UNIVERSIDADE PAULISTA –UNIP

A ADMINISTRAÇÃO NO 18° DIA DA GESTAÇÃO DE RATAS DE LIPOPOLISSACARÍDEO (LPS) MODIFICA A INTERAÇÃO MATERNO-FILHOTE DA GERAÇÃO PARENTAL E DAS GERAÇÕES F1 E F2

Tese apresentada ao Programa de Pós-graduação em Patologia Ambiental e Experimental da Universidade Paulista-UNIP, para obtenção do título de doutor em Patologia Ambiental e Experimental.

SANDRA HELOISA NUNES WHITAKER PENTEADO

SÃO PAULO

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Penteado, Sandra Heloísa Nunes Whitaker

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Aprovado em:

BANCA EXAMINADORA:

_____/__/___
Profa. Dra. Leoni Villano Bonamin
Universidade Paulista – UNIP

_____/__/___
Profa. Dra. Ivana Barbosa Suffredini
Universidade Paulista – UNIP

_____/__/___
Profa. Dra. Elizabeth Teodorov
Universidade Federal do ABC

_____/__/___
Prof. Dr. Luciano Freitas Felicio
Universidade de São Paulo - USP

_____/__/___
Profa. Dra. Maria Martha Bernardi
Universidade Paulista – UNIP

DEDICATÓRIA

Dedico essa tese de doutorado a minhas amadas filhas, Camila e Carolina, que iluminam minha vida; aos meus pais, Luiz Antonio Nunes e Maria Ignez Barbieri Nunes, pelo carinho e apoio; ao meu companheiro, Edmilson, por me ensinar o significado da palavra felicidade; e a minha querida orientadora, Profa. Dra. Maria Martha pelo apoio em todos os momentos e, principalmente, pelo carinho e pela amizade.

“A ciência humana de maneira nenhuma nega a existência de Deus. Quando considero quantas e quão maravilhosas coisas o homem compreende, pesquisa e consegue realizar, então reconheço claramente que o espírito humano é obra de Deus, e a mais notável.”

Galileu Galilei

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RESUMO

Foram estudados os efeitos da administração de LPS no 18° dia de gestação (DG) de ratas na interação materno-filhote das gerações parental, F1 e F2. Para tanto, ratas prenhes receberam no DG18 100 µg/Kg da endotoxina ou seu veículo (geração parental - P) e foram observados os comportamentos de recolher dos filhotes, maternal e maternal agressivo. Na geração F1 foi avaliada a preferência olfatória ao odor materno na infância e após a administração de uma dose adicional de LPS no dia 21 da lactação, observou-se a atividade geral da prole masculina da geração F1 e os níveis séricos de TNFα. Quando adultas, as ratas da geração F1 foram cruzadas com ratos sem qualquer tratamento e observados os seus comportamentos ligados ao cuidado maternal. Na geração F2 testou-se a preferência olfatória pelo odor materno e na idade adulta sua atividade geral em campo aberto e no teste de ansiedade, o labirinto em cruz elevada.

Os resultados mostraram que, em relação ao grupo controle, a geração parental apresentou facilitação no comportamento maternal e redução no comportamento maternal agressivo. Na prole masculina da geração F1 verificou-se que os animais de mães tratadas com a endotoxina tiveram menor preferência pelo odor materno, e redução nos níveis de TNFα. Na atividade geral, os filhotes das ratas cujas mães receberam o LPS não apresentaram alterações se comparadas àquela dos animais do grupo controle.

Na geração F1, após cruzamento e na lactação, verificou-se aumento da latência para assumir a posição maternal do comportamento maternal e do número e latência para os ataques do comportamento maternal agressivo.

Na geração F2 observada na infância, a preferência olfatória pelo odor materno não foi modificada, mas o número de filhotes indiferentes a esse odor do grupo experimental foi maior que daquele do grupo controle. Na idade adulta, estes ratos apresentaram menores índices de ansiedade. Concluiu-se que a exposição no 18° dia da gestação ao LPS interfere na programação da interação mãe-filhote de duas gerações.

Palavras chaves: LPS, comportamento maternal, agressão materna, preferência olfatória, epigenética.

ABSTRACT

The effects of single prenatal LPS administration were investigated on maternal-pups interaction of parental, F1 and F2 generations. Thus, pregnant rats received on DG18 100 µg / kg of LPS or its vehicle (parental generation) and the behaviors of pups retrieval and the maternal and maternal aggressive. In the F1 generation the olfactory preference of pups to maternal odor was assessed. In addition, at a weaning these pups received an additional dose of LPS and the general activity observed in an open field as well as the serum TNFα was measured. When adult female rats of the F1 generation were mated with male rats without any treatment and its maternal care were observed. In the F2 generation the olfactory preference of pups to maternal odor were decreased. In adult age, the pups of F2 generation were examined to their general activity in an open field and in the plus maze.

The results showed that in relation to the control group, the parental generation showed facilitation of maternal behavior and reduction in maternal aggressive behavior. In the male offspring of the F1 generation the animals from mothers treated with endotoxin had less preference for maternal odor, and a decreased levels of TNFα. In the open field behavior, these rats did not showed changes when compared to that of control animals.

In the F1 generation there was increased latency to assume the maternal position and in the number and latency to attack the intruder in maternal aggressive behavior.

In infancy, the olfactory preference of F2 generation for maternal odor was not modified, but the number of pups indifferent to this odor in the experimental group was higher than that of the control group. In adult age these rats showed a decreased anxiety-like behavior in the plus maze.

It was concluded that exposure on day 18 of gestation to LPS acts as an imprinting, which interferes with the maternal programming of the maternal-pups interaction of two generations.

Keywords: LPS, maternal behavior, maternal aggression, olfactory preference, epigenetics.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS

^{125}I-LPS	LPS radiomarcado com iodo
ATV	tegmental ventral
CD14	proteína de membrana periférica da superfície dos macrófagos
CM	comportamento maternal
DG	dia de gestação
DNA	ácido desoxirribonucleico
GABA	ácido gama-aminobutírico
HPA	Eixo hipotálamo-pituitária
i.p.	intraperitoneal
IL-1β	interleucina 1 beta
IL-6	interleucina 6
IRAK	*interleukin-1 receptor associated kinase*
LBP	proteína ligadora de LPS, ou *lipopolysaccharide binding protein*
LPS	Lipopolissacarídeo
MyD88	*Myeloid differentiation primary response gene (88)*
NF-kB	fator de transcrição
P	geração parental
PAG	substância cinzenta periaquedutal
POA	área pré-optica
quinase IkB	inibidor do NF-kB (família do fator de transcrição nuclear kappa-B)
RNA	ácido ribonucleico
RNAm	RNA mensageiro
siRNA	*small interfering RNA*
SNC	Sistema Nervoso Central
TAK-1	*TGF-beta activated kinase 1*
TLR	*toll-like receptor* (receptor semelhante ao Toll)
TNFα	Fator de necrose tumoral alfa (*Tumor necrosis factor alpha*)
TRAF6	*TNF receptor associated factor*

SUMÁRIO

1. INTRODUÇÃO

1.1. Sobre o Lipopolissacarídeo (LPS)

O LPS é uma endotoxina originária da parede celular de bactérias gram-negativas. Consiste num lipídio complexo, denominado lipídio A, ao qual está ligado um polissacarídeo constituído de um núcleo (ou *core*) e de uma série terminal de unidades repetidas (Figura 1). O lipídio A consiste em unidades dissacarídicas de glicosamina fosforilada as quais estão ligadas a vários ácidos graxos de cadeia longa (podendo variar de acordo com a espécie bacteriana). O núcleo do polissacarídeo é semelhante em todas as espécies gram-negativas que possuem LPS, todavia, cada espécie contém uma unidade de repetição particular. Em geral, as unidades de repetição consistem em trissacarídios lineares ou em tetra ou pentassacarídios ramificados [1]. As moléculas de LPS de carga negativa são ligadas de forma não covalente por cátions divalentes, tornando a membrana estabilizada e proporcionando uma barreira contra moléculas hidrofóbicas. As substâncias são termoestáveis, com peso molecular entre 3000 e vários milhões [1].

O LPS é sintetizado na membrana citoplasmática e transportado para sua posição exterior final. É ligado à superfície celular, liberado apenas quando as células são lisadas. Quando o LPS é clivado em lipídio A e em polissacarídeo, toda a interação imune está associada ao lipídio A. A especificidade antigênica é conferida pelas unidades terminais de repetição, que circundam a célula, formando uma camada de polissacarídeos hidrofílicos [1].

A presença do LPS é necessária para a função de muitas proteínas da membrana externa das bactérias [1]. Porém, o LPS pode ser extremamente tóxico para animais. Administrações em doses menores que 1 nM já são capazes de ativar o sistema imune do animal [2]. Os efeitos fisiopatológicos do LPS são semelhantes, independente de sua origem bacteriana [1].

Dentro da área médica e veterinária, o LPS é muito utilizado nas mais diferentes linhas de pesquisa, pelo seu efeito de estímulo do sistema imunológico. É

muito empregado em animais de laboratório, como roedores, por exemplo. Comercialmente, para estudos toxicológicos, neuroimunológicos, dentre outros, uma das principais fontes de LPS é a partir da bactéria gram-negativa *Escherichia coli*, através de um processo de extração fenólica [3].

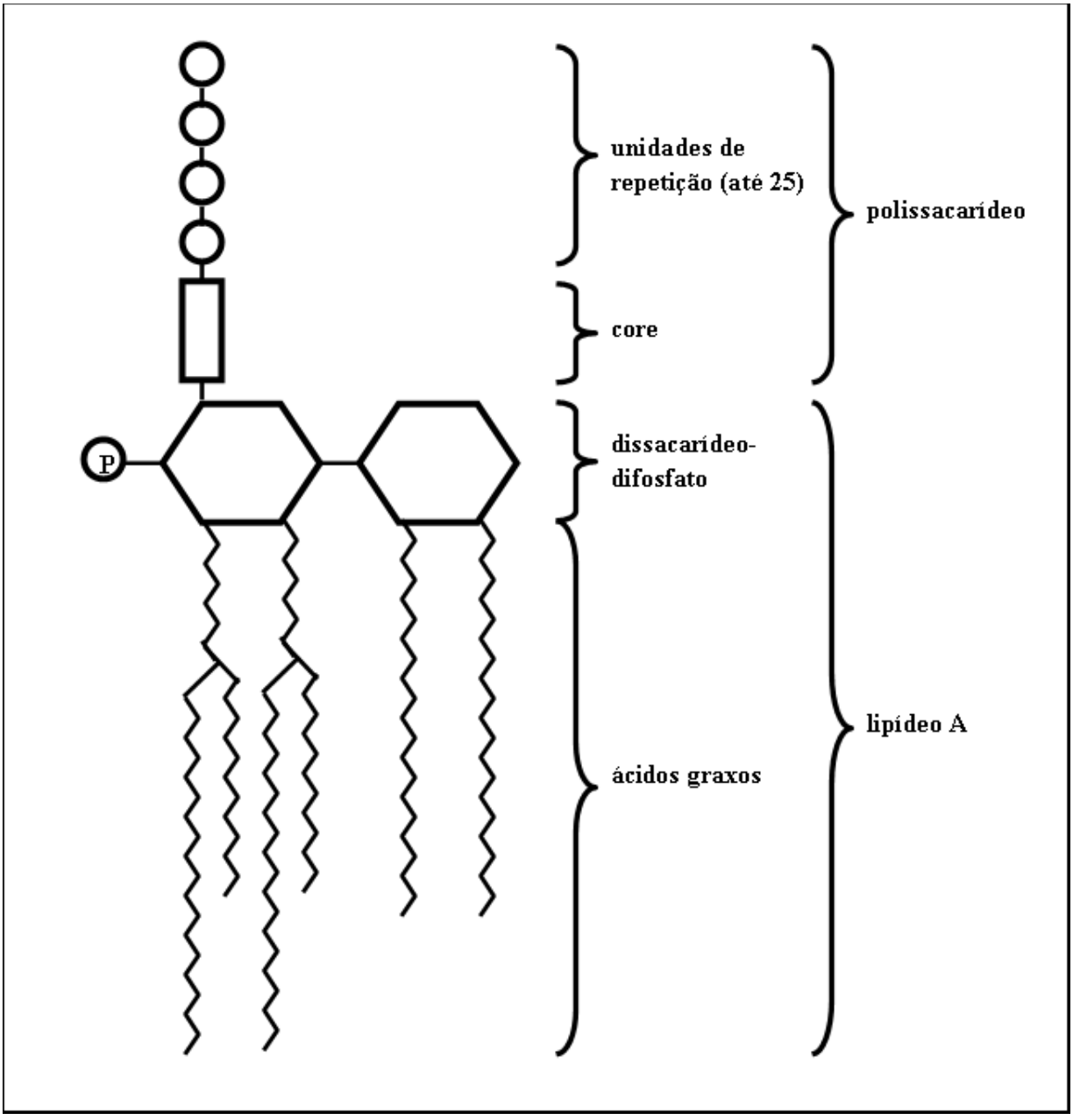
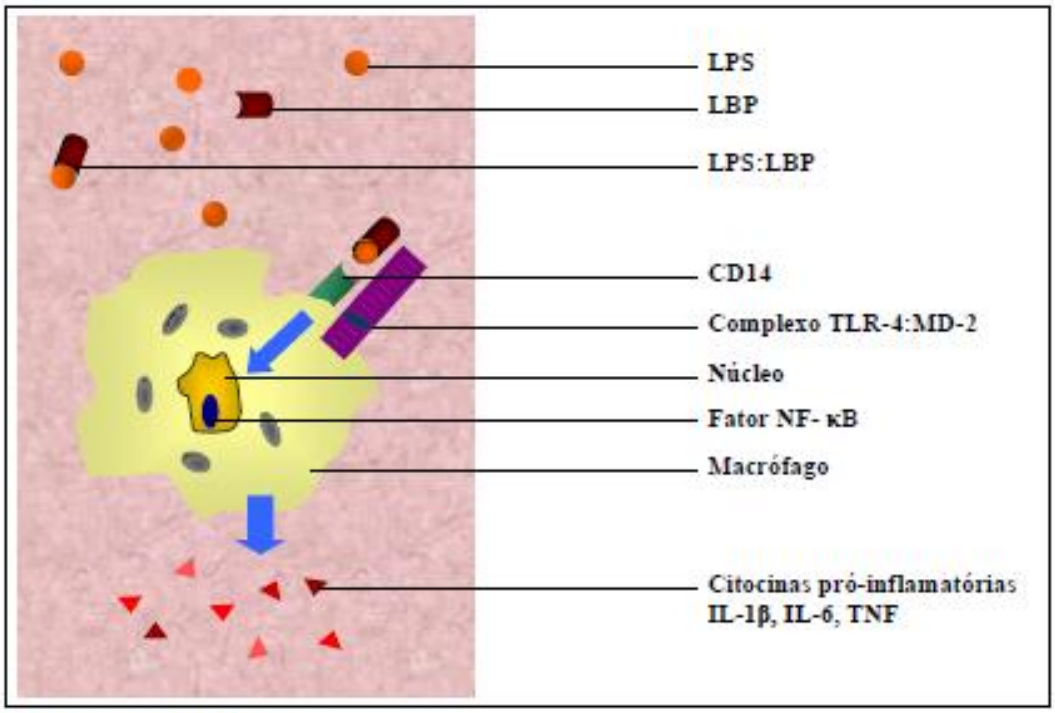


Figura 1 - LPS da parede celular de bactérias gram-negativas. Fonte: adaptado de BROOKS et al., 2000.

1.2. Mecanismo de ação do LPS

Uma vez que o LPS entra em contato com o organismo animal, seja a partir de uma bactéria gram-negativa como a *Escherichia coli*, ou pela administração direta da substância, inicia-se uma série de respostas no organismo infectado. Esta endotoxina pode atuar em macrófagos, monócitos, neutrófilos, plaquetas sanguíneas e células endoteliais [4]. Tomando como exemplo o mecanismo de ação por meio do macrófago, inicialmente, o LPS que se encontra no plasma liga-se a uma proteína de fase aguda do hospedeiro, o LBP (proteína ligadora de LPS, ou *lipopolysaccharide binding protein*), produzida no fígado do animal. A partir deste passo, é formado um complexo chamado de LPS:LBP. O complexo transfere o LPS para a proteína de membrana periférica CD14 na superfície dos macrófagos, iniciando a ativação celular [2,5,6]. A figura 2 ilustra o mecanismo de ação simplificado do LPS no macrófago. O novo complexo formado, chamado de LPS:CD14, ativa a sinalização do receptor semelhante ao Toll (ou *toll-like receptor*, TLR)-4, ao qual é complexada à proteína MD-2. Neste momento é iniciada a geração do sinal transmembranar para o núcleo. Dentro do macrófago ocorre uma série de reações em cascata, incluindo a atuação de MyD88, IRAK,TRAF6, TAK-1, quinase IkB, AP-1, dentre outras (algumas ainda não elucidadas), até a ativação do fator de transcrição NF-kB, que ativa os genes que codificam as proteínas envolvidas na defesa contra a infecção, que são as citocinas pró-inflamatórias [2,7,8]

Entre as citocinas pró-inflamatórias ativadas e liberadas a partir do contato com o LPS, destacam-se a interleucina 1 beta (IL-1β), a interleucina 6 (IL-6) e o TNF-α, além de algumas outras [9]. O LPS é capaz de ativar principalmente a resposta imune inata (inespecífica) com a participação dos macrófagos. Atua também na resposta imune adquirida (ou adaptativa), referente a respostas de linfócitos que reconhecem antígenos microbianos específicos (com atuação dos TLR-4, na ativação de membros da família B7, que ativam células T *naive*) [2,10].



Fonte: KIRSTEN, T. B., 2012

Figura 2 - Mecanismo de ação simplificado do LPS em um macrófago, culminando com a liberação de citocinas pró-inflamatórias.

Em condições normais, o LPS e as citocinas não são capazes de atravessar a barreira hematoencefálica em quantidades significantes [11,12]. Apesar disso, as citocinas liberadas por meio do LPS podem atuar no SNC do animal, onde interferem com sua homeostasia.

As vias pelas quais as citocinas modulam a ação do SNC são:

Através do nervo vago (que é a principal via aferente da cavidade abdominal para o cérebro): as citocinas liberadas podem entrar em contato com terminações de ramificações vagais periféricas, as quais possuem receptores para citocinas. A ativação desses receptores inicia a transmissão de um impulso nervoso pelo nervo vago aferente até sua ligação no encéfalo (no núcleo vagal). A importância dessa via é demonstrada por meio da vagotomia em roedores, e posterior injeção i.p. de IL-1, resultando na ausência do comportamento doentio nestes animais [13].

Outra via envolve os órgãos circunventriculares – regiões desprovidas da barreira hematoencefálica: as citocinas liberadas chegam até a circulação sanguínea e assim acessam o encéfalo do animal, onde são barradas pela barreira

hematoencefálica. Para adentrar o cérebro, elas acessam os órgãos circunventriculares. É possível confirmar a importância desta via, pois são detectados níveis elevados de certas citocinas pró-inflamatórias após infecção nos órgãos circunventriculares como a área postrema, eminência mediana e órgão vasculoso da lâmina terminal, comparados a outras áreas do cérebro[12].

As citocinas atuam ainda a partir do contato com células endoteliais do organismo: o LPS e as citocinas, que em contato com os receptores das células endoteliais, induzem a ativação da enzima ciclooxigenase (COX), iniciando uma resposta no organismo, que leva à produção de eicosanoides (mediadores inflamatórios de origem lipídica), como as prostaglandinas, leucotrienos e tromboxanos. Esses eicosanoides têm propriedades físico-químicas que os possibilitam, via corrente sanguínea, acessar o cérebro, atravessando a barreira hematoencefálica, podendo assim induzir processos patológicos. Para mostrar a relevância desta via, trabalhos utilizam inibidores de eicosanoides, como por exemplo, inibidores da enzima COX-2, resultando na supressão do comportamento doentio [4,12,14-16].

Outras vias também podem contribuir para a atuação das citocinas no cérebro, como quando as citocinas cruzam a barreira hematoencefálica usando sistemas de captura específicos, porém, especialistas consideram a capacidade desses sistemas relativamente baixa [12].

Finalmente, o LPS é ainda capaz de produzir a enzima óxido nítrico sintase, que leva à produção do óxido nítrico, que é um importante mediador inflamatório, com ação vasodilatadora, podendo agir também no SNC [15,17]. Provavelmente, esses distintos mecanismos atuam simultaneamente, de forma integrada, quando da liberação de citocinas [12].

Diversas regiões do cérebro expressam receptores para diversas citocinas (incluindo IL-1β, TNF-α e IL-6) tanto na glia quanto nos neurônios [18]. Aventa-se que a micróglia em contato com as citocinas liberadas pelo LPS, estimula a produção de novas citocinas no próprio cérebro, potencializando o seu efeito. Neste sentido, a micróglia é considerada um análogo dos macrófagos e “órgão imune” do cérebro, com função de combater infecções e a inflamação [9,16,19].

1.3. Citocinas

Normalmente, as citocinas atuam no organismo a fim de combater diversos patógenos. No sistema imune elas participam de respostas adaptativas ou reações homeostáticas [2,18]. Dentre outras funções, as citocinas pró-inflamatórias funcionam como sinalizadores moleculares do sistema imune para informar o cérebro sobre inflamação periférica [20].

Muitos fatores imunes são liberados e participam no sentido de remover o patógeno invasor, agindo localmente além de orquestrar uma complexa difusão de alterações através de todo o organismo [18]. O problema ocorre quando existe liberação excessiva de mediadores pró-inflamatórios, que desencadeiam respostas exacerbadas, tornando-se prejudiciais ao funcionamento do organismo , levando-o à inflamação sistêmica associada com o desenvolvimento de sérias complicações, podendo até mesmo levar ao choque séptico e morte do indivíduo [20].

No SNC as citocinas podem modular neurotransmissores centrais como dopamina, serotonina, noradrenalina, ácido gama-aminobutírico (GABA), acetilcolina, neuropeptídeos, dentre outros. Atuam ainda na diferenciação e crescimento neuronal, na migração dos neurônios para seus alvos e na modificação da plasticidade sináptica. Portanto, em níveis fisiológicos, as citocinas desempenham importantes papéis no cérebro, como, por exemplo, na neurogênese, neuromodulação, na memória e no sono [21,22]. Porém, as citocinas podem causar morte celular durante o desenvolvimento cerebral [12,23,24]. As citocinas podem também ativar o eixo HPA com a liberação do fator liberador de corticotrofina do hipotálamo, que secreta o hormônio adrenocorticotrópico da glândula pituitária, resultando em aumento de glicocorticoides na corrente sanguínea periférica. Esses glicocorticoides têm função básica de frear a ativação do sistema imune. Em níveis elevados no SNC e em exposições crônicas são prejudiciais ao indivíduo,sendo conhecidos como os hormônios do estresse, podendo estas substâncias podem causar danos, como por exemplo, a morte de neurônios ([25].

Além disso, as citocinas podem inibir o eixo hipotálamo-pituitária-gonadal, por reduzir a secreção de hormônios sexuais (hormônio gonadotrófico, hormônio

luterizante, hormônio folículo estimulante e esteroides ovarianos), interferindo na modulação do comportamento reprodutivo [26,27]. Em resposta a infecções periféricas, células imunes inatas produzem citocinas pró-inflamatórias que agem no cérebro produzindo uma série de alterações comportamentais que se enquadram e podem ser definidas como o comportamento doentio [13,28]. O comportamento doentio é geralmente acompanhado pela diminuição da atividade exploratória, da interação social, do interesse sexual, perda de apetite, anedonia e prejuízos cognitivos e no aprendizado [29]. Essa série de alterações é uma estratégia comportamental e adaptativa do organismo, visando o combate ao microrganismo invasor e a cura rápida [30].

1.4. Mecanismo de ação do LPS na infecção pré-natal

O LPS normalmente não é capaz de chegar até o feto. Já se verificou que após a administração de LPS radiomarcado com iodo (^{125}I-LPS) em ratas prenhes, ele foi detectado no sangue, fígado, rins e placenta das mães entre 1-8 h, porém nada foi encontrado no feto. Observou-se, no entanto, a indução de citocinas em um período de 2-8 h no plasma materno. Este fato, somado à presença de LPS na placenta, sugere que o LPS deve agir diretamente nas células placentárias para induzir a expressão de mediadores inflamatórios. Portanto, as alterações encontradas na prole não são produzidas diretamente pela endotoxina, pois o LPS parece não sofrer passagem transplacentária [11].

Ainda foram encontrados, níveis elevados de citocinas na placenta, no fluído amniótico, no sangue e cérebro fetal (inclusive com a indução de genes de citocinas pró-inflamatórias no cérebro fetal após administração de LPS na mãe), bem como a ocorrência de inflamação de membranas fetais, após infecções e a inflamação materna. Sabe-se também da existência de TLRs na placenta e em membranas fetais [26,31-33].

As citocinas acessam o cérebro fetal por diferentes maneiras: a maioria vem do lado materno produzidas no útero e placenta durante a infecção intrauterina, atravessando a barreira hematoencefálica imatura do feto e acessando o SNC. Além

disso, as citocinas podem ser produzidas na micróglia e nos astrócitos do cérebro fetal a partir da estimulação de citocinas oriundas da mãe [31]. Essas citocinas podem interferir na homeostasia do ambiente fetal, alterando o desenvolvimento de seu eixo neuroimune [16,34].

Além das citocinas, os glicocorticoides também podem atuar nos filhotes, sendo capazes de atravessar a barreira hematoencefálica e influenciar processos de Desenvolvimento cerebral dos fetos. Assim, já foi documentada a liberação de hormônio corticotrópico no cérebro fetal após administração de LPS em ratas gestantes, sugerindo a possibilidade de indução da resposta estressora no feto [35].

Portanto, citocinas e glicocorticoides podem ser os responsáveis indiretos pelos danos encontrados nos filhotes expostos prenatalmente ao LPS e essas alterações podem perdurar até mesmo na idade adulta do animal [9,32].

2. SOBRE O COMPORTAMENTO MATERNAL

Durante o desenvolvimento, o SNC é extremamente plástico para as intervenções do ambiente. A experiência é essencial durante as primeiras semanas pós-natal em que as atividades sensoriais se refinam e estabelecem conexões neurológicas estáveis. Sabe-se que, os filhotes de mãe que passaram por algum evento ou stress durante a gestação podem apresentar alterações no seu desenvolvimento global, desta forma o comportamento maternal se apresenta como fundamental para o desenvolvimento inicial de recém-nascidos [36].

O comportamento maternal é um comportamento complexo, instintivo, com características específicas para cada espécie e que consiste em uma série de cuidados que as fêmeas maduras realizam em torno dos indivíduos imaturos para auxiliar na propagação de sua espécie, sendo um fator determinante no desenvolvimento neurológico [37].

Os cuidados maternais se expressam desde a preparação da mãe para o nascimento da prole e se mantêm por todo o período de lactação dos filhotes. Esse comportamento vai se modificando de acordo com o tempo e crescimento dos filhotes. Durante este período, o principal objetivo da fêmea é garantir a sobrevivência dela e dos seus filhotes [37,38].

De acordo com os autores, na preparação para o parto, a mãe prepara o ninho para acolher os filhotes na hora do parto. Nos primeiros 10 dias após o parto, as mães permanecem mais tempo no ninho e à medida que os filhotes crescem se tornando mais independentes em relação à mãe e à própria independência, os cuidados maternos tendem a decrescer e a mãe se torna menos responsiva em relação aos filhotes. Com o ganho de independência dos filhotes e a diminuição da responsividade materna, o desmame tende a acontecer naturalmente.

Em ratas, os cuidados maternais são observados e registrados quando os comportamentos são relacionados aos filhotes como a busca, o agrupamento, ficar sobre os filhotes aquecendo-os e os alimentando, além de comportamentos indiretos como agressividade e construção do ninho.

A figura abaixo mostra alguns parâmetros típicos do comportamento maternal em ratas, como a recuperação, o agrupamento, a postura de amamentação em que a coluna fica arqueada facilitando assim a amamentação (“*crouching*”) e o comportamento maternal total de acordo com Numan [37]. Além desses comportamentos, a mãe passa um tempo significativo lambendo para limpar seus filhotes, pois a estimulação na área genital estimula a defecação e micção assim como a diferenciação sexual do cérebro da prole masculina [39].

O comportamento maternal ocorre e é controlado pela interação de diversos fatores neuroendócrinos, neuroanatômicos, ambientais e comportamentais. As mudanças hormonais, principalmente os níveis de estrógeno, prolactina, progesterona, vasopressina, colecistocinina e β-endorfina estão diretamente relacionadas à preparação pré e pós-parto e ao comportamento maternal. A ocitocina também está envolvida no processo, porém relacionada ao reflexo de ejeção de leite [38,40].

Com relação aos fatores neuroanatômicos envolvidos no comportamento maternal são descritos na literatura, sobretudo a área pré-optica (POA), área tegmental ventral (ATV) e substância cinzenta periaquedutal (PAG). Essas estruturas são responsáveis pela motivação, controle motor e modulação da transmissão proprioceptiva entre outras [38].

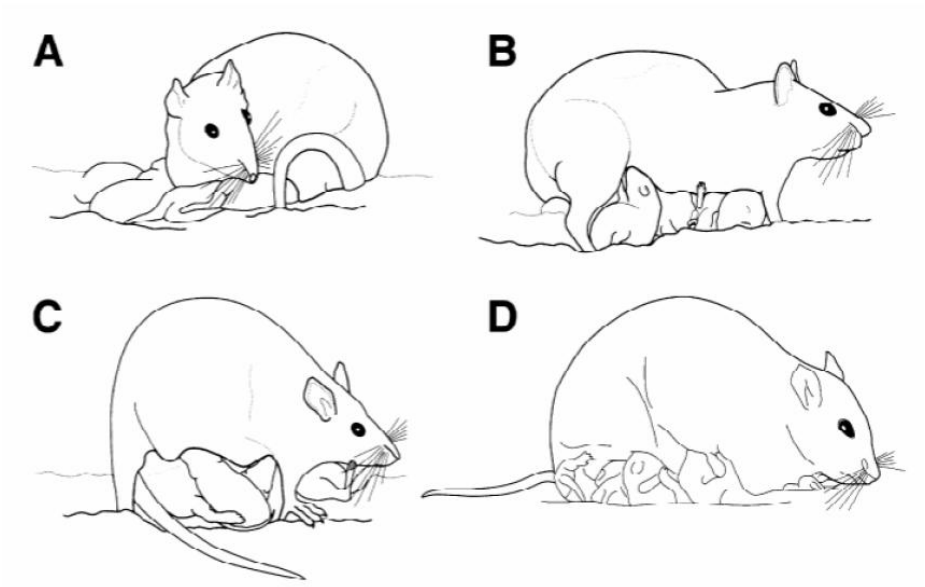


Figura 3 - Parâmetros do comportamento maternal em ratas. Agrupamento de filhotes (A), preparo da postura de amamentação (B), “crouching” ou cifose fisiológica (C) e comportamento maternal total (D) (NUMAN, 1994 apud TEODOROV, 2008).

O controle do comportamento maternal envolve fatores neuroendócrinos e neuroanatômicos. Os hormônios gestacionais preparam o animal para agir de forma maternal para com o filhote, já os neurotransmissores regulam o comportamento maternal durante a fase de manutenção e lactação [41]. A primeira fase da regulação do comportamento maternal determina o início rápido deste no pós-parto, sendo controlada por hormônios relacionados com a gestação e lactação (estrógeno, progesterona, prolactina e ocitocina). A segunda fase, a de manutenção durante a lactação, é controlada principalmente por fatores não hormonais, na qual o estímulo proveniente do filhote se mostra o mais importante [42].

O comportamento maternal, portanto, é resultado da interação entre diversos fatores maternos e é fundamental para o desenvolvimento e sobrevivência do filhote. Desta forma, as alterações no período gestacional podem alterar o comportamento maternal interferindo no desenvolvimento neurológico, comportamental e sexual do filhote, além das possíveis alterações provenientes do período gestacional [37,38,40]. Interferências no cuidado materno promovidas são cruciais no desenvolvimento e na expressão comportamental da prole por alterar a programação do seu desenvolvimento.

2.1. Sobre a relação materno-filhote

Infecções prenatais interferem com o sistema sensorial dos animais. Por exemplo, a exposição ao LPS ou ao polyriboinosinic-polyribocytidilic acid (polyI:C, que mimetiza infecções virais) em ratos e camundongos prejudica a aquisição de informações cognitivas e sensoriais, em modelo de inibição da resposta do reflexo acústico [43].

O sistema olfatório dos ratos, assim como dos mamíferos, é capaz de detectar e discriminar milhares de diferentes moléculas no ambiente e essa habilidade é crucial para o seu desenvolvimento e sobrevivência. Apesar de estar presente desde o nascimento, o sistema olfatório se desenvolve durante a vida do animal e se aprimora de acordo com as informações olfatórias adquiridas e armazenadas. Os filhotes de ratos são menos sensíveis a odores do que ratos

adultos, possivelmente pela quantidade limitada de inervações sensoriais formadas nesta etapa, porém, mesmo na infância, os filhotes já são capazes de se guiar e utilizar as pistas olfatórias para sua sobrevivência [44].

Kirsten et al [45] mostram que a exposição no 9,5 dia da gestação não modifica o comportamento maternal de ratas, porém reduz a preferência dos filhotes para se encaminhar para a maravalha com odor da mãe quando comparados àqueles filhotes do grupo controle. Além disto, verifica-se decréscimo da dopamina do bulbo olfatório destes filhotes. Portanto a exposição a endotoxina leva ao prejuízos no reconhecimento materno pelos filhotes e, este fato, não foi consequência de interferências com o cuidado materno das fêmeas tratadas com LPS.

O estudo das consequências da exposição ao LPS nas demais gerações, torna-se importante quando infecções ocorrem no período perinatal uma vez que podem interferir tanto no cuidado maternal como no reconhecimento da mãe pelo filhote.

Nesta revisão foram enfocados diversos aspectos da interação entre o sistema imune e o sistema nervoso central, em particular durante a gestação e do processo do desenvolvimento dos seus descendentes. São várias a linhas que investigam estas relações cujo âmbito é interdisciplinar, sendo a área denominada de neuro-endócrino-imunomodulação. A visão ampliada dos diferentes aspectos e efeitos de processos inflamatórios durante o período do desenvolvimento abre espaços para o entendimento de diversas doenças, em particular àquelas ligadas às doenças mentais.

3. OBJETIVO GERAL

Este trabalho tem como objetivo investigar a interação materna- filhotes em duas gerações quando a geração parental recebeu no GD18 o LPS.

3.1. Objetivos específicos

1. Avaliar os efeitos da exposição de dose única do lipopolissacarídeo na interação materno-filhote e nos níveis de TNF-α séricos da prole após desafio com a mesma endotoxina.
2. Avaliar os efeitos transgeracionais da exposição antenatal no comportamento maternal e dos filhotes em duas gerações.

4. CONSIDERAÇÕES GERAIS E DISCUSSÃO

Na avaliação dos efeitos da exposição de dose única do lipopolissacarídeo no GD18 na interação materno-filhote das gerações parental e F1 observou-se facilitação do comportamento maternal e redução do comportamento maternal agressivo. No entanto, este tratamento não modificou o desempenho reprodutivo das fêmeas. Em relação aos filhotes, no LD21, a administração de uma dose desafio da mesma endotoxina aumentou o peso corporal e a duração de imobilidade no teste do campo aberto. Além disto, esta dose desafio mostrou que a exposição pré-natal ao LPS induziu tolerância à mesma endotoxina, expressa por menor aumento nos níveis séricos de TNFα .

No comportamento maternal das fêmeas tratadas prenatalmente com LPS observou-se redução na latência para a busca do primeiro filhote quando comparada àquela do grupo controle. Em contraste, no comportamento maternal agressivo, verificou-se nestes animais redução no número de ataques e na duração do tempo de briga. Estes dados, aparentemente contraditórios, foram interpretados como resultado do comportamento doentio induzido pelo LPS. De fato, esta endotoxina promove febre e no caso, a administração do LPS no GD18 pode ter sinalizado para a fêmea que o ambiente estaria frio aumentando a motivação materna para a busca do filhote. Neste sentido, ratos ao nascer não controlam a temperatura corporal, sendo provável que a menor latência para a busca do primeiro filhote tenha sido causada pela sinalização nas fêmeas e necessidade de buscar e aquecer sua prole. Além disto, como a fêmea cuidou mais de sua prole, ela selecionou este comportamento em detrimento da proteção contra um macho invasor.

Com relação aos filhotes, verificou menor preferência pelo odor materno na prole exposta ao LPS sugerindo menor reconhecimento materno. Desde que, a atividade geral destes filhotes não foi modificada pela exposição pré-natal ao LPS, interpretou-se este dado como devido à redução na motivação da prole promovida pela endotoxina pré-natal. De fato, estudos anteriores mostraram que ratos expostos prenatalmente ao LPS tinham sua interação social reduzida por apresentar menor motivação [46].

Para testar a sensibilidade destes filhotes ao LPS foram avaliadas a atividade geral em campo aberto e os níveis séricos de TNFα aos 21 dias de idade após desafio com uma dose adicional da endotoxina.

Notou-se que ocorreu aumento na imobilidade nos animais tratados prenatalmente com salina e aos 21 dias com LPS, tendo sido sugerido que este fato tenha sido consequência do desenvolvimento de comportamento doentio. Por outro lado, ratos tratados prenatalmente com LPS e desafiados com a mesma endotoxina não apresentaram alterações na atividade geral bem como atenuação dos níveis séricos de TNFα, fato interpretado como tolerância aos efeitos do LPS.

Na geração F1 notou-se que a exposição pré-natal ao LPS modificou o comportamento maternal e maternal agressivo sem, no entanto, alterar sua atividade geral. Na geração F2, a exposição antenatal reduziu o peso corporal no desmame e a preferência pelo odor maternal. Na idade adulta, tanto a prole tratada antenatalmente com LPS como aquela tratada com salina foram subdivididas em dois grupos, dois dos quais foram isolados por uma semana enquanto que os demais grupos permaneceram agrupados. Este procedimento teve como objetivo revelar se o tratamento antenatal com LPS teria promovido alterações no sistema nervoso central dos mesmos uma vez que o isolamento representa um estresse para os animais. De fato, embora, a atividade geral dos ratos do grupo LPS+ LPS (tratados antenatalmente com LPS e desafiados na idade adulta com a mesma endotoxina) não tenha sido modificada, no teste do labirinto elevada estes animais apresentaram menores índices de ansiedade.

No comportamento maternal notou-se redução na latência para o “crouching”, mas ocorreu aumento no tempo de emissão deste comportamento. O comportamento de “crouching” é uma postura quiescente e, geralmente, ocorre em resposta à estimulação das crias. Fêmeas lactantes tendem a reduzir outras atividades e apresentar uma postura característica com suas extremidades abertas e costas arqueadas. A finalidade desta postura é permitir que o filhotes tenham acesso às tetas e ao leite, para regular a sua temperatura, e para protegê-los de agressões ambientais. Desta forma, mesmo com redução na latência para o comportamento de “crouching”, a maior duração do mesmo pode ter compensado este atraso no cuidado maternal. Portanto, é improvável que a redução de peso ao

final do desmame se deva á disponibilidade das fêmeas em amamentar sua prole. Duas hipóteses podem explicar estes efeitos. Primeiro, o atraso na expressão do " crouching" pode ter sido causado por uma menor estimulação da cria em relação á sua mãe, pois notou-se que no teste de preferência olfatória estes filhotes apresentaram menor atividade voltada à sua mãe. Em segundo lugar, não se pode descartar que as mães da geração F1 podem ter tido redução na disponibilidade de leite e com isto os filhotes apresentaram redução no peso corporal ao no desmame. Note-se que logo após o nascimento estes filhotes não tinham alterações no peso corporal indicando que *intra-útero* não houve prejuízos no aporte de nutrientes à prole. Não se pode ainda descartar que o atraso na expressão do "crouching" possa ter sido motivado pela menor estimulação promovida pelas crias e a maior duração do mesmo pela tentativa de fornecer mais leite à prole.

Estes dados indicam que a exposição pré-natal e antenatal ao LPS promove alterações na interação materno-filhote que se reflete na geração F2 em menor desenvolvimento corporal e redução do estímulo da prole em relação à sua mãe.

Na idade adulta destas proles foram analisadas a atividade geral em campo aberto e a resposta no teste do labirinto elevado. Neste caso, tanto os animais do grupo antenatalmente tratados com LPS ou solução salina foram subdivididos em dois grupos e obtiveram-se quatro grupos, a saber: S+AS (tratados com salina antenatalmente que receberam solução salina na idade adulta agrupados), S + SI (tratados com salina antenatalmente que receberam solução salina na idade adulta isolados), LPS + LPSA (tratados com LPS antenatalmente que receberam outra dose de LPS na idade adulta agrupados) e LPS + LPSI (tratados com LPS antenatalmente que receberam outra dose de LPS na idade adulta isolados).

A análise dos efeitos em longo prazo da prole masculina das ratas da geração F2 indicou menores níveis de ansiedade da prole antenatalmente tratada com o LPS que foi isolada. Neste caso, o isolamento revelou alterações na emocionalidade dos animais antenatalmente expostos a endotoxina as quais, por adaptação em condições normais da vida do animal não se expressariam. No caso da atividade geral, o isolamento aumentou a atividade geral quer seja em animais do grupo controle quer seja no caso dos animais do grupo experimental. Portanto, este modelo não foi capaz de revelar as alterações promovidas pela exposição antenatal

ao LPS.

É fato conhecido que o estresse em períodos precoces da vida leva a alterações na reatividade ao estresse a qual persiste ao longo da vida até a idade adulta. De fato, intervenções severas maternas como, por exemplo, a separação materna, e mesmo a manipulação pré-natal, que representa um estresse menos severo, sensibilizam o eixo hipotálamo-hipófise-adrenal e levam a um fenótipo resiliente ao estresse. Este efeito pode se refletir nas demais gerações por mecanismos denominados de epigenéticos.

O termo epigenética refere-se a todas as mudanças reversíveis e herdáveis no genoma funcional e que não alteram a sequência de nucleotídeos do DNA [47]. De acordo com Hunter [48] o controle epigenético é a soma dos fatores genéticos e não genéticos que agem sobre as células de forma a controlar seletivamente a expressão dos genes, produzindo assim o aumento da complexidade fenotípica durante o desenvolvimento. Dessa forma, seu estudo direciona-se a compreensão dos padrões de expressão transmitidos aos descendentes, sua mudança de expressão de genes durante a diferenciação de um tipo de célula e como os fatores ambientais podem modificar a expressão de genes.

Os principais mecanismos de alterações epigenéticas são: (1) metilação do DNA; (2) modificações de histonas e (3) ação de RNAs não codificadores. Os padrões de metilação de DNA são os mais conhecidos destes três mecanismos, embora modificações de histonas também sejam bastante discutidas [48].

A metilação do DNA está relacionada normalmente ao silenciamento de genes. As acetilações, fosforilações e ubiquitinações são modificações de histonas já melhor estudadas. Já a ação de RNAs não codificadores está relacionada ao silenciamento póstranscricional de genes através do mecanismo de RNA de interferência onde ocorre o bloqueio da tradução ou degradação do RNAm alvo. Além, da ação bloqueadora da transcrição, os siRNA podem ser associados à metilação de seqüências de DNA. Todos estes mecanismos parecem estar interligados para a organização estrutural da cromatina tornando-a mais acessível ou não aos fatores de transcrição[49].

As mudanças epigenéticas são fortemente influenciadas pelo ambiente, de forma que alterações no mesmo, promovidas por ataques de patógenos, tipo de alimentação, etc., podem acarretar mudanças epigenéticas (Figura 4). Ou seja, é um processo pelo qual o genótipo de um organismo interage com o meio ambiente para produzir o seu fenótipo. Sendo assim, a epigenética está intimamente relacionada com o aumento de variabilidade fenotípica dos indivíduos resultando em uma relevante importância para a evolução [50].

Uma vez que o ambiente tem suma importancia na forma como o organismo irá se desenvolver, estressores que prejudiquem o desenvolvimento pré e pós-natal podem ter efeitos profundos na vida adulta desse organismo. No caso de mamiferos, o cuidado materno e a nutrição são fatores ligados a qualidade do ambiente no início da vida. Em roedores, o cuidado materno é caracterizado por comportamentos complexos e que influenciam fortemente o desenvolvimento de respostas comportamentais, tais como o nível de resposta à ansiedade e ao stress [51]. Estressantes na vida adulta também possuem um forte impacto. Experimentos em ratos, tal como o teste do nado forçado aumenta a fosforilação no giro denteado de ratos e camundongos. Entretanto, essa alteração não é encontrada em outros testes que induzem estresse tal como a exposição ao éter ou baixa temperatura. Embora exista uma herança epigenética em vertebrados ela é considerada uma herança leve e pode ser dividida em dois tipos: modificações que influenciam a aparência morfológica, e modificações relacionadas à susceptibilidade à doenças, e que podem ser alterada por fatores ambientais [51].

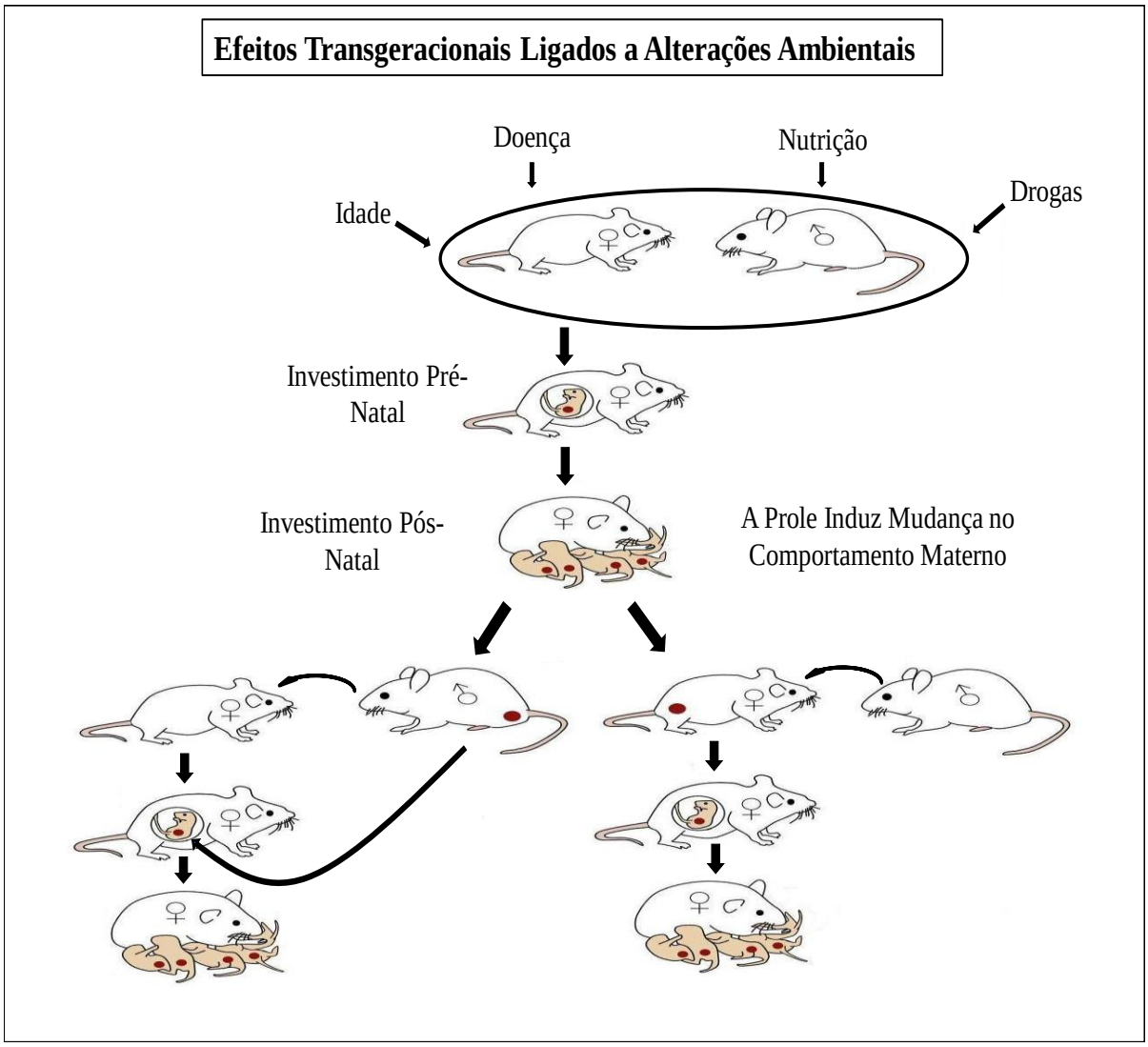


Figura 4 - Vias não genômicas de alterações no desenvolvimento. Fatores como drogas, nutrição, toxina e idade podem pode levar a alterações epigenéticas (círculo vermelho), que são então transmitidos à prole com consequências para a variação fenotípica. Esta alteração pode conduzir a diferencial pré-natal e / ou pós-natal no investimento materno, afentado o desenvolvimento das crias gerado a partir deste cruzamento com consequências para variação da prole fenotípica. Investimento materno também pode variar como uma função das variações paternalmente mediadas no fenótipo descendentes durante períodos tanto o pré-natal e / ou pós-natal. Investimentos diferenciais maternos como uma função de experiências paternas ou traços descendentes poderão servir tanto para aumentar a transmissão de exposições paternas ou compensar défices de funcionamento que são induzidas por estas experiências ambientais (adaptado de CURLEY, MASHOODH, CHAMPAGNE[52]).

Catalani et al. [53] observaram que ratas expostas durante a lactação á corticoesterona apresentavam melhor desempenho em testes de memória espacial e aprendizado de evitação condicionada do desmame até os 15 meses de vida, mas não no período pré-desmame. Além disto, esta exposição atenuou o medo

condicionado do primeiro até os 15 meses de vida. Estes dados evidenciaram que tanto a corticoesterona como o estresse pré-natal têm impacto pronunciado epigenético tanto em seres humanos como em modelos animais e que a relação entre a resposta ao stress e epigenética no cérebro é bidirecional [48]. Neste trabalho, a exposição pré-natal ao LPS melhorou alguns aspectos de cuidados maternos da geração F1 relacionados à amamentação e sobrevivência da prole, mas não na motivação materna, provavelmente devido a estimulação do filhotes em relação a mãe. De fato, na geração F2, a exposição antenatal ao LPS reduziu o reconhecimento materno na infância. Além disso, observou-se efeitos transgeracionais conduzindo a um fenótipo mais resistente a ansiedade. Se estes fenômenos são derivadas de um mecanismo de epigenético ainda esta por ser melhor investigado.

5. CONCLUSÃO

1- Na avaliação dos efeitos da exposição de dose única do lipopolissacarídeo no GD18 na interação materno-filhote das gerações parental e F1 observou-se facilitação do comportamento maternal e redução do comportamento maternal agressivo. No entanto, este tratamento não modificou o desempenho reprodutivo das fêmeas. Em relação aos filhotes, no LD21, a administração de uma dose desafio da mesma endotoxina aumentou a o peso corporal e a duração de imobilidade no teste do campo aberto. Além disto, esta dose desafio mostrou que a exposição pré-natal ao LPS induziu tolerância à mesma endotoxina, expressa por menor aumento nos níveis séricos de TNFα .

2- Na geração F1 notou-se que a exposição pré-natal ao LPS modificou o comportamento maternal e maternal agressivo sem, no entanto, alterar sua atividade geral. Na geração F2, a exposição antenatal reduziu o peso corporal no desmame e a preferência pelo odor maternal. Na idade adulta, tanto a prole tratada antenatalmente com LPS como aquela tratada com salina foram subdivididas em dois grupos, dois dos quais foram isolados por uma semana enquanto que os demais grupos permaneceram agrupados. Este procedimento teve como objetivo revelar se o tratamento antenatal com LPS teria promovido alterações no sistema nervoso central dos mesmos uma vez que o isolamento representa um estresse para os animais. De fato, embora, a atividade geral dos ratos do grupo LPS+ LPS (tratados antenatalmente com LPS e desafiados na idade adulta com a mesma endotoxina) não tenha sido modificada, no teste do labirinto elevada estes animais apresentaram menores índices de ansiedade.

Estes resultados mostram que a administração de LPS na geração parental levou a efeitos transgeracionais na interação mãe-filhote em duas gerações. Estes dados foram atribuídos a alterações epigenéticas induzidas pela endotoxina.

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ANEXOS

ANEXO 1 – APROVAÇÃO PELO COMITÊ DE ÉTICA

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Vice-Reitoria de Pós-Graduação e Pesquisa

CERTIFICADO

CERTIFICAMOS, que o protocolo nº 048/09CEP/ICS/UNIP, sobre o projeto de pesquisa intitulado "EFEITOS TRANSGERACIONAIS DA ADMINISTRAÇÃO DO LIPOPOLISSACARÍDEO (LPS) NO COMPORTAMENTO MARTERNO DE RATOS", sob a responsabilidade de MARIA MARTHA BERNARDI E SANDRA HELOISA NUNES WHITAKER PENTEADO está de acordo com os Princípios Éticos, seguindo as diretrizes e normas regulamentadoras de pesquisa envolvendo animais, conforme a Lei Estadual nº 11.977/05 que institui o Código de Proteção aos Animais do Estado de São Paulo e foi aprovado por este Comitê de Ética em Pesquisa.

Universidade Paulista, em São Paulo-SP, aos 12 dias do mês de novembro de 2009.

Hailey Barros F. Gonçalves

Hailey Barros F. Gonçalves
Secretária do Comitê de Ética
em Pesquisa da UNIP

Comitê de Ética em Pesquisa – UNIP – R. Dr. Bacelar, 1212 CEP 04026-002 São Paulo-SP

Campus: INDIANÓPOLIS
Rua: Doutor Bacelar, 1212 – Vila Clementino – São Paulo – SP – CEP: 04026-000
Fone: (11) 5586-4091 – Fax: (11) 5586-4073
E-mail: cep@unip.br – http://www.unip.br

ANEXO 2 – ARTIGO

Prenatal lipopolysaccharide (LPS) increases maternal behavior, decreases maternal odor preference and induces endotoxin hyporesponsiveness

Penteado, S. H. N. W [a]; Massoco, C.O. SG[c]., Kirsten, T. B. [c]; Reis-Silva, T. M. [c] ; Melo Acenjo, M. K [a]; Queiroz-Hazarbassanov, N.[c]; Bernardi, M. M. [a, b]

[a] Health Sciences Institute, Paulista University, Rua Dr. Bacelar, 1212, 04026-002, Sao Paulo, SP, Brazil.

[b] Centro de Matemática, Computação e Cognição, Universidade Federal do ABC.Rua Santa Adélia, 166, Torre 2, Bloco A, sala 508-2, Santo André, SP, Brasil,09210-580

[c] Department of Pathology, School of Veterinary Medicine, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, 05508–270, São Paulo, SP, Brazil

Corresponding author:

Centro de Matemática e Cognição, Universidade Federal do ABC.Rua Santa Adélia, 166, Bloco A, 5°andar, Santo André, SP, Brasil, cep: CEP 09.210-170.

Tel/Fax: +55 11 4996-7950

e-mail: marthabernardi@gmail.com

Abstract.

This study investigated whether late maternal inflammation disrupts the mother/pup interaction, resulting in long lasting effects on pups' behavior and altering the biological pathways, thereby programming prepubertal behavior and the pups' inflammatory responses after an additional bacterial endotoxin treatment. Female rats received 100 µg/kg of LPS or saline solution 0.9% on gestation day 18. At birth the reproductive performance was observed. On lactation days (LD) 5 and LD 6, respectively, the maternal behavior and maternal aggressive behavior were performed. In pups, maternal odor preference (LD 7), open field behavior (LD 21), and the serum levels of TNF-α after an LPS challenge (LD 21) were also investigated. The results showed that prenatal LPS exposure improved maternal care and reduced maternal aggressive behavior but did not alter the maternal reproductive performance. The male offspring exhibited increased body weight at birth and reduced the maternal odor preference. The LPS challenge increased immobility duration in the open field behavior and induced a weak increased response of serum TNF-α levels. Prenatal exposure to LPS in late pregnancy improves maternal care but in pups, reduces the maternal olfactory preference and induces hyporesponsiveness to a single dose of the endotoxin on serum TNF-α levels.

Key words: prenatal inflammation, LPS, maternal behavior, maternal olfactory perception, TNFα.

Running title: Prenatal LPS and maternal-pups interaction

Introduction

Intrauterine infection and inflammation are known risk factors for brain injuries in neonates. Intrauterine inflammation leads to a dysregulation of the developing brain, irrespective of the gestational age(Burd, Balakrishnan, & Kannan, 2012), known as fetal inflammatory response syndrome(de Moura, Lisboa, & Passos, 2008). The maternal LPS (lipopolysaccharide) response leads to a fetal inflammatory response mediated by cytokines that has been implicated in the development of a spectrum of neurodevelopmental disorders such as autism and schizophrenia (Meyer, 2011; Meyer, et al., 2006).

The endotoxin, lipopolysaccharide, an endotoxin that originates from the cell wall of Gram-negative bacteria, mimics bacterial infections and is a potent inducer of inflammation (Avitsur, Pollak, & Yirmiya, 1997; Saluk-Juszczak & Wachowicz, 2005). Prenatal injections of LPS impair short and long-term behavior and central nervous system activity of neonates (Boksa, 2010; Golan, Lev, Hallak, Sorokin, & Huleihel, 2005; Schwendener, Meyer, & Feldon, 2009). Specifically, previous investigations from our group have shown that prenatal treatment with LPS (100 µg/kg, intraperitoneally on gestational day [GD] 9.5) in male offspring reduces social behavior in infancy and adulthood, decreases dopamine (DA) and metabolite levels in the striatum, and decreases the general activity in an open field after an LPS challenge, without signs of permanent neuroinflammation (Kirsten, et al., 2011; Kirsten, Taricano, Florio, Palermo-Neto, & Bernardi, 2010b; Kirsten, Taricano, Maiorka, Palermo-Neto, & Bernardi, 2010a) . Interestingly, our research has also shown that maternal behavior is slightly improved in pregnant rats treated with LPS on GD 9.5(Kirsten, et al., 2010a), whereas after treatment on GD 21, maternal care is reduced (Bernardi, et al., 2010). In addition, prenatal exposure to LPS (GD 14 to GD

20) decreases adult neurogenesis in the dentate gyrus, persistent microglial activation, and TGFβ_1 downregulation in the hippocampus and impairs performance in the Novel Object Recognition test (Graciarena, Depino, & Pitossi, 2010).

Our hypothesis is that late maternal inflammation may disrupt the programming prepubertal behavior and the immune responses after additional inflammatory stimulus. In addition, the maternal care was examined since alterations in the maternal behavior could thus also make a strong contribution to the long-term effects of stress on the pups' programming behaviors.(Carlos, Lemonica, de Grava Kempinas, & Marques Pereira, 1996; Darnaudery & Maccari, 2008). .

Thus, 100 µg/kg LPS was administered to dams on GD 18, and the following maternal care (parental generation) was observed: (1) maternal behavior (LD 5), and (3) maternal aggressive behavior (LD 6). Pups (F1 generation) were evaluated for (1) maternal odor preference, (2) open field behavior, and (3) the serum levels of the cytokine TNFα after an LPS challenge.

The maternal care was evaluated on LD 5 and LD 6 of the F1 generation because, at these times, the degree of maternal behavior gradually decreases, and changes in maternal care can be revealed (Teodorov, Felício, & Bernardi, 2010). Examining the maternal odor preference evaluates a sensory system critical for mother/pup interactions (Slotnick & Restrepo, 2005) because we previously observed that prenatal LPS (GD 9.5) impaired maternal odor preference as well as cat odor aversion, both of which are related to decreased dopamine levels in the olfactory bulb (Kirsten, et al., 2011) Finally, we administered an LPS dose (50µg/kg, i.p.) on LD 21 of the F1 generation to challenge the pups' immune system and assess whether prenatal exposure to the endotoxin altered the behavioral response and level of a proinflammatory cytokine, the TNF-α.

Methods

Subjects.

Thirty-two pregnant Wistar rats (parental generation) between 12 and 13 weeks of age and weighing 230–255 g were used (GD 0 was defined as the day when spermatozoa were detected in the vaginal smear). The dams were individually housed in polypropylene cages (38 x 32 x 16 cm) at a controlled temperature (22±2°C) and humidity (65–70%) with artificial lighting (12-hour light/12-hour dark cycle, lights on at 6:00 AM). The animals had free access to Nuvilab® rodent chow (Nuvital Co., Sao Paulo, SP, Brazil) and filtered water. Sterilized and residue-free wood shavings were used for the animal bedding. Two experiments were performed. In the first experiment, the maternal performance, the maternal aggressive behavior and pups maternal odor preference were performed. In this experiment, dams were divided into control (saline-treated) and experimental (LPS-treated) groups (n=8 dams/group). The dams were allowed to give birth and nurture their offspring normally. The day of birth was recorded as postnatal day (PND) 1. No handling was performed on PND 1 to avoid maternal cannibalism. On PND 2, after weighting the entire litters and record the number of all pups, the litters were culled to eight offspring (four males and four females), randomly selected (by anogenital differences, greater in males). No cross-fostering procedure was used. In the second experiment, 8 dams/group were treated as in the experiment 1. On PND 21, littermates were separated and co-housed by sex under the same conditions as their parents. In this day, two male from each litter (F1 generation) received either 50 µg/Kg of LPS or saline solution and were employed to open field behavior and the serum TNF-α levels studies. The testing of the control and the LPS-treated rats was

intermixed. The experimental design is summarized in figure 1. The rats used in this study were kept in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources of Paulista University, Brazil (protocol No. 014/09, CEUA-UNIP). These guidelines are similar to those of the National Institutes of Health, Bethesda, MD. Experiments were carried out in accordance with the good laboratory practice protocols and with quality assurance methods.

Treatment.

LPS (from *Escherichia coli*, Sigma®, Saint Louis, MO, USA, serotype 0127: B8) was dissolved in sterile saline (50 µg/mL LPS in a 0.9% NaCl solution) and administered intraperitoneally to pregnant dams at a dose of 100 µg/kg on GD 18 (n= 16 /group). This dose was chosen because it has been shown to (1) elicit sickness behavior, (2) induce endocrine alterations in dams, (3) increase cytokines at the placental level, and (4) impair the offspring birth rate and reduce the social behavior of male offspring during infancy and adulthood (Kirsten, Taricano, Maiorka, Palermo-Neto, & Bernardi, 2010a; Spencer SJ, Mouihate A, Galic MA, Ellis SL, & QJ., 2007; Wang, Rousset, Hagberg, & Mallard, 2006) The control group consisted of pregnant rats that received only sterile saline (0.9% NaCl) with the same treatment schedule as the LPS animals. Each control dam was treated with 0.1 mL/100 g saline solution.

Maternal studies.

Maternal reproductive performance.

The maternal reproductive performance was observed on LD 2 of the F1 generation of dams that received LPS during gestation (n=8 dams/group). The following parameters were assessed: number of pregnant females, total number of

pups, and number of male and female pups, number of pup deaths/litter and litter weight.

Maternal behavior.

Maternal behavior was analyzed as previously described (Bernardi, et al., 2010). Briefly, on LD 5 of the F1 generation (n=8 dams/group), between 08:00 AM and 11:00 AM, maternal behavior was observed in female rats of the parental generation exposed to LPS on GD 18. These dams were employed previously in the maternal retrieval test. Pups were removed from the dam, placed in another cage and kept warm. Immediately following the separation, the presence of a nest in the home cage was evaluated. Sixty minutes following maternal separation, all pups were returned to the cage of their mother, and examination of the maternal behavior began. The retrieval of the first pup (time, s), the retrieval of all pups (s), grouping (s), full maternal behavior (s) and latency to assume maternal behavior were recorded. Dams were scored as displaying full maternal behavior if they transferred all pups to the nest and displayed nursing behavior with their back arched over the pups for 3 consecutive min. If animals were not scored as displaying full maternal behavior following 30 min of continuous observation, they were checked every 15 min for 60 min and then hourly thereafter until full maternal behavior was observed.

Maternal aggressive behavior.

The same dams observed to maternal behavior were employed in this test. On LD 6 of the F1 generation (n=8 dams/group), between 08:00 AM and 11:00 AM, the maternal aggressive behavior test was performed in female rats exposed to LPS on GD 18. These rats were subjected to a 10-min maternal defense test (Wilkins, Logan,

& Kehoe, 1997). A male Wistar rat – the intruder – was introduced into the home cage of the dam and offspring. Intruder rats were only used once. Behaviors during the maternal defense test (against the intruder) was recorded via a remote digital camcorder and later analyzed for offensive behavior by the resident: latency (s) to first attack, attack frequency, total time (s) of attacks, and frequency and time (s) of boxing. Furthermore, the maternal behavior in the presence of the intruder was analyzed: frequency of carrying and hiding the pups and frequency of the intruder sniffing pups.

Pup Studies

Maternal odor preference test.

Maternal odor preference testing was performed on PND 7 as described elsewhere (Kirsten, et al., 2011) in male pups. Briefly, one male pup from each experimental and control litter (n=8 for each group) was examined. The test design was based on studies of associative olfactory learning and consisted of a two-odor choice between areas with nest material or fresh bedding. A polypropylene cage (38×32×16 cm) divided in half by a 2-cm wide neutral zone running the length of the box was used. In each area, 300 mL of fresh or nest bedding was placed in adjacent corners. The pup was placed in the 2-cm neutral zone at the end of the box facing the opposite target beddings. During the 1-min trial, the amount of time the pup (the head or the whole body) spent over each of the two areas was recorded. In addition, the number of pups that reached the area with odor or without odor in each trial was assessed. A time of 60 s was recorded when the pup did not reach one of the sides. Animals were tested in five trials between 2:00-4:00 PM, with an inter-test interval of 2 min, during which the pup was placed in the home cage. In each trial, beddings

were switched between the sides of the box. Following each test period, the box was cleaned with 5% ethanol to remove trace odors. Experiments were recorded with a video camera for later analysis. The pups' total time in each area was obtained by combining the number of times from the five trials.

. Pups' general activity in the open field after an LPS challenge.

The general activity test was performed in male pups that were prenatally exposed to LPS or saline solution, i.e., the F1 generation, on LD 21 (n=8 pups/group) as previously described (Broadhurst, 1960). A challenge dose of LPS (50 µg/Kg, i.p.) was administered 90 min before experiments. This dose and time were chosen because they have been reported to cause sickness behavior and the release of proinflammatory cytokines and glucocorticoids(Corrreia, Fernandes, & Bernardi, 2008). Thus, the following groups were formed: prenatal saline and postnatal saline group (S+S group), prenatal saline and postnatal LPS group (S+LPS group), prenatal LPS and postnatal saline group (LPS+S group) and prenatal LPS and postnatal LPS group (LPS+LPS group). The open-field apparatus has been described in detail elsewhere (Broadhurst, 1960). Briefly, it consists of a round wooden arena (40 cm in diameter, 40 cm high walls), painted black and divided into 25 parts. The apparatus was elevated 100 cm above the floor. For the observations, each rat was individually placed in the center of the apparatus between 2:00-4:00 PM. The following parameters were measured: locomotion frequency (number of floor units entered), rearing frequency (number of times the animals stood on its hind legs) and the immobility time (total seconds of lack of movement). The device was washed with a 5% alcohol/water solution before the animals were placed in it to negate possible biasing effects due to odor clues left by previous rats.

TNFα levels by ELISA.

The sera of the pups that were observed in the open field and challenged with LPS or saline were used in this experiment. For this, a 96-well high-binding plate (Costar, Corning, USA) was coated with mouse anti-rat TNF-α antibodies (R&D Systems, 4.0 µg/mL in PBS) overnight at room temperature (RT). Subsequently, the plate was washed three times with PBS containing 0.05% Tween-20 (Synth, Brazil) after each step. Nonspecific binding was blocked with PBS containing 1% BSA (Sigma-Aldrich, Germany). Samples (100 µL of animals' sera) or standards (0-4000 pg/ml recombinant rat TNFα, R&D Systems) diluted in PBS with 1% BSA were incubated for 2 h at RT. Immunodetection was performed with biotinylated goat anti-rat TNFα antibodies (225 ng/mL in PBS/1% BSA, R&D systems) for 2 h at RT, followed by incubation with streptavidin-HRP for 20 minutes (1:200, R&D Systems). The substrate solution was then applied for 15 minutes (OptEIA™, BD). The plate was read at 450 nm, and values were calculated in pg/ml.

Statistical analysis

Mother was the experimental unit, and one or two males from each litter were used for each experiment, thus with different animals in each experiment. The results are expressed as mean± SEM. Homoscedasticity was verified using an F-test or Bartlett's test. Normality was verified by a Kolmogorov-Smirnov test. Student's t-test (unpaired, two-tailed) was used to compare the parametric group data (maternal reproductive performance, pups body weight, maternal behavior, maternal aggressive behavior). For percentage data the χ_2 test was used. A two-way analysis of variance, followed by the Bonferroni post-hoc test were used to analyze data from

the time to reach one of the sides in the maternal odor preference test, in the open field test and TNF-α levels. In all cases, values of P less than 0.05 were considered statistically significant. Statistical analyses were carried out using GraphPad Prism software, version 5 (GraphPad, San Diego, California, USA).

Results

No effects were observed for all reproductive parameters in females that were or were not prenatally treated with LPS (data not show). However, compared to the control group, the litter weight measured on LD 2, before the litters standardization, was increased in the experimental group relative to control (control group- 49.58 ± 1.16; experimental group- 53.45 ± 1.30, P = 0.03, mean ± SEM). No differences were observed between the number of pups born in each litter (control group – 9.18 ±0.66, experimental group=7.83 ± 1.16, p= 0.29, mean ± SEM).

In maternal behavior, LPS-treated female rats retrieved the first pup faster than females of the control group (p = 0.044). The remaining parameters were not different between the control and experimental groups (Table 1).

In maternal aggressive behavior female rats prenatally treated with LPS exhibited a decreased number of attacks (p = 0.004) and in the time of attacks (P< 0.0001) relative to control group. The remaining parameters were not different between the control and experimental groups (Table 1).

As depicted in Fig. 2A, prenatal treatment with LPS reduced the number of pups that reach the odor, clean and neutral zone. The ANOVA shows significant differences between treatments [$F_{1/42} = 8.20$, p = 0.0065] and side chosen [$F_{2/42} = 39.18$, p< 0.0001] with interaction between factors [F 2/42 = 24.6, p< 0.0001]. Compared to control group experimental group showed a decreased number of pups

that reach the odor area and a largest number of pups remained in the neutral zone. Thus, pups prenatally exposed to LPS had an impaired odor preference to the maternal odor. The time [Fig.2B] to reach the odor side in five trials was decreased in experimental pups relative to controls ($p< 0.029$). In addition, a two way ANOVA revealed that pups of the LPS group took an increased amount of time to reach one of the sides only in the first session relative to the control group [treatment- $F_{1/70} = 13.98$, $p = 0.0004$; sessions- $F_{4/70} = 0.77$, $p=0.54$; interaction – $F_{4/70} = 1.98$, $p=0.11$, Bonferroni test, session 1 - $p<0.05$ - fig. 2C]. Thus, only in session 1 did pups of the LPS group exhibit a decrease in locomotion function.

Fig. 3A-C shows the general activity in an open field of pups prenatally treated (or not) with LPS and challenged (or not) on LD 21 with 50 µg/Kg of LPS.

A two-way ANOVA revealed that locomotion [postnatal treatment- $F_{1/28} = 0.02$, $p=0,896$; prenatal treatment – $F_{1/28} = 0.12$, $p = 0.734$; interaction – $F_{1/28} = 0.74$, $p = 0.395$, fig. 3 A] and rearing [postnatal treatment- $F_{1/28} = 0.45$, $p=0,501$; prenatal treatment – $F_{1/28} = 0.08$, $p = 0.773$; interaction – $F_{1/28} = 1.29$, $p = 0.256$ – fig. 3B] frequencies were not modified by the prenatal or postnatal treatments. The immobility duration (Fig. 3C) was modified by postnatal treatment [$F_{1/28} = 7.28$, $P = 0.02$], but prenatal treatment did not affect immobility [$F_{1/28} = 0.23$, $p = 0.63$]; no interaction was observed between the factors [$F_{1/28} = 0.14$, $p = 0.75$]. A Bonferroni test determined that the immobility duration was increased in the S+LPS group in relation to all other groups.

Fig. 4 shows the TNF-α levels evaluated by ELISA. A two-way ANOVA revealed that prenatal [$F_{1/27} = 10.12$, $p = 0.0038$] and postnatal [$F_{1/27} = 11.05$, $p = 0.0026$] treatments affected the results; a significant interaction between factors was detected [$F_{1/27} = 9.98$, $p = 0.0039$]. A Bonferroni test found significant differences

between the LPS+S and LPS+LPS groups [p< 0.001] but not between prenatal treatments, i.e., between the S+S AND LPS+S groups [p> 0.05]. Thus, the S+LPS group showed increased TNFα levels relative to the S+S group, as expected. However, the LPS+LPS group exhibited a weak increase in cytokine levels compared to the S+ LPS group, indicating a decreased sensitivity to LPS when pups were exposed prenatally to the endotoxin. In addition, only pups exposed to prenatal LPS but not exposed to postnatal LPS (the LPS+S group) did not show detectable levels of TNFα in their sera.

Discussion

The present findings show that prenatal LPS (100 µg/kg on GD 18) exposure improved maternal care and reduced maternal aggressive behavior but did not alter maternal reproductive performance. When considering the male offspring, prenatal LPS increased the body weight at birth and reduced the olfactory perception of maternal odor preference. In addition, on LD 21, a challenge dose of LPS increased immobility duration in the open field apparatus. Also, this challenge dose induces tolerance revealed by a weak increases on serum TNF-α levels in pup rats of LPS+LPS group compared to LPS+ saline group.

The improvement of maternal care in females that were prenatally exposed to LPS was observed in maternal behavior. These females presented a reduced time to retrieve the first pup when compared to the control group. In contrast, in maternal aggressive behavior, these females had fewer numbers of attacks with a reduced duration in the time spent fighting than females of the control group. These apparently contradictory data may be explained by the effects of LPS.

Several authors have reported that LPS also affects central nervous system activity, leading to sickness behavior in many species (Aderem & Ulevitch, 2000;

Avitsur, et al., 1997; Saluk-Juszczak & Wachowicz, 2005). The innate immune system is responsible for many of the acute symptoms of sickness that are related to systemic inflammation or infection (Medzhitov & Janeway, 1999; Rivest, 2003). LPS-induced sickness behavior is generally accompanied by a decrease in exploratory activity, social behavior, ingestive behavior, and sexual behavior and induced anhedonia, as well as poor learning and cognitive functions (Corrreia, et al., 2008). Among the signals of LPS-induced sickness behavior, fever has also been reported (Hart, 1988). Fever, an adaptive reaction to pathogens (Mackowiak, 1998), results from a complex reaction at the hypothalamic centers, which inform the organism that the environmental temperature is low and thereby induce an increase in body temperature(Voltarelli & Loughran Junior, 1994). At birth, rodent neonates have not yet developed thermoregulation mechanisms, and maternal care is critical to maintain pup survival. Both retrieving and nest building are involved in the thermoregulation of pups. Retrieving behavior is directly linked to the survival of the litter and plays a major role in increasing the dam's inclusive fitness, defined by the number of offspring that survive and reproduce (Aubert, Goodall, Dantzer, & Gheusi, 1997)

Recent studies from our group found that 100 µg/Kg of LPS administered on LD 3 induces fever and increases maternal behavior (Nascimento, Felício, & Bernardi, 2011) with a peak on 48 and 72 h after the endotoxin administration and no fever was observed 120 h after treatment. In our experiment, the maternal behavior was observed 192 h after the endotoxin administration. Thus, it is possible that maternal fever during pregnancy signals to mothers that the environmental temperature is low, and this information specifically increases the motivation of the dam to retrieve her pups.

The data from the maternal aggressive behavior test are apparently contradictory when compared to the data from the maternal behavior test. However, the reduced number of attacks and the reduced fight duration can be interpreted as an effect of LPS on motivation, motivation being taken here as a central state that organizes perception and action (Spencer, Martin, Mouihate, & Pittman, 2006). We suggest that LPS induces a competing motivational state that is characterized by reduced attention toward external events and/or an increased sensitivity threshold to external cues, such as pup vocalizations, leading to a preference to take care of the pups and a reduced aggressive response toward the intruder. In addition, direct behavioral observation of the intruder shows no aggressive behavior directed toward the pups.

In the present study, LPS that was injected on GD 18 impaired the preference of male pups for maternal odor. We studied the olfactory maternal preference only in male offspring because maternal behavior is much higher in male than in female pups. Therefore, any deficit in maternal behavior could be better visualized. In addition, transient motor effects induced by prenatal LPS were observed because only in the first session was the time to reach one side decreased in experimental pups compared to the control group. Thus, the impairment of olfactory preference was likely uncorrelated with impairment in motor behavior. This hypothesis is strengthened with the data measured at weaning, when the locomotor behavior of prenatal LPS group (LPS+ saline group) did not differ from the control group.

Another explanation for the present finding in addition to olfaction impairment is that rats prenatally treated with LPS had impaired motivation. Supporting evidence for this hypothesis comes from previous findings that prenatal exposure to LPS impaired the social interest of rats in infancy and adulthood due to motivational

impairments (Kirsten, Taricano, Maiorka, Palermo-Neto, & Bernardi, 2010b). For these reasons, and because maternal olfactory stimuli are linked to rats' survival and are therefore difficult to ignore, we accept the reduced motivational hypothesis.

To test the sensitivity of pups to LPS-induced sickness behavior, we administered an additional dose of LPS and observed the exploratory behavior in an open field. Prenatally saline-treated pups challenged with LPS at weaning had increased immobility duration when compared to other groups. No alterations were observed in the remaining open field parameters when compared to the other groups. This effect could be correlated to a development of sickness behavior. However, prenatally LPS-treated pups that were also treated with LPS at weaning (LPS+ LPS group) showed no changes in immobility, suggesting a low sensitivity to LPS-induced sickness behavior. Thus, it is possible that an activation of the immune system at the end of pregnancy decreases the response to LPS-induced sickness behavior in prepubertal rats.

To verify this hypothesis, we measured the serum levels of TNF-α. Surprisingly, pups prenatally treated with LPS did not respond to LPS-induced immune system activation at weaning. Thus, it is possible that prenatal exposure to LPS induces tolerance of the immune system to pathogens early in life. This result further demonstrates that endotoxin in late pregnancy could negatively regulate LPS-signaling during endotoxin tolerance development and anti-inflammatory cytokines might play important roles. *In vitro* tolerance of human monocytes can be partially mimicked by IL-10 and TGF-β, and the use of anti-IL-10 and anti TGF-β antibodies during the step of tolerization can prevent the phenomenon of endotoxin tolerance (Randow, et al., 1995).

In conclusion, prenatal exposure to LPS in late pregnancy improves maternal

care but reduces the maternal olfactory preference of the pups by interference with dams and pups motivation. In addition, tolerance to a challenge dose of LPS was observed in pups prenatally exposed to the endotoxin.

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Figure Captions

Fig. 1. Experimental design.

Fig. 2. Effects of prenatal LPS exposure (100 µg/kg on GD 18) on the number of pups that choose the odor side (A), time (sec) to reach the odor side in five trials (B) and the time (sec) spent to reach the nest bedding area (C) in the maternal odor preference test in infant male rat pups on PND 7. N=8 pups/group. Data of the number of pups choose de odor, neutral and clean side and time spent to reach the nest were analyzed by the Two way ANOVA followed by the Bonferroni test. The time to reach the odor side was analyzed by the Student t test. * $p< 0.05$ in relation to control group. Values are represented as means ± SEM.

Fig. 3. Open field behavior observed on LD 21 of male pup rats prenatally exposed to LPS on GD 18 and challenged with 50 µg/Kg of the same endotoxin. A- locomotion frequency; B- rearing frequency and C- immobility duration (sec). n = 8/group. * $p<0.05$ compared to the control group (two-way ANOVA followed by the Bonferroni test). Values are represented as means ± SEM.

Fig. 4. Serum TNF-α (pg/mL) of male pups on LD 21 prenatally exposed to LPS on GD 18 and challenge with 50 µg/Kg of the same endotoxin. * $p<0.05$ compared to the control group (two-way ANOVA followed by the Bonferroni test). Values are represented as means ± SEM.

Table 1. Maternal care of dams prenatally exposed to LPS (100 µg/Kg, i.p.) Maternal behavior and maternal aggressive behavior were observed, respectively, on postnatal (PND)5 and PND 6. N = 8/group. Data are presented as mean ± SEM or percentage.

Parameter	Control group	LPS group	P
Maternal behavior			
Retrieval 1st pup (s)	*40.38 ± 11.60*	*13.25 ± 3.89*	*0.044*
Retrieval all pups(s)	202.63 ± 43.26	176.75 ± 38.43	0.662
Maternal position (min)	21.26 ± 3.93	19.55 ± 1.37	0.687
Maternal position (%)	87.5	100	1.0
Grouping pups (%)	87.5	100	1.0
Presence of the nest (%)	100	100	-
Maternal aggressive behavior			
First attack (s)	147.73 ± 24.00	166.60 ± 25.00	0.595
Number of attacks	*6.65 ± 0.70*	*3.50 ± 0.60*	*0.004*
Total number of attacks	4.00 ± 1.26	3.00 ± 1.40	0.604
Time of fight (s)	*1.80 ± 0.02*	*1.00 ± 0.05*	*<0.0001*
Number of bits	0.36 ± 0.20	0.40 ± 0.24	0.074
Number of times of retrieval pups	0.27 ± 1.00	3.80 ± 1.80	0.109
Number of times hidden the pups	1.54 ± 0.66	3.80 ± 2.60	0.414
Number of times the intruder sniffed the pups	0.82 ± 0.42	0.40 ± 0.24	0.399

Student *t* test.

Fig.1

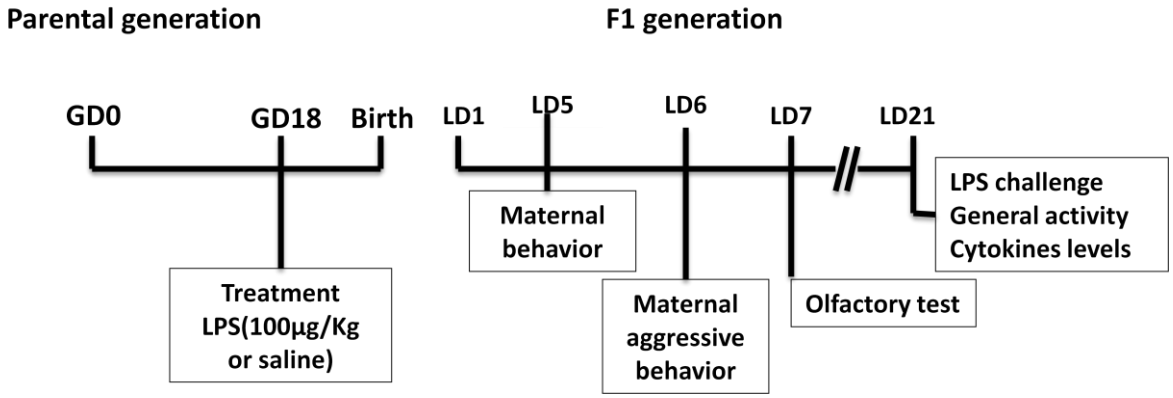

Parental generation
F1 generation
GD0
GD18
Birth
LD1
LD5
LD6
LD7
LD21
Maternal behavior
LPS challenge
General activity
Cytokines levels
Treatment
LPS(100µg/Kg
or saline)
Maternal
aggressive
behavior
Olfactory test

Fig.2

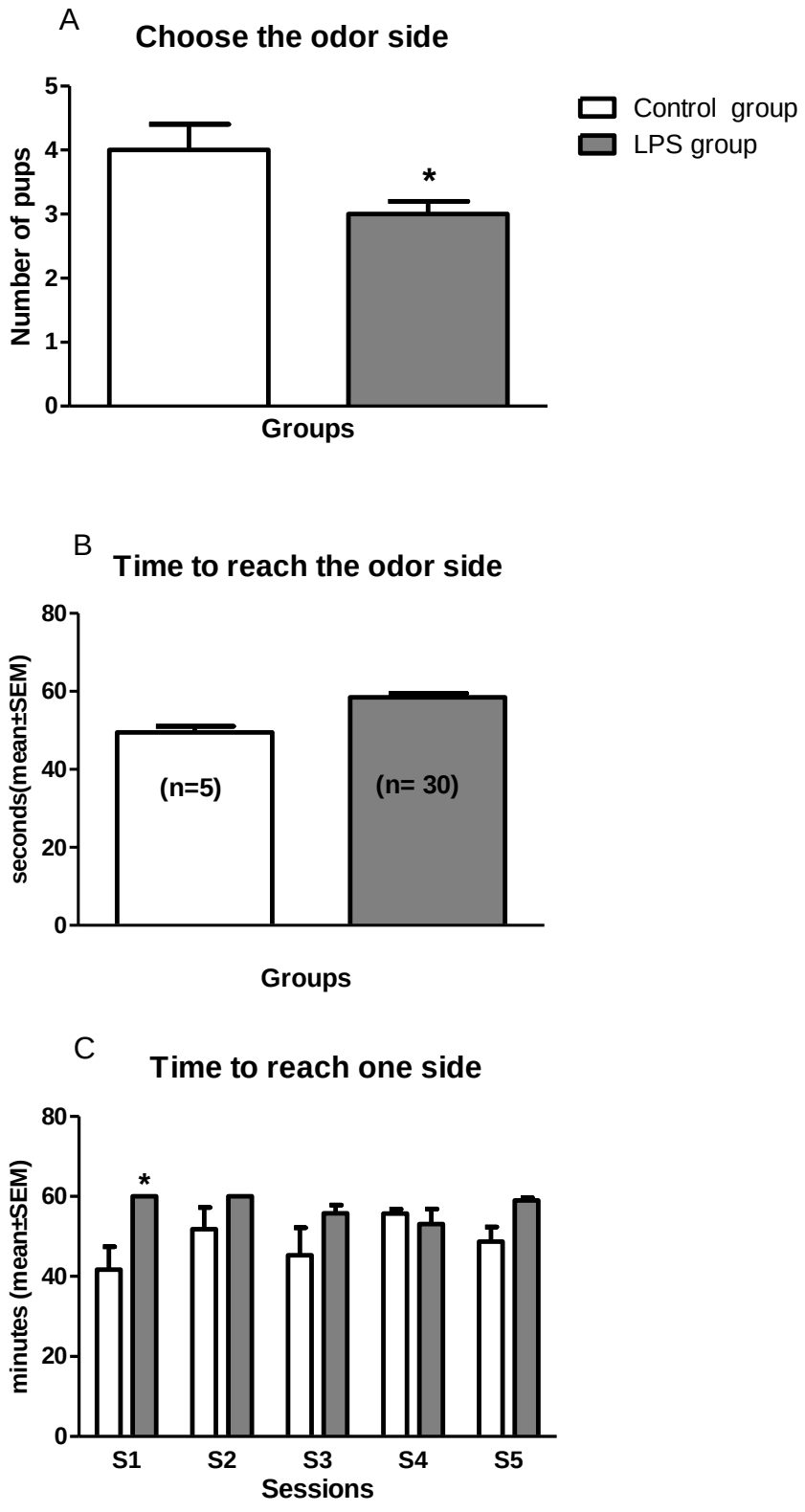

A
Choose the odor side
Number of pups
Groups
Control group
LPS group
*
B
Time to reach the odor side
seconds(mean±SEM)
(n=5)
(n= 30)
Groups
C
Time to reach one side
minutes (mean±SEM)
*
S1
S2
S3
S4
S5
Sessions

Fig.3

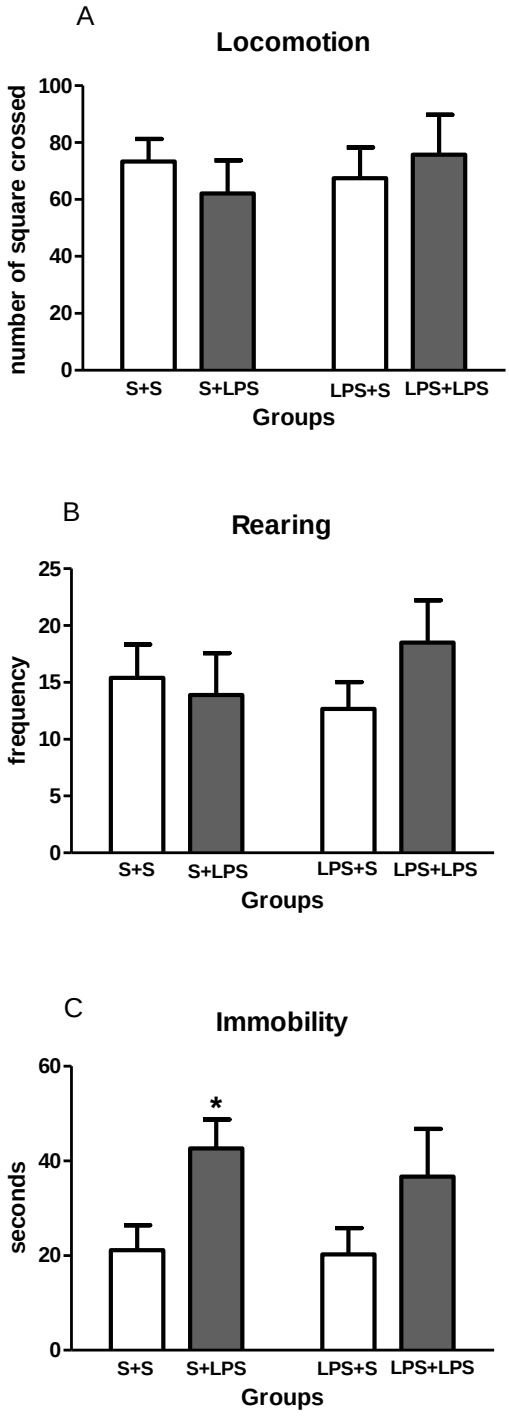
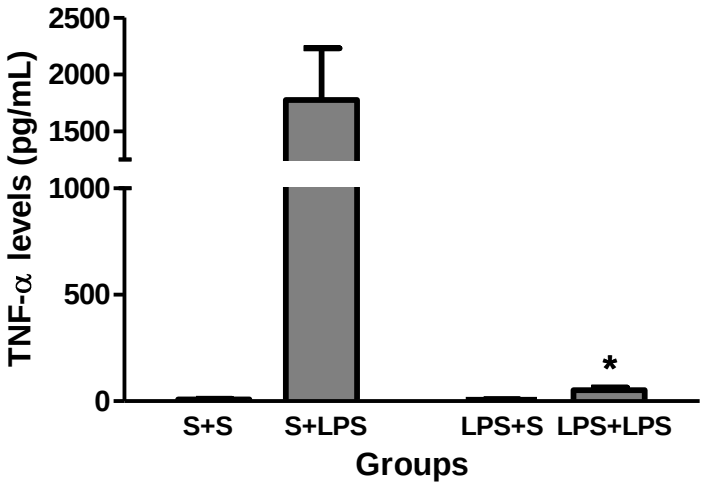

A
Locomotion
number of square crossed
100
80
60
40
20
0
S+S
S+LPS
LPS+S
LPS+LPS
Groups
B
Rearing
frequency
25
20
15
10
5
0
S+S
S+LPS
LPS+S
LPS+LPS
Groups
C
Immobility
seconds
60
40
20
0
*
S+S
S+LPS
LPS+S
LPS+LPS
Groups

Fig.4


2500
2000
1500
1000
500
0
TNF-α levels (pg/mL)
*
S+S
S+LPS
LPS+S
LPS+LPS
Groups

ARTIGO 2

Transgenerational effects of antenatal LPS exposure on maternal and pups behaviors: studies in two generations.

Penteado, S. H. N. W [a]; Kirsten, T. B. [b]; Eluf,B.[a ;] Reis-Silva, T. M. [b] ; Acenjo, M. K [a]; Melo, R.[a]R.[a;] I ,Bernardi, M. M. [a,c]

[a] Health Sciences Institute, Paulista University, Rua Dr. Bacelar, 1212, 04026-002, Sao Paulo, SP, Brazil.

[b] Department of Pathology, School of Veterinary Medicine, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, 05508–270, São Paulo, SP, Brazil.

[c] Centro de Matemática e Cognição, Universidade Federal do ABC, Rua Santa Adélia, 166, Santo André, SP, Brasil

Corresponding author:

Maria Martha Bernardi

Department of Pathology, School of Veterinary Medicine, University of Sao Paulo

Av. Prof. Dr. Orlando Marques de Paiva, 87

São Paulo, SP 05508-270, Brazil

Tel. +55 11 3091 7657, Fax +55 11 3091 7829

e-mail: marthabernardi@gmail.com

Key words: prenatal inflammation, LPS, maternal behavior, maternal aggressive behavior.

Running title: Transgenerational LPS effects.

Abstract

Objective: The present experiment was designed to analyze possible transgenerational lipopolysaccharide (LPS)-induced effects on maternal care of the F1 generation and on behavior of the F2 generation of rats exposed antenatally to LPS (100 μg/kg LPS on GD 18). ***Methods:*** The following parameters of the F1 generation were observed: reproductive performance, maternal behavior, maternal aggressive behavior, and open field general activity in adult age. In the F2 generation, body weight at birth and weaning, maternal olfactory preference, and, in adult age, general activities in an open field and in the plus maze were assessed. ***Results:*** In the F1 generation relative to controls, antenatal exposure to LPS showed that 1) the latency to full maternal behavior was delayed 2) a slightly increased maternal aggression; and 3) no interference of reproductive performance and general activity. In the F2 generation antenatally treated with the endotoxin, it was observed in relation to the control group 1) a decreased body weight at weaning and in the olfactory recognition of maternal odor; 2) no differences in the open field behavior; 3) a decreased percentage in time of open arms and in time in the center while an increase in closed arms were observed. ***Conclusion***: These data reveal that antenatal LPS exposure modified certain aspects of maternal care of the F1 generation related to nursing and pups' survival, but not on maternal motivational parameters. In the F2 generation, antenatal LPS exposure reduces maternal recognition in infancy as well as body weight at weaning. In addition, later prenatal exposure to LPS induces transgenerational effects in the F2 generation, resulting in a less resilient phenotype to anxiety.

Introduction

Lipopolysaccharide (LPS), an endotoxin that originates in the cell wall of gram-negative bacteria and mimics bacterial infection, activates the immune system to release proinflammatory cytokines (Avitsur, Pollak, & Yirmiya, 1997; Saluk-Juszczak & Wachowicz, 2005). Viral and bacterial infections, including those caused by prenatal LPS exposure, induce short- and long-term changes in behavior and central nervous system activity (Boksa, 2010; Golan, Lev, Hallak, Sorokin, & Huleihel, 2005; Meyer, Feldon, & Fatemi, 2009b). Previous investigations by our group have shown that prenatal LPS treatment (100 μg/kg, given intraperitoneally on gestational day [GD] 9.5) reduces the social behavior of F1 males both in infancy and in adulthood and decreases their striatal dopamine (DA) and DA metabolite levels in the absence of signs indicative of neuroinflammation (Kirsten, et al., 2011b; Kirsten, Taricano, Florio, Palermo-Neto, & Bernardi, 2010; Kirsten, Taricano, Maiorka, Palermo-Neto, & Bernardi, 2010). Interestingly, our model also showed that maternal behavior was slightly improved in pregnant rats treated with LPS on GD 9.5 (Kirsten, et al., 2011b), whereas treatment on GD 21 decreased this behavior (Bernardi, et al., 2010).

It has been suggested that the effects of maternal LPS exposure on the developing fetal brain are not directly mediated by LPS, but are instead indirectly induced via increases in proinflammatory cytokines and glucocorticoid levels within the maternal circulation, placenta, and fetal brain (Ashdown, et al., 2006; Cai, Pan, Pang, Evans, & Rhodes, 2000; Gayle, et al., 2004; Urakubo, Jarskog, Lieberman, & Gilmore, 2001). Infections associated with immunological events that occur in early/middle life (e.g., GD 8–10 in rats and mice) have a stronger impact on fetal neurodevelopment than do late-pregnancy infections. Immune activation during the early/middle stages of pregnancy was shown to modify fetal cell proliferation and differentiation, cell migration, target selection, and synapse maturation (Ghiani, et al., 2011; Meyer, et al., 2006; Meyer, Yee, & Feldon, 2007; Samuelsson,

Jennische, Hansson, & Holmang, 2006; Shi, Fatemi, Sidwell, & Patterson, 2003). Multiple brain injuries and behavioral abnormalities persisting through adulthood were also reported after early/middle stage pregnancy infections (Meyer, et al., 2007).

Environmental information received by a mother can induce a phenotypic change in her offspring, commonly known as a maternal or transgenerational effect (Agrawal, Laforsch, & Tollrian, 1999; Curno, Behnke, McElligott, Reader, & Barnard, 2009). Certain cues in the maternal environment, e.g., the prevalence of predators or maternal infection, can lead to behavioral, morphological, and immunological changes in the following generation (Agrawal, et al., 1999; Grindstaff, et al., 2006).

The present experiment was designed to analyze possible transgenerational LPS-induced effects on maternal care of the F1 generation and on behavior of the F2 generation of rats exposed antenatally to LPS (100 μg/kg LPS on GD 18). The following parameters of the F1 generation were observed: reproductive performance, maternal behavior, maternal aggressive behavior, and open field general activity in adult age. In the F2 generation, body weight at birth and weaning, maternal olfactory preference and, in adult age, general activities in an open field and in the plus maze were assessed.

Material and methods

Animals

Sixty pregnant Wistar rats (parental generation) between 12 and 13 weeks of age and weighing 216–263 g were used (GD0 was defined as the day when spermatozoa were detected in the vaginal smear). The dams were individually housed in polypropylene cages (38×32×16 cm) at a controlled temperature (22±2 °C) and humidity (65–70%) with artificial lighting (12-hour light/12-hour dark cycle, lights on at 6:00 AM). The animals had free access to Nuvilab® rodent chow (Nuvital Co., São Paulo, SP, Brazil) and filtered water. Sterilized

and residue-free wood shavings were used for the animal bedding. The animals were divided into control (saline-treated) and experimental (LPS-treated) groups (n=16 dams/group). The dams were allowed to give birth and nurture their offspring normally. The day of birth was recorded as postnatal day (PND) 1. No handling was performed on PND1, but on PND2, 8 offspring (4 males and 4 females) were randomly selected for the following studies. No cross-fostering procedure was used. The 8 randomly selected pups remained with each dam until weaning (PND21). On PND21, littermates were separated and co-housed by sex and treatments under the same conditions as their parents and named the F1 generation. In adult age, the females of F1 generation (8/group) were mated with naïve males to produce the F2 generation. One female (F1 generation) and one male from each litter (F2 generation) were used for the experiments; therefore, the litter was used as a unit. The testing of the control and experimental groups was intermixed. The rats used in this study were kept in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources of Paulista University, Brazil (protocol No. 014/09, CEUA-UNIP). These guidelines are similar to those of the National Institutes of Health, Bethesda, MD. Experiments were carried out in accordance with good laboratory practice protocols and with quality assurance methods.

Treatments

LPS (from *Escherichia coli*, Sigma®, Saint Louis, MO, USA, serotype 0127: B8) was dissolved in sterile saline (50 µg/ml LPS in a 0.9% NaCl solution) and administered intraperitoneally to pregnant dams of the parental generation at a dose of 100 µg/kg on GD18 (n= 16 /group). This dose was chosen because it has been shown to (1) elicit sickness behavior, (2) induce endocrine alterations in dams, (3) increase cytokines at the placental level, and (4) impair the offspring birthrate and reduce the social behavior of male offspring during infancy and adulthood (Kirsten, et al., 2011a; Kirsten, Taricano, Maiorka, et al., 2010;

Spencer, Mouihate, Galic, Ellis, & Pittman, 2007) The control group consisted of pregnant rats that received only sterile saline (0.9% NaCl) with the same treatment schedule as the LPS animals. Each control dam was treated with 0.1 ml/100 g saline solution. The rats of F1 and F2 generations did not receive any treatment.

Studies in the F1 Generation

Reproductive Performance of F1 Generation

The maternal reproductive performance was observed on PND2 of the F1 generation prenatally exposed to LPS or saline solution 0.9% on GD18 (n=8 dams/group). The following parameters were assessed: number of pregnant females, total number of pups, numbers of male and female pups, number of pup deaths/litter, and pups' weights.

Open Field Behavior of Lactating Females

On LD5, female pups of the F1 generation were observed in an open field as previously described by Broadhurst (1960). This test was applied to 8 female rat pups/group prenatally treated with LPS or saline solution 0.9% the day before maternal behavior observation, i.e., LD4. The device is a round arena (96 cm in diameter) surrounded by a 25-cm high wall, painted white, and subdivided into 25 parts by black stripes. During the experiments, a 40-W white bulb placed 72 cm above the floor provided continuous illumination of the arena. Handheld counters and stopwatches were employed to score locomotion (number of floor units entered) and immobility (total time without spontaneous movements). Female rats were individually placed in the center of the open-field arena, and behavioral parameters were observed for 3 min. The open-field apparatus was then rinsed in 5% ethanol before introducing the next animal to preclude the possible cueing effects of odors left by previous subjects. To minimize the possible influences of circadian rhythmicity on rat behavior in the open field, control and experimental animals were intercalated. Animals were observed between 2 and 5 pm, in the light phase of the cycle.

Maternal Behavior

Maternal behavior was analyzed as previously described (Teodorov, Felício, & Bernardi, 2010). Briefly, on lactation day (LD) 5 between 08:00 AM and 11:00 AM, the maternal behavior of the F1 generation (8 dams /group) was observed. Pups were removed from the dam, placed in another cage, and kept warm. Immediately following the separation, the presence of a nest in the home cage was evaluated. Sixty minutes following maternal separation, all pups were returned to the cage of their mother, and examination of the maternal behavior began. The latencies to retrieval of the first pup(s), to retrieval of all pups and to full maternal behavior (s) were recorded. The percentage of dams that retrieved the 1st pup, all pups, grouping pups and presenting maternal behavior were calculated.

Maternal Aggressive Behavior

On LD6 between 08:00 AM and 11:00 AM, the maternal aggressive behavior test was performed in female rats of the F1 generation. These rats were subjected to a 10-min maternal defense test (Teodorov, et al., 2010; Wilkins, Logan, & Kehoe, 1997). A male Wistar rat—the intruder—was introduced into the home cage of the dam and offspring. Intruder rats were only used once. Behaviors during the maternal defense test (against the intruder) were recorded via a remote digital camcorder and later analyzed for offensive behavior by the resident: latency(s) to first attack, attack frequency, total time(s) of attacks, frequency of bites, and time of fight(s). Furthermore, maternal behavior in the presence of the intruder was analyzed: frequency of carrying and hiding the pups and frequency of the intruder sniffing pups. The female was used only once in this test.

Studies of F2 Generation

In these studies only the male pups of F2 generation were employed to avoid hormonal interferences on behavior that occurs in female rats.

Maternal Odor Preference Test

Maternal odor preference testing of the F2 generation was performed on PND7 as described in Kirsten et al. (2011a) in male pups whose mothers were tested on PND5 for maternal behavior. Briefly, one male pup from each experimental or control litter (n=8 for each group) was examined. The test design was based on studies of associative olfactory learning and consisted of a two-odor choice between areas with nest material or fresh bedding. A polypropylene cage (38×32×16 cm) divided in half by a 2-cm-wide neutral zone running the length of the box was used. In each area, 300 mL of fresh or nest bedding was placed in adjacent corners. The pup was placed in the 2-cm neutral zone at the end of the box facing opposite the target beddings. During the 1-min trial, the number of times the pup (the head or the whole body) moved toward each of the two areas (odor and without odor) was recorded. In addition, the number of pups that reached the area with odor or without odor in each trial was assessed. A time of 60 s was recorded when the pup did not reach one of the sides. Animals were tested in five trials between 2:00–4:00 PM, with an inter-test interval of 2 min, during which the pup was placed in the home cage. In each trial, beddings were switched between the sides of the box. Following each test period, the box was cleaned with 5% ethanol to remove trace odors. Experiments were recorded with a video camera for later analysis. The pups' total time in each area was obtained by combining the number of times from each of the five trials.

Open Field Studies

At 70–75 days of age, male pups of the F2 generation were observed in the open field. The control and experimental groups were subdivided into two groups: two groups were isolated for one week (control isolated and LPS isolated) and the others remained grouped (control grouped and LPS grouped). This procedure was employed as a challenge to show if the parental exposure to LPS could modify the behavior of the second generation. The open

field test was performed similarly to those described for the female rats.

Plus Maze Test Studies

The plus maze behaviors of offspring were measured on PND 70–75 of the F2 generation. The same rats employed in the open field test were used to perform this experiment. The device consisted of two opposite open arms (50 cm long x 10 cm wide) and two opposite closed arms (50 cm long x 10 cm wide x 40 cm high) arranged at 90° angles. The floor of the maze was made of wood, painted gray (with acrylic washable covering) and located 50 cm above the floor. The center of the maze was open and the walls of the closed arms started 2 cm from the center of the maze. Each rat was observed using a video camera mounted above the arena to record the behavioral data. For the observations, each animal was individually placed in the center of the maze with the head facing one of the open arms, and the following parameters were measured over a period of 5 min: number of entries into the open arms, number of entries into the closed arms, time spent in the open arms, time spent in the closed arms. The measures that reflect stress/anxiety levels here employed were the percentage of time spent in the open arms versus closed arms (% time in the open arms = [time in the open arms/(time in the open arms + time in the closed arms)] x 100).

To minimize the influence of possible circadian changes on plus maze behaviors, control and experimental animals were alternated. The device was washed with a 5% alcohol/water solution before placing the animals on it to obviate possible biasing effects of odor clues left by previous rats. Observations were made between 2:00 and 5:00 p.m.

Statistical Analyses

The mother was the experimental unit, and one female from each litter was used for each experiment; thus, different animals were used in each experiment. The results are

expressed as mean ±SEM. Homoscedasticity was verified using an F-test or Bartlett's test. Normality was verified by a Kolmogorov-Smirnov test. Student's t-test (unpaired, two-tailed) was used to compare the parametric group data of two variables. The two-way ANOVA followed by the Bonferroni test was employed to analyze the open field and plus maze data. In all cases, the results were considered significant at $P < 0.05$.

Results

Studies in the F1 Generation

Reproductive Performance

The statistical analysis applied on reproductive performance parameters of female rats from the F1 generation prenatally exposed to 100µg/Kg of LPS or saline solution on GD18 did not show differences between controls and experimental groups (data not shown).

Maternal Behavior

Table shows the data of the F1 generation maternal behavior. The latency to full maternal behavior was increased in experimental females in relation to the control group. No significant differences were observed between the latency to retrieve the first pup [Fig.1C, $t=0.46$, $df =14$, $p = 0.65$], in the percentage of female that retrieve the 1^{st} pups retrieved, in the total time to retrieve all pups, and percentage of female that retrieved all pups and presenting full maternal behavior of experimental group in relation to control group.

Open Field Behavior of Lactating Female

No significant differences were observed between the locomotion frequencies and immobility times in lactating females of the F1 generation prenatally exposed to 100µg/Kg of LPS or saline solution on GD18 (data not shown).

Maternal Aggressive Behavior

Fig. 2 A–F shows the data of maternal aggressive behavior of the F1 control and experimental female rats. The Student's t-test indicates an increased number of bites [Fig. 2 E, t=1.93, df=14, p = 0.03] and frequency of retrieval of the pups [Fig. 2 G, t=1,79, df=14, p = 0.04] in experimental female rats compared to controls. There were no differences between both groups in the remaining parameters of maternal aggressive behavior.

Studies of F2 Generation

Fig. 3 A shows that the body weight on PND2 of experimental male pup rats of the F2 generation did not differ from those of the control groups [t=0.22, df =14, p= 0.82]. However, on PND21, i.e., at weaning, the experimental pups showed a reduced weight compared to the control pups [t=2.13, df =14, p =0.05].

As depicted in Fig. 3 B, the F2 generation whose parental generation received LPS showed an impaired maternal odor preference, because these animals spent less time over the nest, than the controls in the first [t=2.28, DF =8, P= 0.039] and second [t=3.13, DF=8, P = 0.007] trials and in total time [t= 5.09, df = 14, p = 0.002] obtained by the sum of the five trials.

Data of the open field test performed at adult age (Fig. 4 A) revealed that isolation increased locomotion frequency [$F_{1/28} = 4.76$, p = 0.03] but not the treatment [$F_{1/28} = 0.46$, p = 0.50]; no interaction was observed between factors [$F_{1/28} = 0.37$, p = 0.55]. The Bonferroni test shows that locomotion frequencies of isolated rats were increased in relation to control isolated and grouped groups. No statistical differences were detected between the immobility times of grouped or isolated groups [Fig. 4 B].

The % of open arms entries [Fig. 4 C] was also affected by the treatment [$F_{1/28} = 4.69$, p = 0.03], but the isolation did not influence the results [$F_{1/28} = 0.39$, p = 0.53]; no interaction was found between factors [$F_{1/28} = 0.24$, p = 0.63]. The Bonferroni test shows a decrease in

the % of time in open arms of the isolated experimental group, indicating a reduced anxiety in these rats. As depicted in Fig. 4 D, the treatment affected the time in closed arms [$F_{1/28} = 4.87$, $p = 0.03$], but not isolation [$F_{1/28} = 0.11$, $p = 0.74$]; an interaction was observed between factors [$F_{1/28} = 4.20$, $p = 0.04$].The Bonferroni test indicates that isolated rats from the experimental group had an increased time in the closed arms. The time in the plus maze center (Fig. 4 E), was affected by the treatment [$F_{1/28} = 4.28$, $p = 0.04$]; no significant interference was found by the isolation [$F_{1/28} = 0.01$, $p = 0.94$] and no interaction between factors was detected [$F_{1/28} = 0.50$, $p = 0.48$. The Bonferroni test indicates a significant decrease in this parameter in the experimental isolated group. No differences were detected between groups on the number of entries in the open and closed arms (data not shown).

Discussion

The present study reveals the novel finding that exposure to the bacterial endotoxin in the late prenatal period modifies the maternal behavior of the F1 generation and their offspring's behaviors in infancy and adulthood. Thus, the present study demonstrated that prenatal administration of LPS was able to modify the behavior of two generations of rats. In fact, both the maternal behavior and maternal aggressive behavior of the F1 generation were affected by the endotoxin exposure. In addition, in the F2 generation antenatally exposed to LPS, the body weight at weaning was decreased, and the maternal olfactory preference was impaired. In adult age, a decreased anxiety and increased locomotor behaviors were observed in these rats.

Prenatal exposure to LPS on GD18 did not affect the reproductive performance of female rats. No differences were observed in the number of pups or in the body weight of the pups at birth; likewise, perinatal deaths did not occur. A previous study of our group also found that prenatal exposure to LPS on GD 9.5 did not alter either the pups' body weight at birth or their neurodevelopment. However, in the present study, at weaning the pups' body

weight of the F1 generation prenatally treated with LPS decreased in relation to the control group, suggesting that prenatal exposure *in utero* to LPS reduces the overall physical development of the pups.

Immune challenge during pregnancy is associated with preterm birth and poor perinatal development. The mechanisms of these effects are not known. LPS did not cross the placental barrier, but induced the release of proinflammatory cytokines (Ashdown, et al., 2006). Several pieces of evidence linked elevated cytokine levels triggered by maternal infection to influence various neurodevelopmental processes, including cell differentiation, maturation, and survival (Deverman & Patterson, 2009; Ghiani, et al., 2011; Paris, Brunton, Russell, & Frye, 2011; Zhao & Schwartz, 1998). Hence, fluctuations in their maternal and fetal levels, for instance, because of a maternal infection, signify a disturbance that can impede the ongoing of neurodevelopmental processes, and subsequently affect proper neural cell maturation (Jonakait, 2007; Meyer, Feldon, & Fatemi, 2009a; Meyer, Feldon, & Yee, 2009; Meyer, et al., 2007).

Also, the maternal behavior of the F1 generation prenatally exposed to LPS or saline solution 0.9% on GD18 was influenced by the prenatal endotoxin exposure. By the way, increases in latency to presents the full maternal behavior was observed in the F1 female rats of the LPS group relative to controls. No significant differences between the remained parameters in both experimental and controls female rats were found. So, although there was a delay to perform crouching, the females of the F1 generation exposed to LPS prenatally spent more time on their offspring. The delay to crouching the pups could not be attributed to a motor impairment, since any effects were found in the open field test. In addition, no motivational impairment was presently observed in F1 maternal behavior because pup retrieval was not modified by prenatal LPS (Pedersen & Boccia, 2003; Pedersen, Vadlamudi, Boccia, & Amico, 2006; J. M. Stern, 1990; J. M. Stern & Protomastro, 2000).

Crouching is considered a quiescent maternal posture and usually occurs in response to sufficient stimulation by pups. A mother rat tends to stop other activities and develops a characteristic posture with her extremities spread out and back arched (Teodorov, et al., 2010). The purpose of the crouching posture is to allow the pups access to teats and milk, to regulate their temperature, and to protect them from environmental elements. Thus, female rats of the F1 generation prenatally exposed to LPS, provided their offspring the opportunity to feed and to regulate their body temperature. However, the latency to present the full maternal behavior was delayed in experimental female rats when compared to the control group. Differences in the effects of prenatal LPS on F1 generation nursing (full maternal behavior) in comparison to its effects on retrieval and nest behaviors may be explained by the understanding that retrieving and nest building behaviors may be more indicative of maternal motivation, whereas the nursing behavior may be indicative of a more reflexive maternal response (Kristal, 2009; J.M. Stern, 1996). In other words, retrieving may represent an active voluntary response, which reflects interest and attraction toward pup-related stimuli, and nursing may be transiently activated as a reflex when the female wanders near pups and they crawl under her.

Despite the delay in the latency to full maternal behavior, these females guaranteed protection of their pups from environmental threats. In fact, during maternal aggressive behavior, these females presented more frequency of bites to intruders and retrieved their pups more frequently than did the control female rats. In a previous experiment (not published) we observed that prenatal exposure on GD18 to the same dose of LPS reduced the maternal olfactory preference in pup rats. Thus, it is possible that a reduced stimulation of pups toward their mothers delayed the onset of full maternal behavior.

It has been known for some time that stressful manipulations in early life contribute to changes in stress reactivity that persists into adulthood. More severe interventions like maternal separation have a sensitizing effect on the stress axis, and milder ones such as

neonatal handling promote a more resilient phenotype (Francis & Meaney, 1999).

LPS administration to pregnant rats upregulates mRNA expression of the stress-related peptide, corticotrophin-releasing hormone, in the fetal brain (Iqbal, Moisiadis, Kostaki, & Matthews, 2012), suggesting the possibility of inducing a fetal stress response. The activation of the stress response during pregnancy was shown to have long-term consequences on the response of adult offspring to stressful situations, as demonstrated in rodents, primates, and humans (Matthews & Phillips, 2010). Environmental information received by a mother can induce a phenotypic change in her offspring, commonly known as a maternal or transgenerational effect (Agrawal, et al., 1999; Curno, et al., 2009). Certain cues in the maternal environment, e.g., the prevalence of predators or maternal infection, can lead to behavioral, morphological, and immunological changes in the following generation (Agrawal, et al., 1999; Grindstaff, et al., 2006).

In the F2 generation at birth, the offspring body weight from the antenatal LPS group did not differ from that of the control group. At weaning, however, a decrease in this parameter was observed in the experimental pup rats, indicating a decreased overall development in these rats, despite the improved maternal care of their respective mothers. In addition, the males of these pup rats had an impaired preference for maternal odor. We studied the olfactory maternal preference only in male offspring, because maternal care is much higher in male than in female pups(Teodorov, et al., 2010). Therefore, any deficit in maternal behavior could be better visualized. The olfactory impairment occurred in the first and second trials, and this impairment was reflected in the total time of olfactory preference.

Maternal–pups interaction involves the maternal olfactory recognition and ultrasonic vocalization. In pups, until the opening eyes (PND 11–16) (Fox, 1965), maternal recognition is made through olfactory cues. Thus, olfaction impairment could be a consequence of impairment in pups' motivation. Supporting evidence for this hypothesis comes from previous

findings that prenatal exposure to LPS impaired the social interest of rats in infancy and adulthood because of motivational impairments (Spencer, et al., 2007). Moreover, prenatal exposure to LPS on GD 9.5 reduced the pups' maternal odor perception in infancy; this impairment was also observed in adult age in the aversive cat odor (Kirsten, et al., 2011a). The most important data here observed is that a reduced pup–mother interaction occurs in the F2 generation of rats antenatally exposed to LPS, showing a transgenerational effect of the endotoxin.

To study if antenatal exposure to LPS had long-term effects on pups' behavior, these rats were observed in an open field and in the plus maze apparatus in adult age. In this case, we subdivided the rats exposed antenatally to LPS or saline solution into two new groups: two groups were isolated for one week, and the others remained grouped. This procedure was employed as a challenge to show if the antenatal exposure to LPS could modify the behavior of this generation.

In the open field test an increased locomotion frequency was observed in both the control and experimental rats submitted to isolation, suggesting an increased exploratory behavior, because no immobility differences between groups were observed. In the plus maze, a clear increased anxiety in the antenatal rats isolated and treated with LPS was observed relative to their control group. In fact, a reduced percentage in open arms was detected. This decrease resulted from an increase in the time of closed arms and a reduced time in the center of the apparatus. Thus, the isolation revealed the deficits produced by the antenatal exposure to LPS. Antenatal exposure to LPS also induced long-term effects on the rats' behavior.

Epigenetics was initially referred to as the "interactions between genes and their products which bring the phenotype into being" (Waddington, 1942). Today, the term refers to molecular or cellular alterations that influence gene expression and, by extension, physiology and behavior, without causing alterations to the DNA sequence itself (Hunter,

2012). These alterations are generally construed to include DNA methylation, non-coding RNAs, and covalent histone modifications or "marks," which include acetylation, phosphorylation, methylation, ubiquitination, and a growing host of ever more exotic moieties (Hunter, 2012).

It has also become apparent that both corticosteroids and stress have a pronounced epigenetic impact in both humans and animal models and that the relationship between the stress response and epigenetics in the brain is bidirectional (Hunter, 2012). Presently, antenatal LPS exposure improved certain aspects of maternal care of the F1 generation related to nursing and pups' survival, but not on maternal motivational parameters, probably because of a reduced maternal stimulation by the pups. In fact, in the F2 generation, antenatal LPS exposure reduces maternal recognition in infancy. In addition, later prenatal exposure to LPS induces transgenerational effects in the F2 generation resulting in a less resilient phenotype to anxiety. Whether these phenomena are derived from an epigenetic mechanism remains to be investigated.

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Caption to figures

Fig.1. Effects of prenatal LPS exposure (100 μg/kg on GD 18) or saline solution (NaCl 0.9%) on maternal aggressive behavior of F1 generation. (A) latency first attack; (B) number of attacks; (C)total of attacks ; (D) time of fight; (E) bites; (F) hiding the pups;(F) retrieve pups ; (G) sniffing pups . Data are presented as means ± SEM. N = 8/ group. * $p < 0.05$ in relation to control group (Student t test).

Fig.2. . Effects of antenatal LPS exposure (100 μg/kg on GD 18) or saline solution (NaCl 0.9%) on body weight (A) and maternal odor preference (B) of pup rats from F2 generation. Data are presented as means ± SEM. N = 8/ group. ANOVA two way analysis followed by the Bonferroni test. ** $p < 0.01$ in relation to control group.

Fig.3. Effects of antenatal LPS exposure (100 μg/kg on GD 18) or saline solution (NaCl 0.9%) on open field behavior and on plus maze of adult rats from F2 generation. Data are presented as means ± SEM. N = 8/ group. ANOVA two way analysis followed by the Bonferroni test. * $p < 0.05$ in relation to control group.

Table.1. Effects of prenatal LPS exposure (100 μg/kg on GD 18) or saline solution (NaCl 0.9%) on maternal behavior of F1 generation. Data are presented as means ± SEM or pecentage. N = 8/ group

Parameters	Control group	Experimental groups	p
Pup retrieval			
1st pup,s	5.09 ± 1.48	6.17 ± 1.80	0.65
1st pup,%	100	100	-
All pups,s	64.20 ± 29.6	77.33 ± 18.00	0.71
All pups, %	100	100	-
Grouping %	87.5	100	0.87
Latency to full maternal behavior,s	388.2 ± 126	936,00 ± 18*	0.0007
Full maternal behavior%	100	100	-

. * $p < 0.05$ in relation to control group (Student t test).

Fig.1

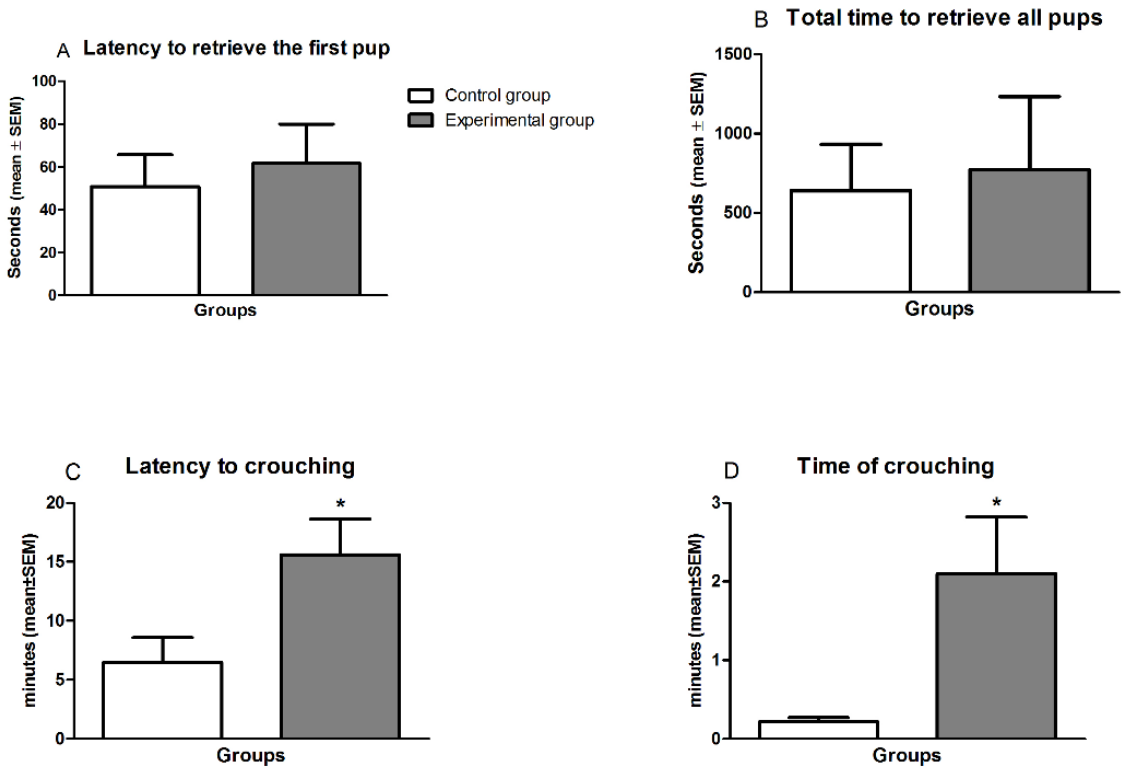

A Latency to retrieve the first pup
Seconds (mean ± SEM)
100
80
60
40
20
0
Groups
Control group
Experimental group
B Total time to retrieve all pups
Seconds (mean ± SEM)
1500
1000
500
0
Groups
C Latency to crouching
minutes (mean±SEM)
20
15
10
5
0
*
Groups
D Time of crouching
minutes (mean±SEM)
3
2
1
0
*
Groups

Fig.2

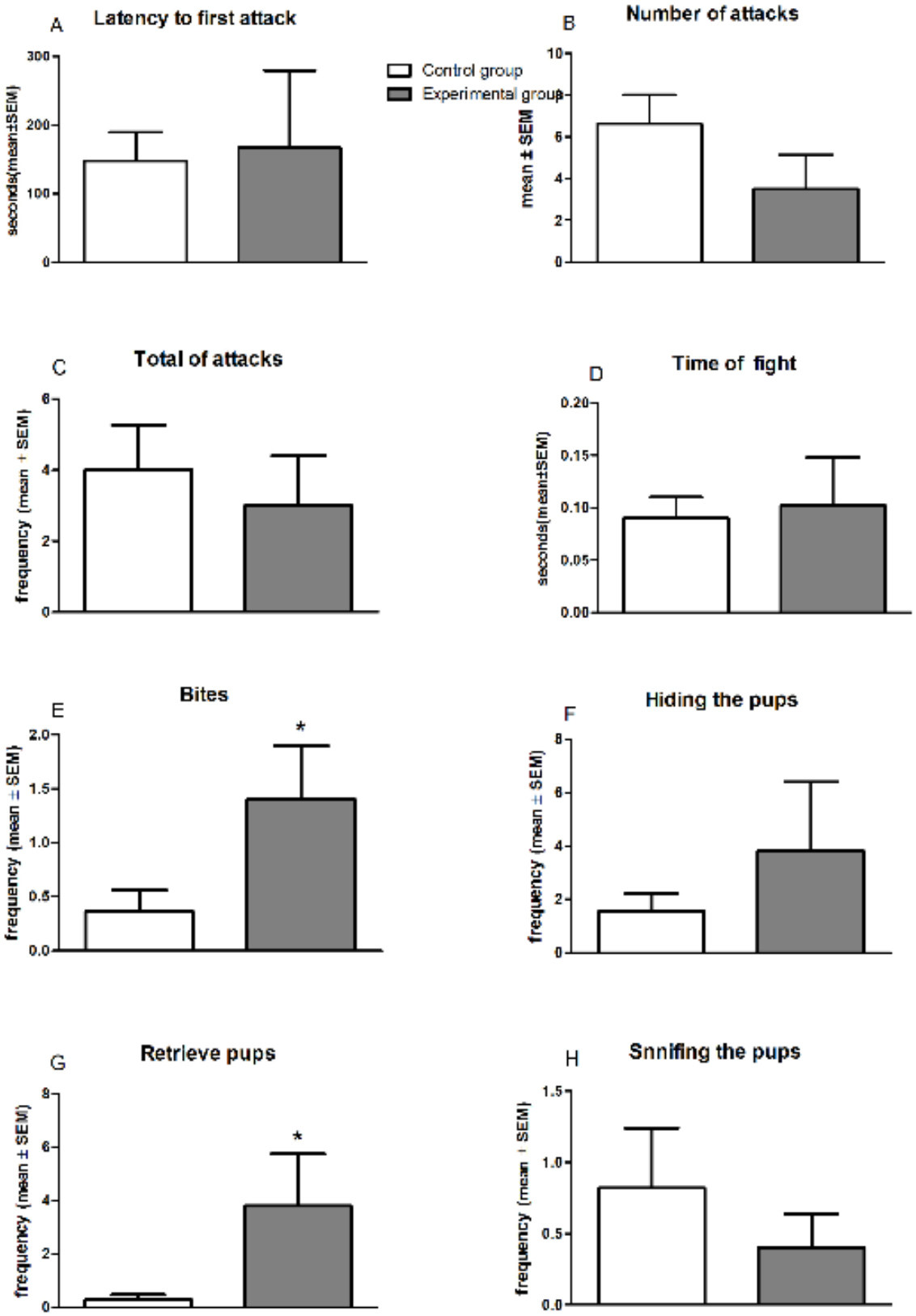

A
Latency to first attack
seconds(mean±SEM)
300
200
100
0
Control group
Experimental group
B
Number of attacks
mean ± SEM
10
8
6
4
2
0
C
Total of attacks
frequency (mean + SEM)
6
4
2
0
D
Time of fight
seconds(mean±SEM)
0.20
0.15
0.10
0.05
0.00
E
Bites
frequency (mean ± SEM)
2.0
1.5
1.0
0.5
0.0
*
F
Hiding the pups
frequency (mean ± SEM)
8
6
4
2
0
G
Retrieve pups
frequency (mean ± SEM)
8
6
4
2
0
*
H
Snnifing the pups
frequency (mean + SEM)
1.5
1.0
0.5
0.0

Fig.3

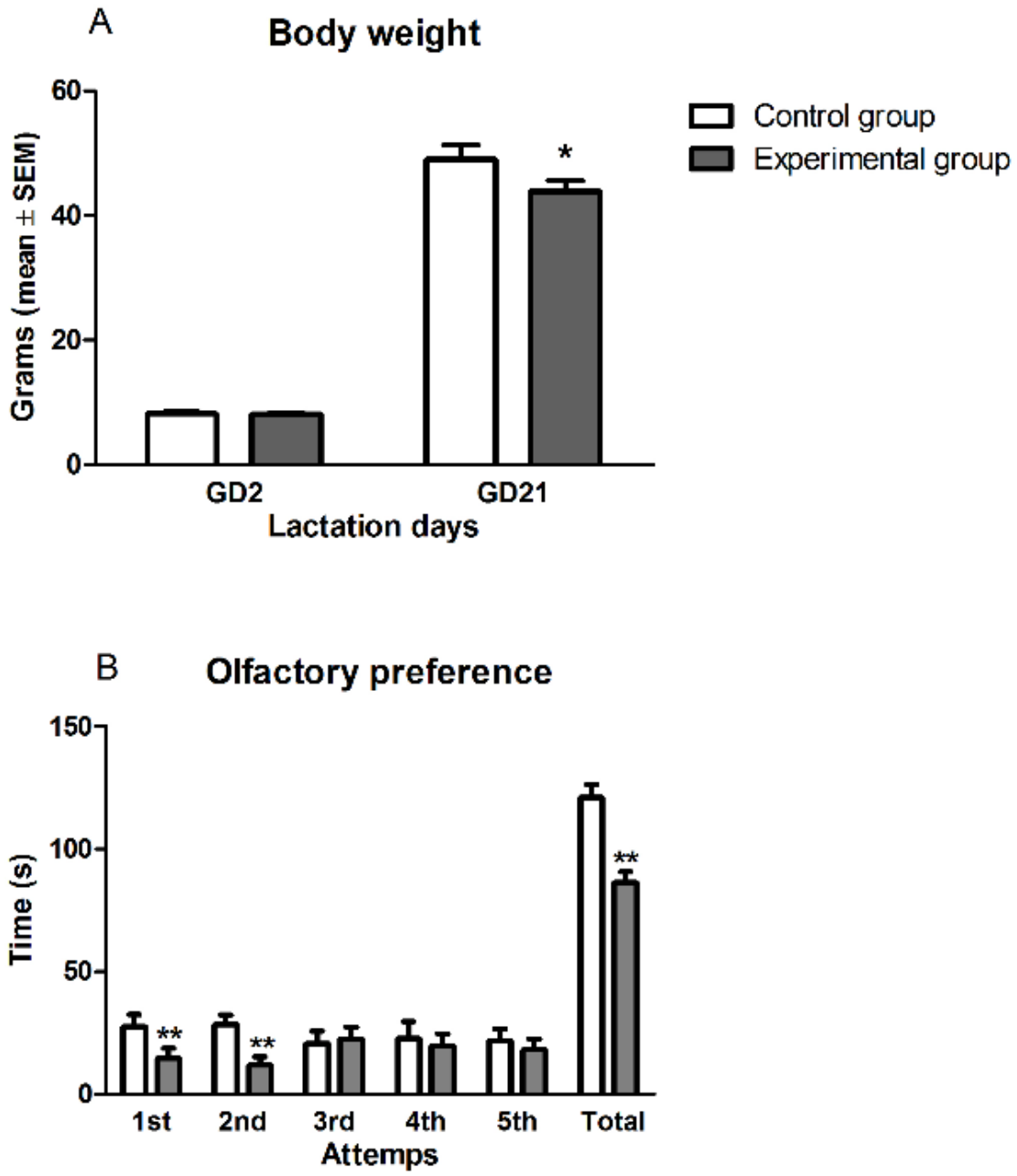
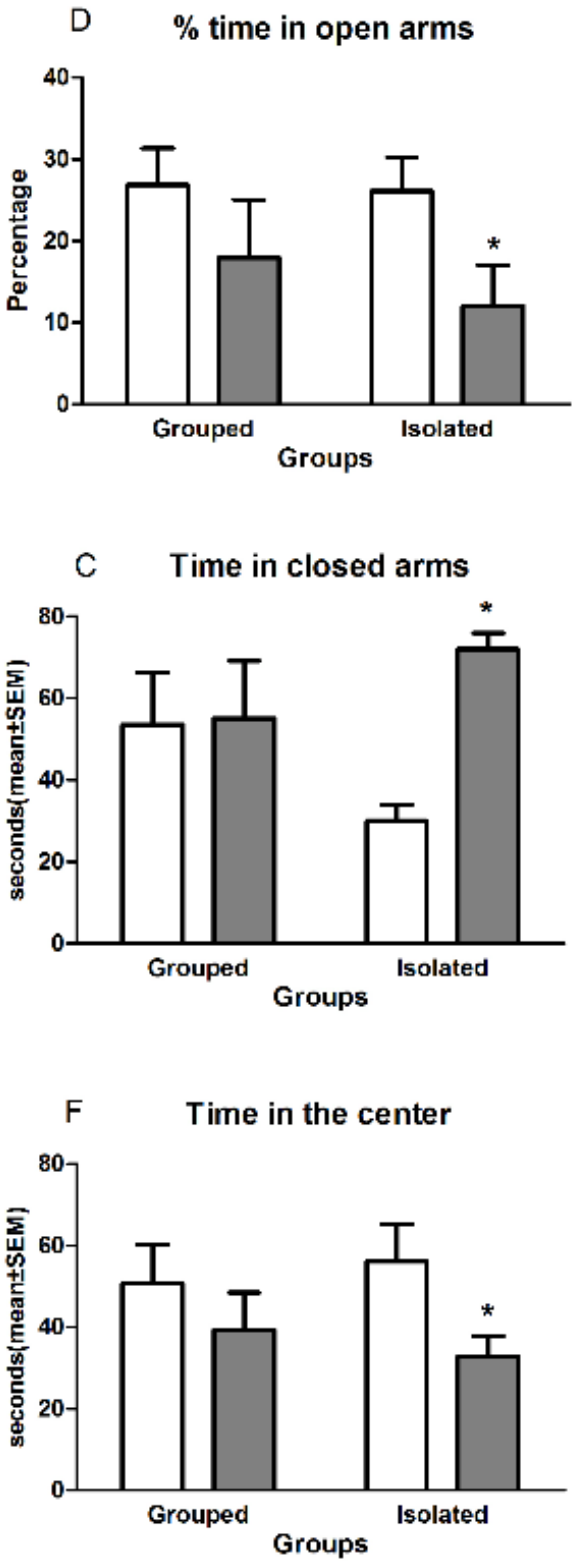

A
Body weight
Grams (mean ± SEM)
60
40
20
0
*
GD2
GD21
Lactation days
Control group
Experimental group
B
Olfactory preference
Time (s)
150
100
50
0
**
**
**
1st
2nd
3rd
4th
5th
Total
Attemps

Fig.4


D
% time in open arms
Percentage
40
30
20
10
0
*
Grouped
Isolated
Groups
C
Time in closed arms
seconds(mean±SEM)
80
60
40
20
0
*
Grouped
Isolated
Groups
F
Time in the center
seconds(mean±SEM)
80
60
40
20
0
*
Grouped
Isolated
Groups