UNIVERSIDADE PAULISTA - UNIP

PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA AMBIENTAL E EXPERIMENTAL

EFEITOS TRANSGERACIONAIS DA DIETA MATERNA: INFLUÊNCIA DA RESTRIÇÃO ALIMENTAR E DA DIETA HIPERCALÓRICA NO FENÓTIPO DE RATOS

Tese apresentada ao Programa de Pós-Graduação em Patologia Ambiental e Experimental da Universidade Paulista – UNIP para obtenção do título de Doutora em Patologia Ambiental e Experimental

ANDRÉIA DE OLIVEIRA JOAQUIM SILVA

São Paulo

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Orientadora: Profa. Dra. Maria Martha Bernardi
Co-orientadora: Leoni Villano Bonamin

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DEDICATÓRIA

Dedico esta tese a toda equipe envolvida no “projeto-mutirão”,
o trabalho, esforço e generosidade de cada um de vocês
tornou este sonho possível.

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“Nenhum de nós é tão bom
quanto todos nós juntos”

Ray Kroc

RESUMO

O desenvolvimento da prole é influenciado de muitas maneiras pela geração parental, incluindo não apenas a transmissão de DNA, mas também suas marcas epigenéticas, ambientais e comportamentais. Dentre os fatores implicados no controle epigenético está a dieta. O histórico alimentar das fêmeas, incluindo infância e adolescência, também pode impactar em mudanças fenotípicas da futura prole. Neste trabalho, demonstramos que ratos nascidos de mães alimentadas com dieta hipercalórica (HD) líquida e altamente palatável (Ensure ®), durante a puberdade (23-65 dias de idade), apresentaram alterações fenotípicas adaptativas tardias, como tendência a sobrepeso na idade adulta, pelo aumento de gordura retroperitoneal (RPF) e do número e área de adipócitos hipodérmicos, e dos níveis plasmáticos de fator de necrose tumoral (TNF-alfa), ocorrendo, porém, redução da área de processos astrocitários no hipotálamo. Curiosamente, esses animais também apresentaram resposta diferenciada do sistema nervoso central (SNC) ao desafio por lipopolissacarídeo (LPS), sendo mais resistentes a desenvolver comportamento doentio. Também demonstramos que ratos de duas gerações (F1 e F2) com dieta normal, cujas avós (geração F0) foram submetidas a 40% de restrição alimentar entre os dias 15 -18 da gestação, apresentaram alterações hipotalâmicas fenotípicas, na prole, compatíveis com maior sensibilidade à neuroinflamação, indicando claramente a tendência desses em acumular gordura subcutânea, principalmente na geração F2.

Palavras chaves: adipócitos, astrócitos, sistema imune, intergeracionais, nutrição materna, comportamento.

ABSTRACT

The development of the offspring is affected in many ways by the parental generation, comprising not only transmission of DNA, but also their epigenetic marks, environmental and behavioral. Diet is one of the factors related to epigenetic control. The dietary history of females, including childhood and adolescence, it can also impact on phenotypic changes in future offspring. In this study we demonstrate that rats born to mothers fed with liquid and highly palatable hypercaloric diet (Ensure ®) during puberty (23-65 days old) presented delayed adaptive phenotypic alterations, such as tendency to overweight in adulthood caused by increase of retroperitoneal fat and the number and area of hypodermic adipocytes, and plasma levels of tumor necrosis factor (TNF-alpha), but reducing the area of astrocytic processes in the hypothalamus. Interestingly, these animals also presented different responses in the central nervous system (CNS) related to challenge by lipopolysaccharide (LPS), being more resistant to developing sickness behavior. We also demonstrated that rats of two generations (F1 and F2) on a normal diet, which the grandparents (F0 generation) were submitted to 40% food restriction between 15 -18 days of gestation showed phenotypic changes in hypothalamic offspring compatible with higher sensitivity neuroinflammation and clearly indicating the trend of accumulating subcutaneous fat mainly in the F2 generation.

Key words: adipocytes, astrocytes, immune system, intergenerational, maternal nutrition, behavior.

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1. INTRODUÇÃO

1.1 Efeitos transgeracionais e epigenética

O desenvolvimento da prole é influenciado de muitas maneiras pela geração parental, incluindo não apenas a transmissão de DNA, mas também suas marcas epigenéticas, ambientais e comportamentais. Os mecanismos pelos quais os pais afetam o desenvolvimento da prole, além da transferência de DNA, são conhecidos como efeitos parentais ou herança não genética. Esses mecanismos incluem a transferência de padrões epigenéticos, nutrientes, anticorpos, hormônios e, também, as interações comportamentais pós-natais. Tal fenômeno pode ser observado também em plantas, portanto sua compreensão é igualmente útil para o gerenciamento da saúde ambiental (SZYF e BICK, 2013; ENGLISH *et al.*, 2015; SCHWINDT, 2015).

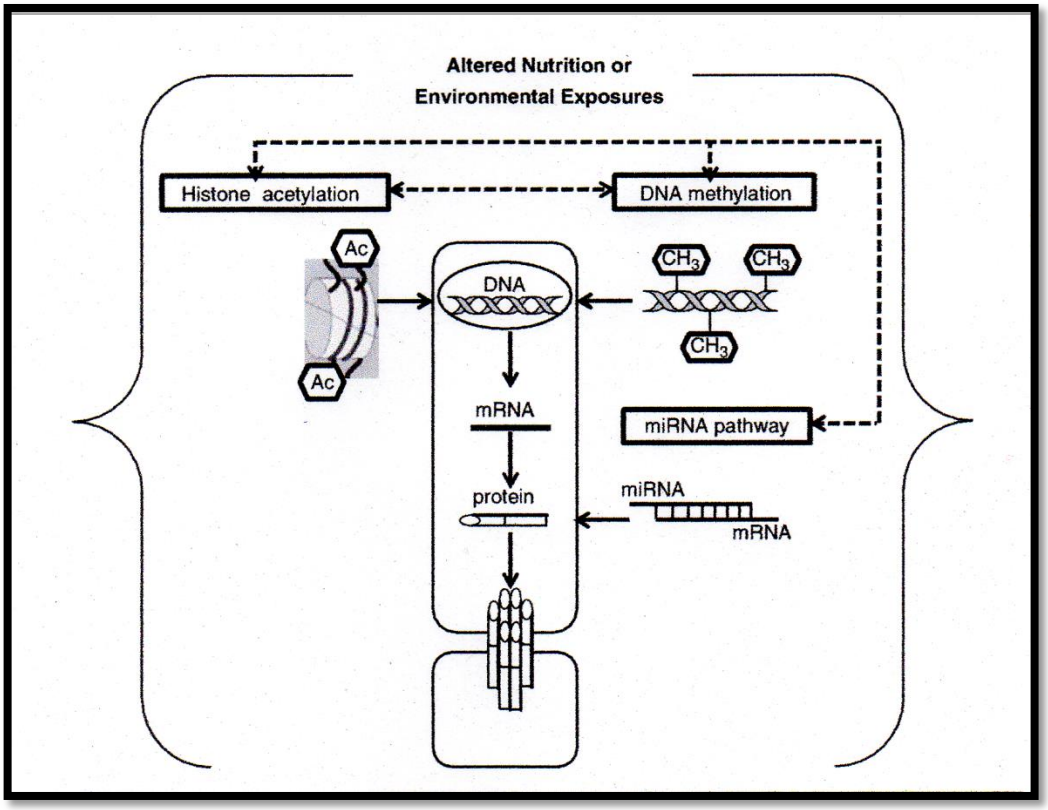
Uma parte significativa da herança não genética se deve aos mecanismos epigenéticos. Historicamente, os estudos sobre epigenética começaram com Waddington, em 1942, Nanney, em 1957, e, nos meados dos anos setenta, com Riggs e Holiday (BURGGREN, 2015). Esses pesquisadores descreviam epigenética como alterações fenotípicas que podiam ou não ser transmitidas entre gerações, sem mudanças na sequência dos genes (BURGGREN, 2015).

Atualmente, o termo epigenética é usado para descrever modificações químicas da cromatina, que incluem as modificações das histonas (isto é, acetilação, metilação, fosforilação), a metilação e desmetilação do DNA (BLAZE e ROTH, 2015), bem como a atividade regulatória da expressão gênica por microRNA ou RNA não codificador (JABLONKA e LAMB, 2005; ROSS e DESAI, 2013). Assim, o controle epigenético é a soma dos fatores genéticos e não genéticos que controlam seletivamente a expressão dos genes, produzindo, assim, o aumento da complexidade fenotípica durante o desenvolvimento (SILVA, 2013; SZYF e BICK, 2013; BURGGREN, 2015).

O mecanismo primário epigenético é a metilação do DNA. No embrião, o DNA é hipometilado e, em resposta à sinalização ambiental à metilação, aumenta progressivamente, o que implica silenciamento transcricional. O mecanismo secundário, por sua vez, corresponde a modificações de histonas, e o terciário está

relacionado à atividade de RNA não codificante. Ambos são capazes de modular a expressão gênica (ROSS e DESAI, 2013; YAN, 2014).

Figura 1- Ilustra os três principais mecanismos de regulação epigenética. I- Metilação de DNA, II- Modificação de Histonas e III- Atividade de RNA não codificante



Fonte: Ross e Desai (2013).

Os efeitos da epigenética podem ser classificados em duas categorias: “contexto-dependente” e “linha germinativa-dependente”. A primeira deriva da herança epigenética que afeta o fenótipo, como resultado da exposição direta e contínua, dentro ou entre gerações, a um estressor ambiental. Enquanto o estressor está presente, o fenótipo continua modificado. E a segunda deriva da modificação direta da linha germinativa e suas modificações fenotípicas persistem através de gerações, mesmo sem a presença direta de um agente estressor ambiental (SZYF e BICK, 2013; BURGGREN e CREWS, 2014; BURGGREN, 2015; FINEGERSH *et al.*, 2015).

1.2 Efeitos transgeracionais da dieta

Dentre os fatores estressores implicados no controle epigenético está a dieta. Exposições nutricionais precoces afetam o fenótipo da prole, incluindo desde situações restritivas até diferentes fontes de carboidratos de alta disponibilidade, como o álcool (RAO *et al.*, 2012; VICKERS, 2014; DESAI *et al.*, 2015; FINEGERSH *et al.*, 2015).

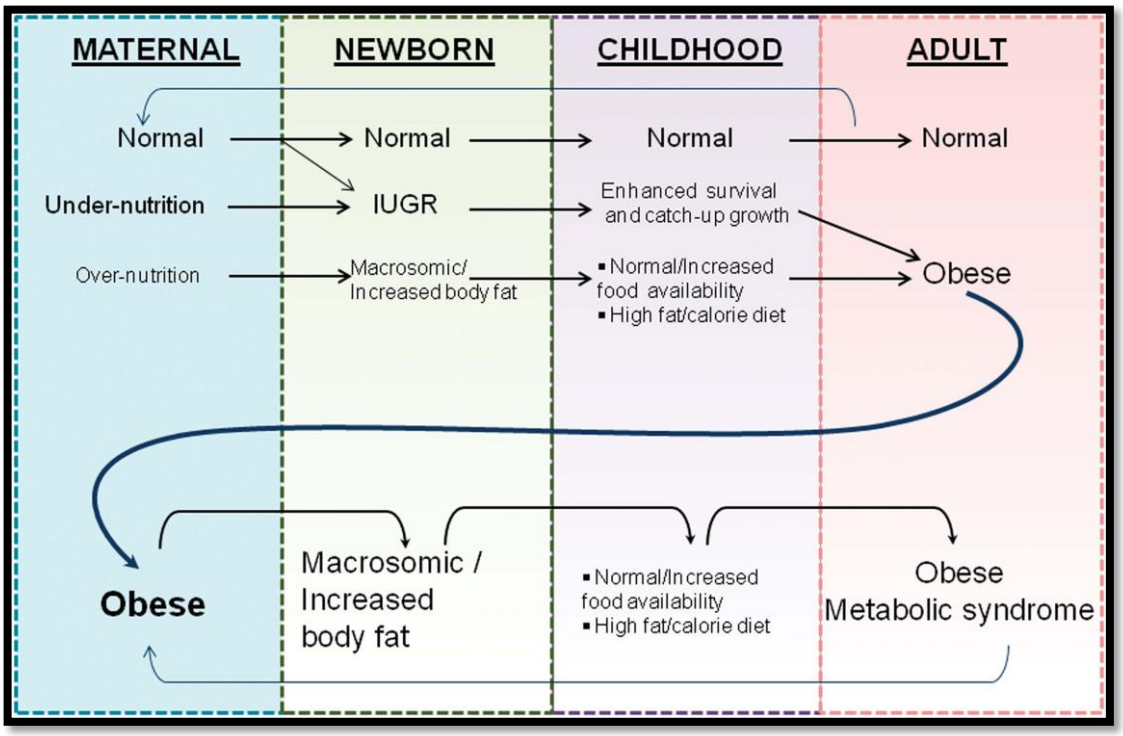
Durante a gravidez e o período neonatal, o indivíduo responde ao seu ambiente por meio da criação de trajetórias anatômicas, fisiológicas e bioquímicas, que moldam sua futura saúde. Por exemplo, a subnutrição perinatal leva à redução do peso do encéfalo e do corpo, conforme descrito por Perez-Torrero e colaboradores, em 2003 (PEREZ-TORRERO *et al.*, 2003). Nesse período, estabelece-se uma plasticidade de processos fisiológicos capaz de preparar o indivíduo para possíveis agressões ambientais relacionadas à alimentação, levando ao risco de obesidade e outros problemas correlatos (MACAULAY *et al.*, 2014). Tanto a desnutrição como a supernutrição materna podem programar na prole a obesidade e a síndrome metabólica (ROSS e DESAI, 2013; AIKEN e OZANNE, 2014; DESAI *et al.*, 2015; SKINNER, 2015). A maior parte dos artigos constantes na literatura mostram os efeitos transgeracionais da alimentação materna durante a gestação; contudo, o histórico alimentar das fêmeas, incluindo infância e adolescência, também pode impactar em mudanças fenotípicas da futura prole. É o que demonstramos neste trabalho.

Por outro lado, problemas de prematuridade e deficiência de crescimento intrauterino também podem potencializar desvios metabólicos nas gerações seguintes. Assim, a sobrevida de recém-nascidos prematuros e com crescimento deficiente favorece o desenvolvimento de obesidade no adulto, que pode ser potencializada com dieta excessiva, respeitando o padrão de fenótipo econômico, conforme descrito por Hales e Barker, 2001. As gerações seguintes, por consequência, também apresentarão desvios metabólicos por efeito transgeracional, o qual pode se dar por via materna ou paterna (MARTINEZ *et al.*, 2012; HALES e BARKER, 2013; VICKERS, 2014; DESAI *et al.*, 2015).

O tamanho do corpo, bem como a estruturação do sistema endócrino e do perfil metabólico na prole, é resultante de uma combinação de estímulos, que incluem a qualidade do ambiente pós-natal e o fluxo transplacentário de mediadores

químicos, que informam o feto sobre o estado nutricional da mãe, permitindo a adaptação do indivíduo às condições do meio para enfrentar ambientes com restrição nutricional ou alto gasto de energia. Quando submetidos a ambientes diferentes do programado, tais indivíduos podem desenvolver doenças na fase adulta, como diabetes e síndrome metabólica (RIBEIRO *et al.*, 2015).

Figura 2- Ilustra a programação gestacional da população que desloca para a obesidade e síndrome metabólica (DESAI *et al.*, 2015). IUGR = restrição de crescimento intrauterino.



Fonte: Desai (2015).

1.3 Restrição alimentar, sobrepeso, obesidade e SNC

Taxas globais de sobrepeso e obesidade têm aumentado rapidamente nos últimos 30 anos, afetando o sistema de saúde pública, a economia e a sociedade. Mundialmente, a prevalência de sobrepeso e obesidade é de 27,5% nos adultos e 47,1% nas crianças nascidas entre 1980 e 2013 (HAIDAR e COSMAN, 2011; BERENSON, 2012; KITZINGER e KARLE, 2013; MACAULAY *et al.*, 2014). Isto afeta a qualidade de vida e eleva os índices de mortalidade (FORMIGUERA e CANTON, 2004; RODRIGUEZ-HERNANDEZ *et al.*, 2013), pois os indivíduos que estão com sobrepeso e obesos têm risco aumentado para várias doenças, incluindo diabetes tipo 2, doenças respiratórias, doenças cardiovasculares, distúrbios endócrinos,

disfunção imune, certos tipos de câncer, transtornos psiquiátricos e diminuição da fertilidade (FORMIGUERA e CANTON, 2004; HASLAM e JAMES, 2005; ABALLAY *et al.*, 2013; ALEMAN *et al.*, 2014).

A obesidade crônica está geralmente associada a um processo inflamatório permanente (BUCKMAN *et al.*, 2013). Adipócitos hipodérmicos (HAS) produzem várias adipocinas (leptinas, adiponectina, resistina, etc.) e mais de 50 hormônios, de forma autócrina, parácrina ou endócrina, que atuam tanto positiva como negativamente em processos inflamatórios sistêmicos, com a participação de macrófagos, linfócitos e citocinas diversas, tais como fator de necrose tumoral alfa (TNF alfa) e interleucina 6 (IL 6). As citocinas e alguns hormônios, como a leptina, também regulam a expressão gênica envolvida no controle metabólico. Assim, o TNF alfa e a leptina são marcadores epigenéticos deste processo (MARTINEZ *et al.*, 2012; MAKKI *et al.*, 2013; RODRIGUEZ-HERNANDEZ *et al.*, 2013). Os efeitos sistêmicos da obesidade, em geral, levam à síndrome metabólica, que é caracterizada por inflamação crônica de baixo grau em vários tecidos, como fígado, pâncreas, músculos, vasos, coração, sistema reprodutivo e encéfalo, incluindo o hipotálamo (ANDEL *et al.*, 2009; LI *et al.*, 2011; GARCIA-CACERES *et al.*, 2013).

A inflamação hipotalâmica ocorre mesmo antes do ganho de peso corporal (BW) (GARCIA-CACERES *et al.*, 2013; GUYENET *et al.*, 2013). No hipotálamo, a micróglia e os astrócitos produzem citocinas que induzem respostas inflamatórias. Devido a sua proximidade física com os vasos sanguíneos e seu papel no transporte de substâncias, os astrócitos são diretamente afetados pelo excesso de nutrientes (GARCIA-CACERES *et al.*, 2013). Os astrócitos são as células mais abundantes no sistema nervoso central (SNC), e suas funções incluem a modulação da atividade neuronal, o armazenamento e fornecimento de energia para os neurônios e a regulação da barreira hematoencefálica (BUCKMAN *et al.*, 2013; GUYENET *et al.*, 2013; MAYO *et al.*, 2014). Em resposta a estímulos nocivos, os astrócitos podem entrar num estado reativo caracterizado por aumento no número e tamanho de células (astrogliose e astrocitose, respectivamente) e por outras alterações morfológicas associadas ao aumento da expressão de proteínas do citoesqueleto, tais como vimentina, nestina e proteína glial fibrilar ácida (GFAP) (GUYENET *et al.*, 2013). Assim, os astrócitos podem desempenhar papel único na promoção de respostas inflamatórias hipotalâmicas na obesidade (GARCIA-CACERES *et al.*, 2013; GUYENET *et al.*, 2013; RODRIGUEZ-HERNANDEZ *et al.*, 2013), as quais

podem, também, modificar aspectos comportamentais e cognitivos (LI *et al.*, 2011). Sabe-se que a depressão e a ansiedade podem estar associadas à neuroinflamação (SINGHAL *et al.*, 2014).

Após a exposição a dietas hipercalóricas, os neurônios do núcleo arqueado e eminência média, assim como os astrócitos e a micróglia dessas áreas, expressam vários marcadores de dano celular, como TNF alfa, IL 6, supressor de sinalização de citocina 3 (SOCS 3), etc. A expressão de GFAP e do receptor do tipo Toll 4 (TLR 4) também acompanha o processo de gliose. Esses efeitos são seguidos de expansão da massa corporal, caracterizando a antecipação da neuroinflamação em relação ao sobrepeso e obesidade, descritos anteriormente. Dessa forma, os astrócitos participam da neuroproteção, limitando a extensão da inflamação e da perda de neurônios subsequente (THALER *et al.*, 2012). Ao contrário, animais submetidos a dieta restritiva expressam menos GFAP em comparação àqueles que recebem dieta normal *ad libitum*. A privação alimentar parcial e constante de 50-70% previne a astrogliose excessiva em situações de dano cerebral, por meio da regulação da expressão de proteínas envolvidas na plasticidade neural, como a GAP-43 e a sinaptofisina (SYP). Ocorre, portanto, a neuroproteção (LONCAREVIC-VASILJKOVIC *et al.*, 2009).

O período perinatal, em particular, é especialmente sensível à restrição alimentar, pois é quando ocorre a maior parte dos processos de maturação do hipotálamo. Mudanças hormonais nesse período podem ser o gatilho de várias alterações duradouras no circuito hipotalâmico relacionadas com atividade metabólica e balanço de energia corporal. Os neurônios do núcleo arqueado e neurônios produtores de pró-opiomelanocortina (POMC) e de peptídeos anorexígenos e orexígenos são os mais envolvidos nesse processo. A restrição alimentar antes do desmame leva à maior expressão de transdutor de sinal e ativador de transcrição 3 (pStat-3) e c-Fos no núcleo arqueado, com consequente aumento na sinalização à leptina e aumento da longevidade (SADAGURSKI *et al.*, 2015). Ao contrário dos efeitos neuroprotetores da restrição alimentar no período pós-natal precoce, a restrição alimentar durante a gestação pode induzir alterações hipotalâmicas fenotípicas na prole compatíveis com maior sensibilidade à neuroinflamação, como demonstramos neste estudo.

1.4 Modelos animais

Vários modelos animais têm contribuído para a construção do conhecimento acerca dos processos transgeracionais relacionados à dieta. Os roedores são os animais mais utilizados para este tipo de estudo, mas há relatos de pesquisas feitas em carneiros que mostram os efeitos da obesidade no padrão fenotípico da cria (fenótipo econômico). Também em primatas babuínos, a dieta hipercalórica (HD) da mãe gera hipertrofia de células adiposas e esteatose hepática na cria, as quais permanecem até a idade adulta (TAYLOR e POSTON, 2007; LI *et al.*, 2011). O modelo clássico para demonstração da ocorrência de obesidade em ratos nascidos de mães privadas de alimento foi descrito em 1984 (JONES *et al.*, 1984). Parte do presente trabalho foi baseada nesses estudos, assim, esta tese é dividida em duas partes: uma corresponde ao artigo enviado à publicação sobre os efeitos transgeracionais da dieta hipercalórica da mãe durante a puberdade e a outra descreve os efeitos da privação alimentar parcial durante a gestação nas gerações seguintes. Segue abaixo a descrição sucinta de ambos os artigos.

2. OBJETIVOS

2.1 Objetivo do Artigo 1

Verificar se a dieta hipercalórica administrada na puberdade de ratas Wistar promove efeitos transgeracionais fenotípicos na prole relacionados com acúmulo de gordura abdominal, comportamento e neuroinflamação.

2.2 Objetivo do Artigo 2

Verificar se a privação alimentar materna durante a gestação promove efeitos transgeracionais fenotípicos, nas gerações seguintes (F1 e F2), relacionados com acúmulo de gordura abdominal, comportamento e neuroinflamação.

3. RESUMO DOS TRABALHOS

3.1 Artigo 1 – Transgenerational effects of hypercaloric diet

A obesidade crônica está associada a processo inflamatório central e periférico persistente. A inflamação hipotalâmica é um dos fatores que antecedem a obesidade, envolvendo células como micróglia e astrócitos, sendo esses últimos particularmente afetados pelo excesso de nutrientes. Adicionalmente, evidências experimentais e clínicas mostram que a programação do desenvolvimento corporal pode ser vista como fenômeno transgeracional adaptativo, cujos mecanismos são epigenéticos. A alimentação excessiva da mãe pode definir padrões específicos de gordura corporal e de parâmetros relacionados ao processo inflamatório e à atividade metabólica de sua prole.

Nesse trabalho demonstramos que ratos nascidos de mães alimentadas com dieta hipercalórica líquida e altamente palatável (Ensure ®) durante a puberdade (23-65 dias de idade), porém alimentadas com dieta normal durante a gestação, apresentam alterações fenotípicas adaptativas tardias.

As fêmeas da geração parental (F0) foram submetidas à eutanásia após o desmame e foram analisados os seguintes parâmetros: peso da gordura retroperitoneal (RPF), análise histométrica da gordura hipodérmica e análise histométrica dos astrócitos hipotalâmicos. Os machos, filhos da geração F0, denominados F1, foram pesados no segundo dia de vida e o ganho de peso durante a lactação também foi mensurado. Após o desmame, foram avaliados quanto à atividade geral no campo aberto (CA) e alimentados com ração comercial balanceada até o dia da eutanásia (50 dias de vida). Quatorze horas antes, metade dos animais da geração F1 foi desafiada com 100 microgramas/quilo de LPS, via intraperitoneal, e a outra metade foi tratada com solução salina 0,9%. Uma nova análise do comportamento no CA foi realizada imediatamente antes da eutanásia para avaliação do comportamento doentio e parâmetros motores.

Na necropsia, a gordura retroperitoneal, a hipoderme abdominal, o encéfalo e o sangue foram colhidos de cada animal. As amostras de sangue foram utilizadas para a dosagem de diferentes citocinas, neuropeptídios e hormônios. A hipoderme abdominal e o encéfalo foram avaliados histologicamente. Os adipócitos hipodérmicos foram quantificados segundo duas categorias: células pequenas (até 9

mil pixels) e células grandes (acima de 9 mil pixels). O encéfalo foi seccionado látero-lateralmente na altura do hipotálamo para avaliação quantitativa da área correspondente aos astrócitos periventriculares positivos ao GFAP.

Houve aumento do peso corpóreo nas fêmeas (F0) após o período de alimentação hipercalórica, acompanhado de aumento muito significativo (P<0,0001) da gordura retroperitoneal e da área dos grandes adipócitos hipodérmicos (p<0,0001). Nenhuma alteração comportamental foi observada na geração F0, mas houve aumento significativo (p=0,001) na positividade dos astrócitos hipotalâmicos ao GFAP.

Na geração F1, observou-se aumento do peso, ao nascimento, nos animais nascidos de mães alimentadas com dieta hipercalórica durante a puberdade, contudo esses animais apresentaram significante perda de peso ao desmame, a qual foi normalizada na idade adulta, exceto aqueles que foram desafiados com LPS. Esses animais também apresentaram aumento do peso da gordura retroperitoneal em relação ao controle, e esse efeito foi igualmente revertido após o tratamento com LPS. A razão entre número de adipócitos grandes e pequenos neste grupo também apresentou aumento significativo (p<0,0001), reforçando a constatação de que esses animais tendem a acumular gordura periférica. Não houve alteração comportamental nesses animais sem desafio; todavia o comportamento doentio induzido pelo LPS no grupo controle foi revertido no grupo experimental.

Em relação ao perfil inflamatório central, os animais nascidos de mães alimentadas com dieta hipercalórica mostram redução significativa na trama de processos astrocitários em reação ao controle, mesmo após desafio com LPS. Da mesma forma, este grupo apresenta aumento nos níveis plasmáticos de TNF-alfa, os quais são igualmente independentes do estímulo com LPS.

Conclui-se que a geração F0 reproduziu, nesse modelo experimental, os dados da literatura pertinente ao assunto. Os resultados da prole são inéditos e mostram padrão adaptativo às condições alimentares das mães mesmo antes da gestação. Esses padrões são caracterizados por tendência a sobrepeso no adulto, pelo aumento de gordura retroperitoneal, e aumento do número e da área de adipócitos hipodérmicos, bem como aumento nos níveis plasmáticos de TNF-alfa e redução da área de processos astrocitários no hipotálamo. Curiosamente, esses animais também apresentaram resposta diferenciada do SNC ao desafio por LPS, sendo mais resistentes a desenvolver comportamento doentio.

3.2 Artigo 2 – Maternal food deprivation increased the retroperitoneal fat, the number and size of adipocytes and induced periventricular astrogliosis in F1 and F2 generations.

Sabe-se, pela literatura, que alterações da dieta durante a gestação interferem no controle metabólico da prole na idade adulta, levando à resistência insulínica e tendência ao ganho de peso. Isso pode ser percebido também na capacidade de certas estruturas do SNC de desenvolver respostas inflamatórias, como ocorre no hipotálamo. A hipertrofia hipotalâmica observada por meio da expressão de GFAP é um indicador importante desse processo.

Nesse estudo, ratas Wistar gestantes foram submetidas à privação alimentar em 40%, entre os dias 15 -18 da gestação. Esse período foi escolhido para evitar canibalismo e perda da prole no período pós-natal. As fêmeas controle foram alimentadas normalmente com ração comercial balanceada. Todas as fêmeas (privadas ou não) deram origem às gerações F1 e F2, as quais também foram alimentadas normalmente.

Aos 21 dias de vida, as fêmeas da geração F1 de cada grupo foram divididas em dois subgrupos, sendo que metade foi submetida às análises de ganho de peso corporal, gordura retroperitoneal, número e tamanho de adipócitos hipodérmicos e expressão de GFAP em astrócitos hipotalâmicos até a idade adulta (90-95 dias de idade) e a outra metade foi encaminhada à reprodução para dar origem à geração de F2.

Os machos da geração de ambos os grupos foram submetidos às mesmas análises, na puberdade (50 dias de idade). As fêmeas de F2 foram usadas em outro experimento.

Os adipócitos hipodérmicos foram analisados histometricamente e divididos em duas populações distintas: uma composta de células pequenas (até 9 mil pixels) e a outra composta de células grandes (acima de 9 mil pixels). Tanto o tamanho quanto o número dessas células foram contabilizados. Os astrócitos e seus processos foram mensurados quanto a área de positividade de GFAP a partir de cortes histológicos da região periventricular do hipotálamo.

Durante a gestação, as mães que sofreram privação tiveram redução de mais de 50% no ganho de peso. Nos filhotes, o ganho de peso foi menor ao nascimento e durante a lactação em ambas as gerações, F1 e F2. Na idade adulta, o peso dos

animais da geração F1 nascidos de mães privadas foi equivalente ao dos respectivos controles, mas na geração F2 o peso foi maior que o controle, indicando tendência à obesidade ainda mais acentuada. O peso da gordura retroperitoneal foi o dobro do controle na geração F1, mas também estava significantemente aumentado na geração F2.

A geração F1 apresentou aumento expressivo no número de pequenos adipócitos, cujo tamanho médio foi estatisticamente menor que o controle. A geração F2, por sua vez, apresentou aumento no tamanho médio de pequenos adipócitos e aumento no número de grandes adipócitos, indicando claramente a tendência desses em acumular gordura subcutânea.

Em relação à positividade ao GFAP por astrócitos hipotalâmicos, observou-se aumento nos descendentes de mães privadas, em ambas as gerações.

Os dados obtidos nesse trabalho evidenciam a adaptação estrutural fenotípica entre gerações em resposta à privação alimentar materna durante a gestação, incluindo a capacidade de armazenamento lipídico e a neuroinflamação.

4. CONSIDERAÇÕES FINAIS

A alimentação hipercalórica da geração F0 modificou os níveis plasmáticos de TNF-α, ocorrendo mudanças de comportamento após a exposição LPS e alterações na reatividade de astrócitos na geração F1. Especulamos que o efeito transgeracional de obesidade ocorreu, pelo menos em parte, por mudanças persistentes na programação do sistema imunológico.

A privação de alimentos das mães durante a gravidez na geração F0 induziu, em duas gerações (F1 e F2), o fenótipo transgeracional à tendência de sobrepeso e obesidade. Esse padrão hereditário também foi observado no SNC, refletido por astrogliose no hipotálamo periventricular, em ambas as gerações, e os efeitos parecem aumentar ao longo de gerações.

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6. ANEXOS

6.1 Transgenerational effects of a hypercaloric diet

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The effects of a maternal hypercaloric diet (HD) during puberty and early adulthood on neuroimmune aspects in offspring were investigated. In female rats of the F_0 generation and male rats of the F_1 generation, bodyweight (BW) gain, retroperitoneal fat (RPF) weight, the number of hypodermic adipocytes (HAs) and expression of glial fibrillary acidic protein (GFAP) were measured in hypothalamic astrocytes. On Postnatal Day 50, the F_1 pups were challenged with lipopolysaccharide (LPS , 100 µg/kg, sc) or an equal volume of saline (S), and behaviour in the open field test was evaluated, as were plasma neuropeptide and cytokine concentrations. The maternal HD caused the female F_0 rats to become overweight. The F_1 offspring of dams fed the HD and injected with saline (HDS group) exhibited increases in BW gain, RPF weight and in the number of large HAs and a decrease in GFAP immunoreactivity. F_1 offspring of dams fed the HD and injected with LPS (HDLPS group) exhibited decreases in BW gain, RPF weight and GFAP immunoreactivity, but no differences were observed in the number of larger and small HAs. Plasma tumour necrosis factor-α concentrations were high in the HDS and HDLPS groups. Thus, the maternal HD during puberty and early adulthood caused the F_1 generation to become overweight despite the fact that they received a normocaloric diet. These results indicate a transgenerational effect of the HD that may occur, in part, through permanent changes in immune system programming. The attenuation of neuroinflammation biomarkers after LPS administration may have resulted in a decrease in the number of adipocytes, which, in turn, reduced cytokine, adipokine and chemokine levels, which are able to recruit inflammatory cells in adipose tissue.

Additional keywords: adipocytes, astrocytes, behaviour, immune system, intergenerational relationship, maternal nutrition.

The maternal diet can alter the bodyweight and immune programming of subsequent generations. A maternal hypercaloric diet (HD) during puberty and early adult age induces overweight in the F_1 generation and increases peripheral inflammation despite the feeding of a normocaloric diet to the F_1 generation. It also induces adaptative patterns of the hypothalamic glial response towards a state of neuroprotection. These data reveal a transgenerational effect of the HD that may occur, in part, through permanent changes in immune and nervous system programming.

Introduction

The incidence of obesity and overweight has increased substantially in recent decades, and obesity is now a major global health problem (Haidar and Cosman 2011; Berenson 2012; Kitzinger and Karle 2013). The considerable health burden of obesity and overweight has negative effects on many health outcomes, leading to disability, mortality and increased healthcare use (Formiguera and Cantón 2004; Rodríguez-Hernández et al. 2013). Individuals who are overweight and obese have an increased risk of several diseases, including Type 2 diabetes, respiratory disorders, cardiovascular disease, endocrine disorders, immune dysfunction, certain types of cancer, psychiatric disorders and decreased fertility (Formiguera and Cantón 2004; Haslam and James 2005; Aballay et al. 2013; Alemán et al. 2014).

Chronic obesity is generally associated with a permanent inflammatory process (Buckman et al. 2013). Increased adiposity is related to systemic low-grade inflammation during pubertal growth and is important for detecting early signs of obesity-related metabolic disorders (Wen et al. 2014). Hypodermic adipocytes (HAs) produce several adipokines that act both positively and negatively in systemic inflammatory processes (Makki et al. 2013; Rodríguez-Hernández et al. 2013). Obesity is characterised by chronic low-grade inflammation in several tissues, including the hypothalamus (Andel et al. 2009;García-Cáceres et al. 2013; Calvo-Ochoa et al. 2014). Hypothalamic inflammation is an early factor in the onset of obesity, which occurs even before bodyweight (BW) gain (García-Cáceres et al.

2013; Guyenet et al. 2013). In the hypothalamus, microglia and astrocytes produce cytokines that drive inflammatory responses (García-Cáceres et al. 2013). Because of their physical proximity to blood vessels and role in transporting nutrients, astrocytes are directly affected by excess nutrients (García-Cáceres et al. 2013). Astrocytes may play a unique role in promoting hypothalamic inflammatory responses in obesity (García-Cáceres et al. 2013; Rodríguez-Hernández et al. 2013; Guyenet et al. 2013; Calvo-Ochoa et al. 2014).

A wide range of nutritional factors during pregnancy and lactation, including undernutrition and maternal obesity, can lead to a range of metabolic disorders in offspring (Li et al. 2011; Vickers 2014). Experimental and human evidence suggests that developmental programming should be regarded as a transgenerational phenomenon and it is therefore often viewed as a form of epigenetic inheritance via either maternal or paternal lineage (Taylor and Poston 2007; Ross and Desai 2013; Vickers 2014).

However, little is known about the long-term effects of a maternal hypercaloric diet (HD) during puberty and early adulthood on behavioural, endocrine and immune aspects in offspring. Thus, in the present study we examined the effects of a maternal HD in puberty and early adulthood on the behaviour, BW gain, retroperitoneal fat (RPF) weight, the number of HAs and glial fibrillary acidic protein (GFAP) immunoreactivity in astrocytes in the hypothalamus of the F1 generation. Knowledge about the interrelationship between adiposity and systemic low-grade inflammation during pubertal growth is important for detecting early signs of obesity-related metabolic disorders (Wen et al. 2014). Thus, F1 generation rats were challenged with lipopolysaccharide (LPS) at 50 days of age to investigate responses to immune activation.

Materials and methods

Ethics statement

The animal procedures in this study were performed in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources and those of the Brazilian Institutional Ethics Committee, Universidade Paulista (Protocol no. 130/12, CEUA/ICS/UNIP, 28/11/2012). The experiments were performed in accordance with good laboratory practice protocols and quality assurance methods. All efforts were made to minimise the suffering of the animals.

Animals

Twenty female Wistar rats, 23 days of age (F0 generation), from the School of Veterinary Medicine (University of São Paulo, SP, Brazil) were used. Rats were housed four per cage in microisolator cages under controlled temperature (22–26°C) and humidity (50–65%) conditions in artificially lit rooms on a 12-h light–dark cycle (lights on at 0700 hours) with free access to sterilised water and irradiated food. Sterilised, residue-free wood shavings were used for animal bedding.

Hypercaloric diet

Rats in the HD group (F0 generation) were given free access to the HD (Ensure; Abbot Brasil, São Paulo, Brazil; total of 1 kcal mL–1 in addition to laboratory chow (Nuvilab; Sogorb Ind. and Com., São Paulo, Brazil); values per 100 g solid food item: 4.2 kcal g–1, 56% carbohydrate, 19% protein, 4.5% cellulose, 5% vitamins and 3.5% g total fat). Ensure is a highly palatable liquid diet supplement and each 231 kcal bottle contained 1.7 g polyunsaturated fat, 3.59 g monounsaturated fat and 2.2 g saturated fat. It did not contain any trans-fat. It was presented in a graduated cylinder with a stopper, with 600 mL per bottle. Rats in the HD and normocaloric diet (ND) groups were housed four per cage and Ensure and laboratory chow were made available to each cage as a whole. The consumption of both diets was measured daily. Both diets were changed daily.

Experimental design

Female pups of the F0 generation were weighed at weaning (Postnatal Day (PND) 21) and randomly divided into two groups. One group received the HD from PND23 to PND65 (HD group; n = 10). The other group received normocaloric laboratory chow over the same period (ND group; n = 10). In the HD group, the HD was changed to normocaloric laboratory chow after PND65. BW gain in these female rats was recorded from PND23 to PND65. On PND90–95, female rats in both groups were mated with sexually experienced male rats to obtain the F1 generation. At weaning, the F0 females were killed and RPF was weighed. Portions of the hypodermis and brain were collected to count the number of small and larger HAs (see below) and for immunohistochemical analyses, respectively.

The litters (F1 generation) from both ND and HD dams (F0 generation) were weighed on PND2 and the pups from each treatment were distributed to respective ND and HD dams (four male pups and four female pups per dam). The individual BW of one male pup per litter was also recorded at weaning on PND21. At this age, general

activity in the open field was assessed in one pup per litter. On PND50, male pups from the ND and HD dams were divided into two groups and injected with either 100 µg kg–1, i.p., LPS as described previously (Kirsten et al. 2012; n = 6 per group; NDLPS and HDLPS, respectively) and two groups injected with 0.9% saline (1 mL kg–1, i.p.; n = 6 per group; NDS and HDS). Fourteen hours after LPS or saline injections, the F1 rats were observed in an open field test to evaluate sickness behaviour (Dantzer et al. 1998). The time of observation was based on previous observations that reported a febrile response 10–24 h after administration of 100 µg kg–1 LPS (Nascimento et al., 2013). This LPS challenge was administered to evaluate possible adaptive changes in the offspring that were caused by the maternal HD in puberty and early adulthood, which is associated with chronic mild inflammation (García-Cáceres et al. 2013). When a developing organism suffers maternal immune activation, such as from prenatal LPS exposure, the adult offspring may react differently after an immune challenge (Bernardi et al. 2010; Penteado et al. 2014). After behavioural evaluation, the offspring were weighed and killed by decapitation. RPF, a portion of the hypodermis, the brain and blood were collected from each rat. The RPF was weighed and the RPF/BW index calculated (RPF weight/BW × 100). The number of small and larger HAs was determined. Abdominal fat weight and the number of HAs were examined in the F1 generation to determine whether a maternal HD affects the tendency of pups to develop obesity. The the brain was fixed by buffered 10% formalin. This the conventional method and the most used for GFAP IHC. Hypothalamic GFAP-positive astrocytes area were evaluated after the LPS challenge to determine possible neuroinflammatory mechanisms. Blood was collected to determine plasma concentrations of cytokines and neuropeptides. Female pups of the F1 generation were used a different altogether, to be published in another paper. The experimental design is shown in Fig. 1.

Bodyweight

BW was evaluated in the F0 generation during HD feeding and in adulthood. In the F1 generation, total litter weight, litter weight at birth divided by the number of pups and the individual weight of one pup per litter at weaning and on PND50 were measured.

Histopathology and immunohistochemistry

Rats of the F0 generation were killed by decapitation on PND90–95. The F1 generation was killed by decapitation 12 h after completion of the behavioural observations. All rats underwent necropsy. Retroperitoneal adipose tissue was harvested and weighed. Abdominal skin, including the hypodermis and abdominal muscle near the umbilical scar, was removed. A 2 × 2 cm fragment was fixed on a thin piece of paper and immersed in 10% buffered formalin for fixation. The skin was stained with haematoxylin–eosin and 10 serial photomicrographs were taken from randomly chosen microscopic fields of the hypodermis using a Nikon (Kanagawa, Japan) E200 microscope (×10 objective) equipped with a Digital Coolpix Camera (Kanagawa, Japan) linked to a liquid crystal display monitor. The area of each entire adipocyte present in each field was measured in pixels using ImageJ software (National Institutes of Health, Bethesda, MD, USA). In the first analysis of the area of adipose cells, two clearly distinct populations were identified: (1) small cells, with an area measuring □9000 pixels; and (2) larger cells, with an area measuring >9000 pixels. The frequency of small and larger cells per field was determined and the significance of differences analysed using the $\chi 2$ test. Two-sided P □ 0.05 was considered significant.

The brain was also collected and fixed in 10% buffered formalin for at least 48 h. A single coronal section was then obtained from each brain, including the parietal cortex, limbic structures and the hypothalamus (see Fig. S1 a available as Supplementary Material to this paper). The samples were processed according to conventional histological procedures. Brain sections were mounted on silane-treated slides and subjected to GFAP immunohistochemical procedures using the avidin–biotin peroxidase complex (ABC) method. The immunohistochemical protocol was initiated by deparaffinization the histological sections in xylene, followed by rehydration in a crescent graded series of ethanol solutions (50%,70% and absolute). Antigen retrieval was performed by transferring the slides to 10 mM sodium citrate buffer (pH 6.0) at 95°C for 20 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min at room temperature. Between incubations, sections were washed twice with Tris-HCl buffer (pH 6.0; ×10 wash buffer; S3006; Dako, Glostrup, Denmark). Sections were incubated for 16 h at 4°C with the primary antibody, polyclonal rabbit anti-GFAP immunoglobulin (1:1000; Z0334; Dako), followed by application of horseradish peroxidase-conjugated biotinylated secondary

antibody (Dako Universal LSAB 2 System; K0690) according to the manufacturer's instructions. Immunoreactivity was visualised by incubating the sections in a solution that contained 0.1% diaminobenzidine (DAB; K3467; Dako). Sections were then counterstained with Harris' modified haematoxylin solution, dehydrated and mounted in Entellan (Merck, Darmstadt, Germany).

Six photomicrographs were taken of each individual hypothalamic periventricular area section using a ×40 objective, three from the right side and three from the left side of the third ventricle, covering approximately 60% of the total counting area (Fig. S1b). The area of astrocytes and their processes, marked in brown, was calculated automatically, in pixels, using Metamorph software Molecular Devices (Sunnyvale, CA, USA), which was calibrated with digital colour filters that regulated red, green and blue bits such that only positive cells were included and background staining was excluded from the measurement. This area reflected the size and positivity of astrocytes to GFAP. Thus, reactive hypertrophic astrocytes were quantified.

Open field behaviour

Behaviour was evaluated in the F1 generation at two time points, namely at weaning (PND21; 3 min observation) and PND50 (14 h after LPS or saline administration; 5 min observation). The open field apparatus for the PND21 rat pups was described previously by Faggin and Palermo-Neto (1985), whereas the apparatus for PND50 rats was described by Bernardi and Palermo-Neto (1984). Testing was performed in a small room with dim lighting. Each rat was placed individually in the centre of the apparatus and total locomotion, peripheral locomotion and immobility time (in seconds) recorded. The locomotor frequency and immobility were recorded to verify any interference with motor and exploratory behaviour (Bernardi et al. 1981). Peripheral locomotion is considered an index of anxiety (Campos et al. 2013). The apparatus was washed with a 5% alcohol–water solution before placement of the animals to obviate possible bias caused by odour cues left by previous rats.

Biochemical analysis

Blood was collected to determine plasma concentrations of cytokines and neuropeptides. Serum concentrations of peptides involved in obesity, including leptin (Friedman and Halaas 1998; Ahima and Osei 2004), insulin (Baskin et al. 1999), oxytocin (Blevins and Ho 2013), β-endorphin (Giugliano and Lefebvre 1991; Wilding 2002), neurotensin (Arora and Anubhuti 2006) and substance P (Miegueu et al. 2013), were measured. Chronic obesity is considered a chronic inflammatory

process, so plasma concentrations of tumour necrosis factor (TNF)-α, interleukin (IL)-6 (Singhal et al. 2014) and monocyte chemotactic protein (MCP)-1/CCL2 (a key factor in proinflammatory effects; Christiansen et al. 2005) were also evaluated. Plasma concentrations of brain-derived neurotrophic factor (BDNF) were measured because this neurotrophin may regulate food intake and be involved in the risk of developing obesity, diabetes and cardiovascular disease and neuroplasticity (Nagahara and Tuszynski 2011).

Trunk blood was collected from rats of the F1 generation on PND50 in conical tubes containing EDTA. Samples were centrifuged and plasma was obtained (centrifuged during 15 min. 1,000-2,000 g in a refrigerated centrifuge at a 40C). Plasma BDNF levels were determined in duplicate using a commercially available ELISA kit (Catalogue no. G7610; Promega, Madison, WI, USA) according to the manufacturer's instructions. Free mature BDNF (i.e. non-acidified samples) was evaluated. Mature BDNF is the active form of this neurotrophin that binds to the TrkB receptor , , a receptor of tyrosine kinase family that regulates synaptic strength and plasticity of the mammalian nervous system (Je et al. 2012). Plasma concentrations of IL-6, insulin, leptin, MCP-1, TNF-α (MILLIPLEX MAP Rat Metabolic Hormone Magnetic Bead Panel-Metabolism Multiplex Assay, Catalogue no. RMHMAG-84K , Millipore, Billerica, MA, USA); β-endorphin, neurotensin, oxytocin and substance P (rat/mouse neuropeptide; Catalogue no. RMNPMAG-83K; MILLIPLEX MAP Rat Metabolic Hormone Magnetic Bead Panel-Metabolism Multiplex Assay, Millipore, Billerica, MA, USA) were determined using the LUMINEX/Magpix system (RSH69K03; Millipore, Billerica, MA, USA). The range of detection of the peptides was 1.6–400000 pg mL–1. Samples were analysed in duplicate and the concentrations were estimated using a five-parameter polynomial curve (Xponent software; Millipore). All results are expressed in pg mL–1.

Statistical analyses

Homoscedasticity was verified using an F-test or Bartlett's test. Normality was verified using the Kolmogorov–Smirnov test. Student's t-test (unpaired, two-tailed) and the Mann–Whitney test were used to compare parametric and non-parametric data, respectively, between groups. One-way analysis of variance (ANOVA) was used to analyse morphometric data. Two-way ANOVA followed by Bonferroni's test was used to analyse data with two factors. Percentage data were analysed using the

$\chi 2$ test. Unless indicated otherwise, results are expressed as the mean ± s.e.m. or as a percentage. In all cases, results were considered significant at $P < 0.05$.

Results

F0 generation studies

Mean Ensure consumption over 20 days in cages of HD rats was 600 mL day–1, with chow consumption in the same cages over the same period being 44.11 ± 9.56 g day–1. Mean chow consumption in cages of ND rats was 126.30 ± 10.10 g day–1. Thus, a total of 800 kcal was consumed per day per cage in the HD group, compared with a total of 530 kcal per day per cage in the ND group.

Characteristics of the F0 generation fed the HD during puberty and early adulthood are given in Table 1. F0 females in the HD group exhibited an increase in BW gain at the end of the period of HD consumption (PND65) compared with the control ND group. However, in adulthood (PND90–95), these females exhibited a decrease in BW compared with the ND group. The weight of RPF and RPF/BW index was greater in the HD compared with ND group. The morphometry of adipose tissue in F0 rats relative to small HA was not significantly different between the ND and HD groups. However, as expected, the number of larger HAs increased in the HD compared with ND group. Morphometric analysis of astrocytes in the periventricular hypothalamus revealed an increase in the area of GFAP-positive astrocyte processes per microscopic field in the HD compared with ND group, indicating astrogliosis. Photomicrographs of the periventricular area of the hypothalamus from rats of the F0 generation are shown in Fig. 2.

F1 generation studies

In the F1 generation, litter weight at birth and litter weight/number of pups increased significantly in the HD compared with ND group (Table 2). Individual BW in both groups did not differ at weaning (Table 2). General activity in the open field test at weaning in pups born from HD and ND F0 dams was not altered by maternal treatment (Supplementary Fig. 2)

The BW of the F1 generation from ND and HD dams on PND50 (Fig. 3a) was modified by the maternal diet ($F_{1,20} = 35.54$, $P < 0.0001$) but not by LPS treatment ($F_{1,20} = 0.06$, $P = 0.809$), and an interaction was found between these two factors ($F_{1,20} = 12.20$, $P < 0.0005$). The Bonferroni test indicated a decrease in BW in the HDLPS compared with NDLPS group ($P < 0.001$). RPF weight was not affected by

either the maternal diet (F1,20 = 0.592) or LPS administration (F1,20 = 0.30, P = 0.592; Fig. 3b). However, a significant interaction was found between these two factors (F1,20 = 17.17, P = 0.0005). The Bonferroni test revealed an increase in RPF weight in the HDS compared with NDS group and a decrease in RPF in the HDLPS compared with HDS group. The RPF/BW index was not affected by the maternal diet (F1,20 = 3.2, P = 0.089) or LPS treatment (F1,20 = 0.08, P = 0.78), but a significant interaction was found between these two factors (F1,20 = 7.86, P = 0.01; Fig. 3c). The Bonferroni test revealed an increase in the RPF/BW index in the HDS compared with NDS group.

One-way ANOVA revealed significant differences between small HAs (F3,36 = 11.15, P < 0.0001) and larger HAs (F3,36 = 29.31, P < 0.0001) in all groups. The number of small HAs was reduced in the HDS compared with NDS group (P < 0.0001; Fig. 3d), whereas the number of larger HAs increased in the HDS compared with NDS group (P < 0.0001; Fig. 3e). No differences were found in the number of small and larger HAs between the NDLPS and HDLPS groups.

An inverse relationship was observed in the area of GFAP-positive astrocytes between both generations. In the F0 generation, GFAP immunoreactivity and the area of astrocyte processes were greater in the HD-fed group than the ND group (mean (± s.d.) 19077 ± 6524 vs 12872 ± 2695, pixels.respectively; P = 0.001, Mann–Whitney test). In the F1 generation, there was less pronounced GFAP-immunoreactivity in astrocytes and shorter astrocyte processes in the HDS compared with NDS group (mean (± s.d.) 30865 ± 15396 vs 42436 ± 12909 pixels, respectively; P = 0.001, Mann–Whitney test). LPS treatment partially reversed this decrease in the GFAP-positive area and the area of GFAP-positive astrocyte processes appeared to be a little greater in the NDLPS and HDLPS groups although the differences did not reach statistical significance (mean (± s.d.) 40986 ± 16679 vs 36934 ± 16186 pixels, respectively; P = 0.275, Mann–Whitney test). The morphology of these cells is shown in Fig. 2.

Fig.4 shows general activity in the open field test on PND50 of F1 rats born to ND and HD dams and treated with either LPS or saline. Locomotion frequency (Fig. 4a) was modified by LPS administration (F1,20 = 8.42, P = 0.009) but not by the maternal diet (F1,20 = 1.01, P = 0.327), and there was no interaction was between these two factors (F1,20 = 0.98, P = 0.334). Locomotion frequency decreased in the NDLPS compared with the NDS and HDS groups, indicating typical sickness

behaviour. Locomotion frequency was significantly greater in the HDLPS compared with NDLPS group ($P < 0.01$), indicating that sickness behaviour was minimised. No differences were observed among the NDS, HDS and HDLPS groups. Peripheral locomotion frequency (Fig.4b) was also altered by LPS treatment ($F1,20 = 6.25$, $P = 0.021$), but not by maternal diet ($F1,20 = 3.15$, $P = 0.09$), and an interaction was found between the two factors ($F1,20 = 6.51$, $P = 0.019$). The NDLPS group exhibited decreased peripheral locomotion frequency compared with the NDS and HDS groups, but the HDLPS group did not ($P < 0.05$). The HDLPS group exhibited a significant increase in peripheral locomotion compared with the NDLPS group, which was similar to that in the saline-treated group. Immobility time (Fig.4c) was altered by LPS administration ($F1,20 = 6.80$, $P = 0.017$), but not by the maternal diet ($F1,20 = 0.80$, $P = 0.383$), and no interaction was found between the two factors ($F1,20 = 0.32$, $P = 0.578$). The HDLPS group exhibited increased immobility compared with the NDLPS group.

Fig. 5 shows plasma TNF-α concentrations in the F1 generation. No interaction was detected between the diet and LPS treatment ($F1,20 = 0.36$, $P = 0.55$), with no effect of LPS treatment ($F1,20 = 0.36$, $P = 0.56$). However, the diet affected TNF-α levels ($F1,20 = 5.64$, $P = 0.03$). Both the HDS and HDLPS groups exhibited increased TNF-α concentrations compared with the NDS and NDLPS groups, respectively. Neither diet nor LPS had any effect on plasma BDNF, leptin, insulin, oxytocin, neurotensin, β-endorphin, substance P, IL-6 or MCP-1/CCL2 concentrations (Table S2).

Discussion

Female rats of the F0 generation in the HD group exhibited significant increases in BW gain during early development (from weaning to PND65) compared with controls. However, only a 5% increase was observed, indicating that the rats were overweight but not obese. The HD provided 800 kcal. When the HD was switched to the ND until adulthood, the BW of the HD group decreased faster compared with controls, although the RPF weight, RPF/BW index and number of larger HAs remained elevated. This reduction in BW in the HD group could be attributable to a change in body mass or fat distribution. RPF is a very lightweight fat and the increase in RPF was not mirrored by significant changes in BW. The most important outcome of the consumption of the HD during puberty was the increase in both abdominal fat and the number of larger HAs in females in adulthood.

Robust data indicate that prevalent forms of metabolic syndrome are found among individuals with excess abdominal fat (Després and Lemieux 2006; Després et al. 2008). Metabolic syndrome has been defined as a constellation of atherothrombotic inflammatory abnormalities, of which insulin resistance is a central component and most often found among individuals with excess abdominal fat (Huang 2009). Studies using sophisticated metabolic markers have shown that such a cluster of metabolic abnormalities is predictive of an increased risk of Type 2 diabetes and cardiovascular disease (Ridker et al. 2003; Wilson et al. 2005; Schmidt and Bergström 2012). Thus, although the HD did not induce obesity in the present study, the long-term increases in the RTF/BW index and the number of large HAs suggest the accumulation of fat in HD-treated rats that may be predictive of metabolic syndrome.

The ability of an HD to cause individuals to become obese and overweight is associated with hypothalamic gliosis and inflammation (Lee et al. 2001; García-Cáceres et al. 2013; Calvo-Ochoa et al. 2014). Hypothalamic inflammation occurs in specific hypothalamic nuclei (Buckman et al. 2013) before systemic inflammatory markers are detected (Thaler et al. 2012; Chowen et al. 2013). This high-fat diet-induced gliosis indicates that the astrocyte-mediated transport of nutrients and other factors is most likely affected (Chowen et al. 2013). The increases in RPF weight, the RPF/BW index and the number of larger HAs and hypothalamic astrogliosis in the present study suggest that HD intake during the course of pubertal maturation can modify neuroendocrine circuits in adulthood, mainly those involved in the control of metabolism, such as leptin and cytokines, and hypothalamic regulation (Chowen et al. 2013; García-Cáceres et al. 2013). Visceral fat is a functional endocrine organ with important effects on numerous metabolic and hormonal responses. Visceral adipose tissue is known to produce inflammatory adipokines, regulatory adiponectin and other regulatory molecules, such as leptin, cocaine- and amphetamine-regulated transcript and nuclear factor-κB, which play important causal roles in these chronic diseases (McGown et al., 2014). Thus, the results of the present study are consistent with the persistent hypothalamic inflammatory process that is caused by HD feeding during puberty and early adulthood.

At birth, the F1 litters from F0 dams in the HD group had a greater BW than litters from F0 dams in the ND group. The litter weight/number of pups was greater in the HD compared with ND group. However, no differences in pups BW were observed at weaning between groups.

Maternal obesity and being overweight negatively affect fetal offspring and their postnatal phenotype, including brain development, exploratory, emotional and cognitive behaviours and metabolic dysfunction. RPF is a visceral fat depot that is associated with metabolic dysfunction (Chau et al. 2014). These metabolic changes caused by the maternal HD may be responsible for the increase in litter weight of the F1 generation at birth.

In the present study, no differences were found in general activity among F1 groups at weaning, suggesting that the maternal HD during puberty and early adulthood did not affect exploratory or motor activity in the pups at this age. In adulthood, no differences in general activity or BW were found between the NDS and HDS groups.

In the HDS group, increases were observed in RPF weight, the number of larger HAs and plasma TNF-α concentrations, which were associated with a decrease in the area of GFAP-positive astrocytes in the hypothalamus. Thus, the maternal HD during puberty and early adulthood predisposes their offspring to developing obesity associated with hypothalamic and peripheral inflammatory processes in adulthood, even if they are fed on an ND.

The intake of an HD increases inflammation within the periphery and hypothalamus, which may be the key to metabolic changes that occur in obesity (Becskei et al. 2008; Velloso et al. 2008; García-Cáceres et al. 2013). Astrocytes respond to changes in the central nervous system (CNS) by undergoing morphological and functional alterations that are anatomically specific and affect neuronal activity (García-Cáceres et al. 2013). When the CNS undergoes a particular insult, astrocytes can become hypertrophic or assume a reactive phenotype, termed astrogliosis. Astrogliosis is characterised by the upregulation of specific structural proteins, such as GFAP and vimentin (Ridet et al. 1995). Astrocytes then produce inflammatory mediators, including TNF-α, IL-1β and IL-6 (Mayo et al. 2014). These inflammatory factors can affect microglia, neurons and astrocytes themselves to control CNS inflammatory processes and immune reactions (Singhal et al. 2014). In a review, García-Cáceres et al. (2013) commented that a prolonged HD induces hypothalamic inflammation and leads to gliosis associated with neuronal apoptosis in the hypothalamus.

There was no significant difference in BW between rats in the HDS and control groups, although HDS rats did exhibit increases in RPF weight and the number of larger HAs and a decrease in small HAs. The increase in RPF in rat offspring from

HD-fed dams and the switch from small HAs to larger HAs could indicate an increase in the inflammatory process in these animals. Importantly, the F1 generation only received an ND. Therefore, the maternal HD appeared to reprogram offspring adipogenesis towards obesity.

An increase in HAs, which is observed in individuals who are obese or overweight, produces and releases large amounts of cytokines, adipokines and chemokines that are able to recruit inflammatory cells to adipose tissue. Macrophages that infiltrate adipose tissue amplify the inflammatory response in concert with HAs (Makki et al. 2013). Thus, we suggest that the neuroinflammation observed herein resulted from an increase in adiposity in the HDS group.

LPS also affects CNS activity, leading to sickness behaviour in many species (Dantzer et al. 1998; Costa-Pinto et al. 2009), generally accompanied by a decrease in exploratory activity, a decrease in social and sexual behaviour, anhedonia, poor learning and a decrease in cognitive function (Bernardi et al. 2010; Kirsten et al. 2010, 2012; Pimentel et al. 2013; Soto et al. 2013; Penteado et al. 2014). As expected, LPS administration induced sickness behaviour in the NDLPS group, in which both total and peripheral locomotion decreased compared with the NDS group. The increase in sickness behaviour that was observed in the NDLPS group could be a consequence of neuroinflammation that was induced by the higher number of larger adipocytes. In addition, high plasma concentrations of TNF-α were observed.

BW and RPF weight in rats in the HDLPS group decreased faster compared with offspring that were not challenged with LPS. There was an equal number of small and larger HAs in the HDLPS group, in contrast with the predominance of larger HAs in rats that were not challenged with LPS. Central control of food and water consumption depends on the activity of hypothalamic areas. Consistent with our data, Riediger et al. (2010) showed that LPS markedly suppressed food and water consumption, beginning approximately 2 h after injection and lasting over the entire 23-h period of observation. Riediger et al. (2010) also reported an association between nitric oxide and LPS-induced symptoms, such as disease-related anorexia and other disease-related symptoms (e.g. adipsia, inactivity and fever). Thus, the decrease in BW and lack of differences in the distribution of small and larger HAs in the HDLPS group could be consequences of LPS-induced sickness, although general activity in the open field test was lower in the HDLPS than NDLPS group. This observation is consistent with the cachectin-like effect that is seen in sepsis,

which is caused by increases in peripheral plasma TNF-α concentrations. The lack of differences in the distribution of small and larger HAs in the HDLPS group could be related to the attenuation of hypothalamic inflammation.

General activity increased in the HDLPS compared with NDLPS group, suggesting an attenuation of sickness behaviour, but the HDLPS group also exhibited increases in locomotion in the peripheral area and immobility time. Thus, we suggest that these rats had high levels of 'anxiety' that masked LPS-induced sickness behaviour. In short, the offspring of dams that were overweight in puberty and early adulthood exhibited changes in behavioural patterns that were induced by immune activation in early adulthood. These data suggest that a transgenerational adaptive process occurred.

Relative to hypothalamic astrocytes, an inverse relationship between generations was observed in the area of GFAP-positive astrocytes in both generations. The HD group of the F0 generation exhibited an increased area of GFAP-positive astrocytes, whereas a decrease was observed in the HDS group of the F1 generation compared with their respective control group, despite the fact that peripheral plasma TNF-α concentrations increased. These data reinforce the relationships between hypothalamic neuroinflammation and the number of larger adipocytes.

These features are very well known effects of obesity, but little is known about the effects of these changes on subsequent generations. Our results showed that although the dams that were fed an HD during puberty and early adulthood had more reactive astrocytes, their F1 offspring exhibited the opposite effects, with small and hyporeactive astrocytes compared with control rats, suggesting an adaptive rebound process in response to the dam's dietary pattern (Rao et al. 2012). However, this 'memory' was deleted after LPS injection.

Metabolic disorders and adverse environmental effects may be perpetuated in the F2 and F3 generations via both the maternal and paternal lineages (Youngson and Morris 2013; Vickers 2014). In the present study, maternal HD exposure during early life predisposed the F1 generation (i.e. the HD group) to an increase in RPF weight, despite being fed an ND. In addition, a higher number of larger versus small HAs and astrogliosis were observed. LPS administration changed these RPF and hypodermic fat weights and astrocyte area patterns. At the biochemical level, these adaptations did not occur with regard to peripheral plasma TNF-α concentrations.

Although interesting data were obtained herein, the diet that was administered only caused marginal increases in BW. Further studies are required that use a better model of diet-induced obesity to examine possible transgenerational effects.

Conclusions

The HD that was administered during puberty and early adulthood caused the rats to become overweight, reflected by increases in BW, RPF, the RPF/BW index and the number of large HAs. This state of being overweight persisted in the F1 generation, although the offspring were fed only an ND. Thus, we found a transgenerational effect of the HD. Based on the increase in plasma TNF-α concentrations and changes in biomarkers of neuroinflammation, we speculate that this transgenerational effect of the HD occurred, at least in part, through permanent changes in immune system programming. The attenuation of biomarkers of neuroinflammation after LPS administration likely resulted from the decrease in the number of adipocytes, which, in turn, reduced the amount of cytokines, adipokines and chemokines that were able to recruit inflammatory cells in adipose tissue.

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Fig. 1. Experimental design. BDNF, brain-derived neurotrophic factor; RPF, retroperitoneal fat.ND- normocaloric diet group; HD- hypercaloric diet group.

Fig.4. General activity in the open field test on Postnatal Day 50 of rats from dams fed the normal (ND) or hypercaloric (HD) diet following injection of 100 µg kg–1, i.p., lipopolysaccharide (LPS) or 1 mL kg–1, i.p., of 0.9% saline solution (S). (a) Locomotion frequency, (b) peripheral locomotion frequency and (c) immobility time. Data are them mean ± s.e.m. (n = 6 per group). *P < 0.001 compared with the NDS group; †P = 0.05 compared with the HDS group; ‡P = 0.05 compared with the HDLPS group (two-way ANOVA followed by Bonferroni's test).

Fig. 3. (a) Bodyweight in adulthood, (b) retroperitoneal fat (RPF) weight, (c) RPF weight/bodyweight (BW) index (× 100), (d) number of small hypodermic adipocytes (HAs) and (e) number of larger HAs in rats from dams fed the normal (ND) or hypercaloric (HD) diet following injection of 100 µg kg–1, i.p., lipopolysaccharide (LPS) or 1 mL kg–1, i.p., of 0.9% saline solution (S). Data are them mean ± s.e.m. (n = 6 per group). *P < 0.05, ***P < 0.0001 compared with the NDS group (two-way ANOVA followed by Bonferroni's test).

Fig. 2. Photomicrographs of the periventricular area of the hypothalamus from rats in the F0 and F1 generations, showing glial fibrillary acidic protein (GFAP)-positive astrocytes with different degrees of positivity, represented by the brown (DAB stained) area, that is smaller in F1 generation born from HD fed mothers.ND, normal diet; HD, hypercaloric diet; NDS, saline-treated pups born to an ND-fed dam; HDS, saline-treated pups born to an HD-fed dam; NDLPS, lipopolysaccharide (LPS)-treated pups born to an ND-fed dam; HDLPS, LPS-treated pups born to an HD-fed mother. (Original magnification ×40.)

Fig. 5. Tumour necrosis factor (TNF)-α concentrations in rats from dams fed the normal (ND) or hypercaloric (HD) diet following injection of 100 µg kg–1, i.p., lipopolysaccharide (LPS) or 1 mL kg–1, i.p., of 0.9% saline solution (S). n = 6/group. Data are them mean ± s.e.m. (n = 6 per group). *P < 0.05, compared with the NDS or HDS groups, respectively (two-way ANOVA followed by Bonferroni's test).

Table 1. Characteristics of the F0 generation fed either the hypercaloric (HD) or normal (ND) diet during puberty

Data are the mean ± s.e.m. (n = 6 per group). All data were analysed by Student's t-test, except for the glial fibrillary acidic protein (GFAP)-positive area, which was evaluated using the Mann–Whitney test relative to the control (ND) group. RPF, retroperitoneal fat; BW, bodyweight

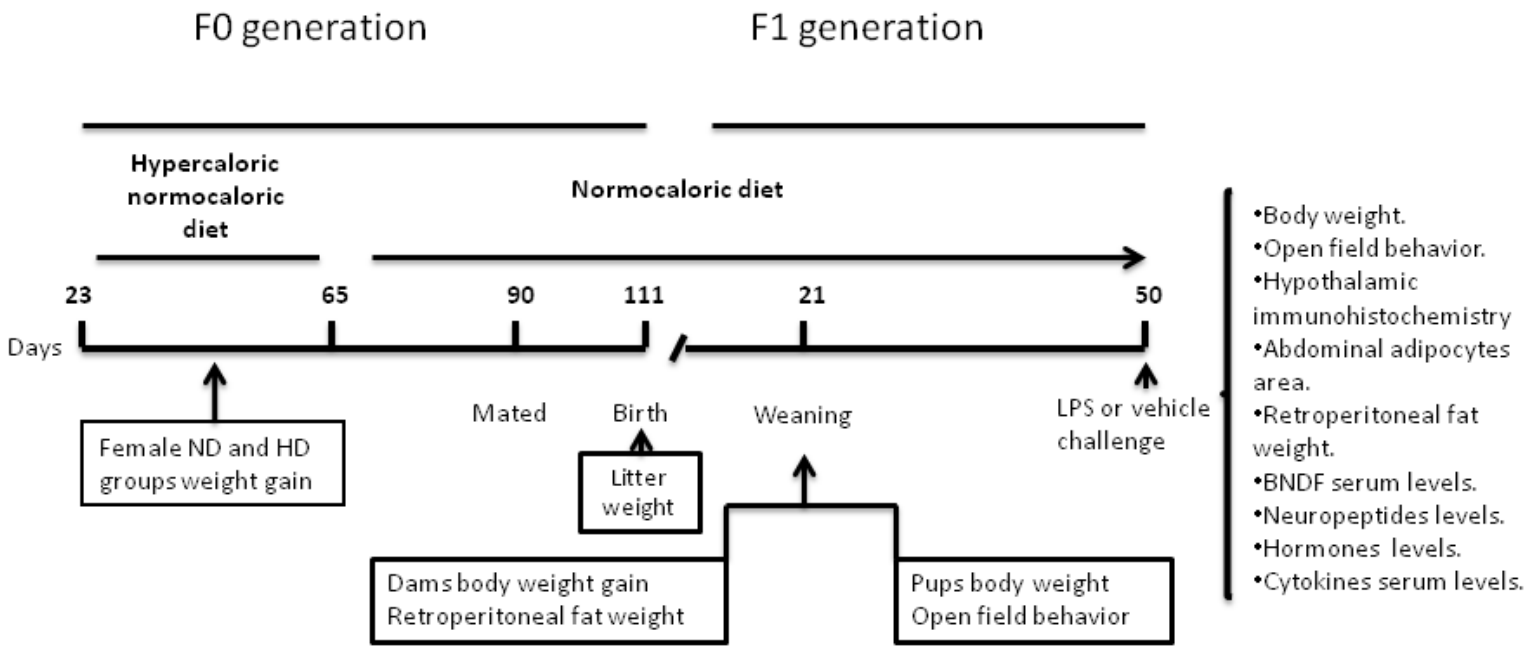
	ND group	HD group	P-value
Weight gain at the end of treatment (g)	201.0 ± 5.1	213.0 ± 1.6	0.036
Weight in adulthood (g)	308.3 ± 14.8	262.7 ± 8.2	0.02
RPF weight (g)	1.45 ± 0.37	8.56 ± 0.56	<0.0001
RPF/BW index	0.70 ± 0.69	3.80 ± 0.26	0.002
Area of small adipocytes (pixels)	3067 ± 181	3032 ± 178	0.13
Area of larger adipocytes (pixels)	10 870 ± 741	17 330 ± 496	<0.0001
GFAP-positive area (pixels)	12 872 ± 2695	19 077 ± 6524	0.001

Table 2. Litter weight, litter weight/number of pups, number of pups at birth and individual weight of pups at weaning of rats born to dams fed either the hypercaloric (HD) or normocaloric (ND) diet

Data are the mean ± s.e.m. P-values were calculated using Student's t-test

	ND group	HD group	No litters per group	P-value
Litter weight (g)	89.92 ± 4.02	171.60 ± 5.02	10	<0.0001
Litter weight/pups (g)	8.74 ± 0.38	17.33 ± 0.71	10	<0.0001
Total no. pups	123	121	–	–
No. pups	10.25 ± 0.49	10.08 ± 0.34	10	0.78
Mean weight of all pups in the ND and HD groups (g)	67.42 ± 3.22	55.50 ± 5.12	10	0.06

Fig.1


F0 generation
F1 generation
Hypercaloric normocaloric diet
Normocaloric diet
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21
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Days
Female ND and HD groups weight gain
Mated
Birth
Litter weight
Weaning
LPS or vehicle challenge
Dams body weight gain
Retroperitoneal fat weight
Pups body weight
Open field behavior
•Body weight.
•Open field behavior.
•Hypothalamic immunohistochemistry
•Abdominal adipocytes area.
•Retroperitoneal fat weight.
•BNDF serum levels.
•Neuropeptides levels.
•Hormones levels.
•Cytokines serum levels.

Fig. 2

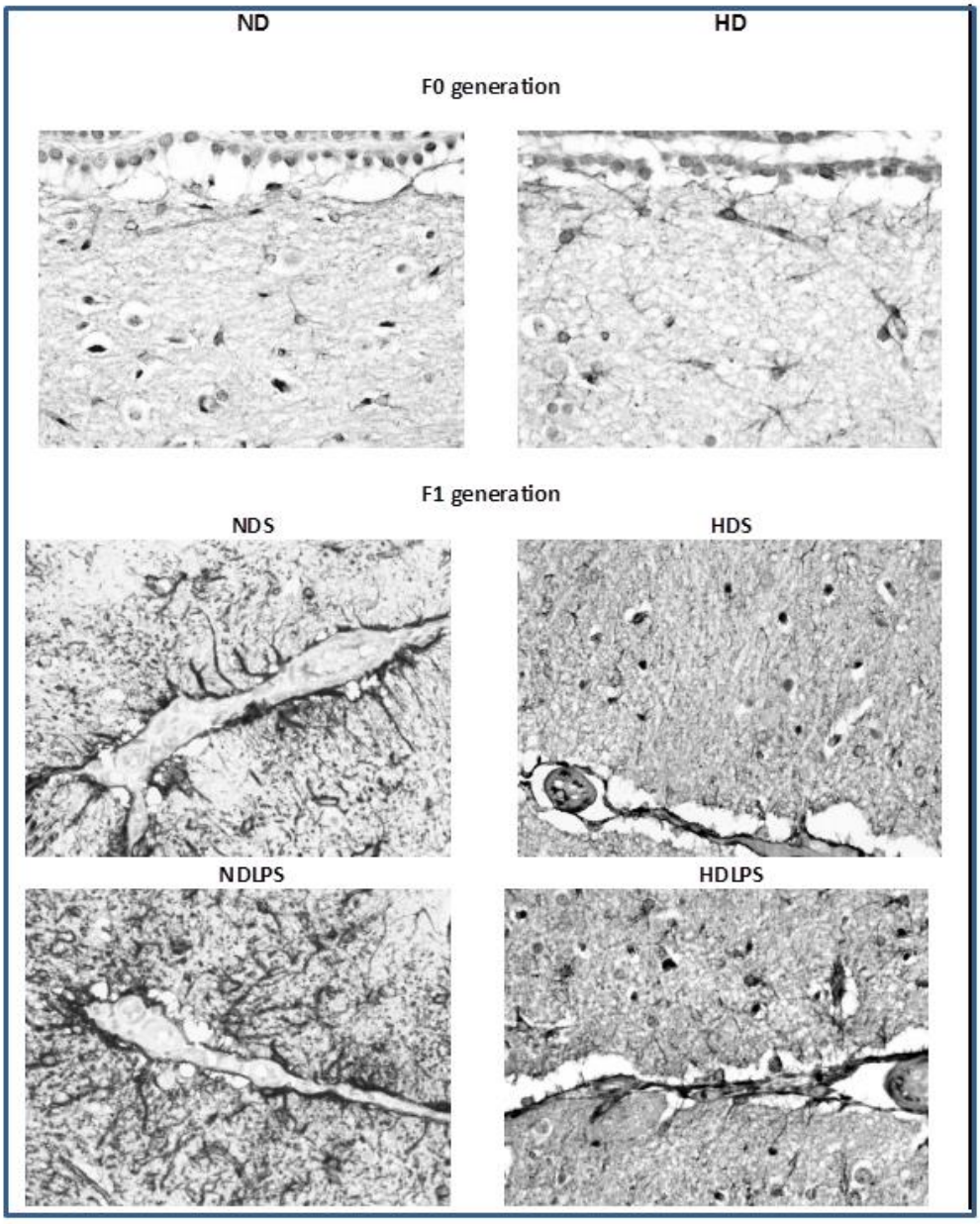
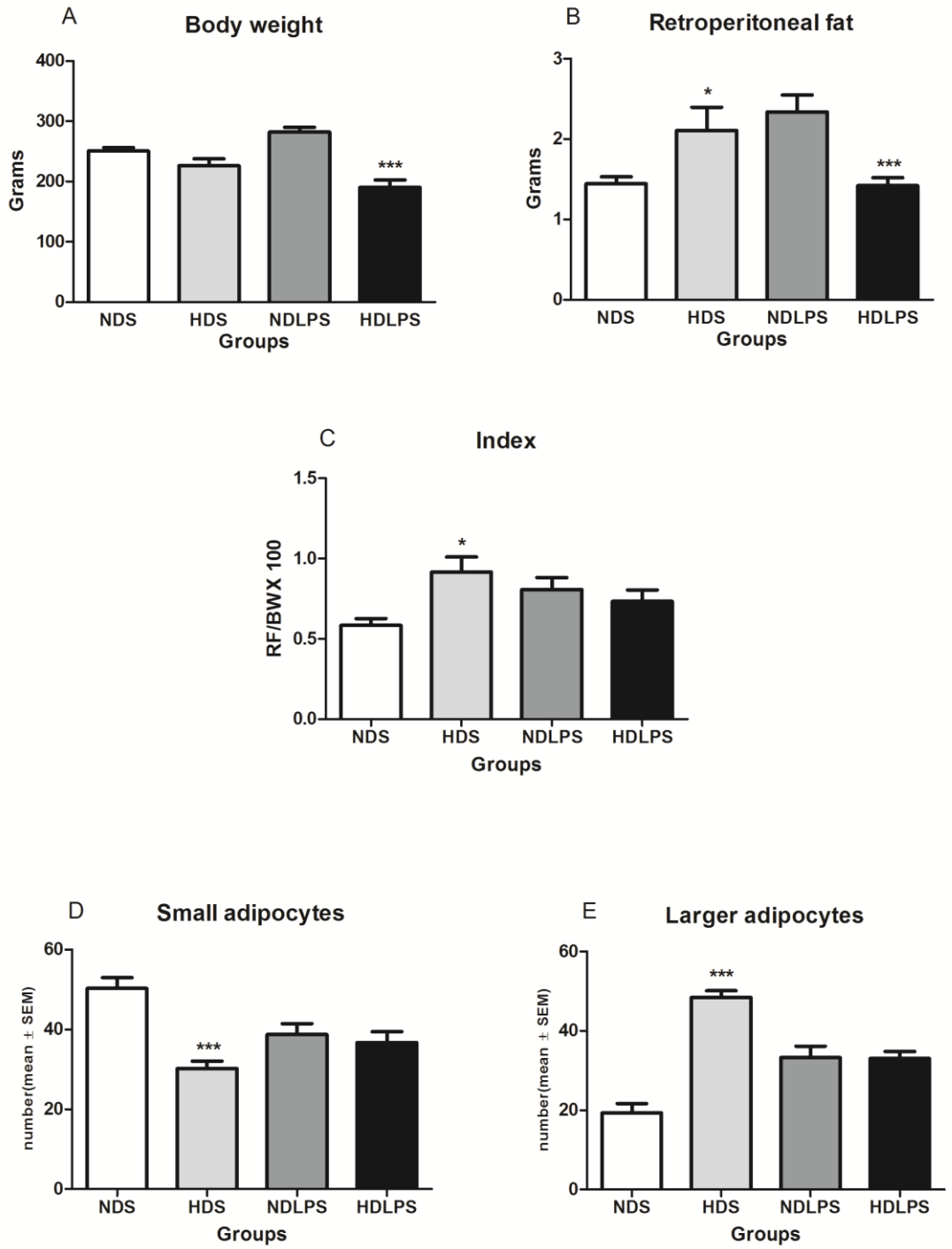


Fig. 3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Retroperitoneal fat
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NDLPS
HDLPS
Groups
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1.0
0.5
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*
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HDS
NDLPS
HDLPS
Groups
D
Small adipocytes
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20
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Larger adipocytes
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Groups

Fig. 4

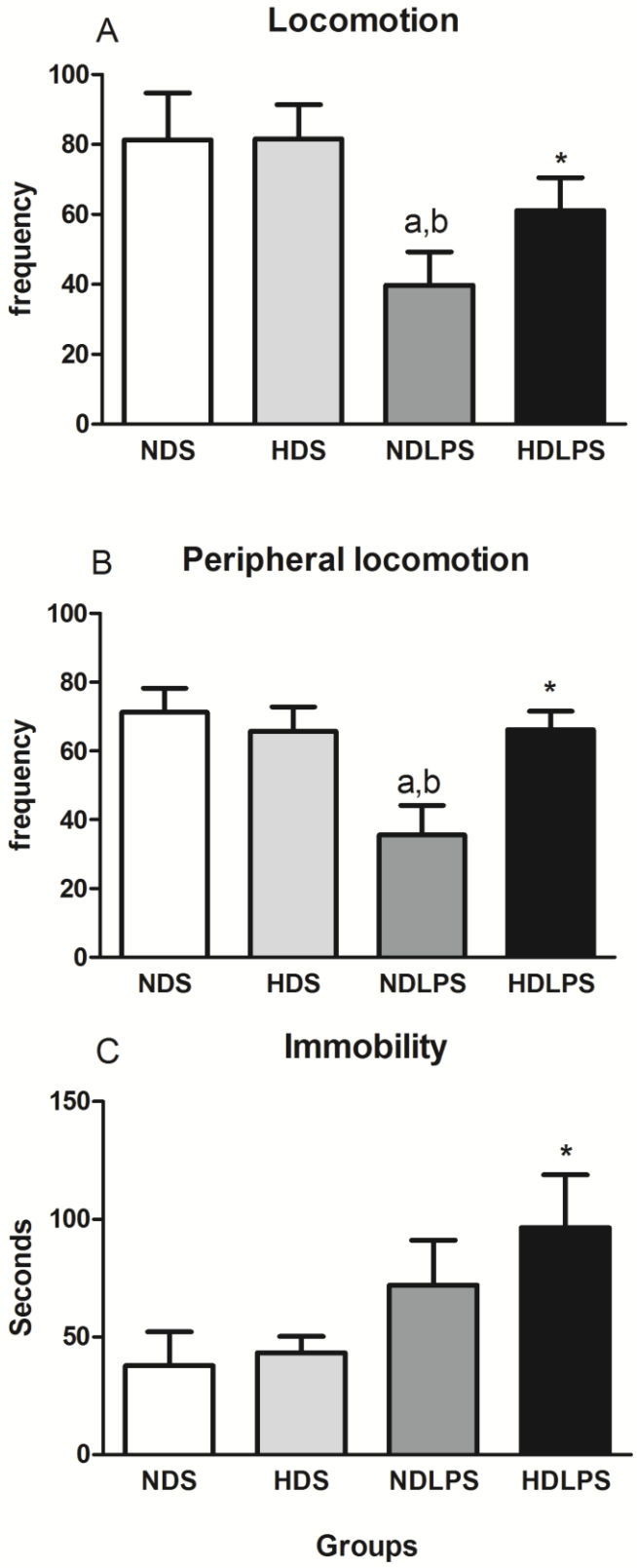
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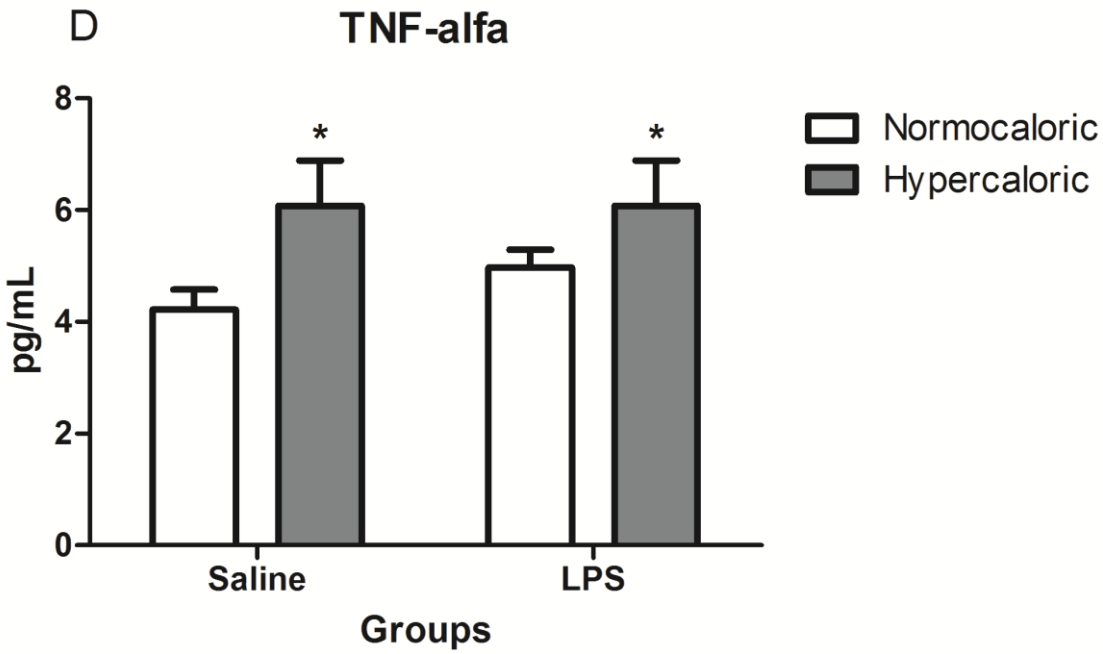
Fig. 5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Normocaloric
Hypercaloric

Supplementary Fig. 1

Figure 1A

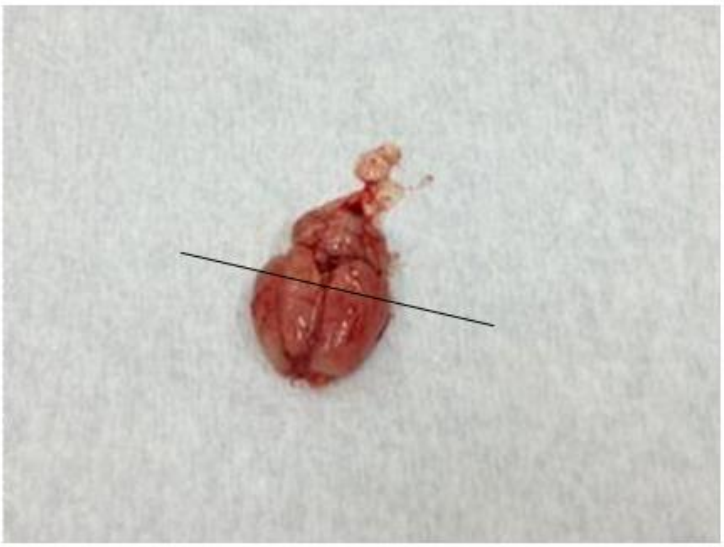
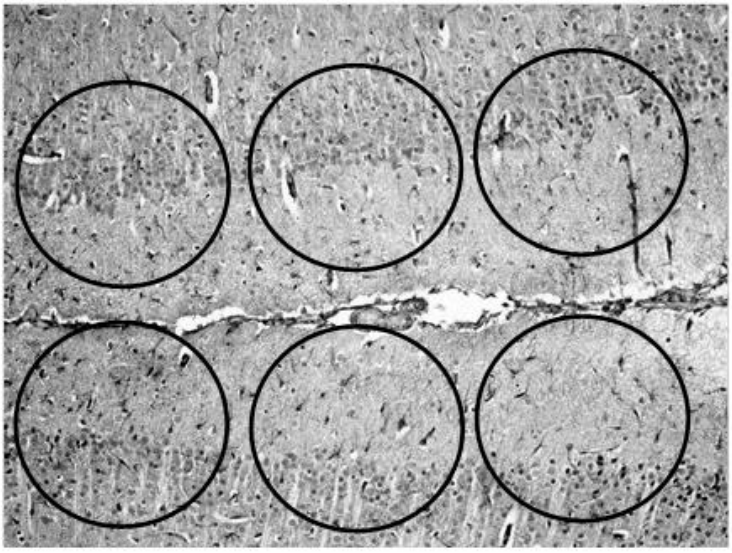


Figure 1B



Supplementary Fig. 2

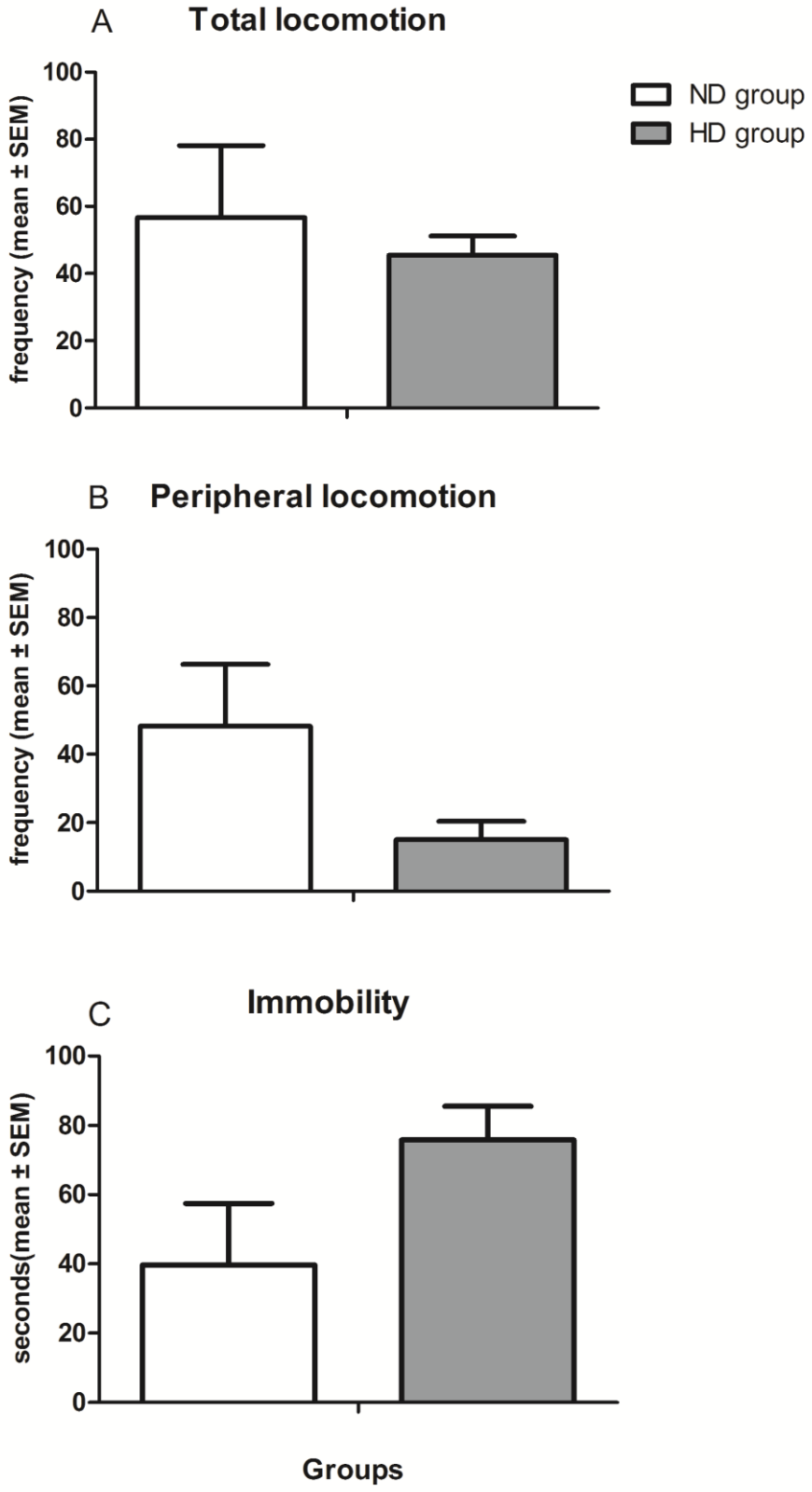


Table 3. BNDF, leptin, insulin, oxytocin, neurotensin, β-endorphin, Substance P, IL-6 and MCP-1/CCL2 plasma levels (pg/ml) of male rats from dams treated during puberty with normocaloric or hypercaloric diet . These male rats received at 50 days of age 100µg/kg of LPS or saline solution and the blood was collected 14 h after this treatment. CNS- pup of dams fed with normocaloric diet and that received at 50 days of age 1 ml/kg of saline solution; HDS- pup of dams fed with hypercaloric diet and that received at 50 days of age 1 ml/kg of saline solution; CNLPS- pup of dams fed with normocaloric diet and that received at 50 days of age 100µg/kg of LPS; HDLPS- pup of dams fed with hypercaloric diet and that received at 50 days of age 100µg/kg of LPS. Data are presented as means ± SEM. N= 6/group.

Groups	CNS	HDS	CNLPS	HDLPS
BNDF	3579 ± 1181	3662 ± 1395	3715 ± 1033	4402 ± 1594
Leptin	1857.0 ± 324.5	1664.0 ± 363.3	2883.0 ± 495.1	2181.0 ± 263.2
Insulin	1887.0 ± 151.90	1591.0 ± 156.2	1780.0 ± 120.7	2056.0 ± 138.80
Oxytocin	11.56 ± 0.80	13.42 ± 1.11	11.55 ± 0.26	12.84 ± 1.32
Neurotensin	64.06 ± 2.58	63.30 ± 6.39	50.87 ± 2.52	53.60 ± 4.36
B-endorphin	95.6 ± 11.0	92.9 ± 8.0	108.9 ± 7.6	87.2 ± 5.9
Substance P	1.74 ± 0.12	2.06 ± 0.15	1.60 ± 0.03	1.76 ± 0.15
IL-6	87.47 ±43.60	200.40 ± 156.90	19.77 ± 6.34	159.60± 80.11
MCP-1/CCL2	347,20 ± 61.14	359.10 ± 96.48	819.50 ± 229.20	457.50 ± 113.70

The two way ANOVA did not show differences between groups.

6.2 Maternal food deprivation in rats increased retroperitoneal fat and the number and size of adipocytes and induced periventricular astrogliosis in the F1 and F2 generations

Reproduction, Fertility and Development

Maternal food-restriction in rats increased retroperitoneal fat and the number and size of adipocytes and induced periventricular astrogliosis in the F1 and F2 generations

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Keyword:	development, fetal programming, hypothalamus, adipose tissue, growth

Maternal food-restriction in rats increased retroperitoneal fat and the number and size of adipocytes and induced periventricular astrogliosis in the F1 and F2 generations

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Running head: Transgenerational effects of maternal food restriction

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Abstract

This study investigated the impact of maternal food- restriction during gestation on rats of the F1 and F2 generations that received a normal diet, the number and size of larger and small hypodermal adipocytes (HAs), retroperitoneal fat weight(RPF), and the expression of glial fibrillary acidic protein (GFAP) in periventricular hypothalamic astrocytes (PHAs) by immunohistochemistry. Dams of the F0 generation were 40% food-restricted during pregnancy. Body weight (BW), (RPF, HAs, and GFAP in PHAs were studied in the F1 and F2 generations. In the F1 generation, we observed a decrease in BW gain only during the juvenile period, a decrease in the size of small adipocytes, increased number of small adipocytes, increased RPF weight, and increased GFAP PHAs in adulthood. In the F2 generation, similar findings were observed, but the effects of maternal food restriction were further enhanced. These data revealed that maternal food-restriction induced a two-generation phenotypic transgenerational tendency toward obesity and being overweight, with effects that were more pronounced in subsequent generations. This heritable pattern was also observed in the central nervous system, reflected by periventricular hypothalamic astrogliosis in both the F1 and F2 generations, suggesting that central neuroinflammatory processes could be related to the thrifty phenotype hypothesis.

Keywords: Reprogramming, Development, adipose tissue, Hypothalamus, growth.

Introduction

Increasing evidence suggests that adverse environmental factors can lead to intrauterine growth retardation, and under- or overnutrition may predispose individuals to certain diseases later in life by altering their fetal programming (Parlee and MacDougald 2014; Williams et al. 2014). The thrifty phenotype hypothesis proposes that poor nutrition in early life results in poor fetal growth and increased susceptibility to type 2 diabetes and metabolic syndrome (Hales and Barker 2013). Adult individuals whose mothers were undernourished early in pregnancy displayed higher rates of intra-abdominal adiposity associated with an increased risk of metabolic disease (Muhlhausler and Ong 2011; Yang and Huffman 2013). Additionally, prenatal maternal undernutrition that is associated with early hypercaloric nutrition in pups may significantly heighten energy balance dysfunction in adulthood (Williams et al. 2014). These adaptations may persist until adulthood and be transmitted to subsequent generations (Holemans et al. 2003; Sébert et al. 2009).

Through its neural circuitry and peptides, the hypothalamus regulates food intake and energy expenditure. Impairments in hypothalamic control can lead to lipid accumulation and body weight (BW) gain (García-Cáceres et al. 2013). Several studies have demonstrated that glial cells are intimately involved in producing hypothalamic inflammation that results from high fat diet-induced obesity and is linked to increased insulin resistance (Argente-Arizón et al. 2015; Thaler et al. 2012) and pathological processes associated with excess weight gain (Horvath et al. 2010; Chen et al. 2008). In addition to hypothalamic inflammation, high fat diet-induced obesity can also result in hypothalamic astrogliosis (Argente-Arizón et al. 2015).

According the to thrifty phenotype hypothesis, early maternal undernutrition increases the rate of intra-abdominal adiposity. Increases in retroperitoneal and

adipocyte fat are related to hypothalamic astrogliosis, as observed in overweight/obese individuals (Buckman et al. 2013). In contrast, the consequences of maternal undernutrition on hypothalamic neuroinflammation in the subsequent two generations that are fed a normocaloric diet remain relatively unexplored. Thus, the main objective of the present study was to explore the impact of maternal food restriction on F1 and F2 generations that were both fed normally and the expression of glial fibrillary acidic protein (GFAP) in periventricular hypothalamic astrocytes (PHAs) by immunohistochemistry, in addition to other peripheral histological changes.

Periventricular hypothalamic astrocytes (PHAs) were chosen because this brain region is one the most important for controlling food intake (Vrang et al. 1999; Lechan and Fekete 2006). Dams of the F0 generation were 40% food-deprived during pregnancy. Body weight, retroperitoneal fat (RPF), the number and area of small and larger hypodermal adipocytes (HAs), and GFAP in PHAs were studied in the F1 and F2 generations to evaluate trends in maternal food restriction-induced obesity or being overweight.

2. Material and methods

2.1. Ethics statement

The animal procedures were performed in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources and Brazilian Institutional Ethics Committee guidelines, Universidade Paulista (protocol no. 130/12, CEUA/ICS/UNIP, 28/11/2012). The experiments were performed in accordance with good laboratory practice protocols and quality assurance methods. All efforts were made to minimize the suffering of the animals.

96 *2.2. Animals*

97 Twenty pregnant Wistar rats, 12-14 weeks of age and weighing 210-260 g, were
98 provided by the School of Veterinary Medicine, São Paulo University, and divided into
99 two groups: non-food-restricted and food-restricted. Upon arrival, the animals were
100 housed in individual microisolator cages with controlled temperature (22-26°C) and
101 humidity (50-65%) in artificially lit rooms on a 12 h/12 h light/dark cycle (lights on at
102 7:00 AM) with free access to water and balanced food (Nuvilab, Sogorb Ind. & Com.
103 Ltda, São Paulo, SP, Brazil; values per 100 g solid food item: 4.2 kcal/g, 56%
104 carbohydrate, 19% protein, 4.5% cellulose, 5% vitamins, and 3.5% g total fat). These
105 females were mated with sexually experienced males to obtain the F1 generation. In
106 adulthood, female rats of the F1 generation were mated with sexually experienced males
107 to obtain the F2 generation. All of the dams were allowed to give birth normally and
108 nurture their offspring. The day of birth was recorded as postnatal day 1 (PND1). No
109 handling was performed on PND1 to avoid cannibalism (DeSantis and Schmaltz 1984).
110 On PND2, eight offspring (four males and four females) were randomly selected. No
111 cross-fostering procedures were used (Chiavegatto and Bernardi 1991). The eight
112 randomly selected pups remained with their dam until weaning on PND21. On PND21,
113 the littermates were separated and co-housed by sex under the same conditions as their
114 parents. In the F1 generation, only females were used (one pup per litter). In the F2
115 generation, only male pups were used (one pup per litter) in the experiments. Males of
116 the F1 generation and females of the F2 generation were used in a different study
117 altogether, to be published in another paper.
118
119 *2.3. Groups and experimental design*
120 Twenty pregnant female rats of the F0 generation were divided into two groups:

experimental (n = 12, F0D) and control (n = 8, F0ND) groups. The experimental group was food-restricted (40%) from gestation day 5 (GD5) to GD18. These females gave birth to the F1 generation (i.e., pups from non-food-restricted dams [F1ND, n = 12] and pups from food-restricted dams [F1D group, n = 16]). In half of each group of F1 female rats, BW during development and in adulthood, RPF, HAs, and GFAP in PHAs were examined. The other half of each group of F1 female rats were mated with sexual experienced males to obtain the F2 generation. At weaning on PND21, RPF, HAs, and GFAP in PHAs were evaluated in male pups of the F2 generation (F2ND, n = 6; F2D, n = 8).

Thus, the group configuration in the present study was the following: F0ND (non-food-restricted dams of the F0 generation), F0D (food-restricted dams of the F0 generation), F1ND (F1 female rats born from F0ND dams), F1D (F1 female rats born from F0D dams), F2ND (F2 male rats born from F1ND dams), and F2D (F2 male rats born from F1D dams). Fig. 1 shows the experimental design.

2.4. Histopathology of adipocytes

Rats of the F1 (PND90-95) and F2 (PND50) generations were euthanized by rapid decapitation. All of the rats underwent necropsy. Retroperitoneal adipose tissue was harvested and weighed. Abdominal skin, including the hypodermis and abdominal muscle near the umbilical scare, was removed. A 2 cm × 2 cm fragment was fixed on a thin piece of paper and immersed in 10% buffered formalin for fixation. The skin was stained with hematoxylin-eosin, and 10 serial photomicrographs were taken from randomly chosen microscopic fields of the hypodermis using a Nikon (Kanagawa, Japan) E200 microscope (10 × objective) equipped with a Digital Coolpix Camera (Kanagawa, Japan) linked to a Liquid crystal display monitor. The area of each entire

adipocyte that was present in each field was measured in pixels using ImageJ software (National Institutes of Health, Bethesda, MD, USA). In the first analysis of the area of adipose cells, two clearly distinct populations were identified: (*i*) small cells with ≤ 9000 pixel area and (*ii*) larger cells with > 9000 pixel area. The number of cells per field in each category was calculated and analyzed using the χ^2 test. Values of $p \leq 0.05$ were considered statistically significant.

2.5. Hypothalamic immunohistochemistry

The brain was also collected and fixed in 10% buffered formalin for at least 48 h. A single coronal section was made of each brain, including the parietal cortex, limbic structures, and the hypothalamus. Samples were processed according to conventional histological procedures. The brain sections were mounted on silane-treated slides and subjected to GFAP immunohistochemical procedures using the avidin-biotin peroxidase complex (ABC) method. The Immunohistochemical protocol was initiated by the paraffin withdrawal of histological sections in xylene and rehydration in a crescent graded series of ethanol solutions (50%, 70% and absolute). Antigen retrieval was performed by transferring the slides to 10 mM sodium citrate buffer (pH 6.0) at 95° C for 20 min. Endogenous peroxidase was blocked by 3% hydrogen peroxide for 10 min at room temperature. Two washes with Tris/HCl buffer (pH 6.0, Wash buffer 10x, S3006, Dako, Glostrup, Danmark) were performed between incubations. Polyclonal rabbit anti-GFAP immunoglobulin (1:1000; Z0334, Dako) was used as the primary antibody, with 16 h incubation at 4°C, followed by the application of biotinylated secondary antibody (Dako Universal LSAB 2 System, HRP, K0690) according to the manufacturer's instructions. Immunoreactivity was visualized by incubating the sections in a solution that contained 0.1% diaminobenzidine (DAB, K3467, Dako). The sections

were then counterstained with Harris' modified hematoxylin solution, dehydrated, and mounted in Entellan (Merck, Darmstadt,Germany).

Six photomicrographs from each individual hypothalamic periventricular area section were made using a 40x objective, with three from the right side and three from the left side of the third ventricle, covering approximately 60% of the total counting area. The area of astrocytes and their processes, marked in brown, was automatically calculated, in pixels, using Metamorph software (Molecular Devices, Sunnyvale, CA, USA), calibrated with digital color filters that regulated red, green, and blue bits such that only positive cells were included and background staining was excluded from the measurement. This area reflects the size and GFAP-positivity of astrocytes. Thus, reactive hypertrophic astrocytes could be quantified.

2.6. Statistical analyses

Homoscedasticity was verified using Bartlett's test. Normality was verified using the Kolmogorov-Smirnov test. Student's *t*-test (unpaired, two-tailed) and the Mann-Whitney test were used to compare parametric and nonparametric data, respectively, between two groups. Two-way analysis of variance (ANOVA) followed by the Bonferroni test was used to analyze interactions between factors. The percentage data were analyzed using the χ^2 test. The results are expressed as mean ± SEM or a percentage. In all cases, the results were considered significant at $p < 0.05$.

3. Results

3.1. Body weight

During pregnancy, as expected, maternal BW decreased in F0D dams compared

with the F0ND group ($t = 5.0$, df = 18, $p < 0.0001$; Fig. 2A). At birth, maternal food restriction reduced BW in the F1D group compared with the F1ND group ($t = 7.04$, df = 12, $p < 0.0001$; Fig. 2B). Moreover, the F1D group exhibited a decrease in BW during development, from weaning until PND65, with significant effects of treatment ($F_{1,98} = 59.72$, $p < 0.0001$) and days of life ($F_{6,98} = 506.16$, $p < 0.0001$), with no treatment × days of life interaction ($F_{6,98} = 0.49$, $p = 0.82$; Fig. 2C). No differences in BW were found between groups in adulthood ($t = 1.70$, df = 10, $p = 0.12$; Fig. 2D). In the F2 generation, no differences in BW were found at weaning between groups ($t = 7.04$, df = 12, $p = 0.08$; Fig. 2E). However, in adulthood, BW increased in the F2D group compared with the F2ND group ($t = 3.56$, df = 12, $p = 0.004$; Fig. 2F).

3.2. Retroperitoneal fat

In adulthood, RPF increased in rats of both generations whose mothers were undernourished during gestation compared with the control groups (F1 generation: $t = 10.59$, df = 10, $p < 0.0001$; Fig. 3A; F2 generation: $t = 2.69$, df = 12, $p = 0.02$; Fig. 3B). However, RPF was higher in F1 rats than in F2 rats, with significant effects of maternal food restriction ($F_{1,24} = 6.91$, $p = 0.02$) and generation ($F_{1,24} = 17.93$, $p = 0.0003$) and a significant maternal food restriction × generation interaction ($F_{1,24} = 4.27$, $p = 0.049$).

3.3. Adipocyte area and number

Compared with controls (F1ND group), the area of small HAs in the F1D group was reduced ($t = 2.44$, df = 556, $p = 0.015$; Fig. 4A). Larger HAs were not different between groups ($t = 0.17$, df = 115, $p = 0.87$; Fig. 4A). When considering the total number of small and larger HAs in the analyzed microscopic fields, the small/larger HA ratio (threshold fixed at 9000 pixels) revealed a significant increase in the number of

small HAs relative larger HAs in the F1D group compared with the F1ND group (total number of small HAs: F1ND = 179, F1D = 379; total number of larger HAs: F1ND = 74, F1D = 43; $\chi^2 = 38.780$, $p = 0.0001$; Fig. 4B). In short, the F1D group exhibited more and smaller HAs in subcutaneous tissue compared with the F1ND group.

In the F2 generation, the size of small adipocytes increased in the F2D group compared with the F2ND group ($t = 61.73$, df = 1092, $p < 0.0001$; Fig. 4C). No differences were observed between groups in the size of larger adipocytes (Fig. 4C). The number of larger adipocytes increased in the F2D group compared with the F2ND group, but no differences in small adipocytes were observed between groups ($p < 0.0001$).

With regard to the number of each cell category in all of the analyzed fields (Fig. 3E), the F2ND group presented 513 small HAs and 181 larger HAs, and the F2D group presented 581 small HAs and 414 larger HAs ($\chi^2 = 42.521$, $p = 0.0001$). Thus, the F2D group had more larger adipocytes than the F2ND group.

3.4. Area of hypothalamic GFAP-immunoreactive astrocytes

The area of hypothalamic GFAP-immunoreactive astrocytes in the F1D group significantly increased compared with controls (F1ND group; $t = 12.95$, df = 52, $p < 0.0001$; Fig. 5A), suggesting possible hypothalamic neuroinflammation. In the F2 generation, food restriction in the F0 generation increased the area of immunoreactive astrocytes in the F2D group ($t = 560$, df = 86, $p < 0.0001$; Fig. 5B). The morphology of these cells is illustrated in Fig. 6.

4. Discussion

In the present study, we first investigated BW in the F1 and F2 generations from dams of the F0 generation that were food-restricted until adulthood. Retroperitoneal fat and the number of HAs indicated that maternal food restriction induced a trend toward transgenerational obesity. As expected, maternal food restriction reduced female pups' BW at birth and during development. Body weight in the F1ND and F1D groups did not differ in adulthood, suggesting that compensation for the effects of maternal food restriction on BW occurred in adulthood (Desai et al. 2007). The number of small adipocytes and RPF weight increased in the F1D group. No difference was seen in larger adipocytes in the F1 generation. These data may suggest enhanced susceptibility to obesity, depending of the quality of food according of the thrifty phenotype hypothesis of Hales and Barker (1992). This hypothesis was confirmed in the F2 generation, in which males in the F0D group presented higher BW and RPF than the F2ND group. Interesting, the F2 generation exhibited lower RPF weight than the F1 generation.

Obesity and being overweight are sometimes characterized by chronic, low-grade inflammation in several tissues, including the hypothalamus (García-Cáceres et al. 2013) Buckman et al. 2013; Krasowska-Zoladek et al. 2007). Hypothalamic inflammation is an early factor in the onset of obesity, which occurs even before BW gain (García-Cáceres et al. 2013; Guyenet et al. 2013). In the hypothalamus, microglia and astrocytes produce cytokines that drive inflammatory responses. Because of their physical proximity to blood vessels and role in transporting nutrients, astrocytes are directly affected by excess nutrients (García-Cáceres et al. 2013). Astrocytes might play a unique role in promoting hypothalamic inflammatory responses in obesity (Tzanavari et al. 2010; García-Cáceres et al. 2013; Guyenet et al. 2013; Rodríguez-Hernández et al. 2013). The present results showed that maternal food restriction induced

transgenerational effects on GFAP expression in PHAs in both the F1 and F2 generations (i.e., maternal food restriction increased GFAP expression in subsequent generations). These data suggest the presence of neuroinflammation in the F1 and F2 generations, despite the fact that these rats received normal laboratory rat chow. We attribute this effect to interference with the pups' developmental programming, as proposed by the *Developmental Origins of Health and Disease* paradigm (Gluckman and Hanson 2006). Epidemiological data and several animal models have shown that maternal undernutrition results in increased risk of obesity and metabolic disease in offspring later in life (Vickers 2014; Rao et al. 2012; Gluckman et al. 2009; Gluckman and Hanson 2006). The development of transgenerational obesity is an important adaptive phenomenon for a programmed thrifty phenotype which is appropriate for a subsequent poor nutritional environment.

The increase in periventricular hypothalamic GFAP (a biomarker of neuroinflammation) in both the F1D and F2D groups suggested that maternal food restriction predisposed subsequent generations to the development of obesity and being overweight associated with hypothalamic inflammation, even if the rats received a normocaloric diet during their entire life. This finding reinforces the adaptive transgenerational thrifty phenotype hypothesis (Hales and Backer, 1992). Interestingly, the expression of the area of GFAP immunoreactivity was higher in the F2 generation than in the F1 generation, suggesting increased neuroinflammation in the F2 generation.

As proposed by Holemans et al. (Holemans et al. 2003), these adaptations in fetal metabolism to the altered intrauterine environment have consequences for offspring that can persist into adulthood and impact the next generation. Individuals that were conceived, born, or raised in crowded environment continue to exhibit physiological and behavioral responses to the stressful environment in which they were

294 born (Boonstra et al. 1998; (Batzli 1996). In fact, we observed similar profiles in the F2
295 and F1 generation with regard to architectural adaptive features of both hypodermal
296 adipose cells and HAs, although no changes in BW occurred during adulthood.
297 The choice of using females of the F1 generation in the present study was based
298 on the possibility of following their histopathology related to metabolic changes during
299 pregnancy. Conversely, male offspring were used for the F2 generation to avoid
300 putative reproductive cycle interference with the induction of inflammation (Bernardi et
301 al. 2014). Interestingly, similar histological features of adipocytes and astrocytes were
302 seen in both generations, despite any putative sexual dimorphism between F1 and F2
303 rats and genotype heritages.
304 Finally, the number of larger adipocytes increased in the F2 generation, and the
305 F2 generation had higher expression of periventricular hypothalamic GFAP compared
306 with the F1 generation, suggesting that the effects of maternal food restriction increased
307 over generations.
308
309 **Conclusion**
310 Maternal food restriction during pregnancy in the F0 generation induced a two-
311 generation phenotypic transgenerational tendency toward obesity and being overweight.
312 Our findings are consistent with the thrifty phenotype hypothesis of Hales and
313 Barker (1992). This heritable pattern was also observed in the central nervous system,
314 reflected by periventricular hypothalamic astrogliosis in both the F1 and F2 generations.
315 This transfer of information across generations occurred in the F1 and F2 generations,
316 despite the fact that the rats were fed normally, and the effects appeared to increase over
317 generations. The present findings reveal the transfer of hypothalamic

318 neuroinflammation susceptibility and associated changes in morphology across
319 generations.
320
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328

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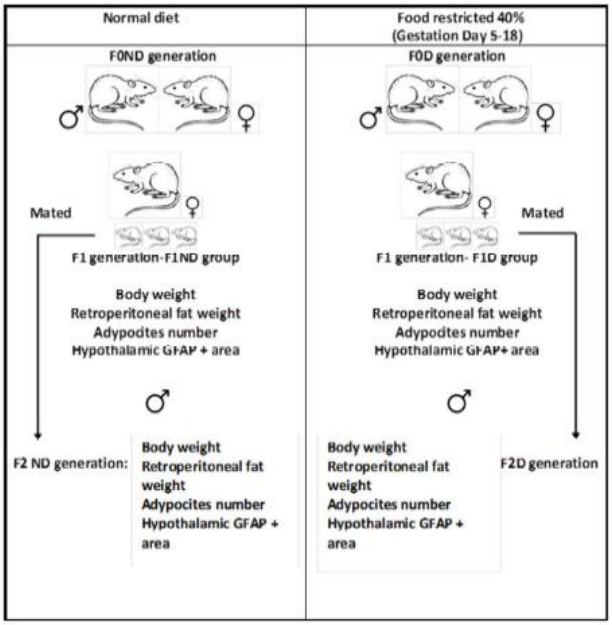

Fig. 1. Experimental design. F0ND, non-food-restricted F0 dams; F0D, food-restricted F0 dams; F1ND, female F1 rats from non-food-restricted F0 dams; F1D, female F1 rats from food-restricted F0 dams; F2ND, male F2 rats born from F1 generation from non-food-restricted F0 dams; F2D, male F2 rats born from F1 generation from food-restricted F0 dams.
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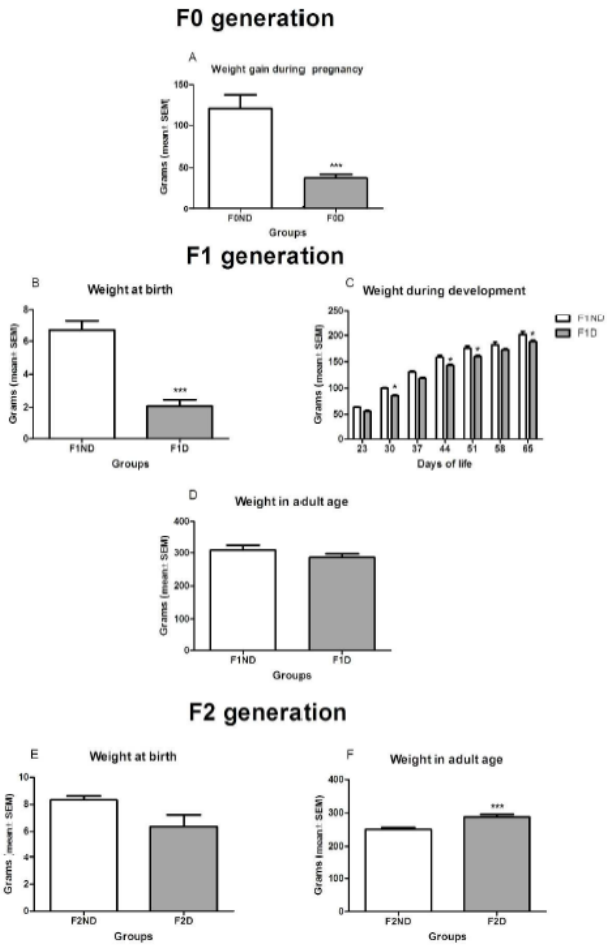

Fig. 2. (A) Body weight of females of the F0 generation that were subjected to 40% food restriction during pregnancy until gestation day 18. (B) Body weight of the F1 generation at birth. (C) Body weight of the F1 generation from PND23 to PND65. (D) Body weight of the F1 generation in adulthood. (E) Body weight of the F2 generation at weaning. (F) Body weight of the F2 generation in adulthood. F0ND, non-food-restricted F0 dams; F0D, food-restricted F0 dams; F1ND, female F1 rats from non-food-restricted F0 dams; F1D, female F1 rats from food-restricted F0 dams; F2ND, male F2 rats born from F1 generation from non-food-restricted F0 dams; F2D, male F2 rats born from F1 generation from food-restricted F0 dams. The data are expressed as mean ± SEM. n = 6-8/group. The F1 body weight development data were analyzed by two-way ANOVA followed by the Bonferroni post hoc test. The remaining data were analyzed by Student's t-test. *p < 0.05, ***p < 0.001, compared with control group.
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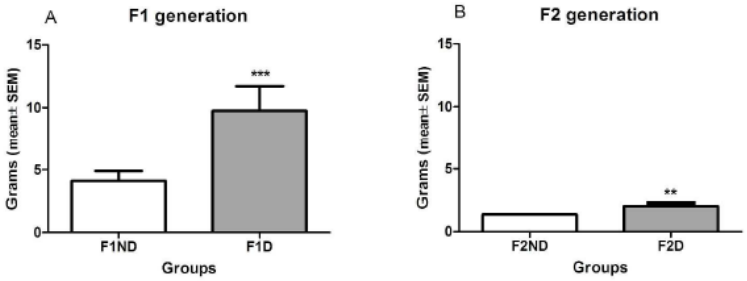

Fig. 3. Retroperitoneal fat of the (A) F1 and (B) F2 generations of rats from F0 dams that were subjected to 40% food restricition until gestation day 18. F1ND, female F1 rats from non-food-restricted F0 dams; F1D, female F1 rats from food-restricted F1 dams. F2ND, male F2 rats born from F1 generation from non-food-restricted F0 dams; F2D, male F2 rats born from F1 generation from food-restricted F0 dams. The data are expressed as mean ± SEM. n = 6-8/group. **p < 0.01, ***p < 0.001, compared with control group (Student's t-test).
267x106mm (300 x 300 DPI)

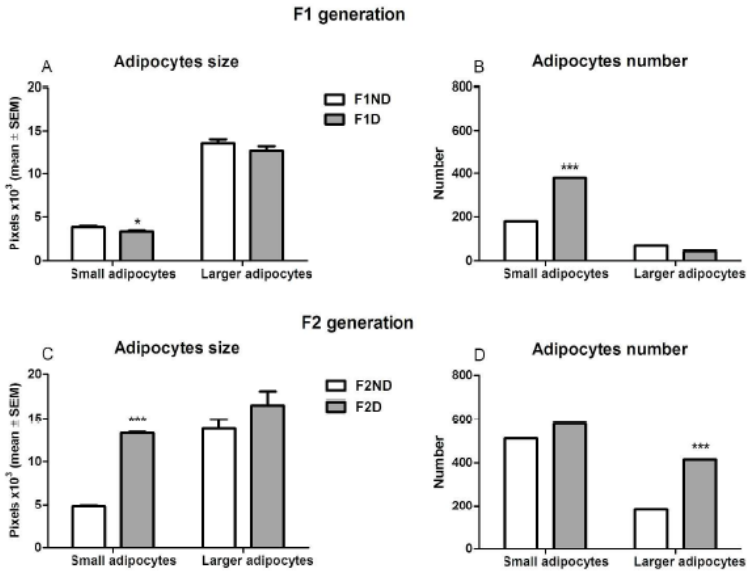

Fig. 4.Adipocyte area (A, C) and number of small and larger adipocytes (C, D) in F1 and F2 generations from F0 dams that were subjected to 40% food restricition until gestation day 18. F1ND, female F1 rats from non-food-restricted F0 dams; F1D, female F1 rats from food-restricted F0 dams; F2ND, male F2 rats born from F1 generation from non-food-restricted F0 dams; F2D, male F2 rats born from F1 generation from food-restricted F0 dams. n = 6-8/group. **p < 0.01, ***p < 0.001, compared with control group (Student's t-test).
254x188mm (300 x 300 DPI)

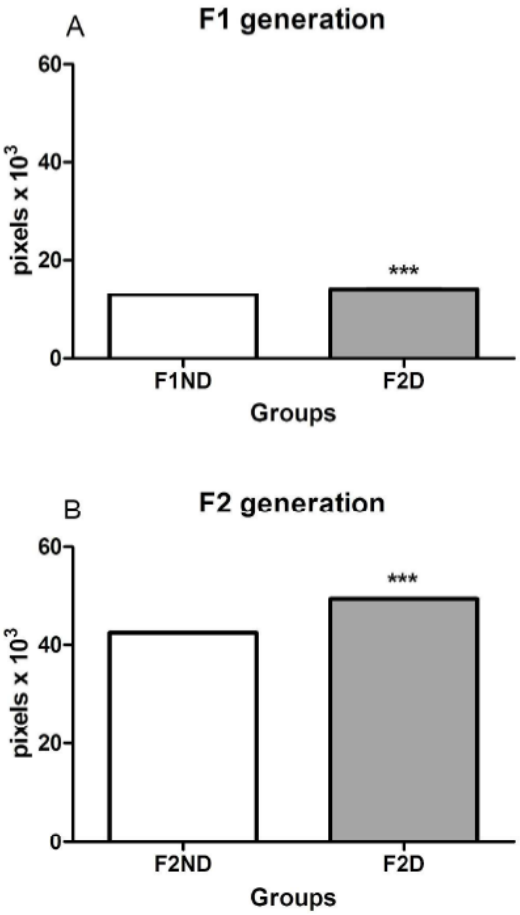

Fig. 5. Area of GFAP-immunoreactive astrocytes in the periventricular hypothalamus in rats of the F1 (A) and F2 (B) generations. F1ND, female F1 rats from non-food-restricted F0 dams; F1D, female F1 rats from food-restricted F0 dams; F2ND, male F2 rats born from F1 generation from non-food-restricted F0 dams; F2D, male F2 rats born from F1 generation from food-restricted F0 dams. n = 6-8/group.
174x270mm (300 x 300 DPI)