolvidos na esquizofrenia. Contudo a mensuração da tirosina hidroxilase é bastante controversa, é possível encontrar na literatura trabalhos mostrando uma diminuição de sua atividade / quantidade em pacientes esquizofrênicos post-mortem (Akil *et al.* , 2000) e em resposta a citocinas (Shi and Habecker, 2012), como também trabalhos mostrando elevação em modelos de

inflamação (Nolan *et al.* , 2000) e doenças psíquicas (Toru *et al.* , 1982). Neste estudo encontramos uma indução região específica (somente em córtex) desta proteína, este achado está de acordo com estudos que mostram uma hiperfunção dopaminérgica na patologia da esquizofrenia.

Sabe-se que esquizofrenia envolve distúrbios neuroquímicos, alterações da atividade metabólica e de conectividade entre várias regiões cerebrais como núcleo accubens, córtex pré-frontal e hipocampo (Cui *et al.* , 2009, Lisman *et al.* , 2008). Cada vez mais se observa que desequilíbrio de fatores neurodegenerativos e neuroprotetores pode desempenhar um papel fundamental em distúrbios do SNC. Dentre os fatores que modulam o sutil equilíbrio entre morte e sobrevivência celular, o papel das citocinas tem sido consistentemente relatado em esquizofrenia (Mansur, Zugman, 2012). S100B é uma proteína de ligação ao cálcio, secretada por astrócitos, que participa da plasticidade sináptica (Nishiyama *et al.* , 2002), sendo neurotrófica em níveis nanomolares e neurotóxica em micromolares (Donato, Sorci, 2009). Nós observamos que a S100B tem um padrão de expressão dependente de idade, uma vez que é reduzida no hipocampo da prole LPS DPN (dia pós-natal) 30 e aumentada no córtex e no hipocampo do grupo LPS DPN 60, quando comparado aos ratos controle. Esta observação de que a expressão de S100B é diminuída na prole LPS juvenil e aumentada na idade adulta, é particularmente interessantes quando se leva em conta que os primeiros sintomas da esquizofrenia ocorrem justamente no início da idade adulta (Monji, Kato, 2009) e que a S100B tem sido encontrada elevada em pacientes durante o primeiro surto de esquizofrenia (Steiner, Bielau, 2006).

GFAP e S100B são moduladas por citocinas pró-inflamatórias (Edwards e Robinson, 2006) e desempenham um papel crítico na neuroinflamação e em doenças neurodegenerativas (Maragakis and Rothstein, 2006, Sheng *et al.* , 2003). No entanto,

enquanto os níveis aumentados de S100B em pacientes com esquizofrenia têm sido interpretados como um marcador de danos estruturais, ou ainda, como um sinal de ativação astrocítica (Wiesmann *et al.* , 1999), o papel da GFAP, um marcador clássico de astrogliose, em doenças psiquiátricas permanece controverso (Hao *et al.* , 2010, Samuelsson *et al.* , 2006). Encontramos uma modulação da GFAP dependente de idade, sexo e região do cerebral: observamos uma diminuição da GFAP no córtex frontal de LPS- descendentes juvenis, e aumentada no hipocampo de ambos DPN 30 e DPN 60, além de uma redução significativa na quantidade de GFAP em fêmeas DPN 30 em comparação com os machos da mesma idade. Estes resultados podem ajudar a explicar as discrepâncias encontradas em estudos sobre a quantificação de GFAP em doenças neurológicas, uma vez que nem sempre é possível controlar todas estas variáveis, especialmente em estudos com humanos.

Vários estudos encontraram aumento da concentração sérica e em LCR da S100B na fase aguda da esquizofrenia (Rothermundt, Falkai, 2004a, Schroeter and Steiner, 2009). Surpreendentemente, vimos apenas uma diminuição dos níveis de S100B no LCR de fêmeas com exposição pré-natal ao LPS, no entanto, embora os ratos aos 60 dias estejam na faixa etária equivalente a do aparecimento das primeiras manifestações da esquizofrenia em humanos, não é possível determinar se o aumento da S100B está implicado na causa ou resulta do surto esquizofrênico. A fim de avaliar as possíveis alterações neuronais paralelas, NeuN, proteína nuclear neuronal específica, foi quantificada em hipocampo, nenhuma alteração entre ratos pré-natalmente tratados e não tratados foram observadas. No entanto, verificou-se uma interessante diferença ontogenética do conteúdo desta proteína entre machos e fêmeas.

Apesar de resultados prévios mostrarem uma elevação de estresse oxidativo e diminuição de defesas antioxidantes na patologia e em modelo de esquizofrenia

(Dadheech *et al.* , 2008, Do *et al.* , 2009), nossos dados mostram uma elevação de NO somente em fêmeas adultas expostas ao LPS, além de uma surpreendente elevação de GSH em hipocampo de ratos DPN 30 e em córtex frontal de DPN 60. Mostrando assim, que provavelmente o mecanismo regulador de defesas antioxidantes e de estresse oxidativo dependa de diferentes fatores como sexo, região cerebral e idade, porém mais estudos são necessários, com uma gama maior de radicais livres e / ou EROs para a elaboração de um painel mais representativo.

CONCLUSÕES

- Secreção de S100B foi aumentada pelas citocinas pró-inflamatórias (IL-1β, TNF-α, IL-6 e IL-8) em glioma C6 e em fatias hipocampais através da via MAPK, e estresse oxidativo pode ser um componente desta modulação.

- IL-6 foi capaz de induzir um aumento no conteúdo de S100B e GFAP em glioma C6. Além disso, os antipsicóticos, haloperidol e risperidona, foram capazes de inibir a secreção de S100B estimulada por IL-6.

- O modelo de alteração imune maternal provocou alterações comportamentais na prole adulta, especialmente no comportamento social dos ratos afetados.

- A exposição maternal ao LPS modificou o padrão de secreção da proteína S100B e da captação de glutamato em fatias *ex vivo* da prole adulta.

- Conteúdo de Glutationa e de NO são modificados de forma idade/gênero dependente.

- Desafio pré-natal com LPS parece levar a anormalidades neuroquímicas de marcadores gliais na vida pós-natal, como diminuição do conteúdo de S100B na prole juvenil e aumento em ratos adultos.

- Foi observada uma pronunciada astrogliose particularmente hipocampal. A proteína GFAP apresentou-se aumentada em ratos juvenis e adultos em resposta a exposição imune maternal.

PERSPECTIVAS

- Quantificar as cinases da via MAPK, tanto no modelo *in vitro*, quanto no modelo *in vivo*.
- Avaliar outros parâmetros comportamentais, tais como memória (de trabalho e aversiva), inibição latente, inibição pré-pulso.
- Avaliar alterações comportamentais também em fêmeas e ratos jovens para o modelo experimental de ativação imune maternal.
- Avaliar outros parâmetros de estresse oxidativo, para uma avaliação mais completa dos efeitos da exposição do LPS sobre o desequilíbrio entre defesas antioxidantes e geração de espécies reativas.
- Investigar os mecanismos pelo qual o LPS está agindo, por exemplo, efetuando dosagem de citocinas nas mães, fetos, prole juvenil e adulta.
- Investigar possíveis modificações na citoarquitetura cerebral dos ratos expostos a LPS, utilizando marcadores gliais e neuronais em diferentes áreas cerebrais, em tempos diferentes do desenvolvimento da prole.

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