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Sistema dopaminérgico e dieta: uma investigação de variantes genéticas em humanos e expressão gênica em roedores

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SUMÁRIO

ABREVIATURAS

DNA: ácido desoxirribonucleico

RNA: ácido ribonucleico

miRNAs: micro-RNAs

mRNA: RNA mensageiro

GABA: neurotransmissor ácido gama-aminobutírico

DA: neurotransmissor dopamina

ATV: área tegmentar ventral

HPT: hipotálamo

TNF: fator de necrose tumoral

IMC: índice de massa corporal

L-DOPA: L-3,4-diidroxifenilalanina

TH: enzima tirosina hidroxilase

DDC: enzima dopa descarboxilase

DAT: transportador específico da dopamina

MAO: enzima monoaminooxidase (A e B)

COMT: catecol-O-metiltransferase

AMPc: monofosfato cíclico de adenosina

FAD: dinucleotídeo de flavina e adenina

MSN: *medium spiny neurons*

ARC: núcleo arqueado do hipotálamo

NTAD: cluster dos genes *NCAM1-TTC12-ANKK1-DRD2*

RIP: proteína serina-treonina-quinases de interação com receptores

NF-κB: fator nuclear *kappa* B

LISTA DE FIGURAS

RESUMO

A ingesta de alimentos ricos em gordura e açúcar, tanto em humanos quanto em animais, aumenta drasticamente a síntese e secreção de dopamina em áreas do sistema de recompensa. Em humanos, essa sensação de recompensa após a ingestão alimentar, é particular e mais intensa em alguns indivíduos, em comparação a outros. Variantes genéticas, portanto, principalmente em genes do sistema dopaminérgico, podem influenciar na resposta de diferentes pessoas expostas aos mesmos fatores ambientais. Para investigarmos a relação do sistema dopaminérgico com a ingesta alimentar e como a dieta pode alterar esse sistema, dois tipos de estudo foram utilizados. Inicialmente, em uma coorte de crianças acompanhadas desde o nascimento, investigamos a associação de variantes no gene *ANKK1/DRD2* com padrões de ingestão alimentar e parâmetros de adiposidade. O alelo **A1* da variante *Taq*IA (rs1800497) foi relacionada com o aumento da ingestão alimentar de lipídeos em crianças de 7-8 anos, mas o polimorfismo -141C Ins/Del (rs1799732) não foi associado a nenhuma das variáveis analisadas. Em modelo animal, as dietas restritiva e hipercalórica modificaram a expressão gênica na área tegmentar ventral (ATV) e no hipotálamo (HPT) de genitoras e de sua prole. Na ATV, ambas as dietas foram relacionadas a uma elevação nos níveis de mRNA dos genes *Th, Drd1, Drd2* e *Drd3,* no entanto, o gene *Slc6a3/Dat1* teve sua expressão aumentada apenas nas fêmeas que receberam a dieta hipercalórica. Na prole dessas fêmeas, observamos, pelo contrário, uma redução generalizada na expressão gênica *Th, Ddc, Drd2, Drd3* e *Slc6a3/Dat1*, devido a interação entre a dieta materna e a dieta da prole. Já no HPT, demonstramos que a alteração na expressão gênica ocasionada pela dieta foi mais expressiva nas genitoras. As dietas restritiva e hipercalórica elevaram os níveis de mRNA dos genes *Drd1, Drd3, Maoa* e *Th,* mas apenas as fêmeas que receberam dieta hipercalórica tiveram expressão aumentada de *Comt* e *Ddc*. Em contrapartida, as dietas reduziram a expressão do gene *Drd4*. A expressão gênica da prole foi menos alterada pela dieta nessa área, uma vez que a interação entre a dieta materna e a dieta materna foi relacionada com a redução dos níveis de mRNA apoenas do gene *Maob*. A dieta da prole aumentou a expressão do gene *Drd3,* e reduziu a expressão de *Comt.* Através dos dados apresentados, observa-se a importância do sistema dopaminérgico e sua relação com a alimentação, tanto em humanos quanto em camundongos. Variantes em genes relacionados ao sistema dopaminérgico podem

modificar a transmissão dopaminérgica, provocando maior ou menor sensação de recompensa após a ingestão alimentar, e ocasionando desfechos como sobrepeso e obesidade. Além disso, alterações na expressão de genes do sistema dopaminérgico podem ser ocasionadas tanto pela alimentação materna durante a gestação e a lactação, quanto pela alimentação na vida adulta.

Palavras-chave: Sistema dopaminérgico, dieta, variantes genéticas, expressão gênica.

ABSTRACT

High-fat and high-sugar food intake, both in humans and in animals, dramatically increases the synthesis and secretion of dopamine in areas of the reward system. In humans, the rewarding sensation after food intake is very particular and more intense in some individuals compared to others. Genetic variants, therefore, especially in genes of the dopaminergic system, can influence the response of different people exposed to the same environmental factors. To investigate the relationship between the dopaminergic system and dietary intake, and how diet can alter this system, two types of study were used. Initially, in a cohort of children followed since birth, we investigated the association of variants in *ANKK1/DRD2* gene with food intake and adiposity parameters. The **A1* allele of the *Taq*IA variant (rs1800497) was related to increased lipid food intake in children aged 7-8 years, but the -141C Ins/Del polymorphism (rs1799732) was not associated with any of the variables analyzed. In the animal model, restrictive and hypercaloric diets modified gene expression in ventral tegmental area (VTA) and hypothalamus (HPT) of mice dams and their offspring. In VTA, mRNA levels of *Th, Drd1, Drd2* and *Drd3* genes were increased in mice dams who received a restrictive and hypercaloric diet, but the *Slc6a3*/*Dat1* gene had its expression increased only in females receiving a hypercaloric diet. In the male offspring of these females, we observed, on the contrary, a generalized reduction in the expression of *Th, Ddc, Drd2, Drd3* and *Slc6a3/Dat1* genes. In HPT, we demonstrated that the alteration in the gene expression caused by the diet was more expressive in mice dams. Restrictive and hypercaloric diets elevated the mRNA levels of *Drd1, Drd3, Maoa* and *Th* genes, but only females receiving a hypercaloric diet had increased *Comt* and *Ddc* genes expression. In contrast, diets reduced *Drd4* gene expression. Offspring's gene expression was less affected by diet in this area, since the interaction between the maternal and the offspring diets was related with a reduction in the mRNA levels of *Maob* gene only. The offspring diet elevated the expression of *Drd3* gene and reduced the expression of *Comt* gene. Therefore, with the presented data, we can observe the importance of the dopaminergic system and its relation with feeding, both in humans and mice. Variants in dopaminergic-related genes may modify dopaminergic transmission, causing increased or decreased reward sensation after food intake, which may lead to outcomes such as overweight and obesity. In addition, alterations in

dopaminergic gene expression can be caused by maternal nutrition during pregnancy and lactation, and by ingested diet in adult life.

Keywords: Dopaminergic system, diet, genetic variants, gene expression.

1. INTRODUÇÃO

1.1 Necessidade nutricional

Os seres vivos necessitam de energia para manter todas as funções biológicas, visto que a ação contínua do cérebro, pulmões e coração tem um gasto energético elevado [1]. A necessidade nutricional pode ser definida pela quantidade de energia que um indivíduo sadio deve ingerir para satisfazer suas necessidades fisiológicas normais, além da energia adicional para crescimento, gravidez e lactação [1, 2]. Portanto, as necessidades nutricionais são valores fisiológicos individuais que são expressos em médias de calorias, por exemplo, para grupos semelhantes (mulheres, homens, crianças) [2].

A energia para as funções metabólicas e fisiológicas é derivada dos alimentos e seus macronutrientes: carboidratos, gorduras e proteínas [1]. Manter níveis adequados de macronutrientes na dieta (consumo energético), e balanceá-los com o gasto energético é essencial em uma dieta saudável [3]. Uma dieta balanceada previne a desnutrição, a obesidade e o aumento do risco de desenvolvimento de doenças crônicas como diabetes, doenças cardiovasculares e câncer [3].

1.2 Nutrição na gestação e lactação

A nutrição materna no período gestacional é essencial para o correto desenvolvimento do embrião, o qual necessita de água, aminoácidos, lipídeos, carboidratos, minerais e vitaminas [4]. Durante o crescimento fetal, ocorrem diversos processos biológicos que necessitam de energia, principalmente proveniente de macronutrientes (carboidratos, lipídeos e proteínas). A glicose é essencial para as hemácias, cérebro, células da retina, e células da medula renal, enquanto que o lactato é utilizado como fonte de energia para o coração, tanto da gestante quanto do feto [4]. A oxidação do glutamato, aspartato e glutamina geram energia para o intestino delgado de ambos. Ácidos graxos são os substratos energéticos do fígado, músculo esquelético, coração e rins da gestante, já o feto oxida ácidos graxos a gás carbônico em uma quantidade expressiva. Portanto, a alimentação materna deve ser ajustada de acordo com as necessidades nutricionais da gestante e do feto para esse período [5].

Os carboidratos da dieta têm como função o fornecimento de energia para as células, principalmente no cérebro, que é o único órgão glicose-dependente [2]. Portanto carboidratos são macronutrientes essenciais na fase do desenvolvimento do cérebro fetal e também para o cérebro materno [2]. A maioria dos países desenvolvidos e em desenvolvimento tem como problema de saúde pública a supernutrição, ocasionada por dietas ricas em carboidratos e gorduras [6]. Em modelo animal, dietas ricas em carboidratos simples (como dextrose e maltodextrina) durante a gestação tiveram como desfecho nos filhotes: ganho de peso, aumento do percentual de gordura, desregulação na expressão de genes lipogênicos e adipogênicos, hiperinsulinemia, hiperleptinemia, resistência à insulina, hiperglicemia e aumento de triglicérides [7-9].

Proteínas são macronutrientes essenciais durante a gestação, uma vez que têm um importante papel estrutural (queratina, colágeno) e funcional (enzimas, proteínas de transporte, hormônios) [10]. A gestação é um período excepcional da vida, caracterizado por um rápido crescimento e desenvolvimento do feto, além de diversas alterações fisiológicas na mãe, sendo, portanto, necessário um aporte maior de proteínas [10]. Desse modo, a restrição proteica é extremamente prejudicial para o feto, e já foi associada a diversas consequências, inclusive na vida adulta da prole. Em modelo animal, a restrição proteica durante a gestação e a lactação foi associada a diversos desfechos na prole, como restrição do crescimento, aumento da pressão arterial, alteração nos níveis de insulina e lipídeos, além de alterações na expressão de diversos genes [10]. Quando adultos, a prole submetida à restrição proteica durante o desenvolvimento fetal apresenta aumento no ganho de peso, aumento do peso corporal e elevação da adiposidade, além de um aumento do comportamento do tipo ansioso [11].

Os lipídeos são a principal fonte de energia da dieta, além de essenciais para a absorção de vitaminas lipossolúveis e carotenoides. No entanto, as recomendações sugerem a redução da ingestão de colesterol, ácidos graxos *trans* e gorduras saturadas, em função da relação dessas moléculas com aumento do risco de doenças cardiovasculares [2]. Em humanos, dietas ricas em gorduras (mais de 30% das calorias provenientes de gorduras) já foram associadas com obesidade e resistência à insulina nas mães submetidas a essa dieta durante a gestação e a lactação [12]. Na prole, os efeitos da dieta hiperlipídica

materna, em humanos são: aumento do crescimento fetal, aumento do peso ao nascer, além de desenvolvimento de obesidade e diabetes tipo 2 durante a vida adulta [13].

1.3 Desenvolvimento intrauterino e efeitos da dieta materna na prole

Early life programming, ou as origens fetais e pueris das doenças adultas, também referidos como a hipótese de Barker [14, 15], postula que a exposição a certos fatores ambientais durante períodos críticos no desenvolvimento e crescimento podem levar a adaptações no feto ocasionando consequências significativas em sua saúde tanto a curto quanto a longo prazo [16, 17]. A exposição a um ambiente intrauterino hostil, ocasionado por nutrição inadequada, infecções e perturbações químicas, metabólicas e hormonais, resulta em adaptações (respostas adaptativas preditivas) que são desenvolvidas pelo feto e que permitem a sua viabilidade imediata, além de prepará-lo para exposições semelhantes que possam ocorrer ao longo da vida[18]. Durante o desenvolvimento fetal, o epigenoma sofre modificações consideráveis, devido a ondas de deleção e reestabelecimento de marcadores epigenéticos. A desregulação epigenética tem sido proposta como um potencial mecanismo para explicar a programação fetal anormal[19].

O período em que o feto é exposto ao estímulo é crítico para determinar o desfecho, sendo que os períodos de maior sensibilidade são os de maior crescimento e maturação [16]. O órgão mais suscetível durante a gestação é o cérebro, devido ao seu extenso crescimento e o desenvolvimento de novas vias neuronais que se prolongam até os anos iniciais da infância [16]. A formação do sistema de recompensa ocorre durante o desenvolvimento perinatal e, portanto, alterações do ambiente intrauterino durante períodos críticos podem programar mecanismos imperfeitos de apetite e saciedade, alterando assim o comportamento da ingestão alimentar durante toda a vida do indivíduo [20]. Estudos epidemiológicos têm observado associações entre alterações do ambiente intrauterino com padrões de crescimento e alterações metabólicas na vida adulta [20]. A fome holandesa durante o inverno de 1944-45 oportunizou a determinação dos efeitos da subnutrição durante o período perinatal em bebês que posteriormente receberiam alimentos em abundância. Observou-se que os filhos cujas mães foram expostas à fome durante os primeiros dois trimestres da gestação tiveram maior incidência de obesidade do que a população em geral [14, 21-23]. Recentemente, estudos têm analisado os desfechos gerados pela dieta ocidental e pela ingesta materna excessiva durante a gestação, por meio

de modelos animais. Em porcos, assim como em humanos, a subnutrição materna modifica permanentemente o crescimento e desenvolvimento da prole, além de promover defeitos na maturação de diversos órgãos e sistemas, principalmente no sistema imune, pâncreas e intestinos [24, 25]. A restrição da dieta materna, em roedores, para 50% das calorias durante a última semana de gestação, causa danos no desenvolvimento pancreático da prole [26]. Outro estudo em ratos indica que a redução de 30% das calorias da dieta padrão durante os primeiros 18 dias de gestação resulta em crescimento intrauterino restrito, predominantemente em machos, além de associações com hiperinsulinemia, hiperfagia, obesidade e hipertensão [27].

Fatores externos que modificam a expressão de determinados genes, sem modificar a sequência do DNA genômico, são denominados de fatores epigenéticos. Essas modificações descritas no sistema de recompensa, que ocorreram durante o desenvolvimento fetal, podem estar relacionadas a processos epigenéticos que ativam ou silenciam certos genes, como a modificação de histonas, regulação por RNAs não-codificantes e metilação [28]. Além disso, o perfil epigenético de um indivíduo é, ao mesmo tempo, herdado e modificável, uma vez que os padrões de expressão do DNA podem ser transmitidos dos pais para a prole, podendo também ser modificados em resposta a fatores ambientais [29, 30].

Nutrientes provenientes da dieta materna tem grande importância no desenvolvimento fetal por modificarem processos epigenéticos. Essas mudanças induzidas por fatores externos parecem ser mantidas durante a vida, levando a alterações permanentes no fenótipo [31]. O melhor exemplo de como a dieta pode alterar o fenótipo é observado em estudos com abelhas [31]. Larvas fêmeas, dependendo do tipo de dieta que recebem, desenvolvem-se em abelhas operárias estéreis ou abelhas-rainha férteis, mesmo sendo geneticamente idênticas [32]. Estudos com humanos e animais mostram que a deficiência de folato resulta em uma redução dos níveis de S-adenosilmetionina, cofator enzimático envolvido na transferência de grupamentos metil, levando à hipometilação do DNA genômico [33]. O consumo elevado de vitamina B12 na gestação foi associado à diminuição da metilação global de DNA em recém-nascidos [34], enquanto que a vitamina D foi relacionada a modificações de histonas através do recrutamento de acetilases de histona [35]

A dieta, portanto, é essencial para o desenvolvimento do perfil epigenético do feto. Qualquer alteração no ambiente intrauterino ocorrida durante esse período pode modificar essas marcações no genoma, afetando a expressão gênica até mesmo na vida adulta. Apesar de não avaliarmos a quantificação de marcadores epigenéticos diretamente, nesta tese, as alterações na expressão de determinados genes podem ser decorrentes de modificações epigenéticas no genoma da prole.

1.4 Ingesta alimentar e recompensa

A recompensa resultante da ingesta alimentar é um processo composto pelo "gostar" de determinado alimento, o que nos leva a "querer" esse alimento [36]. "Gostar" está envolvido com a palatabilidade dos alimentos, ou seja, eles evocam uma resposta hedônica prazerosa, enquanto que o "querer" corresponde ao apetite e ao desejo de consumir determinado alimento [36, 37]. Apesar de serem definições que muitas vezes se confundem, são diferentes áreas e sistemas cerebrais que estão envolvidos no "gostar" e no "querer", visto que não há um centro da fome nem um centro do prazer no cérebro [36, 38]. Áreas como núcleo *accumbens* (NAc), globo pálido ventral e tronco encefálico, através de neurotransmissores como opioides endógenos, ácido gama-aminobutírico (GABA), canabinoides e orexina, medeiam o "gostar" através de uma atividade coordenada [37]. No entanto, para o "querer", o sistema necessário é o sistema dopaminérgico mesocorticolímbico [37]. Portanto, a sensação de recompensa ocasionada após a ingestão do alimento pode ser elevada ou reduzida, dependendo do estado metabólico do indivíduo, além das variações interindividuais [37]. Catellanos et. al (2009) [39] observou que pessoas obesas respondem mais fortemente a sugestões de alimentos do que pessoas magras quando saciados. Diferenças genéticas podem, portanto, estar relacionadas com essas diferenças interindividuais.

A ingesta de alimentos ricos em gordura e açúcar, tanto em humanos quanto em animais, aumenta drasticamente a síntese e secreção de opioides e dopamina (DA) em áreas do sistema de recompensa, que envolve as vias nigroestriatal, mesocorticolímbica e tuberoinfundibular (ver figura 1) [40-43]. Esse tipo de alimento altamente palatável estimula áreas cerebrais relacionadas à motivação e recompensa, de uma maneira muito semelhante ao álcool e drogas de abuso, e levam a ingestão alimentar excessiva [44, 45]. A sensação de recompensa ocasionada após o consumo de drogas de abuso e de alimentos

palatáveis está associada à intensificação na sinalização dopaminérgica [46]. Portanto, a ingestão de alimentos palatáveis estimula a síntese de opioides endógenos, que se ligam a receptores μ-opiodes em neurônios GABAérgicos inibitórios, localizados na área tegmentar ventral (ATV) [47]. Esses neurônios inibitórios são então bloqueados e deixam de exercer sua função inibitória em neurônios dopaminérgicos nessa área cerebral, intensificando, dessa forma, a sinalização dopaminérgica [48]. As porções terminais desses neurônios dopaminérgicos se projetam para diversas áreas do cérebro, relacionadas com o sistema de recompensa, como amígdala, NAc, estriado e hipocampo [47].

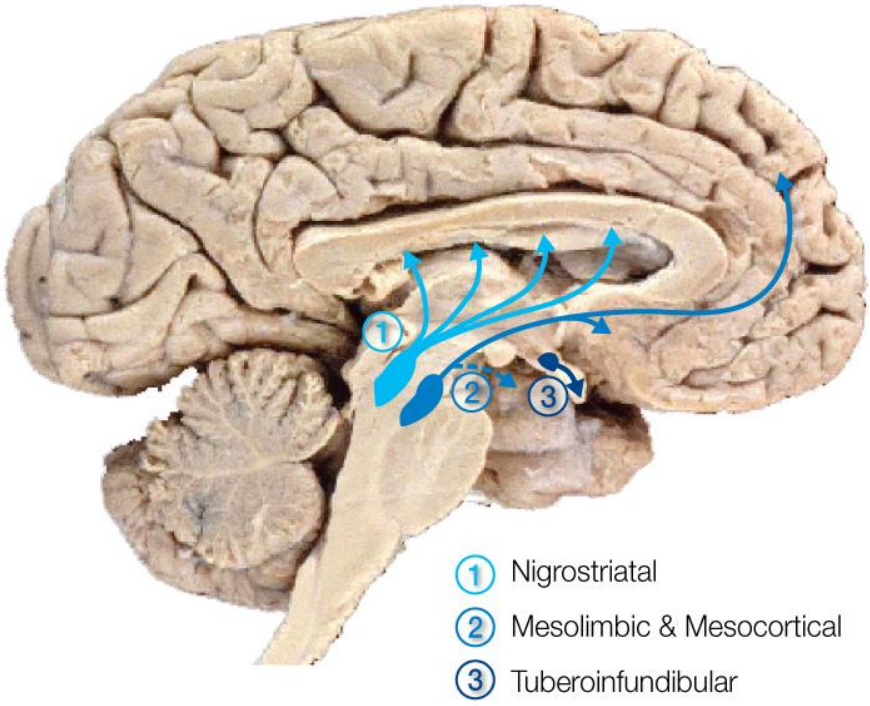

Figura 1. As vias dopaminérgicas. (1) Via nigroestriatal: neurônios que se projetam da subtância negra para o estriado; (2) Via mesolímbica e mesocortical, ou via mesocorticolímbica: projeções da ATV para NAc, amídala, hipocampo e córtex pré-frontal; (3) Via tuberoinfundibular: projeções dopaminérgicas do hipotálamo (HPT) até a glândula pituitária. Fonte: Genetic Science Learning Center. (2013, August 30) Beyond the Reward Pathway. Disponível em https://learn.genetics.utah.edu/content/addiction/beyond/. Acesso em 05 de fevereiro de 2019.

Em humanos, a impossibilidade de controlar o real consumo dietético de mulheres durante a gravidez e lactação, devido a diversos fatores intrínsecos e extrínsecos que podem afetar a quantificação, acaba por dificultar o estudo da relação entre a dieta materna e suas consequências a curto e longo prazo na prole. Um estudo prospectivo de 5717 pares de mãe-filho e 3009 pares pai-filho denominado *Avon longitudinal Study of Parents and Children*, avaliou a relação entre os macronutrientes ingeridos pela mãe e pelo pai antes e durante a gravidez, e os macronutrientes ingeridos pelas crianças aos 9-10 anos de idade. Esse estudo mostrou que há uma correlação muito forte entre a ingestão materna de lipídeos durante a gravidez e as preferências da criança aos 9-10 anos de idade. No entanto, não foi observada nenhuma relação entre a ingesta de lipídeos do pai em nenhuma fase do desenvolvimento da criança [49]. Estudos em outros períodos do desenvolvimento da criança indicam que a exposição a certos sabores (como por exemplo, cenoura e alho), tanto durante a gestação quanto durante a amamentação, aumentam a preferência da criança para o mesmo sabor após o desmame [50-52]. Além disso, crianças alimentadas com fórmulas preparadas com soja, que possuem um sabor mais amargo, preferem alimentos mais amargos e ácidos ao 4-5 anos de idade, em comparação com crianças que foram alimentadas com fórmulas preparadas com leite, que são mais adocicadas [53]. Portanto, a exposição perinatal a alimentos ricos em gorduras e açúcares pode estar relacionada com o desenvolvimento de preferências a sabores de alimentos mais palatáveis [47]. Estudos farmacológicos em humanos buscam relacionar o sistema dopaminérgico com a ingesta e preferências alimentares. A administração de naloxona, um antagonista de receptores opióides, reduz a ingesta de alimentos palatáveis, sem alterar a ingesta dos demais alimentos [54].

A fome holandesa de 1944/45, como anteriormente citado, possibilitou a determinação de efeitos perinatais da desnutrição em bebês que, futuramente, estariam expostos a calorias em excesso. Ao completarem 60 anos de idade, esses indivíduos expostos à fome durante as duas últimas semanas de gestação possuíam metilação alterada de alguns genes relacionados à obesidade, em comparação com seus irmãos do mesmo sexo, não expostos à fome [55, 56]. Essa associação foi específica para uma exposição periconcepcional, reforçando que as fases iniciais do desenvolvimento são cruciais para o estabelecimento e manutenção de marcadores epigenéticos [55, 56]. A obesidade materna e o ganho de peso durante a gravidez estão associados a elevado peso ao nascer e aumento

do risco do desenvolvimento de obesidade e diabetes na vida adulta [57-59]. Mais recentemente, estudos associaram características maternas de ganho de peso e diabetes gestacional com elevada metilação do gene da leptina em placenta [60, 61]. A metilação de histonas específicas (H3K4 e H3K9) e certos miRNAs foram positivamente associados com índice de massa corporal (IMC) [62-64]. Outro estudo analisou a metilação dessas histonas em células primárias de adipócitos humanos com e sem diabetes do tipo 2, e descreveram que, em comparação com indivíduos de peso normal, indivíduos com sobrepeso e não-diabéticos apresentavam 40% menos dimetilação de K4, e 40% mais trimetilação de K4 em adipócitos do que indivíduos com sobrepeso e diabéticos. Além disso, indivíduos obesos e normais apresentam variação nos níveis de metilação do DNA em genes específicos e seus pré-adipócitos e adipócitos maduros em cultura possuem diferente expressão de miRNAs [63]. Similarmente, um estudo de miRNAs de tecido adiposo de biópsias de humanos mostra uma expressão diferencial e desregulada de miRNAs quando se compara indivíduos obesos com indivíduos normais [64].

1.5 Sistema dopaminérgico

Responsável por diversas funções fisiológicas (como atividade locomotora, cognição, emoção, recompensa, sono e alimentação), a DA é a principal catecolamina no cérebro de mamíferos [65]. Projeções dopaminérgicas partem da ATV para diferentes áreas do cérebro como amígdala, NAc, estriado e hipocampo, que estão relacionadas com a recompensa [66]. Além disso, essas áreas controlam a motivação para comer, tanto através fome fisiológica quanto pela fome hedônica [66].

A síntese de DA inicia com uma etapa limitante da velocidade que consiste na conversão de tirosina a L-3,4-diidroxifenilalanina (L-DOPA) pela enzima tirosina hidroxilase (TH), seguida de descarboxilação à DA pela enzima dopa descarboxilase (DDC) (Figura 2). Após sua liberação nos terminais nervosos, esse neurotransmissor é amplamente recaptado por um transportador específico da DA (DAT) e metabolizado pelas enzimas monoaminooxidases (MAO) A e B e catecol-O-metiltransferases (COMT), localizadas principalmente em células gliais. Sua ação ocorre após ligação a receptores presentes nas células-alvo, os ativadores (classe D1) ou os inibidores (classe D2) da adenilciclase [65, 67]. Os receptores da classe D1 (subtipos D1 e D5) aumentam o

monofosfato cíclico de adenosina (AMPc) intracelular quando ativados, enquanto que os receptores da classe D2 (subtipos D2, D3 e D4) o inibem [67-69].

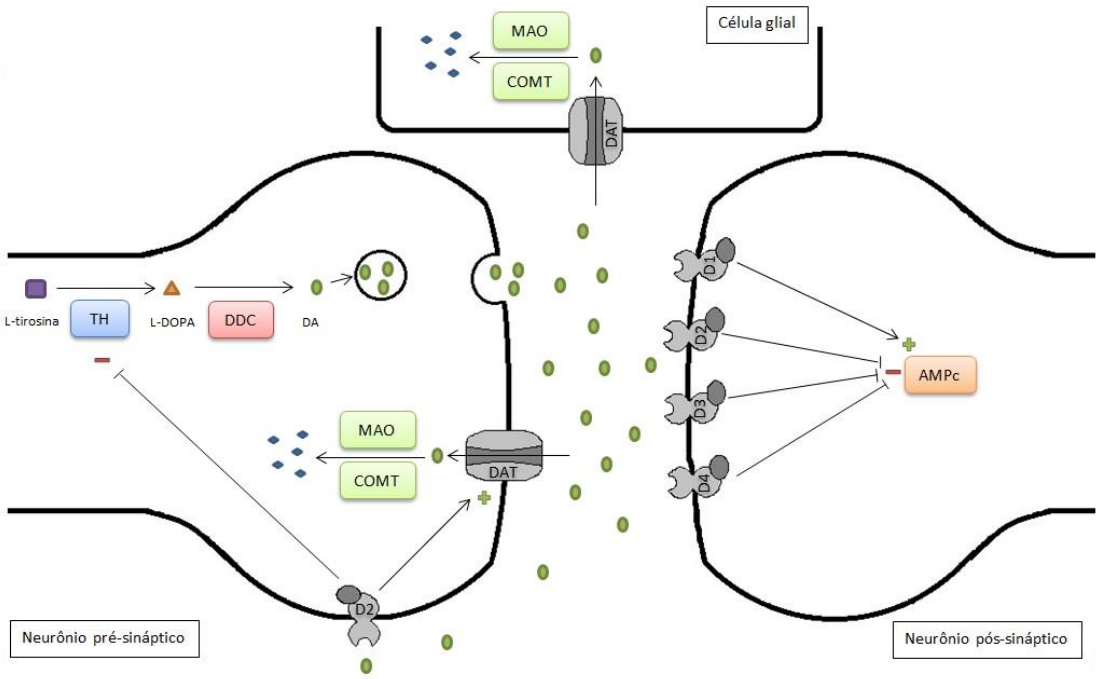

Figura 2. Sinapse dopaminérgica. TH, tirosina hidroxilase; L-DOPA, L-3,4-diidroxifenilalanina; DDC, dopa descarboxilase; DA, dopamina; MAO, monoaminooxidases; COMT, catecol-O-metiltransferases; DAT, transportador de dopamina; D1, receptor de dopamina D1; D2, receptor de dopamina D2; D3, receptor de dopamina D3; D4, receptor de dopamina D4; AMPc, monofosfato cíclico de adenosina.

A TH catalisa a hidroxilação da cadeia lateral de seu substrato, a tirosina, através da reação de tetrahidrobiopterina, oxigênio molecular e tirosina para formar L-DOPA [70]. Essa é a etapa limitante da reação de síntese de DA, uma vez que, quando há liberação diminuída de catecolaminas pelo terminal axonal ocorre também um aumento da sua concentração no citosol, levando à inibição da enzima TH pelo produto final da reação, a DA [71]. A TH está localizada nos corpos dos neurônios dopaminérgicos, principalmente na ATV e substância negra [70]. O gene *TH* está localizado, em humanos, no cromossomo 11p15.5 e contém 14 éxons, que codificam 4 isoformas de TH, através de *splicing*

alternativo [71, 72]. Em camundongos, o gene *Th* está localizado no cromossomo 7 F5, possui 13 éxons separados por 12 íntrons [72].

A DA age em cinco diferentes receptores pertencentes à família de receptores ligados à proteína G, dividida em subtipo D1 (D1 e D5) e subtipo D2 (D2, D3 e D4). Cada receptor possui propriedades únicas, incluindo a distribuição, a afinidade por DA e a especificidade pela proteína G [73]. A afinidade dos receptores D2 pela DA é 10 a 100 vezes maior do que receptores do tipo D1, sendo que os receptores D3 e D4 apresentam a maior afinidade e D1 a menor afinidade pela DA [74, 75]. Os receptores do tipo D1 são observados apenas nos neurônios pós-sinápticos, enquanto que os do tipo D2 são expressos em neurônios pré e pós-sinápticos [73].

Receptores de DA D1 estão envolvidos diretamente com recompensa e motivação [74, 76-78]. O gene *DRD1* de humanos está localizado no cromossomo 5q35.1, não possui íntrons na região codificadora e, portanto, não possui variantes de *splicing* [72, 75, 79]. Em camundongos, o *Drd1* está localizado no cromossomo 13 B1 e possui 3 éxons [72]. É amplamente expresso nas áreas nigroestriatal, mesocorticolímbica, como no caudado-putâmen (estriado), NAc, substância negra, bulbo olfatório, amígdala e córtex pré-frontal, bem como, em níveis mais reduzidos, no hipocampo, cerebelo e áreas do tálamo e HPT [75].

Receptores de DA D2 são expressos tanto em neurônios pré-sinápticos quanto em neurônios pós-sinápticos e estão relacionados ao controle do feedback negativo e controle da transmissão dopaminérgica [74, 76-78, 80]. São os principais autorreceptores envolvidos com a regulação pré-sináptica da síntese e liberação de DA, localizados principalmente no soma e dendritos de neurônios dopaminérgicos do mesencéfalo, na ATV e na sustância negra [75, 81]. Sua ação quando ativado pela DA é inibitória, sendo o seu principal papel no mecanismo de *feedback* negativo, ajustando a sinapse dopaminérgica através da redução da síntese e liberação de DA, além de estimular a sua recaptação [81-83]. Esse receptor é um dos mais abundantes do sistema nervoso central, é essencial na coordenação motora, atividade locomotora, motivação/aversão e recompensa [83, 84]. O gene *DRD2* está localizado no cromossomo 11q23.1 em humanos, possui 6 íntrons e duas variantes de *splicing* D2L e D2S [72, 75, 79]. Essas variantes apresentam diferentes distribuições neuronais, sendo o D2S predominantemente pré-sináptico e o D2L pós-

sináptico. Em camundongos, o gene *Drd2* localiza-se no cromossomo 9 A5 e possui 8 éxons [72]. Os maiores níveis de expressão do receptor D2 ocorrem no estriado, NAc e bulbo olfatório. Também são expressos em níveis significativos na substância negra, ATV, áreas corticais, septo, amígdala e hipocampo [74, 85-87].

A função fisiológica específica do receptor de DA D4 no cérebro ainda não foi elucidada. O gene *DRD4* de humanos está localizado no cromossomo 4p16.1, possui 3 íntrons e variantes de *splicing* [72, 75, 79]. Em camundongos, o gene *Drd4* está localizado no cromossomo 7 F5 e possui 4 éxons [72]. É o receptor de DA menos expresso no cérebro, mas é detectado no córtex frontal, amígdala, hipocampo, HPT, globo pálido, substância negra e tálamo [74, 88].

DAT é uma proteína localizada na membrana plasmática do neurônio pré-sináptico, e sua função é transportar ativamente a DA livre na fenda sináptica para o citoplasma. Pertencente à família de transportadores SLC6 (*SLC6A3*), dependentes de Na^+ e Cl^-, regula os níveis de DA, influenciando a ingestão alimentar [89-92]. O gene *SLC6A3* de humanos está localizado no cromossomo 5p15.3 e contem 15 éxons e 14 íntrons [72, 93, 94]. Em camundongos, o gene *Slc6a3* está localizado no cromossomo 13 C1 e possui 15 éxons [72]. Estudos com animais demonstram que ratos obesos apresentam diminuição na densidade de DAT na superfície celular dos neurônios estriatais, além diminuição na expressão do gene *Slc6a3* [95-99].

A MAO é uma amina oxidase contendo um dinucleotídeo de flavina e adenina (FAD), cuja função é a desaminação oxidativa de monoaminas biogênicas e xenobióticas [100]. Possui duas isoformas, a MAO-A e a MAO-B, identificadas de acordo com diferenças em suas especificidades para inibidores e substratos [100, 101]. A DA é substrato para ambas as isoformas em humanos, mas em ratos é preferencialmente substrato da MAOA [100]. Os genes *MAOA* e *MAOB* estão localizados, em humanos, no cromossomo Xp11.23 e Xp22.1, respectivamente, enquanto que em camundongos, os genes *Maoa* e *Maob* estão localizados no cromossomo X A2 e X A1.2, respectivamente [72]. Ambos os genes, em ambas as espécies, possuem uma organização semelhante com 15 éxons [72, 100, 102]. A expressão de MAOA no cérebro ocorre em neurônios catecolaminérgicos, enquanto que MAOB é primariamente encontrada em astrócitos em diversas partes do cérebro como HPT, córtex pré-frontal e amígdala [102, 103]. Estudos

em humanos observaram uma associação do fumo com hipermetilação do promotor de *MAOB,* e a regulação epigenética do gene *MAOA* tem sido relacionada com propensão gênero-específica para dependência de nicotina [104, 105]. As regiões promotoras dos genes *MAOA* e *MAOB* são caracterizados por sequências ricas em GC (ilhas CpG), que interagem com fatores de transcrição [102, 106]. A modificação epigenética da ilha CpG do promotor do gene *MAOB* reduz a expressão da proteína, e o mesmo é esperado do gene *MAOA* [107, 108].

A enzima COMT é encontrada em astrócitos adjacentes a sinapses dopaminérgicas e também em espinhos dendríticos pós-sinápticos, não existindo elevada atividade de COMT em neurônios pré-sinápticos [109]. A rota primária de terminação da sinapse dopaminérgica ocorre através da recaptação da DA excedente na fenda para o interior do neurônio pré-sináptico, onde ocorre a internalização do neurotransmissor em vesículas para reutilização ou então degradação [109]. Existe, no entanto, uma rota secundária que é a recaptação da DA por células glias, seguida de degradação pelas enzimas MAO e COMT [109]. Em humanos, o gene *COMT* fica localizado no cromossomo 22q11.21, enquanto que em camundongos o gene *Comt* está codificado no cromossomo 16 A3. A expressão do gene *Comt* está reduzida em ratos submetidos à dieta rica em gorduras e açúcares [110]. No entanto, outro estudo [111] não observou diferença na expressão de *Comt* em camundongos expostos à dieta baixa em gorduras *versus* camundongos expostos à dieta rica em gorduras, indicando que a alteração da expressão desse gene pela dieta não está bem esclarecida.

1.5.1 Como variantes genéticas no sistema dopaminérgico alteram o comportamento alimentar?

Em humanos, descobriu-se que a sensação de recompensa após a ingestão alimentar, principalmente de alimentos ricos em gorduras e açúcares, era particular para alguns indivíduos, em comparação a outros [112]. A exposição contínua a alimentos palatáveis devido à sensação de recompensa após a ingesta acaba ocasionando diversos desfechos, como, por exemplo, o acúmulo excessivo de gordura e consequentemente o aumento de peso e IMC. Estudos que comparam semelhanças entre gêmeos idênticos e não-idênticos estimam que a herdabilidade do IMC é extremamente alta: genes estão relacionados com 50 até 90% das diferenças individuais no IMC [113]. Variantes

genéticas, portanto, principalmente em genes do sistema dopaminérgico, podem influenciar na resposta de diferentes pessoas aos mesmos estímulos. Estudos com modelos animais demonstraram a relação entre o sistema dopaminérgico e a recompensa em ratos. O uso de agonistas do receptor dopaminérgico D2 diminui o consumo alimentar em ratos [114]. O receptor D2 pode ser um autorreceptor, ou seja, possui um papel importante no *feedback* negativo, ajustando o ritmo dos disparos neuronais, a síntese e a recaptação de DA [75, 81]. Um estudo, em humanos, analisou a densidade desse receptor no estriado, e descreveu que em indivíduos obesos o receptor D2 estava diminuído [115].

O alelo **A1* do polimorfismo *Taq*IA (rs1800497), no gene *ANKK1* localizado 10 kb a jusante do gene *DRD2*, já foi associado a reduzida densidade e atividade do receptor D2 em diferentes áreas do SNC [116-119]. Outro polimorfismo −141C Ins/Del (rs1799732) na região flanqueadora do gene *DRD2* já foi associado a alterações na transcrição gênica *in vitro* [120], além de alterar a ligação da DA ao receptor no estriado [116, 120]. Além disso, polimorfismos do gene *ANKK1/DRD2* são frequentemente associados a ganho de peso e a dificuldade na perda de peso [121, 122]. Dessa forma, o estudo de polimorfismos no gene do receptor de DA D2 torna-se essencial para a melhor compreensão de como os indivíduos de uma população respondem de modo diferente quando expostos ao mesmo ambiente obesogênico, ou seja, aos mesmos fatores ambientais.

1.5.2 Como a dieta modifica a expressão gênica do sistema dopaminérgico?

Alguns estudos demonstraram que a exposição prolongada à gordura e ao açúcar em ratos está associada a adaptações moleculares, como a redução na expressão de receptores de DA D2 e redução dos níveis de DA no NAc, de um modo semelhante ao observado nos casos de dependência em drogas de abuso [40, 123, 124]. Ratos alimentados com dieta de “cafeteria”, que consiste em alimentos processados hipercalóricos e altamente palatáveis, apresentaram uma redução nos níveis de TH, semelhante ao observado após exposição crônica de drogas opioides como a morfina [125, 126]. A exposição a alimentos palatáveis por longos períodos de tempo resultam em mudanças comportamentais indicativas do desenvolvimento de dependência [47]. Ratos com livre acesso a dietas ricas em gordura, ou ricas em açúcar, consomem quantidades cada vez maiores do alimento ao longo do tempo, e exibem clássicos sinais de abstinência quando a dieta é removida [127, 128].

O efeito da dieta materna também é abordado em alguns estudos, que demonstram que expressão de genes do sistema dopaminérgico em áreas relacionadas à recompensa no cérebro da prole. Estudos de restrição proteica, indicam que a exposição a esse tipo de dieta durante a reprodução, gestação e lactação resulta em níveis elevados de mRNA do gene *Th* na ATV e no HPT [129] e do gene *Drd1* no estriado ventral [130]. No córtex pré-frontal, Hehar, Ma [131] identificou uma redução na expressão do gene *Drd2* na prole de genitoras submetidas a uma dieta restritiva de 40% durante a gestação. Diferentes gerações também podem ser afetadas pela dieta restritiva, conforme descrito por Giraudo, Della-Fera [132], em um estudo que submeteu genitoras a uma restrição calórica durante a gestação, e analisou que a dieta rica em gordura alterou a expressão gênica do *Th* no HPT nas três gerações subsequentes. Até mesmo a restrição alimentar apenas na última semana de gestação pode reduzir os níveis de mRNA dos genes *Drd1* e *Drd2* no HPT da prole [133]. No entanto, fica claro que mais estudos são necessários, quando se observa que pode não ocorrer alteração na expressão gênica do *Drd1*, *Drd2* e *Comt* no HPT da prole de genitoras expostas à restrição proteica durante a reprodução, gestação e lactação [129].

Dietas ricas em gordura também afetam a expressão desses genes. Sanchez-Hernandez, Poon [134] descreveu elevação nos níveis de mRNA do gene *Drd1* no hipocampo da prole de genitoras que se alimentaram de dieta rica em gordura durante a gestação. Outro estudo observou aumento da expressão gênica de *Th* no HPT da prole neonata de genitoras expostas à dieta rica em gordura [135]. É notável, portanto, que estudos que analisam a expressão de genes relacionados ao sistema dopaminérgico na prole, são decorrentes de uma alta variedade de protocolos dietéticos, dificultando comparações. Em contrapartida, não há estudos que analisam o efeito da dieta durante a gestação e a lactação na expressão de genes do sistema dopaminérgico na ATV e no HPT das genitoras.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar a associação de variantes do gene do receptor de dopamina D2 na ingestão alimentar em humanos e da dieta na expressão de genes do sistema dopaminérgico em modelo animal.

2.2 Objetivos específicos

- Investigar a associação das variantes *Taq*IA (rs1800497) e -141C Ins/Del (rs1799732) no gene *ANKK1/DRD2* com parâmetros de adiposidade (índice de massa corporal, circunferência da cintura e dobras cutâneas) em humanos.
- Investigar a associação das variantes *Taq*IA (rs1800497) e -141C Ins/Del (rs1799732) no gene *ANKK1/DRD2* com ingestão alimentar (consumo total de calorias, consumo de lipídeos, de açúcares e de alimentos altamente energéticos) em humanos.
- Analisar, em modelo animal, o efeito de diferentes dietas nos comportamentos do tipo ansioso, atividade locomotora, medo inato e memória de curto e longo prazo das genitoras.
- Analisar, em modelo animal, o efeito de diferentes dietas na expressão de genes do sistema dopaminérgico na ATV e HPT das genitoras.
- Analisar, em modelo animal, o efeito de diferentes dietas nos comportamentos do tipo ansioso, atividade locomotora, medo inato e memória de curto e longo prazo da prole.
- Analisar, em modelo animal, o efeito de diferentes dietas na expressão de genes do sistema dopaminérgico na ATV e HPT da prole.

3. DESCRIÇÃO DOS ARTIGOS CIENTÍFICOS

O sistema dopaminérgico está diretamente associado à ingestão alimentar e à recompensa. O estudo dessa relação alimentos-recompensa, em humanos e animais, é extremamente relevante para a melhor compreensão de causas genéticas e ambientais relacionadas com alterações no comportamento alimentar, que podem levar a desfechos como o sobrepeso e a obesidade. Nosso grupo de pesquisa participou do estudo de duas coortes de crianças, que eram acompanhadas desde o nascimento até a adolescência. Variantes em diversos genes já foram relacionadas a parâmetros antropométricos e de adiposidade nesses estudos. Um deles, que faz parte da presente tese, estudou variantes no gene do receptor de DA D2 (gene *DRD2*). Os resultados desta análise compõem o artigo intitulado "*Evaluation of association of DRD2 TaqIA and -141C InsDel polymorphisms with food intake and anthropometric data in children at the first stages of development*" publicado na revista *Genetics and Molecular Biology*.

Apesar das muitas associações com variantes genéticas em humanos, na mais recente coorte analisada pelo grupo, não se pode observar o efeito dos polimorfismos (alelos considerados protetores e alelos de risco), ou seja, apenas alterações na sequência do DNA não estavam sendo suficientes para detectar associações com alterações no padrão de ingestão alimentar. Isso provavelmente ocorreu, porque seus efeitos, por serem pequenos, estavam sendo encobertos pelos efeitos ambientais, visto que atualmente, o acesso a alimentos ricos em gordura e açúcar está facilitado, favorecendo o desenvolvimento da obesidade. Observou-se, portanto, a necessidade de estudar, de uma maneira mais aprofundada, os efeitos ambientais e como eles influenciam na expressão gênica. Recentemente, estudos epigenéticos mostram que diversos fatores, como dieta, medicamentos e alterações no ambiente intrauterino, podem influenciar a expressão gênica sem alterar a sequência do DNA.

Com esse objetivo, nosso grupo de pesquisa passou a estudar, em modelo animal, o efeito da dieta materna, da dieta da prole e da interação de ambas, na expressão de genes do sistema dopaminérgico. Com esse modelo, poderíamos ter maior controle da composição e da duração de exposição à dieta, além de estudar a alteração da expressão

gênica diretamente nas áreas cerebrais que estão relacionadas ao sistema dopaminérgico. Para tal, submetemos fêmeas de camundongo a diferentes dietas (dieta controle, dieta restritiva e dieta hipercalórica), durante a fase reprodutiva, gestação e lactação. A prole também foi dividida entre esses grupos dietéticos, de modo que cada genitora tivesse pelo menos um filhote em cada dieta. Analisamos, portanto, o efeito dessas dietas na expressão de genes relacionados ao sistema dopaminérgico, na ATV que é uma área rica em neurônios dopaminérgicos e fortemente associada à recompensa alimentar. Os resultados desta etapa são apresentados no artigo intitulado "*Restriction and hypercaloric diets modulate the expression of genes related to the dopaminergic system in ventral tegmental area of mice dams and offspring*", submetido à revista *Neuroscience.*

Também, analisamos o efeito dessas dietas no HPT das genitoras e dos filhotes, visto que essa área está relacionada à fome e à alimentação. Estudos recentes indicam a existência de neurônios dopaminérgicos nessa região cerebral, os quais enviam projeções para regiões intra-hipotalâmicas envolvidas na homeostase energética, resultando em ativação de vias orexigênicas e anorexigênicas. Além disso, há projeções para regiões extra-hipotalâmicas, como ATV e NAc, que são áreas ricas em sinapses dopaminérgicas, relacionadas à recompensa alimentar. Para detectarmos o efeito da dieta em outro sistema relacionado à alimentação, não relacionados ao sistema de recompensa, analisamos a expressão de genes do sistema dopaminérgico também no HPT. Os resultados obtidos a partir dessas análises resultaram no artigo intitulado "*Altered expression of dopaminergic system related genes in the hypothalamus of mice dams and male offspring submitted to restriction and hypercaloric diets*", que será submetido para a revista *Molecular and Cellular Neuroscience*.

Para a execução dos trabalhos de expressão, fez-se necessária inicialmente a seleção de genes constitutivos, que tivessem a expressão estável para posterior comparação da expressão com os genes-alvo. Esse trabalho foi realizado durante o doutorado, na coorientação do Trabalho de Conclusão de Curso de Biomedicina, da acadêmica Bruna Nórdio, e anexado à presente tese. O artigo desenvolvido denomina-se "*Study of gene expression in the ventral tegmental area and hypothalamus of mice dams and male offspring: which internal control gene should be used?*", e será submetido para a revista *Einstein.*

Por fim, sabemos que o material coletado é valioso, e desta forma pretendemos complementar os estudos concluídos neste doutoramento. As alterações descritas na expressão de genes na ATV e HPT da prole podem estar relacionadas a fatores epigenéticos, devido a alterações na ingesta de dieta pelas mães no período de reprodução, gestação e lactação. Portanto, fatores epigenéticos de interesse, como metilação da região promotora do gene e miRNAs, serão futuramente analisados por colegas do grupo de pesquisa, com o objetivo de complementar os resultados obtidos na presente tese.

ARTIGO CIENTÍFICO 1

Evaluation of association of DRD2 TaqIA and -141C InsDel polymorphisms with food intake and anthropometric data in children at the first stages of development

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Evaluation of association of *DRD2 Taq*IA and *-141C InsDel* polymorphisms with food intake and anthropometric data in children at the first stages of development

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Abstract

The reward sensation after food intake may be different between individuals and variants in genes related to the dopaminergic system may indicate a different response in people exposed to the same environmental factors. This study investigated the association of *Taq*IA (rs1800497) and *-141C InsDel* (rs1799732) variants in *DRD2/ANKK1* gene with food intake and adiposity parameters in a cohort of children. The sample consisted of 270 children followed until 7 to 8 years old. DNA was extracted from blood and polymorphisms were detected by PCR-RFLP analysis. Food intake and nutritional status were compared among individuals with different SNP genotypes. Children carrying the *A1* allele (*Taq*IA) had higher energy of lipid dense foods (LDF) when compared with *A2/A2* homozygous children at 7 to 8 years old (GLM p=0.004; Mann Whitney p=0.005). No association was detected with *-141C Ins/Del* polymorphism. To our knowledge, this is the first association study of the *DRD2 Taq*IA and *-141C Ins/Del* polymorphism with food intake and anthropometric parameters in children. *DRD2 Taq*IA polymorphism has been associated with a reduction in D_2 dopamine receptor availability. Therefore, the differences observed in LDF intake in our sample may occur as an effort to compensate the hypodopaminergic functioning.

Keywords: Child obesity, DRD2 polymorphisms, food intake.

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Introduction

The prevalence of childhood overweight and obesity had a dramatic increase between 1990 and 2010, rising from 4.2% to 6.7%, and it is estimated that in 2020 the rate will be 9.1%, or approximately 60 million children (de Onis *et al.*, 2010). The obesity prevalence in developed countries is twice higher than in developing countries. However, most of the affected children (35 million) live in developing countries (de Onis *et al.*, 2010). Moreover, the relative increase rate of obesity in recent decades was higher in developing countries (+65%) than in developed countries (+48%) (de Onis *et al.*, 2010; Oggioni *et al.*, 2014). Obese children are more likely to become obese adults, and have higher risk of developing coronary heart diseases and other related diseases, which diminish life expectancy (Must, 1996; Rossner, 1998; Berenson, 2012). Insulin resistance, metabolic syndrome, and type 2 diabetes are also consequences of childhood obesity (Gupta *et al.*, 2012).

Some factors contribute to overweight and obesity, such as low physical activity, high intake of high fat and sugar foods, change from the rural lifestyle to the urban, sociocultural factors, age, gender, and genetic factors (Popkin, 2006; Gupta *et al.*, 2012; Oggioni *et al.*, 2014). The sensation of reward after food intake, especially of palatable foods, may be different among individuals and might cause different amounts of food ingestion (Berridge *et al.*, 2010). The dopaminergic system regulates food intake through a reward system, and although its function in eating disorders is poorly understood, it is known that the use of dopamine D_2 receptors agonists decreases food intake in rats (Terry *et al.*, 1995). A study that analyzed images via

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Positron Emission Tomography (PET) scans shows that obese individuals have low concentration of striatal D2 dopamine receptors as a mechanism of downregulation due to high levels of dopamine, indicating that the reduction of these receptors could be associated with an addictive behavior also observed in drug users (Wang *et al.*, 2001). *DRD2/ANKK1* gene polymorphisms alter the density of dopamine receptors, and thus may explain the different food intake levels in individuals exposed to the same environmental factors (Stelzel *et al.*, 2010).

Several studies have associated the *Taq*IA (rs1800497) polymorphism with obesity, body mass index (BMI), and food intake (Barnard *et al.*, 2009; Winkler *et al.*, 2012; Cameron *et al.*, 2013; Carpenter *et al.*, 2013). However, to our knowledge, there is no study linking the *-141C Ins/Del* (rs1799732) polymorphism to obesity, although it was associated with other pathologies such as alcoholism and schizophrenia (Jonsson *et al.*, 1999a; Johann *et al.*, 2005; Lafuente *et al.*, 2008a,b). Therefore, the objective of the present study was to analyze the association of *Taq*IA and *-141C Ins/Del* polymorphisms with adiposity parameters and food intake of children.

Materials and Methods

Subjects

The sample consisted of 270 children followed until 7 to 8 years old on average. The nutritional and anthropometrics data were collected at 12 to 16 months, 3 to 4 and 7 to 8 years. The children included in the present study participated in a randomized controlled trial of dietary counseling on breast feeding and diet during the first year of life. The trial consisted of 500 children, randomized in a control or intervention group, of which mothers received a dietary advice about breastfeeding and complementary feeding during home visits in children's first year of life. This dietary advice was based on the "Ten steps to Healthy Feeding", a Brazilian national health policy for primary care, supported by World Health Organization (2006). More information of the first phase of the study can be found elsewhere (Vitolo *et al.*, 2010), but in Table 1 we described the main characteristics of the sample. A substantial reduction of the sample occurred throughout the study and the main reason for the losses was the inability to locate the participants' homes, usually due to the family moving to another city. Other reasons for losses were refusal to continue and children or maternal death. This intervention was not the primary objective of the present research and the participation in the intervention or control group was used as a confounding factor in statistical analyses.

Ethnicity was defined by the interviewer by skin color (i.e., whites and non-whites). More details of the traits studied are described elsewhere (Galvão, 2012; Louzada *et al.*, 2012; Fontana *et al.*, 2015; Miranda *et al.*, 2015). This study was conducted according to the guidelines of the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the Universidade Federal de Ciências da Saúde de Porto Alegre (n. 286/06), and all participants provided written informed consent before commencing the study.

Table 1 - Main characteristics of the sample.

Characteristics	
Ethnicity (whites) [a]	210 (77.8)
Sex (boys)[a]	149 (55.2)
12 to 16 months	
Child's BMI (Z-score) [b]	0.59 ± 1.07
Average daily energy intake (kcal)[b]	948.51 ± 398.56
3 to 4 years	
Child's BMI (Z-score) [b]	0.27 ± 1.16
Average daily energy intake (kcal) [b]	1520.21 ± 391.36
Sugar dense food intake (SDF; kcal) [b]	105.94 ± 86.15
Lipid dense food intake (LDF; kcal) [b]	177.18 ± 190.81
Average daily energy intake per kilogram (kcal/kg)[b]	91.23 ± 27.27
7 to 8 years	
Child's BMI (Z-score)[b]	0.41 ± 1.38
Average daily energy intake (kcal)[b]	1564.54 ± 381.34
Sugar dense food intake (SDF; kcal)[b]	230.02 ± 194.97
Lipid dense food intake (LDF; kcal)[b]	85.10 ± 80.33
Average daily energy intake per kilogram (kcal/kg)[b]	59.75 ± 18.10

[a]Data are presented as % (n).
[b]Data are presented as mean ± standard deviation.

Nutritional status assessment

At 12 to 26 months, children were weighted using a portable digital scale (Techline, São Paulo, Brazil) and length was measured by an infant stadiometer (Serwital Inc, Porto Alegre, Brazil). At 3 to 4 and 7 to 8 years, children were weighted using a digital scale (Techline), and height was measured using a stadiometer (SECA, Hamburg, Germany). BMI was calculated [weight (kg)/height^2(m^2)], and the values were transformed into Z-scores.

Dietary data assessment

One 24-hour dietary recall was collected for each child at 12 to 16 months, and two 24-hour dietary recalls, on two nonconsecutive days, were collected for each child at the ages of 3 to 4 and 7 to 8 years. The 24-hour dietary recall was carried out by a trained undergraduate nutrition student, and the child's food intake was recorded on the day before the last home visit. A food portion measurement device and the common household measures (e.g. teaspoons, tablespoons, cups) were used to quantify portion sizes.

Dietary information was entered into the Nutrition Support Program software from the Escola Paulista de Medicina, Federal University of São Paulo, based on the United States Department of Agriculture chemical composition tables. The energy intake was calculated using only one dietary recall or the average of two dietary recalls. The items listed in the response were classified as sugar-dense foods (SDF) if the percentage of simple carbohydrates was higher than 50% (e.g., soda, Jell-O, candies, and artificially flavored juice) and as lipid-dense foods (LDF) if there was more than 30% fat (e.g. fried pastries, cookies with fillings, cold cuts and sausages, fried foods, and chocolate).

DNA analyses

Blood samples for DNA extraction were collected in EDTA tubes (6 mL). Genomic DNA was extracted from peripheral blood leukocytes by the Lahiri and Nurnberger procedure (Lahiri and Nurnberger, 1991). *DRD2/ANKK1 Taq*IA (rs1800497) and *-141C InsDel* (rs1799732) polymorphisms were detected by PCR-RFLP analysis using primer sequences and conditions described by Grandy *et al.* (1993) and Ohara *et al.* (1998), respectively. Primers sequences (IDT Coralville, IA, USA) were as follows: *Taq*IA, forward primer 5'- CACCTTCCTGAGTGTCAT CAA -3' and reverse primer 5'-AGACAACTTGGCCAG CCGTG-3'; *-141C InsDel*, forward primer 5'-ACTGGC GAGCAGACGGTGAGG and reverse primer 5'-TGCGCGCGTGAGGCTGCCGGT. PCR products were digested separately with either *Taq*I (*Taq*IA polymorphism) or *Mva*I (*-141C InsDel* polymorphism*)* enzyme (Fermentas, Glen Burnie, MD, USA), according to the manufacturer's instructions. Genotypes were determined after electrophoresis in 2 or 3% agarose gels that had been stained with ethidium bromide. For the *DRD2/ANKK1 Taq*IA polymorphism, the *C* allele contains a *Taq*I restriction site and is designated as the *A2* allele, while the *T* allele is designated as the *A1* allele. For the *-141C InsDel* polymorphism, the *-141C*Ins* allele contains a restriction site for *Mva*I while the *-141C*Del* allele does not.

Statistical analyses

Allele frequencies were estimated by gene counting. A chi-square test for goodness-of-fit was used to determine whether the observed genotype frequency distributions agreed with those expected under Hardy-Weinberg equilibrium. Linkage disequilibrium was estimated using the Haploview software Version 4.2 (Broad Institute, Barrett *et al.*, 2005).

Pearson's chi-squared or Fisher's Exact Test was used to compare genotype or allele frequencies between white and non-white children. Since the first publication of an association study with the *Taq*IA polymorphism (Blum *et al.*, 1990), and because of the rare occurrence of the *A1* allele, genotypes are normally grouped as *A1* allele presence or *A1* allele carriers (*A1/A1* and *A1/A2*, n=102), *versus A2/A2* homozygotes (n=116). Similarly, due to low frequencies of the *Del* allele, genotypes of the *-141C InsDel* polymorphism were grouped by *Del* allele presence or *Del* allele carriers (*Del/Del* and *Ins/Del*, n=61), *versus Ins/Ins* homozygotes (n=157). All data are presented as mean and standard deviation. Statistical analysis of SDF and LDF variables were performed on natural logarithm transformed data to normalize their distribution. This allowed including these variables in multivariate analysis; non-transformed values are shown in Table 2. Means of food intake (average daily energy intake, SDF, LDF and average daily energy intake per kilogram) and adiposity (BMI Z-score) parameters were compared among genotype groups by a multivariate general linear model (GLM). The multivariate GLM was performed including all dependent continuous variables in one model, using the categorical variables (1) the control or intervention variable of the randomized trial, (2) sex, and (3) ethnicity as covariates, and genotypes of *-141C InsDel* (rs1799732) and *Taq*IA (rs1800497) polymorphisms as fixed factors (see Table 2). This first step of the analysis verified whether the group of dependent continuous variables was significantly affected by the group of independent categorical variables. Only LDF intake at 7 to 8 years old was associated with *Taq*IA polymorphism, and the covariates did not influence this dependent variable. Therefore, to test the association of *Taq*IA polymorphism alone with LDF intake at 7 to 8 years, we performed a Mann-Whitney test. A p-value of < 0.05 was considered significant. All tests and transformations were performed using the Statistical Package for Social Sciences, Version 20.0 (SPSS®, Chicago, IL, USA).

Results

This longitudinal survey sample was composed of 270 children, 149 (55.2%) boys and 121 (44.8%) girls, followed up from 12 to 16 months until 7 to 8 years old (Table 1). Minor allele frequencies (MAF) of the *DRD2/ANKK1* gene variants observed in the sample were 0.14 of *Del* allele of the *-141C InsDel* (rs1799732) polymorphism and 0.28 of *A1* allele of the *Taq*IA (rs1800497) polymorphism, which were intermediary to those described in the 1000 Genomes Project database for European (MAF 0.08 (*Del*) and 0.19 (*A1*)) and African (MAF 0.57 (*Del*) and 0.38 (*A1*)) populations. All genotype frequencies in this sample were in agreement with those expected under the Hardy-Weinberg equilibrium. The two gene variants were not in linkage disequilibrium (D'=0.3, r=0).

In Table 2, anthropometric and food intake variables are shown according to the analyzed polymorphisms. As some children could not be found at the third home visit at 7 to 8 years, and some samples could not be analyzed in the laboratory, the total number of children included in the multivariate analysis is different from the initial sample size. Children carrying the *A1* allele (*Taq*IA rs1800497) had higher energy of LDF when compared with *A2/A2* ho-

Table 2 - Food intake and anthropometrics parameters according to polymorphisms in *DRD2/ANKK1* gene (*-141C Ins/Del* and *Taq*IA) in children at 1, 3 to 4, and 7 to 8 years old.

	-141C Ins/Del Genotypes			*Taq*IA Genotypes		
	Ins/Ins (n=157)	*Del* carriers (n=61)	*p*	*A1* carriers(n=102)	*A2A2* (n=116)	*p*
12 to 16 months						
Average daily energy intake (kcal)	930.95 ± 400.09	957.82 ± 390.89	0.601	917.78 ± 395.82	956.66 ± 398.51	0.845
BMI Z-score	0.60 ± 1.13	0.48 ± 1.04	0.609	0.52 ± 1.05	0.62 ± 1.15	0.436
3 to 4 years						
Average daily energy intake (kcal)	1506.24 ± 396.82	1579.95 ± 419.60	0.175	1476.62 ± 337.72	1571.04 ± 450.73	0.453
SDF (kcal) [a]	112.00 ± 97.91	110.77 ± 77.42	0.969	110.0 ± 86.95	113.11 ± 97.40	0.623
LDF (kcal) [a]	191.55 ± 197.10	162.20 ± 189.60	0.288	168.83 ± 179.06	196.09 ± 208.03	0.884
BMI Z-score	0.27 ± 0.96	0.28 ± 1.62	0.839	0.29 ± 1.17	0.25 ± 1.19	0.774
Average daily energy intake per kilogram (kcal/kg)	91.92 ± 27.74	94.84 ± 29.75	0.448	89.43 ± 24.96	95.65 ± 30.71	0.327
7 to 8 years						
Average daily energy intake (kcal)	1560.73 ± 355.60	1581.21 ± 414.36	0.756	1581.13 ± 354.41	1553.56 ± 388.07	0.481
SDF (kcal) [a]	90.52 ± 75.95	68.03 ± 58.51	0.164	76.97 ± 62.02	90.60 ± 79.62	0.847
LDF (kcal) [a]	238.38 ± 203.75	223.48 ± 181.94	0.782	282.85 ± 207.96	191.77 ± 178.34	0.004[b]
BMI Z-score	0.45 ± 1.26	0.25 ± 1.65	0.514	0.50 ± 1.36	0.30 ± 1.40	0.258
Average daily energy intake per kilogram (kcal/kg)	59.99 ± 17.00	60.97 ± 20.91	0.861	59.83 ± 17.45	60.65 ± 18.78	0.739

Data are presented as mean ± standard deviation.
BMI – body mass index. SDF – sugar dense foods. LDF – lipid dense foods. General Linear Model (GLM) multivariates with the following co-variates: group (intervention or control), sex, ethnicity. [a]Statistical analysis from logarithm form, non-transformed values are shown; [b]Mann-Whitney test p=0.005

mozygous children at 7 to 8 years old (multivariate GLM p=0.004; Mann-Whitney test p=0.005). No association was detected among *DRD2 -141C Ins/Del* polymorphism with food intake and anthropometric parameters.

Discussion

The dopaminergic pathway has been associated with midbrain reward circuit activation (Roth *et al.*, 2013), and individual differences in D_2 receptor expression are hypothesized to contribute to differences in motivated behaviors, such as the motivation to eat (Gluskin and Mickey, 2016). Therefore, polymorphisms of the *ANKK1/DRD2* gene are frequently associated with altered perception of food reward and weight gain (Ariza *et al.*, 2012; Muller *et al.*, 2012; Roth *et al.*, 2013). *Taq*IA is the most commonly tested polymorphism, and is characterized by a single nucleotide change [C(*A2*)/T(*A1*)] located downstream of the termination codon of *DRD2* gene at the *ankyrin repeat and kinase domain containing 1* (*ANKK1)* gene (Dubertret *et al.*, 2004; Neville *et al.*, 2004; Li *et al.*, 2015; Ponce *et al.*, 2016). This SNP produces a Glu713-to-Lys (E713K) substitution in the ANKK1 amino acid sequence, at the eleventh ankyrin, which may alter the affinity of the ANKK1 protein and its substrate (Neville *et al.*, 2004). It is not clear by which molecular mechanisms the ANKK1 protein could be associated with the dopaminergic system and how *ANKK1* polymorphic alleles would impact addiction vulnerability. However, *ANKK1* and *DRD2* genes belong to the same gene cluster, the NTAD cluster, an ancient cluster of which genes are apparently co-regulated and may have emerged when the central nervous system became more complex (Mota *et al.*, 2012). Since genes of related function are sometimes found in the same cluster, it is possible that ANKK1 is somehow involved in the dopaminergic reward processes via a signal transduction pathway (or other cellular response) (Neville *et al.*, 2004). A few *in vitro* studies with *ANKK1* gene mRNAs and proteins were able to show a potential connection between this gene and the dopaminergic system (Hoenicka *et al.*, 2007; Garrido *et al.*, 2011).

In our sample, the *A1* allele (*Taq*IA rs1800497) was found associated with higher intake of LDF when compared with children *A2/A2* homozygous at 7 to 8 years. This allele has been associated with a reduction in D_2 receptor availability (Pohjalainen *et al.*, 1998; Ritchie and Noble, 2003; Eisenstein *et al.*, 2016). Stice *et al.* (2008) found that the dorsal striatum is less responsive to food reward in obese relative to lean individuals, probably because obese individuals have reduced D_2 receptor density that compromises dopamine signaling. This hypodopaminergic functioning or reward deficiency syndrome may induce obese patients to overeat in an effort to compensate for this reward deficit; several studies are consistent with this theory (van Strien *et al.*, 2010; Duran-Gonzalez *et al.*, 2011; Winkler *et al.*, 2012; Cameron *et al.*, 2013). van Strien *et al.* (2010) associated the *A1* allele with an increase in emotional eating in Dutch adolescents. The *A1* allele was also most frequent in young obese Mexican-American subjects than in non-obese, as well as subjects with central-obesity versus subjects with no central-obesity (Duran-Gonzalez *et al.*, 2011). Winkler *et al.* (2012) observed in an intervention study that carriers of the *A1* allele had a higher BMI at all time-points (baseline, after weight loss, and after weight maintenance), and showed less overall weight loss. Similarly, Cameron *et al.* (2013) observed that post-menopausal women carriers of the *A1* allele lost significantly less body weight and fat mass than women with the *A2/A2* genotype after undergoing an intervention-induced weight loss and increased carbohydrate intake. Some studies were not able to find any association of the *DRD2 Taq*IA polymorphism with adiposity parameters (Hardman *et al.*, 2014).

In the present study, no association was detected between *DRD2 -141C Ins/Del* polymorphism with food intake and anthropometric parameters, despite previous findings relating *Del* carriers of the *DRD2 -141C Ins/Del* polymorphism with higher D_2 receptor density (Jonsson *et al.*, 1999b). The *DRD2 -141C Ins/Del* polymorphism corresponds to a deletion of one cytosine from a run of two cytosines at position -141 of the *DRD2* gene (Arinami *et al.*, 1997). This polymorphism has been associated with risk of schizophrenia in different populations (Arinami *et al.*, 1997; Ohara *et al.*, 1998; Jonsson *et al.*, 1999a; Himei *et al.*, 2002; Wu *et al.*, 2005; Lafuente *et al.*, 2008a,b; Cordeiro *et al.*, 2009; Saiz *et al.*, 2010; Xiao *et al.*, 2013; Wang *et al.*, 2016; Zhao *et al.*, 2016), as well as with weight gain (Lencz *et al.*, 2010) and other responses due to schizophrenia drug treatment (Lencz *et al.*, 2006; Zhang *et al.*, 2010). Associations have been described with propensity to alcohol dependence in different populations (Ishiguro *et al.*, 1998; Konishi *et al.*, 2004a,b; Johann *et al.*, 2005; Du and Wan, 2009; Prasad *et al.*, 2010; Lee *et al.*, 2013), suicide attempts (Suda *et al.*, 2009), psychiatric disorders (Kishida *et al.*, 2004; Ujike *et al.*, 2009; Lencer *et al.*, 2014), different responses to medication and higher quit rates in smokers (Lerman *et al.*, 2006). To the best of our knowledge, there is no other study that associated the *DRD2 -141C Ins/Del* polymorphism with anthropometric parameters or food intake.

The lack of associations in the two other phases of development (12 to 16 months and 3 to 4 years) may have occurred because children at these ages have restricted access to food, and depend on adults for meals, despite their own preferences. Notwithstanding, at 7 to 8 years, children have many opportunities to eat without parental supervision (Briefel *et al.*, 2009), and the differences observed in LDF intake in our sample may have occurrred as an effort to compensate hypodopaminergic functioning.

Palatability is the induced sensitive response of foods that are usually rich in lipids and/or sugar (Cansell and Luquet, 2016). The sense of taste during food ingestion is the most important aspect in the decision to consume or avoid foods (Besnard, 2016). Contrary to sugar, oral fat perception was considered dependent only on its textural and olfactory cues, but recent identification of lipid-receptors in taste buds of both rodents and humans strongly suggests that lipids might also be perceived by the gustatory pathway (Besnard, 2016). Stimulation of taste buds triggers a signaling cascade leading to subsequent neurotransmitter releases in different brain areas responsible for taste perception (e.g., anterior insula, frontal operculum, orbitofrontal cortex, and the mesolimbic system) (Besnard, 2016). The exchange between these areas results in information of the hedonic experience related to the food's taste (Berridge, 1996). Therefore, not only sugar, but also lipids generate a hedonic experience, producing a positive reinforcement that stimulates dopamine secretion in the brain (Salamone, 1994, Volkow *et al.*, 2002), which is a stimulus associated with "wanting" (Berridge *et al.*, 2010). "Wanting" is an incentive salience or motivation for reward triggered by reward-related cues, such as LDF (Berridge *et al.*, 2010). The attribution of incentive salience makes a cue and its reward more attractive, or more "wanted", without being necessarily more "liked" (Berridge *et al.*, 2010). Consistent with our findings, other studies of our group detected associations of palatable food intake with another polymorphism related to the dopaminergic system in children of the same cohort at 12 to 16 months and 3 to 4 years old (Galvão *et al.*, 2012; Fontana *et al.*, 2015). However, further research is needed to confirm the association of *DRD2 Taq*IA polymorphism with LDF intake and its potential mechanisms.

In summary, our results showed that *Taq*IA polymorphism may have an influence on the children's eating behavior, due to the presence of the *A1* allele associated with lower D_2 receptor density that may lead children to compensate the hypodopaminergic functioning with palatable foods. To our knowledge, this is the first association study of the *DRD2 Taq*IA and *-141C Ins/Del* polymorphism with food intake and anthropometric parameters in children at the first stages of development. Notwithstanding, it is nec-

essary to replicate this findings in other populations and identify the mechanisms by which the dopaminergic system may influence food intake. Nevertheless, the investigation of other polymorphisms in this and other genes of the dopaminergic system and their relation to food intake and anthropometric parameters may be interesting.

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ARTIGO CIENTÍFICO 2

Restriction and hypercaloric diets modulate the expression of genes related to the dopaminergic system in ventral tegmental area of mice dams and offspring

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Restriction and hypercaloric diets modulate the expression of genes related to the dopaminergic system in ventral tegmental area of mice dams and offspring

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Highlights

- Caloric restriction modulate expression of dopaminergic system genes in VTA of mice dams and their offspring.

- Hypercaloric diet modulate expression of dopaminergic system genes in VTA of mice dams and their offspring.

- Maternal and offspring diets alter Th, Ddc, Drd2 and Slc6a3/Dat1 gene expression in VTA of male offspring.

- Diet modifies the Th, Drd1, Drd2, Drd3 and Slc6a3/Dat1 gene expression in the VTA of dam's mice.

- Diet altered locomotor activity and innate fear in mice dams but the offspring was not affected.

Abbreviations

DA: dopamine

VTA: ventral tegmental area

NAc: nucleus accumbens

OF: open field

CONT: control diet group

RD: restriction diet group

HD: hypercaloric diet group

PPD: postpartum day

CONT-CONT: offspring of control dams fed control diet

TH: tyrosine hydroxylase protein

DDC: DOPA decarboxylase

DAT: dopamine transporter

Abstract

Eating is necessary to maintain physiological processes, but it is also rewarding. The dopaminergic system is related to addictive-like behaviors and food reward. Therefore, we aim to evaluate the effects of restrictive and hypercaloric diet during pregnancy and lactation on the expression of dopaminergic system genes in the ventral tegmental area (VTA) of mice dams and offspring. We also measured diets' effect in locomotor activity and innate fear in the open field (OF) test. Female mice were divided into control (CONT), restriction (RD) and hypercaloric (HD) dietary groups, and mated with isogenic male mice. On the 9th post-partum day, dams were tested in the OF, and on the 22nd PPD VTA was collected. After weaning, the offspring received the same diets provided to the dams. In the 80th PPD, the offspring was tested in the OF, and at 100th PPD, VTA was collected. Gene expression was analyzed by reverse transcription real-time polymerase chain reaction. RD and HD dams had increased lateral locomotion in the OF. HD had reduced innate fear when compared to RD and CONT dams. No alteration was observed in offspring. RD and HD dams had higher mRNA levels of *Th, Drd1, Drd2* and *Drd3*, and HD dams had increased *Slc6a3/Dat1* mRNA. Dam and offspring diets also altered the expression of *Th*, *Ddc*, *Drd1, Drd2, Drd3* and *Slc6a3/Dat1*. Our results show that restriction and hypercaloric diets alter locomotor activity and the expression of dopaminergic system genes in mice dams and in the offspring.

Keywords: Dopamine, reward, pregnancy, lactation, mesocorticolimbic system

INTRODUCTION

Animals eat mainly in order to suffice two main necessities: to acquire essential substances and to obtain energy to preserve physiological process. However, foods may evoke a pleasurable hedonic response, and therefore they become "wanted" (Berridge KC, 1996). A crucial system related to "wanting" is the dopaminergic system (see review in (Higgs S, 2016)). This is relevant because many overweight and obese individuals are unable to control their food intake (Volkow ND et al., 2017), suggesting the existence of an addiction-like process in some cases (Gearhardt AN et al., 2009;Potenza MN, 2014;Volkow ND et al., 2013). The uncontrolled eating lead to an excessive adipose tissue accumulation, that has long been associated with increased morbidity and mortality (Jarolimova J et al., 2013).

Addictive-like behaviors have been associated with the dopaminergic system through reward and reinforcement. In fact, dopamine (DA) is crucial in food reinforcement hence animals with permanent disruption of DA signaling do not survive due to the loss of motivation to eat (Szczypka MS et al., 2001). The dopaminergic pathway that is related to food reinforcement is the mesolimbic pathway, consisting of DA neurons located in the ventral tegmental area (VTA) that project to the nucleus accumbens (NAc) with inputs of various components of the limbic system, including amygdala, hippocampus and hypothalamus, striatum, orbitofrontal cortex and prefrontal cortex (Alonso-Alonso M et al., 2015).

On the other hand, caloric restriction (30 to 50%) seems to be a protective factor, as it has been associated with increased life expectancy, reduced incidence or delay in the appearance of some chronic and neurodegenerative diseases, such as stroke damage, neurotoxic damage and deposition of amyloid-β (see review in (Ingram DK et al., 2007)). Food restriction has also been associated with decreased basal DA activity (see review in (Carr KD, 2011)) as observed by a previous study that submitted rats to a severe food restriction regiment and recorded approximately 50% lower extracellular DA concentration in the NAc when compared to controls (Pothos EN et al., 1995). Moreover, mice maintained in caloric restriction for 14 months had increased locomotor activity in the open field (OF) (Halagappa VK et al., 2007).

Besides the effect of diet on their metabolism, pregnant mice's diet has been described to have a crucial influence in the intrauterine environment. The Developmental Origins of Health and Disease theory indicates that any disturbance in the intrauterine environment, caused by alteration in macronutrient consumption for example, may result in alterations in the fetal development and increased risk of certain metabolic diseases as the offspring age (Kereliuk SM et al., 2017). Thus, fetal programming causes epigenetic modifications, which may be maintained throughout adulthood and result in different eating habits and consequently their metabolic outcomes (Wang J et al., 2012). These permanent changes are induced by epigenetic process like DNA methylation, histone modifications and post-transcriptional regulation (Lillycrop KA, 2011). The brain is the most exposed organ during fetal development due to its extensive growth and the development of new neuronal pathways (Langley-Evans SC, 2015). Therefore, the mesolimbic pathway, especially VTA, may be directly affected by alterations in the intrauterine environment.

In rodents, some studies have already demonstrated the effects of hyperlipidic, cafeteria and restriction diets on metabolic and behavioral parameters of dams and their offspring. However, there is lack of information that correlates dopaminergic system's response to diets, especially during pregnancy and lactation periods, which consists in great metabolic and physiological changes for the dam and the offspring. The aim of the present study was to determine the effects of caloric restriction and hypercaloric diet, during pregnancy and lactation, on the expression of dopaminergic system genes (*Th, Ddc, Drd1, Drd2, Drd3, Drd4, Slc6a3* also known as *Dat1, Comt, Maoa* and *Maob)* in the VTA (area involved in reward mechanisms) of mice dams and their adult male offspring, in addition to locomotion activity and innate fear. Gene expression was altered in VTA of both hypercaloric and restriction dam's groups, and maternal and offspring diet also influenced gene expression in VTA of the offspring. This indicates that diet influence the expression of genes related to the dopaminergic system in dams and their offspring.

EXPERIMENTAL PROCEDURES

Animals

All procedures were performed in conformity with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1996), and the protocols approved by the Institutional Animal Care and Use Committee of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA, protocol number 388/15). Seventy days-old female BALB/c albino mice (N=38), obtained from the animal house of UFCSPA, in Porto Alegre, Brazil, were housed in individual polypropylene cages (30x20x13cm of dimension) whose floor were covered in wood shavings. The access to water was *ad libitum* and food access depended on the dietary group they were assigned. Animals were housed under controlled temperature (23±1°C), humidity (55 ± 5 %) and light (12:12 light-dark cycle with light off at 6:00 pm) conditions. All efforts were made to minimize animal suffering and to use only the necessary number of animals to produce reliable scientific data.

Experimental Design

Experiment I – Dams Evaluation

Isogenic females were randomly placed on three diet groups: control diet group (CONT; n=11) that received standard chow diet (Nuvital, Curitiba, Brazil) with 3.4 Kcal/g of which 63% of calories from carbohydrate, 26% of protein, 11% of fat; restriction diet group (RD; n=14) with a 30% caloric restriction, adjusted according to the consumption of the CONT group; and a hypercaloric diet group (HD; n=13) that received a custom made chow (Pragsoluções Biociências, Jaú, Brazil) with 5.0 kcal/g of which 42.4% carbohydrate, 9.9% protein, 47.7% fat.

After 25 days of adaptation to the diet, isogenic adult male mice were placed with 2-3 females for a maximum of 1 week, in which all mice received a standard diet *ad libitum*. The vaginal smears were taken to detect the presence of sperm, which was the confirmation of the pregnancy. Females were then singly housed and received their respective diet throughout pregnancy and lactation. Thus, females were

fed with control (CONT group), restriction (RD group) and hypercaloric (HD group) diets before mating, during pregnancy and lactation, totalizing 70 days approximately (see Figure 1a). On the first postpartum day (PPD), the number of pups per dams was standardized at 6.

On the 9th PPD, dams were tested in the OF during the light cycle, between 10:00 am to 12:00 pm. The OF consists in a wooden box of 50x50 cm, surrounded by 30 cm high walls. The floor is divided into 25 painted squares, of which 16 were in the lateral area and 9 in the central area. To initiate the test an animal is placed in one of the corners of the apparatus and their behavior recorded in video for a total of five minutes. The behaviors observed were: lateral and central locomotion (the number of line crossings), time spent moving (in seconds) and time spent in the lateral or central zone of the apparatus (in seconds) (Ruthschilling CA et al., 2012). At the end of the test, mice were removed from the OF and the floor was wiped with 70% alcohol and completely dried before the next animal. The innate fear behavior was verified by evaluating the locomotion into the central zone of the open field (Benetti F et al., 2007;Ruthschilling CA,Albiero G,Lazzari VM,Becker RO,de Moura AC,Lucion AB,Almeida S,Veiga AB and Giovenardi M, 2012). All animals of this study were analyzed for this behavior test. The evaluation was realized using the program The Observer (Noldus®, Holland).

Tissue collection was performed on the 22nd PPD. Dams were anesthetized with xylazine (10 mg/kg) and ketamine (90 mg/kg) and euthanized by decapitation. VTA was macroscopically dissected (adapted from Salvatore MF et al. (2012)), using a stereo microscope on ice, and sterile materials and stored in RNA later™ stabilization solution (Invitrogen, São Paulo, Brazil) for subsequent molecular analysis. The samples were always collected in the morning, during the light cycle and in a noiseless room.

Experiment II – Offspring Evaluation

After weaning, on the 22nd PPD, male offspring were randomly sorted into the three dietary groups, independently of their dam's diet. Therefore, offspring of a CONT dam were placed into either CONT, RD, or HD groups, in a way that all the offspring of a given dam group were equally divided into the three different types of diet (see Figure 2). Offspring diets were the same diets provided to the mice dams. Behavioral analysis was performed with the OF test, in the 80th PPD, and animals were euthanized and VTA was collected at 100th PPD, as previously described for the mice dams (see Figure 1b).

Molecular Analysis

Reference Gene Selection

The most commonly used reference genes in rodents were selected in the literature. Peptidylprolyl isomerase A (*Ppia*, also known as cyclophilin A, *CypA*) (Gong H et al., 2016;Gong ZK et al., 2014;Moura AC et al., 2014), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (Gong ZK,Wang SJ,Huang YQ,Zhao RQ,Zhu QF and Lin WZ, 2014;Kraemer N et al., 2012;Xu L et al., 2011) and β-actin (*Actb*) (Gong ZK,Wang SJ,Huang YQ,Zhao RQ,Zhu QF and Lin WZ, 2014;Moura AC,Lazzari

VM,Agnes G,Almeida S,Giovenardi M and Veiga AB, 2014) were described as stable in different rodent tissues, and thus selected for stability analysis in this study.

Primer Design

Target and housekeeping genes primers were designed using Primer3 software, based on mice sequences in GenBank database. The specificity of the primers was checked using the Basic Local Alignment Search Tool (BLAST) search against nucleotide collection (nr) of the National Center for Biotechnology Information (NCBI) database. All primers were from Invitrogen™ (São Paulo, Brazil). The sequences of primers used are listed on Table 1.

RNA isolation and cDNA synthesis

Total RNA was extracted from samples using PureLink™ RNA Mini Kit (Invitrogen, São Paulo, Brazil), according to manufacturer's instructions. Briefly, each brain structure was homogenized with 0.3 mL of Lysis Buffer containing 1% of 2-mercaptoethanol. Then, 70% ethanol was added to the tissue-buffer mixture, vortexed to disperse any precipitate, and transferred to a spin cartridge for centrifugation (12000xg, 15 seconds, at room temperature), in order to bind RNA to the membrane. The flow-through was discarded and the membrane washed three times. After washing, the membrane was dried with the attached RNA by centrifugation (12000xg, 1 minute, room temperature). RNA was eluted with 30 µL of RNase-free water, incubated for 1 minute at room temperature and centrifuged (12000xg, 2 minutes, room temperature).

RNA concentration and A260/A280 ratios were determined using a BioSpec-nano (Shimazu, Kyoto, Japan). cDNA synthesis was performed using M-MLV Reverse Transcriptase kit (Invitrogen, São Paulo, Brazil), with total RNA (0.1-4 µg, until a maximum volume of 10µL) as template. RNA was incubated at 65°C for 5 minutes with 1µL of oligo$(dT)_{12\text{-}18}$ (0.5µg/µL) and 1µL 10mM dNTPs, to complete 12µL. After incubation, the mixture was chilled on ice, briefly centrifuged and incubated at 37°C for 2 minutes with 4µL 5X first-strand buffer, 2µL 0.1 M DTT and 40 units RNaseOUT™ (Invitrogen, São Paulo, Brazil). Finally, 200 units of M-MLV reverse transcriptase were added to a final volume of 20µL and incubated at 37°C for 50 minutes. The reaction was inactivated by heating at 70°C for 15 minutes.

Quantitative PCR assays

Real time PCR was performed with SYBR™ Select Master Mix (Applied Biosystems™, São Paulo, Brazil) in a StepOnePlus™ thermocycler (Applied Biosystems™, Foster City, CA, USA). The reaction was assembled as follows: 7.5 µL of SYBR™ Select Master Mix, 0.33 µM of forward and reverse primers, 5 ng of cDNA and nuclease-free water for a total volume of 15 µL. Amplification was followed by a melting curve analysis to confirm the PCR product specificity.

Determination of Reference Gene Expression Stability

Five samples of each dietary group of the dams and of the male offspring were randomly selected for reference gene expression stability analysis. After RT-qPCR, logarithmic Ct values were transformed into linear values using the formula 2^{-Ct} that more accurately describes the individual variation among replicate reactions (Livak KJ and Schmittgen TD, 2001;Schmittgen TD et al., 2000). Expression stability was analyzed using the publicly available software tool, NormFinder (a Visual Basic application for Microsoft Excel), that ranks the reference genes according to their expression stability using a model-based approach. The program estimates both the intra- and the inter-group expression variation and calculates candidate gene stability values (Andersen CL et al., 2004;Langnaese K et al., 2008). The top ranked gene was *Ppia* for mice dams and *Actb* for male offspring. The selected genes had the smallest NormFinder stability value, and were selected as the most stably expressed in the VTA of the samples.

Target Genes Expression

The Ct value of each reaction was used to calculate the level of mRNA expression of that specific gene, after normalization to the expression of the reference gene that was analyzed in parallel in the same reaction plate. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method using samples from the control group as calibrator samples (Livak KJ and Schmittgen TD, 2001). All samples were analyzed in duplicate, and the mean value of each duplicate was used for all further calculations.

Statistical Analysis

Data were expressed by mean ± S.E.M. Kolmogorov-Smirnov test was used to test normality, and homogeneity of variances was assessed with Brown-Forsythe test. One-way ANOVA followed by Tukey multiple comparisons test analyzed mice dam's variables with normal distribution. Dam's variables that did not have normal distribution were analyzed by Kruskal-Wallis followed by Dunn's multiple comparisons test. Two-way ANOVA was used to analyze offspring's variables. All statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, USA). Differences were considered statistically significant when $p<0.05$.

RESULTS

Experiment I – Dams Evaluation

In the OF (see Table 2), when we analyze locomotor activity in the lateral area of the apparatus, both RD and HD dams spent more time moving ($p<0.001$) and had a higher frequency in number of entries ($p<0.001$) than CONT dams. On the other hand, when we analyze only the central area of the apparatus, HD dams spent more time moving ($p=0.006$) than both CONT and RD groups. In addition, the HD group had a higher frequency in number of entries in the central area, when compared with CONT group ($p=0.008$). Moreover, in the duration of total locomotion, RD and HD dams showed a significant increase when compared to CONT group ($p<0.001$).

Figure 3 shows the gene expression analysis in VTA. Both, RD and HD groups showed a significant increase in *Th*, *Drd1*, *Drd2* and *Drd3* mRNA levels when compared to CONT group. However, only HD dams had a significant increase in *Slc6a3/Dat1* (p=0.005) expression when compared to CONT group. On the other hand, for *Ddc*, *Drd4, Comt, Maoa,* and *Maob* genes, our results indicate that there was no significant difference in mRNA expression among dam groups in the studied area.

Experiment II – Offspring Evaluation

Behavioral analysis indicates that neither the offspring's neither the dam's diet influenced in locomotion and innate fear of the male offspring in the OF (data not shown). We detected a statistically significant interaction between the effects of the dam's and the offspring's diet on the expression of *Th, Drd2,* and *Slc6a3/Dat1* genes (see Figure 3). This diets interaction was not associated with expression of *Ddc*, *Drd1* and *Drd3* genes, but the main effects analysis showed that dam's diet was associated with altered expression of *Ddc* and *Drd3* genes. The offspring diet was also significantly related to lower *Ddc* gene expression, when compared to CONT-CONT group.

DISCUSSION

This study tested mice dams in the OF and found that when exposed to this behavioral test RD and HD dams explored more the lateral area and spent more time moving than CONT dams (see Table 2). DA plays an important role in modulation of locomotor activity (Ryczko D and Dubuc R, 2017;Sharples SA et al., 2014;Speciale SG et al., 1986) and its affective motivational component (Kalivas PW et al., 1983;Sharples SA,Koblinger K,Humphreys JM and Whelan PJ, 2014). Locomotor activity is increased by drugs that enhance transmission at DA synapses and reduced by DA receptor blocking drugs (see review in (Beninger RJ, 1983)). Our study demonstrated that RD and HD dams in addition to the increased locomotion in OF also showed increased *Th, Drd1, Drd2* and *Drd3* mRNA levels and HD dams also showed increased *Slc6a3/Dat1* gene expression in VTA (see Figure 3). The behavioral outcome observed might be explained by dopaminergic modulation inducing hyperactivity in both groups when exposed to the OF test. Other study (Carr KD et al., 2003) corroborate our findings, as it demonstrated that rats submitted to food restriction had increased locomotor activity (compared to *ad libitum* fed animals) in response to intracerebroventricular injections of the D2 receptor agonist quinpirole. These results were later confirmed by another study (Collins GT et al., 2008) that analyzed that food restriction enhances the effect of pramipexole (a DA agonist that enhances locomotor activity), suggestive of an enhanced D2 receptor activity. Therefore, we may infer that the composition and the duration of the diet can interfere in the outcomes of the behavior, in this context, partly mediated by DA. It is worth mentioning that restriction and hypercaloric diets may also have modulated distinct neurotransmitters' systems in other brain regions, besides the DA system analyzed in the VTA in this study, which may have influenced the observed behavioral outcomes.

In addition to the locomotion, we also observed innate fear behavior in the OF (verified by evaluation of the locomotion into the central area of the apparatus), and we demonstrated that HD dams

spent more time in the central area when compared to CONT and RD groups, indicating decreased innate fear in this group (see Table 2). The medial prefrontal cortex, amygdala and NAc are areas related to innate fear (Lauzon NM et al., 2009;Vergara MD et al., 2017), which receive dopaminergic afferents from the VTA through the mesocorticolimbic system, and when stimulated by DA result in lower innate fear response (Brandao ML and Coimbra NC, 2019). In our study, we observed a reduction of innate fear in the HD dams group concomitantly with altered expression of dopaminergic system genes in VTA. However, when analyzing the performance of the offspring in the OF, we observed none statistically significant difference in any of the behaviors analyzed in this test in the studied groups. Besides that, our model consisted of female dams, which underwent substantial hormonal changes during pregnancy, altering physiological and neuroendocrine systems in order to facilitate fetal development and prepare them for parturition and lactation (Stolzenberg DS and Champagne FA, 2016). These hormones also induce short and long-term changes in the maternal brain, contributing to alterations in behaviors like locomotion and innate fear, which are also modulated by the dopaminergic system.

To the best of our knowledge, this is the first study to describe the effects of restriction and hypercaloric diets, during pregnancy and lactation, in the expression of genes related to the dopaminergic system in mice dams and their offspring. Our findings showed that both RD and HD dams had increased *Th, Drd1, Drd2* and *Drd3* expression in VTA when compared to CONT group. HD dams also had increased *Slc6a3/Dat1* expression, compared to CONT group (Figure 3). No change was observed in *Ddc, Comt, Maoa* and *Maob* genes mRNA expression in VTA in the three groups studied (data not shown). In the offspring, the interaction between the dam's and the offspring's diet resulted in reduced expression of *Th, Drd2* and *Slc6a3/Dat1* genes. Also, *Ddc* gene mRNA levels were also reduced due to the dam's and the offspring's diet, although no interaction between them was statistically significant.

Tyrosine hydroxylase (TH) and DOPA decarboxylase (DDC) are enzymes that participate in catecholamine synthesis (see review in (Juárez Olguín H et al., 2016)). Even though *Ddc* gene expression was not altered in any of the mice dams groups, our results showed that RD and HD dams groups had elevated *Th* gene expression, suggesting a reward system response to high and low calorie intake (see Figure 3). No previous studies analyzed gene expression of *Th* and *Ddc* in VTA of mice dams submitted to a hypercaloric or restriction diets. However, a few studies analyzed the effects of these diets in *Th* gene expression in male rodents, demonstrating that food restriction (Lindblom J et al., 2006), high-fat (Li Y et al., 2009;Vucetic Z et al., 2012) or cafeteria (South T et al., 2012) diets can alter mRNA levels of this gene in VTA. In our male offspring sample, *Ddc* gene expression was influenced by the dam's and the offspring diet, both reducing the expression of this gene in VTA when compared to the CONT-CONT group, but we did not detect an interaction effect between them. However, on *Th* gene expression we were able to detected an interaction between the dam's and the offspring's diet, resulting in a reduction of *Th* gene expression in VTA (see Figure 3). In opposition to our findings, a previous study reported elevated *Th* mRNA levels in VTA of the offspring of protein restricted (8.5% protein) dams that received the diet throughout breeding, pregnancy and lactation (Vucetic Z et al., 2010). The alteration of expression in mice dams and their offspring were inversely related, as *Th* gene expression was elevated in RD and HD dams, and reduced in their offspring, independent of the diet they received after weaning. Possibly the elevated levels of this mRNA in mice during the fetal development of the offspring might

have caused alterations in the regulation of gene expression, observed even in adulthood and despite changes in diet.

After release, DA binds to DA receptors that may activate (D1-type) or inactivate (D2-type) adenylyl cyclase (see review in (Surmeier DJ et al., 2010)). Among those that activate adenylyl cyclase, we studied the expression of *Drd1* gene. The local perfusion of D1 receptor agonist in VTA has been described to increase release of glutamate and GABA, neurotransmitters that can control the activity of DA neurons (Adell A and Artigas F, 2004). In our study, we observed higher *Drd1* mRNA levels in VTA of RD and HD dam's groups. However, we were not able to detect any alteration in the expression of this gene in any of the offspring groups. Other studies analyzed the expression of this receptor in male rodents, and described altered *Drd1* gene expression in different brain areas when they were submitted to high-fat (Sanchez-Hernandez D et al., 2015), high-sugar (Choi CS et al., 2015) or low protein (de Melo Martimiano PH et al., 2015) diet during pregnancy. Despite the fact that the studies above analyzed the effect of maternal diet in the offspring, they did not observe the effect in the expression of *Drd1* in the dams, and also concentrated in other brain areas that were not VTA. All this studies demonstrate an effect between the consumed diet and the modulation of *Drd1* mRNA expression, indicating that diet may influence the expression of this gene.

Among D2-type receptors, that inactivate adenylyl cyclase, we measure the mRNA levels of *Drd2*, *Drd3* and *Drd4* genes. Although D2, D3 and D4 receptors have already been described as autoreceptors, the D2 receptor is the one that plays a major role in the inhibition of automatic feedback, providing an important negative feedback mechanism that adjusts the rate of neuronal firing, synthesis and DA release in response to changes in extracellular levels (see reviews in (Beaulieu JM and Gainetdinov RR, 2011;Ford CP, 2014)). Considering that VTA is an area richly dense in bodies of dopaminergic neurons (Juárez Olguín H,Calderón Guzmán D,Hernández García E and Barragán Mejía G, 2016), and that the main localization of autoreceptors is the soma and dendrites of DA neurons, we can presume that our sample is composed mainly of D2, D3 and D4 autoreceptors. These autoreceptors regulate DA transmission by inhibition of TH, which leads to a reduction in DA synthesis, and also suppress DA release and increase reuptake of exceeding DA in the synaptic cleft (see review in (Ford CP, 2014)). Our study is the first to evaluate the effects of restriction and hypercaloric diets during pregnancy and lactation in *Drd2*, *Drd3* and *Drd4* gene expression in VTA of mice dams. Our data indicate that RD and HD dams have an elevation of *Drd2* and *Drd3* genes expression, but no alteration in *Drd4* gene expression in VTA.

We also detect an interaction between the dam's and the offspring's diets in the expression of *Drd2* gene. All male offspring had a reduction in the levels of *Drd2* mRNA in VTA when compared to CONT-CONT group. A study by Hehar H et al. (2016) previously described decreased levels of *Drd2* mRNA, but in the prefrontal cortex of Sprague-Dawley rat offspring of dams submitted to a 40% restriction diet during pregnancy. Different studies described altered *Drd2* gene or D2 receptor protein expression in male rodents fed high-fat (Huang XF et al., 2005;Sharma S and Fulton S, 2013), cafeteria (Johnson PM and Kenny PJ, 2010) and restrictive (Jones KT et al., 2017) diets in other brain areas. To the

best of our knowledge, our study is the first to describe elevated *Drd3* mRNA levels in the VTA of mice dams submitted to restriction and hypercaloric diets during gestation and lactation. We also detected the influence of the dam's diet in the offspring *Drd3* gene expression, as the offspring of HD dams had higher levels of mRNA than the offspring of RD dams. Of the D2-like receptors, *Drd4* was the only gene that had no alteration in expression in the VTA of mice dams and their offspring, when submitted to restriction or hypercaloric diet.

We also analyzed, in mice dams and their offspring, the expression of *Slc6a3*/*Dat1* gene, which encodes the dopamine transporter (DAT). It has already been demonstrated by other studies that diet regulates DAT (Pothos EN,Creese I and Hoebel BG, 1995;Zheng D et al., 2012). Our results demonstrate that HD dams had increased *Slc6a3/Dat1* mRNA levels, and the offspring gene expression was influenced by an interaction of both the dam's and the offspring's diet, which resulted in reduced levels when compared to CONT-CONT. Once again, there is an inversely related gene expression in HD dams and their offspring, which can be a possible result of the intrauterine environment alterations during fetal development. Previous studies (Jones KT,Woods C,Zhen J,Antonio T,Carr KD and Reith ME, 2017;Narayanaswami V et al., 2013;South T and Huang XF, 2008) with adult male rodents submitted to different diets (food restriction and obesogenic diets) in other regions of the brain have been shown to decrease DAT activity, expression and density. Although these studies analyzed male rodents, these findings indicate the effect of diet in the expression of dopaminergic system related genes.

Although previous studies described alteration of *Maoa, Maob* and *Comt* gene expression in different brain areas in male rodents submitted to low-fat and high-fat diets (Kaczmarczyk MM et al., 2013;Reichelt AC et al., 2018), we did not observe any alteration in mice dams and their male offspring submitted to hypercaloric or restriction diets. Therefore, our experimental model of diets had no impact in the DA degradation related enzymes in the studied area.

We demonstrated increased locomotor activity in the OF of HD and RD dams, but only HD dams had decreased innate fear. However, the offspring locomotion parameters were not affected by their diet or the maternal diet. Our study was the first to describe the effects of restriction and hypercaloric diet, during the pregnancy and lactation periods of mice dams, in the expression of genes of the dopaminergic system. We also described augmented *Th, Drd1, Drd2, Drd3* and *Slc6a3/Dat1* gene expression in VTA of mice dams exposed to restriction and hypercaloric diet. Besides that, we showed an interaction between maternal and offspring diet in the expression of *Th, Drd2*, and *Slc6a3/Dat1*. Maternal diet was significantly related to a dowregulation of *Th, Ddc, Drd2* and *Slc6qa3/Dat1* in the VTA of the offspring. Offspring diet was also related to gene expression as the restriction group had lower levels of *Ddc* mRNA. Our results reinforce the effect of restriction and hypercaloric diets in the modulation of dopaminergic system related genes expression in VTA of dams mice and their offspring.

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LIST OF FIGURES AND TABLES

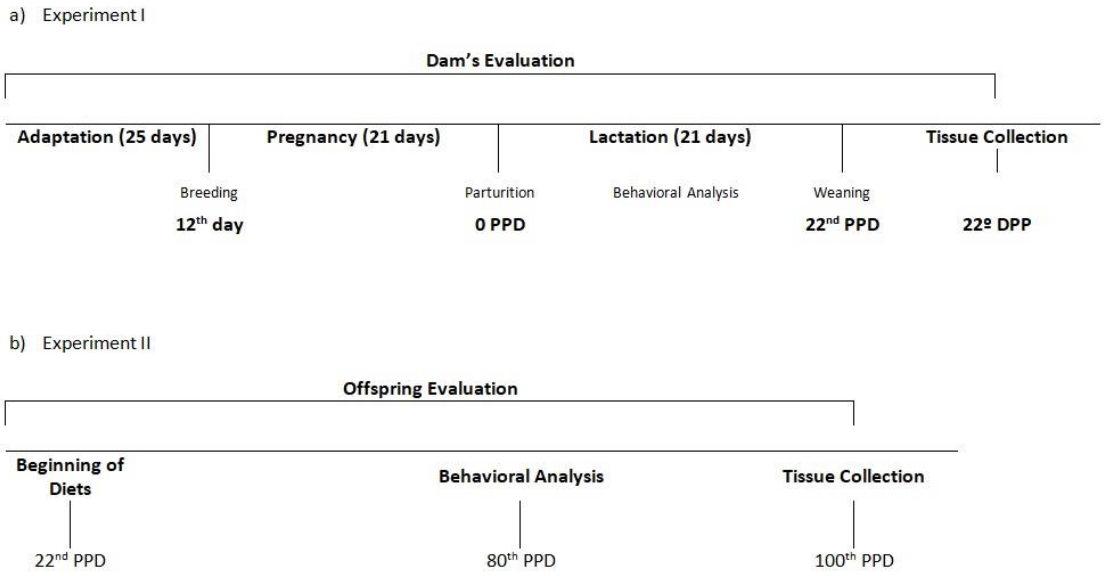

Figure 1. Timeline of experimental procedures of the study. a) Experiment I, representing the timeline of dam's experiment. b) Experiment II, representing the timeline of offspring's experiment. PPD – postpartum day.

EXPERIMENTAL GROUPS

Dams' diet	CONT			RD			HD		
Offspring's diet	CONT	RD	HD	CONT	RD	HD	CONT	RD	HD
Offspring group	CONT-CONT	CONT-RD	CONT-HD	RD-CONT	RD-RD	RD-HD	HD-CONT	HD-RD	HD-HD

Figure 2. Experimental groups used in the study. CONT: control diet; RD: restriction diet; HD: hypercaloric diet.

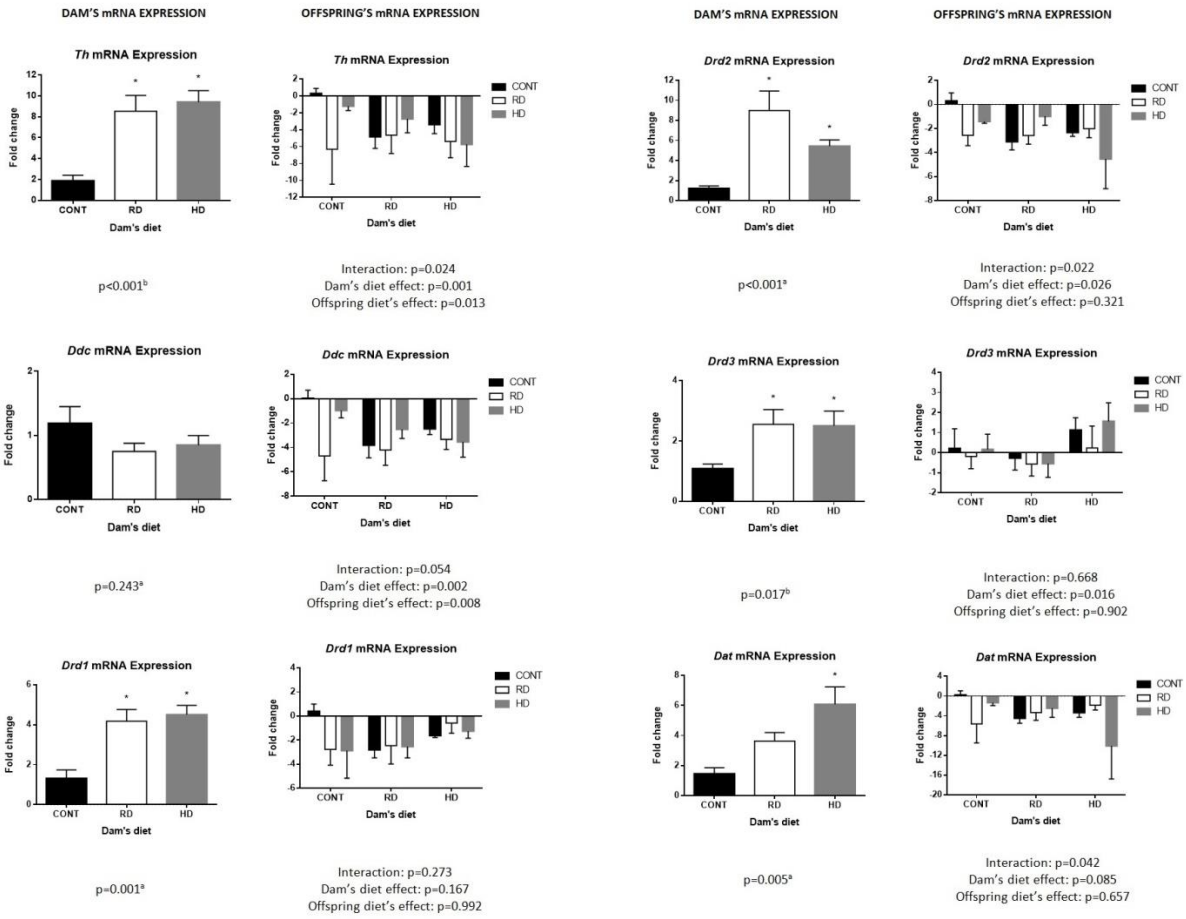

Figure 3. Dam's and offspring's gene expression in ventral tegmental area.

All the offspring gene expression was analyzed by two-way ANOVA followed by Tukey's multiple comparisons test.

[a] Kruskal-Wallis followed by Dunn's multiple comparisons test.

[b] One-way ANOVA followed by Tukey's multiple comparisons test.

* $p<0.05$, when compared to CONT (dams).

CONT: control diet; RD: restriction diet; HD: hypercaloric diet.

Table 1. Reference and target genes selected for this study, their primers and its function.

Gene (ID)[1]	Description	Primer F	Primer R	Function
Ppia/ CypA (268373)	Peptidylprolyl isomerase A	5'GTGTTCTTCGACATCACGGC3'	5'TGTAAAGTCACCACCCTGGC3'	Enzyme that catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides and accelerate the folding of proteins.
Gapdh (14433)	Glyceraldehyde-3-phosphate dehydrogenase	5'CCCTTAAGAGGGATGCTGCC3'	5'TACGGCCAAATCCGTTCACA3'	Glycolytic enzyme that converts D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate.
Actb (11461)	B-actin	5'AGATCAAGATCATTGCTCCTCCT3'	5'ACGCAGCTCAGTAACAGTCC3'	Protein involved in cell motility, structure, and integrity.
Th (21823)	Tyrosine hydroxylase	5'TACAAGCAGGGTGAGCCAAT3'	5'TGGGTAGCATAGAGGCCCTT3'	Conversion of tyrosine to dopamine.
Ddc (13195)	Dopa decarboxylase	GGCGACGATTTCGCTCTTTG	TCATTGGAGCCCTTTAGCCG	Catalyzes the decarboxylation of L-3,4-dihydroxyphenylalanine (DOPA) to dopamine.
Drd1 (13488)	Dopamine receptor D1	5'GTCTCCCAGATCGGGCATT3'	5'AGTCACTTTTCGGGGATGCT3'	Dopamine receptor D1 subtype.
Drd2 (13489)	Dopamine receptor D2	5'GACACCACTCAAGGGCAACT3'	5'TCCATTCTCCGCCTGTTCAC3'	Dopamine receptor D2 subtype.
Drd3 (13490)	Dopamine receptor D3	5'ACGGGACAAATGGAGCACAT3'	5'TGGCAGATGCTGTAGTAGCG3'	Dopamine receptor D3 subtype.
Drd4 (13491)	Dopamine receptor D4	5'TCATCGGCTTGGTGTTGGCA3'	5'CCTGGACCTCGGAGTAGACAAA3'	Dopamine receptor D4 subtype.
Slc6a3/Dat1 (13162)	Solute carrier family 6, member 3	5'CGGGATGCCCCTCTTCTACA3'	5'ACAGTGAAGCCCACACCTTT3'	Dopamine transporter (DAT).
Comt (12846)	Catechol-O-methyltransferase	5'TTCTGGCCCATAAATGCTGTTG3'	5'TGCACGAACTCAAACCAACC3'	Degradation of catecholamine transmitters.
Maoa (17161)	Monoamine oxidase A	5' TTCCAATGGGGGCTGTCATC3'	5' GGGCAAGTATGAAGCCCATGA3'	Enzyme that catalyzes the oxidative deamination of biogenic and xenobiotic amines.
Maob (109731)	Monoamine oxidase B	5'TCAGCCAGTGGGCAAGATTT3'	5'GTCGTGCAGGGACATCCAAA3'	Enzyme that catalyzes the oxidative deamination of biogenic and xenobiotic amines.

[1] Gene Identification Number, available at: http://www.ncbi.nlm.nih.gov/gene.

Table 2. Influence of diet in locomotor activity and innate fear in mice dams in the open field test.

	CONT (n=11)	**RD (n=14)**	**HD (n=13)**	**p**
Duration (s) of lateral locomotion	30.91 ± 3.54	54.78 ± 4.32*	59.31 ± 2.81*	<0.001
Number of entries in lateral area	66.82 ± 5.43	98.43 ± 4.88*	102.54 ± 3.14*	<0.001
Duration (s) of central locomotion	6.36 ± 1.69	9.00 ± 1.85	16.85 ± 2.78*#	0.006
Number of entries in central area	12.00 ± 2.62	21.07 ± 3.42	31.46 ± 5.31*	0.008
Duration (s) of total locomotion	37.27 ± 4.71	63.78 ± 5.02*	76.15 ± 5.41*	<0.001

Data expressed as mean ± S.E.M.
One-Way ANOVA, followed by Tukey's multiple comparisons test.
* when compared to CONT group
# when compared to RD group

ARTIGO CIENTÍFICO 3

Altered expression of dopaminergic system related genes in the hypothalamus of mice dams and male offspring submitted to restriction and hypercaloric diets

A ser submetido ao periódico Molecular and Celullar Neuroscience, fator de impacto 3.312.

Expression of dopaminergic system related genes in the hypothalamus of mice dams and male offspring submitted to restriction and hypercaloric diets

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ABSTRACT

Hunger and satiety neurocircuitry is very complex, and many mechanisms are still unknown. Only recently hunger and energy homeostasis have been related to dopaminergic neurons in the arcuate nucleus in the hypothalamus (HPT). Therefore we aim to investigate the effects of restriction and hypercaloric diets in the expression of DA related genes in the HPT, anxiety-like behavior and memory in mice dams and male offspring. Female mice were divided into control (CONT), restriction (RD) and hypercaloric (HD) dietary groups, and mated with isogenic male mice. After birth, dams were tested in the elevated plus maze (EPM) and with the novel object recognition test (NOR). HPT was collected after pups weaned. The offspring received the same diets provided to the dams, and submitted to the same behavioral tests with 80 days of age. HPT was collected with 100 days of age. Gene expression was analyzed by reverse transcription real-time polymerase chain reaction. RD and HD dams had higher levels of *Th, Drd1, Drd3* and *Maoa* and reduced expression of *Drd4* in the HPT. Hypercaloric diet also elevated *Ddc* and *Comt* mRNA levels. In the offspring, only *Maob* gene showed reduced expression due to the interaction between the maternal and the offspring diet. Restriction and hypercaloric diets also influenced the behavior of mice dams in the EPM, as HD dams had reduced anxiety-like behavior, and of the offspring in the NOR, increasing object recognition.

Keywords: Dopamine, memory, anxiety, pregnancy, lactation

1. INTRODUCTION

The neurocircuitry underlying hunger and satiety is complex, and despite being a much studied system, a lot of mechanisms are still unknown. The arcuate nucleus (ARC) of the hypothalamus (HPT) has an important role in feeding regulation and metabolism (see review in [1]). It is located near the medial eminence, a capillaries-rich region, which enables the transport of peripheral hormones and nutrient signals through the blood-brain barrier (see review in [2]). Therefore, the ARC gathers the peripheral information and central neuronal inputs in order to promote a coordinated feedback response [1].

There are two main neuronal populations in ARC, with opposing effects on food intake: orexigenic (appetite-stimulating) and anorexigenic (appetite-suppressing) neurons [1, 3]. Both types of neurons projects to different hypothalamic nuclei involved in control of appetite, such as dorsomedial nucleus (DMN), paraventricular nucleus (PVN), lateral hypothalamic area (LHA) [3]. Besides internal hypothalamic projections, there are projections to extrahypothalamic brain regions, such as ventral tegmental area (VTA) and nucleus accumbens (NAc), related to the mesolimbic reward system that controls the hedonic aspects of food intake [1]. The hypothalamus has an extensive developmental period, therefore is very susceptible to changes in the intrauterine environment [4]. Maternal undernutrition are related to hyperphagia, reduced saciety and the development of metabolic syndrome which are related to changes in the hypothalamic anorexigenic and orexigenic circuits (see review in [5]), and may be related to epigenetic modifications.

Recent studies have analyzed the role of tyrosine hydroxylase (TH) expressing neurons in the ARC, and their relation to hunger and energy homeostasis [6]. Axons of these neurons project from the ARC to the PVN, the ventromedial (VMH) and dorsomedial (DMH) hypothalamus, which are regions involved in energy homeostasis [6]. The activity of dopamine (DA) depends on the type of receptor in the post-synaptic neuron: D1-like receptors excite orexigenic cells; and D2-like receptors inhibit anorexigenic neurons resulting in an orexigenic action of DA in ARC [6].

DA not only plays a critical role in food intake regulation, as diet modifications (both restriction and high calorie diets) are known to also alter DA related genes expression [7-10]. During pregnancy and lactation, both the dam and the fetus have different metabolic needs, and therefore, different energy necessities [11]. Due to its extensive growth and development of new neuronal pathways, the brain is the

most exposed organ to any disturbances or stimuli during pregnancy, and changes in this period may result in consequences even in adulthood [12]. Thus we aim to investigate the effects of restriction and hypercaloric diets in the expression of DA related genes (*Th, Ddc, Drd1, Drd2, Drd3, Drd4, Slc6a3 also known as Dat1, Comt, Maoa* and *Maob*) in the HPT of mice dams exposed to these diets during pregnancy and lactation. Also, to evaluate the outcome of the dam's and the offspring's diet in the expression of DA related genes in the HPT of the adult male offspring.

2. MATERIALS AND METHODS

2.1 Animals

Adult female BALB/c albino mice (N=39), with 70 days of age, were obtained from the animal house of UFCSPA, in Porto Alegre, Brazil. They were housed in individual polypropylene cages (30x20x13cm of dimension) with wood shavings floor, had free access to water and food consumption varied according the stipulated dietary group. Animals were maintained under controlled temperature (23±1°C), humidity (55 ± 5 %) and light (12:12 light-dark cycle with light off at 6:00 pm) conditions. All efforts were made to minimize animal suffering and to use only the necessary number of animals to produce reliable scientific data. All procedures were performed in conformity with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), and the protocols approved by the Institutional Animal Care and Use Committee of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA, protocol number 388/15).

2.2 Experimental Procedures

2.2.1 Experiment I – Dams Evaluation

Animals were randomly divided into three diet groups: control diet group (CONT; n=12) received standard chow diet (Nuvital, Curitiba, Brazil) with 3.4 Kcal/g of which 63% of calories from carbohydrate, 26% of protein, 11% of fat; restriction diet group (RD; n=14) fed a 30% caloric restriction, adjusted according to CONT group consumption; and a hypercaloric diet group (HD; n=13) provided a custom made chow (Pragsoluções Biociências, Jaú, Brazil) consisting of 5.0 kcal/g of which 42.4% carbohydrate, 9.9%

protein, 47.7% fat. Female mice received their respective diet for 25 days, an adaptation period. After that, they were placed with isogenic adult male mice in a maximum proportion of 3:1 for a period that did not exceeded a week. During this period, all mice received a standard chow *ad libitum*. Confirmation of pregnancy was obtained with the detection of sperm in vaginal smears. When pregnant, mice dams were housed separately and provided with their respective diet throughout pregnancy and lactation. Therefore the animals received either control, restriction or hypercaloric diets before mating, during pregnancy and lactation, totalizing 70 days approximately (see figure 1a). Number of pups per litter was standardized at 6.

Dam's anxiety-like behavior was assessed by elevated plus maze (EPM) test and memory by the novel object recognition (NOR) test during the light cycle, between 10:00 am to 12:00 pm. All animals of this study were submitted to these behavioral tests. Behavioral evaluation was realized using recorded videos and the program The Observer (Noldus®, Holland). On the 8^{th} postpartum day (PPD) mice dams were submitted to the EPM, which consisted of two open arms (25 x 5 cm), and two perpendicular closed arms (25 x 5 cm, with 30 cm high walls), elevated 50 cm above the floor. To initiate the test one mice dam at a time were placed in the central quadrant of the maze with the head facing the open arm of the apparatus. Their behavior was recorded in video for a total of five minutes, in order to asses: the percentage of entries into the open arms [100×open/(open + enclosed)], the percentage of time spent in the open arms [100×open/(open + enclosed)], and the number of head-dipping, as previously described in literature [13]. At the end of the test, the floor was cleaned with 70% alcohol and completely dried before the next animal.

The NOR test was performed on the 10^{th}, 11^{th} and 12^{th} PPD in a plastic box (30.5 cm×19.8 cm×13.4 cm), in which animals were previously habituated for 10 minutes. On the following day, the mice dam was placed in the same arena for the first test, this time containing two identical objects, which they could freely explore for a total of 5 minutes. Three hours later, short-term memory was assessed by the second test, which consisted in introducing the animal to the same object (familiar object) and to a new and unknown object (novel object) in the same arena for 5 minutes. Twenty-four hours after the first test, long-term memory was assessed by repeating the second test. All test were recorded for posterior analysis. Exploration was defined by the amount of time spent actively sniffing or interacting with the object at a maximum distance of 2 cm. Recognition index (RI) of each test (3h and 24h) was calculated by time spent exploring the new object/time exploring both objects multiplied by 100.

Tissue collection was performed on the 22nd PPD, always in the morning, during the light cycle and in a noiseless room. Xylazine (10 mg/kg) and ketamine (90 mg/kg) were used to anesthetize mice dams, which were subsequently euthanized by decapitation. HPT was macroscopically dissected in a stereo microscope, on an ice-cold surface, with sterile materials and stored in RNA later™ stabilization solution (Invitrogen, São Paulo, Brazil) for subsequent molecular analysis.

2.2.2 Experiment II – Offspring Evaluation

On the 22nd PPD, male offspring were weaned and randomly distributed into the three dietary groups (CONT, RD and HD), forming a total of nine new groups according to the dam and the offspring diet. Therefore, offspring of a CONT dam, for example, were divided and submitted to either control (CONT-CONT), restriction (CONT-RD) or hypercaloric (CONT-HD) diet, in a way that all the male offspring of a given dam were equally divided into all dietary groups (see figure 2). Behavioral analysis was performed with the EPM and NOR tests, around the 80th PPD and HPT collected at 100th PPD, as previously described for mice dams (see figure 1b).

2.3 Molecular Analysis

2.3.1 Reference Gene Selection

Reference genes were selected in the literature. The ones that were mostly described as stable in different rodent tissues, and thus selected for stability analysis in our study were: Peptidylprolyl isomerase A (*Ppia*, also known as cyclophilin A, *CypA*) [14-16], glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) [15, 17, 18] and β-actin (*Actb*) [15, 16].

2.3.2 Primer Design

Target and housekeeping genes primers were designed based on mice sequences in GenBank database in Primer3 software. Primers specificity was checked against nucleotide collection (nr) using the Basic Local Alignment Search Tool (BLAST) search of the National Center for Biotechnology Information (NCBI) database. The sequences of primers used are listed on Table 1. All primers were from Invitrogen™ (São Paulo, Brazil).

2.3.3 RNA isolation and cDNA synthesis

Total RNA was extracted from HPT samples using PureLink™ RNA Mini Kit (Invitrogen, São Paulo, Brazil), according to manufacturer's instructions. In summary, 0.3 mL of Lysis Buffer containing 1% 2-mercaptoethanol was used to homogenized brain tissues. Next, 70% ethanol was added and the mixture vortexed to disperse any precipitate. The homogenized was transferred to a spin cartridge and centrifuged (12000xg, 15 seconds, at room temperature), in order to bind RNA to the membrane. The membrane was washed three times, and then dried with the attached RNA by centrifugation (12000xg, 1 minute, room temperature). RNase-free water (30 µL) was used for RNA elution.

BioSpec-nano (Shimazu, Kyoto, Japan) was used to determine sample's total RNA concentration and A260/A280 ratios. Then, total RNA (0.1-4 µg, until a maximum volume of 10µL) samples were used as templates to cDNA synthesis, performed with M-MLV Reverse Transcriptase kit (Invitrogen, São Paulo, Brazil). In the following step, 1µL of oligo$(dT)_{12-18}$ (0.5µg/µL) and 1µL 10mM dNTPs, until a volume of 12µL, were added to total RNA and incubated at 65°C for 5 minutes. After incubation, microtubes were chilled on ice, centrifuged, added 4µL 5X first-strand buffer, 2µL 0.1 M DTT and 40 units RNaseOUT™ (Invitrogen, São Paulo, Brazil) and placed in the thermocycler at 37°C for 2 minutes. Lastly, a final volume of 20 µL was obtained by the addition of 200 units of M-MLV reverse transcriptase and incubated at 37°C for 50 minutes, following by 70°C for 15 minutes for inactivation of the reaction.

2.3.4 Quantitative PCR assays

All quantitative PCR assays were performed with SYBR™ Select Master Mix (Applied Biosystems™, São Paulo, Brazil) in a StepOnePlus™ thermocycler (Applied Biosystems™, Foster City, CA, USA). SYBR™ Select Master Mix (7.5 µL), forward and reverse primers (0.33 µM), cDNA (5 ng) and nuclease-free water for a total volume of 15 µL were the reagents of the real time PCR reaction. After amplification, a melting curve analysis was performed in order to confirm the PCR product specificity.

2.3.5 Determination of Reference Gene Expression Stability

Five samples of each dam and offspring dietary group were randomly selected for reference gene expression stability analysis. NormFinder (a Visual Basic application for Microsoft Excel) was used to analyzed the expression stability. Firstly, logarithmic Ct values provided after RT-qPCR analysis were transformed (with 2^{-Ct} formula) into linear values, that more accurately describes the individual variation

among replicate reactions [19, 20]. Therefore, a model-based approach was used to rank the reference genes according to their expression stability, calculated by an estimation of intra- and inter-group expression variation [21, 22]. In HPT, *Actb* was determined as the top ranked most stable gene for the dams samples and *Ppia* for mice male offspring samples.

2.3.6 Target Genes Expression

Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method using samples from the control group as calibrator samples [19]. After quantitative PCR assays of target genes, Ct values of each reaction were normalized to the expression of the reference gene (run in parallel in the same reaction plate) and used to calculate the level of mRNA expression. All samples were analyzed in duplicate, and the mean value of each duplicate was used for all further calculations.

2.4 Statistical Analysis

Data were expressed by mean ± S.E.M. and tested for normality with Kolmogorov-Smirnov test, and for homogeneity of variances with Brown-Forsythe test. Mice dam's variables with normal distribution were analyzed with One-way ANOVA followed by Tukey multiple comparisons test. If the distribution was not normal, dam's variables were analyzed with Kruskal-Wallis followed by Dunn's multiple comparisons test. Offspring's variables were analyzed with Two-way ANOVA followed by Tukey multiple comparison test. All statistical analysis was performed in GraphPad Prism software (GraphPad Software, San Diego, USA), and differences were considered statistically significant when $p<0.05$.

3. RESULTS

3.1 Experiment I – Dams Evaluation:

In the EPM, HD dams had a higher frequency of head-dips ($p=0.021$) in the open arms of the apparatus, which indicates that these females had reduced anxiety-like behavior when exploring this area of the apparatus (see table 2). Restriction or hypercaloric diet did not alter the recognition of the novel object in mice dams submitted to the NOR test, which indicates that short- and long-term memory were not affected by diet in any of the dams groups (see table 2). The expression of *Th* ($p<0.001$), *Maoa* ($p=0.004$), *Drd1*

(p<0.001), and *Drd3* (p=0.016) genes was increased in RD and HD when compared to CONT. *Drd1* mRNA levels was also higher in HD when compared to RD. *Comt* (p=0.001) and *Ddc* (p=0.018) were upregulated only in HD dams in comparison to CONT group. *Drd4* (p<0.001) was downregulated in RD and HD compared to CONT (figure 3 and supplementary).

3.2 Experiment II – Offspring Evaluation:

The offspring behavior in the EPM had no influence of the dam's nor the offspring's diet. When analyzing the offspring in the NOR test, the short-term memory was influenced only by the offspring diet, as male offspring that received a restriction diet (independently of the dam's diet) had higher 3h-RI when compared to CONT-CONT (offspring diet factor p=0.033), indicating improvement of the short-term memory in the offspring submitted to restriction diet. The long-term memory analysis detected an interaction between the dam's and the offspring's diet, resulting in elevated 24h-RI, indicating a general improvement of the long-term memory in all groups when compared to CONT-CONT (interaction effect p=0.030; see table 3).

In the gene expression analysis, the two-way ANOVA detected only one interaction between the dam and the offspring diet in the offspring gene expression (supplementary). The only gene expression affected by the interaction of both the dam's and the offspring's diet was *Maob* gene (interaction effect p=0.035), in which diet reduced the levels of this gene's mRNA when compared to CONT-CONT (see figure 4). Also the simple main effect of the dam diet in the offspring gene expression was not significant in any of the genes analyzed in the HPT. Therefore, two-way ANOVA data for most genes are presented only in supplementary data, and the other expression data analysis is presented solely with the effect of the offspring diet. The effect of the offspring diet alone had a statistically significant effect on the expression of *Drd3* (p=0.022) and *Comt* (p=0.047) genes (see figure 5). *Drd3* mRNA levels were elevated in male offspring submitted to restriction diet in comparison to the control group. Despite one-way ANOVA indicated there was a significant effect of the offspring diet in the expression of *Comt* gene, the post-hoc analysis did not detect the groups with different expression.

4. DISCUSSION

HPT is an important neural structure that controls long-term energy homeostasis and body weight, due to its extensive synaptic connections with intra and extrahypothalamic areas [23]. Several nuclei, that influence each other by many synapses, are contained in the HPT: VMH, DMH, LHA, PVN and ARC. DA is a neurotransmitter rarely approached in HPT in dietary studies, but the use of dopaminergic agonists and antagonists in this area have already been associated with altered ingestion (see review in [24]). To the best of our knowledge, our study was the first to evaluate the expression of *Th, Ddc, Drd1, Drd2, Drd3, Drd4, Slc6a3 also known as Dat1, Comt, Maoa* and *Maob* genes in the HPT of dams submitted to hypercaloric and restriction diet, and their effect in male offspring submitted to the same diets.

Catecholamine synthesis is mediated by two main enzymes: tyrosine hydroxylase (TH) and DOPA decarboxylase (DDC) (see review in [25]). In our study, we detected elevated levels of *Th* mRNA expression in RD and HD dams compared to CONT (figure 3). Previous studies analyzed the effect of high-fat [7, 26], cafeteria [27] diet in the expression of *Th* in the HPT of male rodents, and described both elevated and reduced expression of this gene. However, in opposition to all this findings, de Leeuw van Weenen, Hu [28] observed no difference in the expression of *Th* mRNA in the HPT of mice maintained in a high fat diet (45 energy% of fat) or a low fat control diet (10 energy% fat) for 4 weeks. None of these studies analyzed the effect of restriction and hypercaloric diets in the expression of *Th* in the HPT of female mice during pregnancy and lactation, which is a period of intense hormonal and metabolic changes.

Ddc gene was upregulated in our study in the HD compared to CONT group (figure 3). No other study to this date has analyzed the effects of diet in the expression of this gene in the HPT of rodent dams. Thus we are the first to describe an influence of diet in the expression of this gene in females during pregnancy and lactation periods. Despite other studies have described altered *Th* expression in the offspring of rodent dams fed restriction [29, 30] and high-fat [31] diets, in our study, neither the dam's nor the offspring's diets had influence in the expression of *Th* and *Ddc* genes in the HPT of the male offspring (supplementary). Therefore, during the adult life, diet had no influence in the expression of dopaminergic related genes in HPT of the male offspring, indicating that possibly the dopaminergic system in this area is minimally affected by diet in adult males.

DA receptors are divided into two main classes that, respectively, activates and inactivates adenylyl cyclase: D1-like (D1 and D5 subtypes) and D2-like (D2, D3 and D4 subtypes) receptors (see review in [24, 32]). The activation of adenylyl cyclase has many actions in the cell which, as a whole, is described as excitatory (see review in [32]). The modulation of HPT DA receptors activity by pharmacological agonists and antagonists indicate that they may be responsible for the regulation of food intake [24]. Increased food intake was related to D1-like receptor agonist administration in the PVN, while a decrease food intake was observed when an antagonist of these receptors was administered in the same area [33]. Although we did not analyze the activity of D1-like neurons in the HPT, we were able to detect the influence of restriction and hypercaloric diets in the expression of *Drd1* gene in mice dams (figure 3). Our results indicate that alterations in diet (low- or high- calorie) in this period of life can induce a hypothalamic response that elevates *Drd1* gene transcription. A previous study investigated diet (high-fat and low-fat) influence in the expression of this receptor in the HPT of male mice and detected no difference in *Drd1* mRNA levels after four week of exposure [28]. Nevertheless, they did not used female lactating mice in their analysis, which may have caused the opposing results. When we analyzed the male offspring, we could not observed any statistical significant influence between the dam's and the offspring's diet and the expression of *Drd1* (supplementary). In agreement with our findings, Vucetic, Totoki [30] also observed no difference in *Drd1* gene expression in the HPT of offspring of dams fed a low-protein diet (8.5%) during breeding, pregnancy and lactation, when compared to controls. These findings indicate that diet, especially restrictive diets, during pregnancy do not affect the offspring expression of D1 receptor gene. However, other study described that prenatal undernutrition (50% food restriction during the last pregnancy week) resulted in a reduction of *Drd1* mRNA levels in different hypothalamic areas, in comparison to the control group [34]. The difference observed in comparison to our findings may be related to the acute exposure in opposition to our chronic exposure, and also the percentage of reduction in calories that in our study was less intense, and had no impact in the offspring birth weight.

D2-like receptors (*Drd2, Drd3* and *Drd4* genes) inactivate adenylyl cyclase, which results in a series of intracellular events that are inhibitory. In our mice dam sample, we were able to detect an overexpression of *Drd3* in RD and HD dams in comparison to CONT dams (figure 3). We also observed the effect of the offspring diet in the expression of this gene, as offspring submitted to a restriction diet had higher expression when compared to the control group (figure 5). We also described a downregulation of *Drd4* gene in RD and

HD dams in comparison to CONT group, but no difference was observed in the male offspring (figure 4 and supplementary). *Drd2* gene expression had no difference in our sample (supplementary), and other studies that submitted mice to a low-protein [30] or a high-fat [28] diet are in conformity with our findings. Nonetheless, in opposition to these findings, a study by Manuel-Apolinar, Rocha [34] showed that prenatal undernutrition (50% food restriction during the last pregnancy week) resulted in elevated *Drd2* mRNA levels in ARC of HPT, when compared to controls. However, the cited study analyzed only the ARC of the HPT in opposition to our total hypothalamic gene expression.

Monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) are enzymes responsible for DA degradation to its inactive metabolites [25]. MAO-A isoform is mainly found in catecholaminergic neurons, and MAO-B is present in astrocytes [25]. In our study, *Maoa* gene expression was elevated in RD and HD compared to CONT dams (figure 3). Neither dam's nor the offspring's diet had influence in the expression of *Maoa* in HPT of the male offspring. *Maob* on the other hand, presented no difference in the mice dam groups, but an interaction between the maternal and the offspring diet was demonstrated (figure 4), indicating that changes in diet results in reduced *Maob* gene expression in the offspring's HPT. In our mice dams sample, *Comt* gene was upregulated in the HD group compared to CONT (figure 3). However, in the offspring, *Comt* mRNA levels were reduced due to the effect of the offspring diet only (figure 5). One other study evaluated the effect of diet in the expression of *Comt* gene in HPT of mice, and observed no difference in the expression of offspring of dams fed a low-protein diet (8.5%) during breeding, pregnancy and lactation, when compared to controls [30]. *Slc6a3/Dat1* gene suffered no alteration in the expression in HPT of mice dams and their offspring when submitted to a hypercaloric or a restriction diets.

In our study, we evaluated the effect of diet in the anxiety-like behavior and short- and long-term memories. We described that HD mice dams, when submitted to the EPM test, had increased head-dipping frequency, indicating lower anxiety-like behavior comparing to CONT group (table 2). Other studies [35, 36] were in conformity with our findings, indicating that high calorie diets are related to a reduction in the anxiety-like behavior, however, they were performed in male rodents. A study that analyzed female rats exposed to chronic high-fat feeding showed that it did not alter their behavior in the EPM [37]. Fountain, Mao [38] that analyzed mice dams, observed that there was an elevation of anxiety-like behavior when submitted to a, omega-3 high concentration diet. However, despite the same use of female lactating mice, the

diet is very different than the ones our dams were fed, which may be responsible for the difference between results. Diet ingestion, especially diets that are high in calories, can influence the anxiety-like behavior in rodents in the EPM test. However, the variety of diet protocols and the use of male and female animals in different periods of life may result in diverse findings. Further, pregnancy and lactation periods consist in profound metabolic changes in the organism, making it difficult to compare the behavior with male rodents, or even female rodents in other periods of life. Despite the effect of diet in the anxiety-like behavior of HD dams, in the adult male offspring diet had no influence in this behavior (table 3). A study by Ware, Voigt [39] also described no effect of maternal and offspring diet in the behavior in the EPM. However, Johnson, Javurek [40] and Winther, Elfving [41] have observed that maternal diet influenced the behavior of the offspring in the EPM. The opposite findings may be related to the different diet protocols, and the use of different rodent species.

The RI in the NOR test indicates the recognition rate of the novel object. If RI is increased, the animal had no difficulty recognizing the object as novel, indicating increased performance in the test. In the studied groups, dam's diets did not alter their performance in the NOR test, indicating no difference in short- and long-term memories (table 2). Similar to our findings, Krishna, Keralapurath [42] described no difference between female mice that received high- or low-fat diet in the performance of the NOR test. When analyzing the offspring of our study, we were able to detect an improvement in the short-term memory of the restriction group, as they presented greater 3h-RI when compared to the control group (table 3). In the 24h-RI, we detected an interaction between the dam's and the offspring's diet indicating that changes in diet improve the long-term memory (table 3). On the other hand, other studies described lower recognition index in the NOR test when male rodents were fed a low-protein[43], low-calorie [44] and high-fat [45] diets, and also in the male offspring of dams fed high-fat diet [46]. No difference in the NOR test was also described in the offspring of mice dams submitted to a high-fat diet [41, 47]. Despite the many different protocols, it is clear that the offspring diet, as well as the maternal diet may influence the short- and long-term memory of male mice offspring.

Our study was the first to report that restriction and hypercaloric diet during gestation and lactation alter the expression of dopaminergic system genes in the HPT of mice dams and their offspring. We described elevated mRNA levels in *Th, Drd1, Drd3* and *Maoa* genes in RD and HD dams; *Ddc* and *Comt*

genes in HD dams; and reduced expression of *Drd4* in RD and HD dams. In the offspring, we detected an interaction of the dam's and the offspring's diet only on *Maob* gene expression, which was reduced both due to the offspring's and due to dam's diet. We also described the effect of these diets in increasing anxiety-like behavior in HD dams, improvement of short- and long-term memory in the offspring. Therefore, our results confirm the effect of restriction and hypercaloric diets in the modulation of anxiety, memory and the expression of dopaminergic system genes in the HPT of mice dams and their offspring.

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LIST OF FIGURES AND TABLES

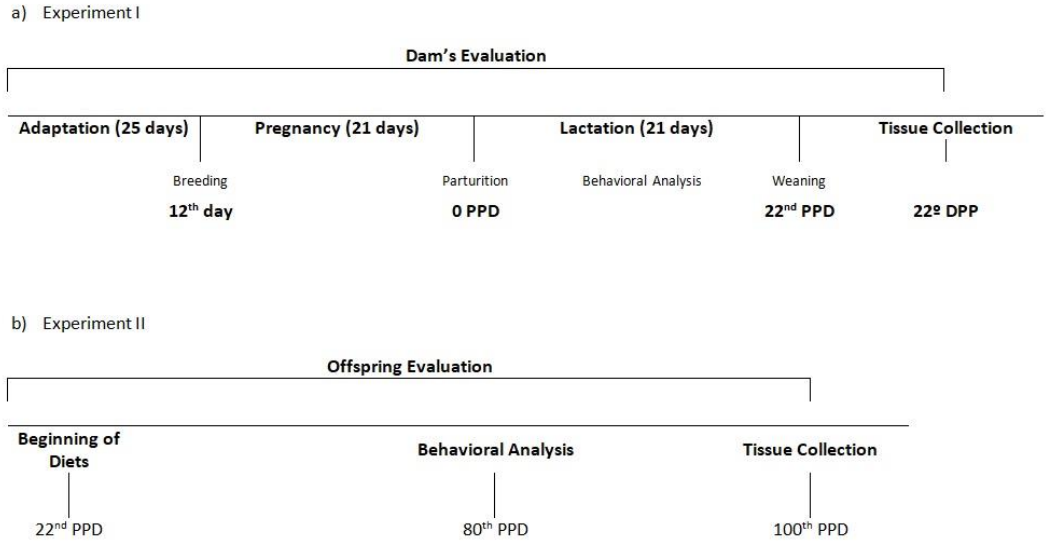

Figure 1. Timeline of experimental procedures of the study. a) Experiment I, representing the timeline of dam's experiment. b) Experiment II, representing the timeline of offspring's experiment. PPD – postpartum day.

EXPERIMENTAL GROUPS

Dams' diet	CONT			RD			HD		
Offspring's diet	CONT	RD	HD	CONT	RD	HD	CONT	RD	HD
Offspring group	CONT-CONT	CONT-RD	CONT-HD	RD-CONT	RD-RD	RD-HD	HD-CONT	HD-RD	HD-HD

Figure 2. Experimental groups used in the study. CONT: control diet; RD: restriction diet; HD: hypercaloric diet.

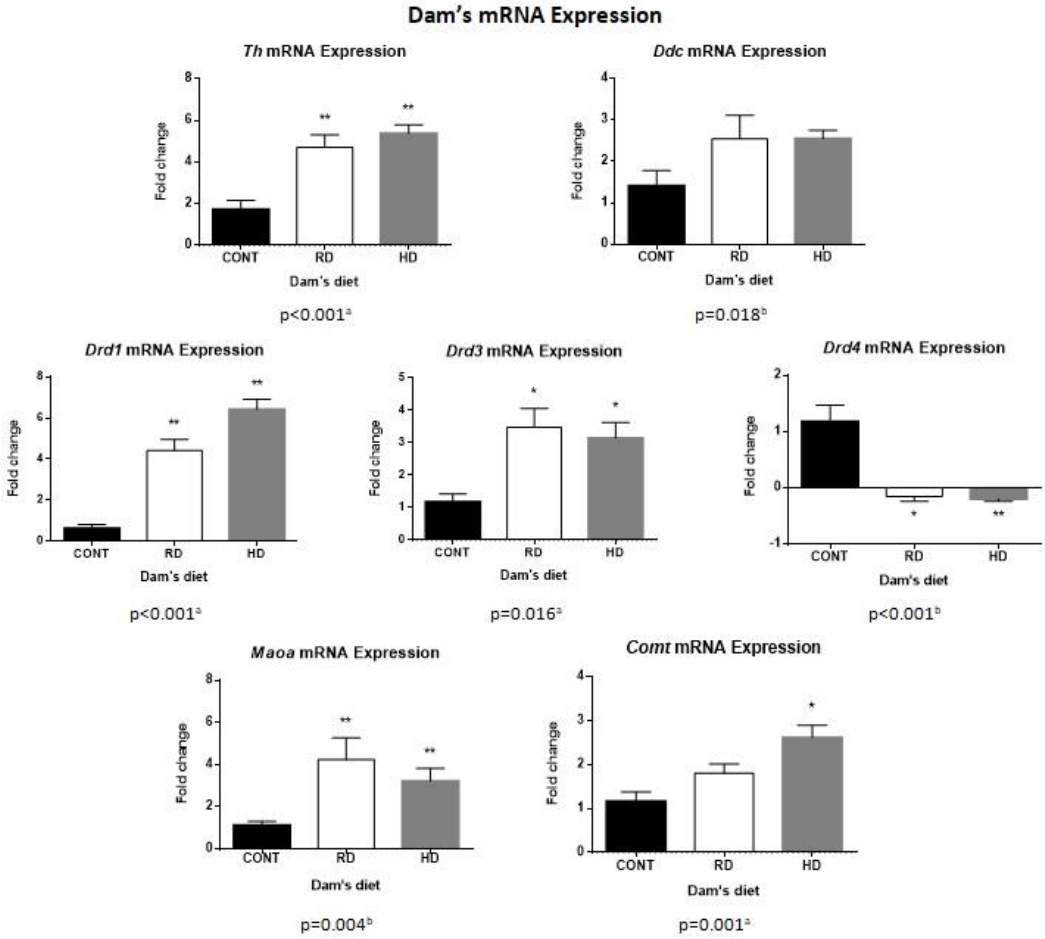

Figure 3. mRNA expression in HPT of mice dams.

[a] One-way ANOVA followed by Tukey's multiple comparisons test.

[b] Kruskal-Wallis followed by Dunn's multiple comparisons test.

* $p<0.05$

** $p<0.001$, when compared to CONT.

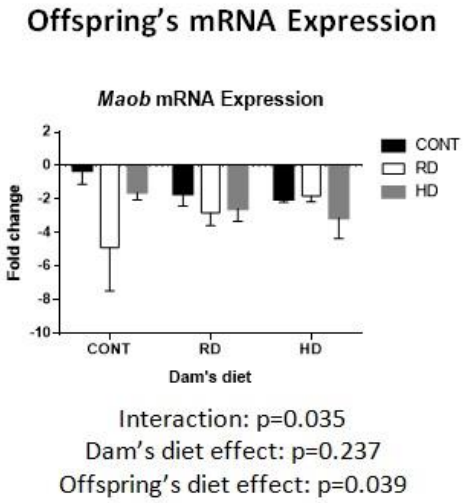

Figure 4. mRNA expression in HPT of male offspring. Interaction between dam's and offspring's diets. Analyzed by two-way ANOVA.

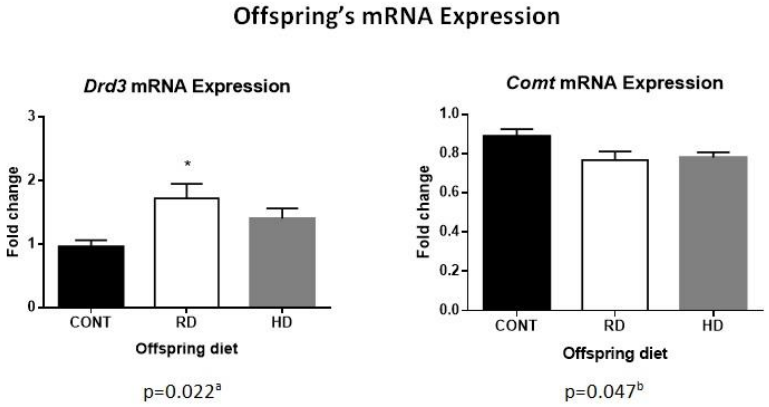

Figure 5. mRNA expression in HPT of male offspring.

[a] Kruskal-Wallis followed by Dunn's multiple comparisons test.

[b] One-way ANOVA followed by Tukey's multiple comparisons test.

* $p<0.05$, when compared to CONT

Two-way ANOVA indicated for *Drd3* gene interaction p=0.745, maternal diet factor p=0.981, offspring diet factor p=0.017; for *Comt* gene interaction p=0.198, maternal diet factor p=0.996, offspring diet factor p=0.031.

Table 1. Reference and target genes selected for this study, their primers and its function.

Gene (ID)[1]	Description	Primer F	Primer R	Function
Ppia/ CypA (268373)	Peptidylprolyl isomerase A	5'GTGTTCTTCGACATCACGGC3'	5'TGTAAAGTCACCACCCTGGC3'	Enzyme that catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides and accelerate the folding of proteins.
Gapdh (14433)	Glyceraldehyde-3-phosphate dehydrogenase	5'CCCTTAAGAGGGATGCTGCC3'	5'TACGGCCAAATCCGTTCACA3'	Glycolytic enzyme that converts D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate.
Actb (11461)	B-actin	5'AGATCAAGATCATTGCTCCTCCT3'	5'ACGCAGCTCAGTAACAGTCC3'	Protein involved in cell motility, structure, and integrity.
Th (21823)	Tyrosine hydroxylase	5'TACAAGCAGGGTGAGCCAAT3'	5'TGGGTAGCATAGAGGCCCTT3'	Conversion of tyrosine to dopamine.
Ddc (13195)	Dopa decarboxylase	GGCGACGATTTCGCTCTTTG	TCATTGGAGCCCTTTAGCCG	Catalyzes the decarboxylation of L-3,4-dihydroxyphenylalanine (DOPA) to dopamine.
Drd1 (13488)	Dopamine receptor D1	5'GTCTCCCAGATCGGGCATT3'	5'AGTCACTTTTCGGGGATGCT3'	Dopamine receptor D1 subtype.
Drd2 (13489)	Dopamine receptor D2	5'GACACCACTCAAGGGCAACT3'	5'TCCATTCTCCGCCTGTTCAC3'	Dopamine receptor D2 subtype.
Drd3 (13490)	Dopamine receptor D3	5'ACGGGACAAATGGAGCACAT3'	5'TGGCAGATGCTGTAGTAGCG3'	Dopamine receptor D3 subtype.
Drd4 (13491)	Dopamine receptor D4	5'TCATCGGCTTGGTGTTGGCA3'	5'CCTGGACCTCGGAGTAGACAAA3'	Dopamine receptor D4 subtype.
Comt (12846)	Catechol-O-methyltransferase	5'TTCTGGCCCATAAATGCTGTTG3'	5'TGCACGAACTCAAACCAACC3'	Degradation of catecholamine transmitters.
Maoa (17161)	Monoamine oxidase A	5' TTCCAATGGGGGCTGTCATC3'	5' GGGCAAGTATGAAGCCCATGA3'	Enzyme that catalyzes the oxidative deamination of biogenic and xenobiotic amines.
Maob (109731)	Monoamine oxidase B	5'TCAGCCAGTGGGCAAGATTT3'	5'GTCGTGCAGGGACATCCAAA3'	Enzyme that catalyzes the oxidative deamination of biogenic and xenobiotic amines.
Slc6a3/Dat1 (13162)	Solute carrier family 6, member 3	5'CGGGATGCCCCTCTTCTACA3'	5'ACAGTGAAGCCCACACCTTT3'	Dopamine transporter (DAT).

[1] Gene Identification Number, available at: http://www.ncbi.nlm.nih.gov/gene.

Table 2. Influence of diet in anxiety-like behavior and short- and long-term memory in mice dams.

		CONT (n=12)	RD (n=14)	HD (n=13)	p
LCE	Entries into the open arms (%)	47.64 ± 3.49	47.20 ± 2.62	50.72 ± 3.82	0.711[a]
	Time spent in the open arms (%)	35.83 ± 9.33	53.60 ± 6.46	56.45 ± 6.69	0.134[a]
	Number of head-dipping	23.08 ± 5.40	29.50 ± 3.74	44.92 ± 6.31[*]	0.021[b]
NOR	3h Recognition Index (%)	64.91 ± 5.53	71.36 ± 3.27	71.08 ± 3.52	0.483
	24h Recognition Index (%)	60.61 ± 4.09	62.05 ± 5.52	59.87 ± 4.68	0.948

Data expressed as mean ± S.E.M.
[a]One-Way ANOVA, followed by Tukey's multiple comparisons test.
[b]Kruskal-Wallis, followed by Dunn's multiple comparisons test.
* when compared to CONT group

Table 3. Influence of maternal and offspring's diet in anxiety-like behavior and short- and long-term memory in male offspring.

Dam's diet	**CONT**			**RD**			**HD**			**p values**		
Offspring's diet	**CONT (n=11)**	**RD (n=11)**	**HD (n=11)**	**CONT (n=11)**	**RD (n=11)**	**HD (n=9)**	**CONT (n=10)**	**RD (n=11)**	**HD (n=10)**	Interaction	Maternal Diet Factor	Offspring Diet Factor
EPM												
Entries into the open arms (%)	44.30 ± 6.09	47.80 ± 3.66	52.33 ± 2.38	46.20 ± 3.66	41.57 ± 2.24	53.39 ± 3.42	47.87 ± 4.72	49.36 ± 2.97	49.38 ± 3.25	0.624	0.874	0.217
Time spent in the open arms (%)	44.90 ± 5.88	44.10 ± 6.48	59.33 ± 5.42	49.12 ± 8.31	37.30 ± 3.62	42.00 ± 7.76	51.12 ± 8.14	56.54 ± 6.14	52.88 ± 6.20	0.346	0.179	0.623
Number of head-dipping	25.50 ± 5.20	30.10 ± 5.25	33.56 ± 6.38	30.50 ± 7.26	23.50 ± 4.54	34.00 ± 4.62	36.00 ± 6.53	48.73 ± 7.32	34.00 ± 4.62	0.235	0.096	0.706
NOR												
3h Recognition Index (%)	63.12 ± 4.60	68.95 ± 3.43	65.04 ± 4.26	57.10 ± 2.96[a]	71.80 ± 3.36[b]	62.12 ± 4.48	55.69 ± 4.10	62.18 ± 5.83	65.11 ± 4.28	0.570	0.392	0.033
24h Recognition Index (%)	41.68 ± 3.82	46.02 ± 3.79	59.15 ± 4.07	54.54 ± 3.84	58.03 ± 4.53	59.38 ± 7.14	40.97 ± 6.61	61.46 ± 5.41	43.81 ± 3.76	0.030	0.045	0.034

Data expressed as mean ± S.E.M.

Two-Way ANOVA, followed by Tukey's multiple comparisons test.

SUPPLEMENTARY MATERIAL

Sup. Table 1. Dam's gene expression (fold change) according to dietary group.

	CONT (n=10)	**RD (n=12)**	**HD (n=13)**	**p**
Drd1	0.62 ± 0.19^{a}	4.41 ± 0.54^{b}	6.41 ± 0.50^{c}	<0.001
Drd2	1.20 ± 0.26	1.67 ± 0.10	1.83 ± 0.11	0.088*
Drd3	1.18 ± 0.24^{d}	3.48 ± 0.58^{e}	3.15 ± 0.48^{e}	0.016
Drd4	1.78 ± 0.29^{f}	0.16 ± 0.20^{g}	0.20 ± 0.04^{g}	<0.001*
Th	1.73 ± 0.42^{h}	4.70 ± 0.61^{i}	5.38 ± 0.40^{i}	<0.001
Ddc	1.41 ± 0.36^{j}	2.54 ± 0.57	2.55 ± 0.20^{k}	0.018*
Slc6a3/Dat1	1.22 ± 0.27	6.60 ± 1.93	4.28 ± 0.96	0.060*
Comt	1.16 ± 0.21^{l}	1.80 ± 0.21^{l}	2.61 ± 0.28^{m}	0.001
Maoa	1.13 ± 0.17^{n}	4.24 ± 1.04^{o}	3.23 ± 0.60^{o}	0.004*
Maob	1.25 ± 0.23	1.81 ± 0.35	1.45 ± 0.19	0.505*

Data expressed as mean ± S.E.M.

One-Way ANOVA, followed by Tukey's multiple comparisons test.

* Kruskal-Wallis, followed by Dunn's multiple comparisons test.

[a-c] Tukey's multiple comparisons test, CONT and RD *p<0.001*; CONT and HD *p<0.001*; RD and HD *p=0.017*.

[d-e] Tukey's multiple comparisons test, CONT and RD *p=0.016*; CONT and HD *p=0.043*; RD and HD *p=0.877*.

[f-g] Dunn's multiple comparisons test, CONT and RD *p=0.002*; CONT and HD *p=0.007*; RD and HD *p=0.999*.

[h-i] Tukey's multiple comparisons test, CONT and RD *p<0.001*; CONT and HD *p<0.001*; RD and HD *p=0.585*.

[j-k] Dunn's multiple comparisons test, CONT and RD *p=0.154*; CONT and HD *p=0.016*; RD and HD *p=0.999*.

[l-m] Tukey's multiple comparisons test, CONT and RD *p=0.213*; CONT and HD *p=0.001*; RD and HD *p=0.054*.

[n-o] Dunn's multiple comparisons test, CONT and RD *p=0.007*; CONT and HD *p=0.020*; RD and HD *p=0.999*.

Supp. Table 2. Offspring's gene expression (fold change) according to dietary group.

Dam's diet	**CONT**			**RD**			**HD**			**p values**		
Offspring's diet	**CONT (n=6)**	**RD (n=6)**	**HD (n=6)**	**CONT (n=6)**	**RD (n=6)**	**HD (n=6)**	**CONT (n=6)**	**RD (n=6)**	**HD (n=6)**	Interaction	Maternal Diet Factor	Offspring Diet Factor
Drd1	1.11 ± 0.21	0.68 ± 0.20	1.16 ± 0.24	0.98 ± 0.12	2.21 ± 0.44	1.70 ± 0.71	1.30 ± 0.27	1.28 ± 0.30	1.13 ± 0.26	0.255	0.110	0.680
Drd2	1.04 ± 0.12	0.75 ± 0.22	1.12 ± 0.14	1.56 ± 0.24	1.40 ± 0.18	1.20 ± 0.39	1.14 ± 0.15	1.13 ± 0.14	1.31 ± 0.35	0.675	0.092	0.709
Drd3	1.12 ± 0.21	1.77 ± 0.59	1.30 ± 0.23	1.06 ± 0.19	1.54 ± 0.34	1.49 ± 0.44	0.72 ± 0.12[a]	1.88 ± 0.38[b]	1.44 ± 0.12[a,b]	0.745	0.981	0.017
Drd4	1.09 ± 0.19	2.49 ± 0.63	1.50 ± 0.38	1.48 ± 0.45	0.73 ± 0.09	0.68 ± 0.14	1.21 ± 0.24	1.53 ± 0.52	1.86 ± 0.96	0.184	0.150	0.691
Th	1.09 ± 0.21	1.21 ± 0.53	1.25 ± 0.31	1.94 ± 0.50	1.60 ± 0.40	1.19 ± 0.51	1.51 ± 0.34	1.50 ± 0.26	1.80 ± 0.78	0.797	0.444	0.962
Ddc	1.13 ± 0.28	0.71 ± 0.07	0.84 ± 0.13	1.03 ± 0.19	0.99 ± 0.18	0.78 ± 0.11	1.06 ± 0.18	1.02 ± 0.14	1.15 ± 0.31	0.671	0.471	0.516
Slc6a3/Dat1	1.26 ± 0.87	0.80 ± 0.30	4.96 ± 2.05	11.68 ± 5.94	7.41 ± 3.36	0.45 ± 0.10	4.95 ± 1.94	7.47 ± 2.62	1.54 ± 0.54	0.081	0.214	0.268
Comt	1.10 ± 0.26[c]	0.64 ± 0.08[d]	0.76 ± 0.05[d]	0.85 ± 0.04	0.89 ± 0.07	0.77 ± 0.05	0.96 ± 0.07	0.77 ± 0.05	0.80 ± 0.05	0.198	0.996	0.031
Maoa	1.05 ± 0.14	0.69 ± 0.06	0.89 ± 0.07	1.01 ± 0.10	1.05 ± 0.13	1.07 ± 0.06	1.08 ± 0.06	1.01 ± 0.18	0.94 ± 0.13	0.406	0.275	0.290
Maob	1.22 ± 0.35[e]	0.38 ± 0.08[f]	0.59 ± 0.09[f]	0.63 ± 0.13	0.43 ± 0.08	0.55 ± 0.13	0.53 ± 0.05	0.64 ± 0.10	0.52 ± 0.12	0.035	0.237	0.039

Data expressed as mean ± S.E.M.

Two-Way ANOVA, followed by Tukey's multiple comparisons test.

[a-b] Tukey's multiple comparisons test, HD/CONT and HD/RD *p=0.023;* HD/CONT and HD/HD *p=0.209;* HD/RD and HD/HD *p=0.558* .

[c-d] Tukey's multiple comparisons test, CONT/CONT and CONT/RD *p=0.007*; CONT/CONT and CONT/HD *p=0.047*; CONT/RD and CONT/HD *p=0.654.*

[e-f] Tukey's multiple comparisons test, CONT/CONT and CONT/RD *p<0.001*; CONT/CONT and CONT/HD *p=0.010*; CONT/RD and CONT/HD *p=0.552.*

4. DISCUSSÃO

A presente tese engloba a avaliação da variabilidade genética em humanos e a expressão gênica em modelo animal. Através dos resultados obtidos, torna-se possível fazer algumas inferências que permeiam os resultados obtidos com as duas abordagens de estudo. Essa seção da tese trará, portanto, a discussão destas intersecções, de modo a unificar os desfechos observados e sua repercussão no sistema dopaminérgico de humanos e roedores.

O polimorfismo *Taq*IA (rs1800497) origina uma variante *missense*, ou seja, proporciona a troca do nucleotídeo timina (**T*; alelo **A1*) para uma citosina (**C*; alelo **A2*), modificando a sequência de aminoácidos ácido glutâmico na posição 713 para lisina (E713K). Essa modificação ocorre no éxon nove do gene *ankyrin repeat and kinase domain containing 1* (*ANKK1*) [136], causando uma substituição de aminoácidos na região C-terminal da proteína [136]. Apesar de localizar-se a aproximadamente 10000 pb do gene *DRD2*, estudos já descreveram sua influência na densidade do receptor de DA D2 no estriado dos indivíduos portadores do alelo **A1* [117]. A associação da proteína ANKK1 com o sistema dopaminérgico ainda não está clara, porém os genes *ANKK1* e *DRD2* pertencem ao mesmo cluster, denominado NTAD (genes *NCAM1-TTC12-ANKK1-DRD2*), que surgiu nos vertebrados, quando o SNC tornou-se mais complexo a mais de 400 milhões de anos [137]. Genes essenciais tendem a permanecer em clusters, em resposta a pressões de seleção natural, que também pode ter sido responsável pela manutenção do cluster NTAD praticamente intacto por todo esse período [137]. Além da possibilidade de alterar a expressão do gene *DRD2* devido a desequilíbrio de ligação, o gene *ANKK1* pertence à família das serina-treonina-cinases *receptor-interacting protein* (RIP), que são descritas como sensores do estresse celular, respondendo a vários fatores ambientais, tais como ingestão alimentar [138]. Essas proteínas ativam fatores de transcrição como o fator nuclear *kappa* B (NF-κB), que induzem a expressão gênica do *DRD2* [139, 140] e, portanto, o polimorfismo *Taq*IA do gene *ANKK1* pode afetar a função dopaminérgica.

Em nosso estudo em humanos, descrevemos que o alelo **A1* em crianças de 7-8 anos estava associado com um maior consumo de alimentos ricos em gordura, quando

comparadas com as homozigotas *A2/A2*. É possível que essas crianças tenham menor densidade de receptores de DA do tipo D2, em áreas relacionadas ao sistema mesocorticolímbico de recompensa. Sabe-se que inicialmente a DA, quando liberada na fenda, ativa receptores do tipo D1 (ativam a adenilil ciclase). Se o estímulo para a liberação de DA na sinapse se mantém, ocorre a ativação de receptores do tipo D2 (inibidores da adenilil ciclase), que podem ser tanto pós quanto pré-sinápticos. Podemos, portanto, analisar a redução da densidade de receptores de duas maneiras: redução de receptores D2 pós-sinápticos e redução de receptores D2 pré-sinápticos, também denominados autorreceptores.

Havendo poucos receptores D2 pós-sinápticos, podemos inferir que haverá maior ativação (receptores D1) do que inibição (receptores D2) dos neurônios, se o estímulo, neste caso a comida, se mantiver. O efeito resultante, portanto, dependerá da área cerebral afetada pela redução de receptores. As projeções da ATV para o NAc no estriado atingem principalmente *medium spiny neurons* (MSN) que expressam tanto receptores D1 quanto receptores D2. Através de técnicas optogenéticas é possível avaliar a ativação de células neuronais específicas. A ativação de D1-MSN, no estriado de animais, ocasionou aumento da sensibilidade à recompensa gerada pela cocaína, enquanto que a ativação dos D2-MSN promoveu dessensibilização e redução da sensação de recompensa gerada por essa droga [141]. Portanto, inicialmente, a DA liberada no estriado, ativa os receptores do tipo D1, o que origina a sensação de recompensa após a ingestão alimentar. No entanto, se esse estímulo permanece, os receptores D2 passam a ser ativados, iniciando um processo de aversão ao alimento. Isso é consistente com os nossos achados, visto que detectamos que crianças consumiam maior quantidade de alimentos ricos em gordura quando portadores do alelo **A1*, associado à reduzida expressão de receptores D2. Como os receptores D2 estão associados à aversão, podemos inferir que essas crianças consumiam maior quantidade de alimentos ricos em gordura, por provavelmente terem retardada a sensação de aversão que se segue à sensação de recompensa.

D2-autorreceptores estão localizados nos neurônios dopaminérgicos pré-sinápticos, levemente afastados da fenda sináptica. Sua função é, quando ativado pela DA que está excedente na fenda, inibir a sinapse através: da diminuição da síntese de DA pela TH; da inibição da liberação da DA na fenda sináptica; e do aumento da recaptação da DA

presente na fenda sináptica. Portanto, a redução de D2-autorreceptores, ocasionada pela presença do alelo **A1* do gene *ANKK1*, pode estar relacionada a um retardamento do auto *feedback* negativo dos neurônios dopaminérgicos, o que resultaria na manutenção da sinapse dopaminérgica por um tempo mais prolongado, ativando mais D1-MSNs, resultando em um estado de recompensa mais intenso. O aumento da ingestão de alimentos ricos em gordura, portanto, estaria relacionada à elevada sensação de recompensa ocasionada pela intensificação da transmissão dopaminérgica, devido à diminuição de D2-autorreceptores que atuariam no auto *feedback*.

A alimentação, de um modo geral, permite que o indivíduo mantenha o equilíbrio energético, através de sensações processadas pelo cérebro, como a fome. No entanto, o consumo de alimentos pode também ser prazeroso, levando a um consumo além das necessidades calóricas, principalmente quando esses alimentos são altamente palatáveis, ou seja, com elevados níveis de carboidratos e lipídeos. O sistema dopaminérgico, principalmente através do sistema mesocorticolímbico, tem um papel essencial na recompensa alimentar. Recompensas naturais como o alimento, estimulam a atividade neuronal na ATV, aumentando a liberação de DA principalmente no NAc, ativando receptores dopaminérgicos e produzindo, portanto, a sensação de recompensa [36, 142, 143]. Ao analisarmos a expressão gênica na ATV em animais, detectamos a alteração dos níveis de mRNA de diversos genes relacionados ao sistema dopaminérgico, tanto nas genitoras quanto na prole. Isso nos leva a crer que essa área cerebral é afetada pela dieta (restritiva ou hipercalórica), independente do período da vida ou do sexo dos animais estudados. Alterações na dieta levam a modificações na sinalização dopaminérgica, e podem influenciar até mesmo a expressão de genes relacionados a esse sistema, como receptores dopaminérgicos, transportador de DA, e enzimas de síntese [129, 130, 144]. Além disso, a existência de interação entre a dieta da genitora e da prole indica que nessa região cerebral, tanto a dieta materna quanto a dieta da prole têm um papel importante na expressão gênica do filhote até mesmo na vida adulta. As alterações observadas na expressão gênica, provavelmente, são decorrentes de fatores epigenéticos, que influenciados por fatores externos podem modificar a transcrição de alguns genes, sem alterar a sequência de bases no DNA.

Efeitos epigenéticos na prole submetida à dieta restritiva (calórica ou de macronutrientes específicos) durante o desenvolvimento intrauterino, já foram abordados em estudos prévios. A redução de 50% das calorias totais ou apenas das proteínas na dieta materna no período final da gestação, leva a modificações sexo-específicas na metilação global, metilação diferencial de miRNAs e diferente expressão de miRNAs [145, 146]. Além de alterar a metilação global no cérebro [147], a restrição proteica durante o desenvolvimento intrauterino, através de alterações nos padrões de metilação da região promotora, modifica a expressão de genes relacionados à DA em regiões cerebrais relacionadas à recompensa [129]. Em compensação, dietas hipercalóricas, que excedem os valores nutricionais necessários para o desenvolvimento fetal adequado também causam alterações no organismo da prole, como por exemplo, o excesso no consumo de alimentos e drogas que geram recompensa [144, 148, 149]. O sistema dopaminérgico, portanto, também é afetado por essas dietas, que, através de fatores epigenéticos, modificam a expressão de genes relacionados a esse sistema [144, 150].

O HPT é a área cerebral responsável pela regulação do peso corporal e pelo balanço energético, principalmente através de peptídeos anorexígenos e orexígenos liberados do núcleo arqueado (ARC) [151]. Tanto em humanos quanto em roedores, o HPT necessita de um período prolongado para seu desenvolvimento, estendendo-se por todo o período fetal, e ainda nas fases iniciais da infância para complementar sua formação [152]. Por esse motivo, o HPT é muito suscetível a alterações ambientais e metabólicas por um extenso período, no qual podem ocorrer diversas alterações epigenéticas que podem ser mantidas até mesmo na vida adulta. No entanto, quando analisamos a expressão de genes do sistema dopaminérgico no HPT, observamos que a alteração dos níveis de mRNA foi mais pronunciada nas genitoras do que na prole. Estes resultados indicam que durante o período de gestação e lactação, a dieta ocasiona alterações fisiológicas mais intensas nessa área do que no período intrauterino, nas fases iniciais da vida e na vida adulta. Possivelmente as alterações hormonais durante esse período promovem modificações mais significativas na expressão gênica no HPT. Isso se evidencia uma vez que a dieta materna não foi associada com alteração na expressão dos genes do sistema dopaminérgico no HPT da prole e que a interação dieta materna e dieta da prole foi associada apenas com a expressão do gene *Maob* na prole.

Além do sistema mesocorticolímbico de recompensa, outro sistema em que a DA está presente é a via tuberoinfundibular, relacionada a liberação hormonal como por exemplo na lactação. É através desse sistema que ocorre o principal meio de inibição da secreção de prolactina pela hipófise [153], que recebe projeções de neurônios dopaminérgicos cujos corpos estão localizados principalmente no ARC do HPT [154], região que também está altamente relacionada à regulação da alimentação [155]. Dietas restritivas maternas promovem alterações no ambiente intrauterino, e modificam padrões de metilação do DNA, que resultam em disfunções (como hiperfagia, redução da saciedade e desenvolvimento de síndrome metabólica na prole), relacionadas ao controle hipotalâmico da fome [20, 156-158]. Outros estudos observaram o efeito da dieta materna rica em gordura na expressão de genes relacionados à DA no HPT da prole, além de alteração nos padrões de metilação nas regiões promotoras e de metilação global nessa área cerebral [144]. Fêmeas roedoras, expostas a estressores durante o desenvolvimento intrauterino, e alimentadas com dieta rica em gordura e açúcar durante a vida adulta, tiveram a expressão gênica de *Slc6a3* alterada devido a modificações no padrão de metilação da região promotora do gene [159]. Observa-se, portanto, que a dieta, como fator ambiental, é muito importante na modificação de perfis epigenéticos que, portanto, levam a alteração da expressão gênica no HPT.

Ao modificar a expressão de genes do sistema dopaminérgico, a dieta também pode influenciar no comportamento do tipo ansioso, na atividade locomotora, no medo inato e na memória dos animais, visto que a DA tem um importante papel na modulação desses comportamentos [160-162]. Em roedores, a utilização de fármacos que estimulam a transmissão dopaminérgica também aumenta a atividade locomotora dos animais, enquanto que fármacos que bloqueiam os receptores dopaminérgicos resultam na redução da locomoção [163]. Nosso estudo demonstrou que a dieta, tanto restritiva como hipercalórica, afetou a locomoção e o medo inato das genitoras, porém não teve influência no comportamento dos filhotes. Quando associamos esses achados comportamentais com a expressão de genes do sistema dopaminérgico, observamos que pode haver uma relação entre eles, visto que o aumento na atividade locomotora dessas fêmeas foi acompanhado de um aumento nos níveis de mRNA de genes que estimulam a transmissão dopaminérgica (*Th, Drd1, Drd2, Drd3* e *Slc6a3/Dat1*). Possivelmente, durante a gestação e a lactação, a fêmea esteja mais suscetível a alterações na atividade locomotora do que os filhotes

submetidos a essas mesmas dietas durante o desenvolvimento intrauterino, as fases iniciais da vida e a vida adulta.

Além da recompensa, consumo alimentar, memória e da atividade locomotora, a DA está relacionada também com o comportamento do tipo ansioso [164-166]. Estudos mostram que situações de estresse ativam o sistema dopaminérgico, inibindo a recaptação de DA pelo transportador DAT, aumentando a DA na fenda sináptica, o que pode induzir efeitos relacionados ao comportamento do tipo ansioso [167, 168]. No entanto, a ativação de neurônios dopaminérgicos é crítica para prevenção de um estado de ansiedade generalizada [169]. Apesar de não detectarmos alteração no gene *Slc6a3/Dat1* no HPT dessas fêmeas, observamos que a dieta influenciou na sinapse dopaminérgica, aumentando a expressão de genes relacionados à ativação dopaminérgica como *Th*, *Drd1* e *Drd3*.

Por fim, pudemos observar que a memória de curto prazo da prole foi afetada apenas pela dieta recebida, enquanto que a memória de longo prazo foi influenciada por uma interação da dieta materna e da prole. A dieta restritiva da prole foi associada a uma melhora na memória de curto prazo, e a interação das dietas das genitoras e da prole resultaram em uma melhora das memórias de longo prazo. Sabe-se que a DA é essencial para a memória de trabalho, demonstrado por estudos em pacientes com doença de Parkinson e de Huntington [170]. Além disso, estudos em primatas indicam que a DA está relacionada à memória associada a sensações de prazer e recompensa [171]. Por alterar a expressão de genes relacionados ao sistema dopaminérgico, a dieta pode também ter sido responsável pela melhora na memória observada nos animais.

A dieta e o sistema dopaminérgico, portanto, estão intimamente relacionados. Alterações na sequência de nucleotídeos no DNA, bem como modificações epigenéticas influenciam na expressão de genes desse sistema, resultando em adaptações importantes na transmissão dopaminérgica, que envolvem diferentes desfechos, como modificação na sensação de recompensa e em padrões comportamentais.

5. CONCLUSÕES

Tendo em vista todos os resultados apresentados, tanto em humanos quanto em animais, podemos observar a relação do sistema dopaminérgico com a ingesta alimentar e como a dieta pode alterar esse sistema. Em humanos, investigamos a associação das variantes *Taq*IA (rs1800497) e -141C Ins/Del (rs1799732) no gene *DRD2* e pudemos relacionar o alelo **A1* da variante *Taq*IA (rs1800497) com o aumento da ingestão alimentar de lipídeos em crianças de 7-8 anos. A variante -141C Ins/Del (rs1799732) não foi associada a nenhum parâmetro de ingestão alimentar, nem com parâmetros de adiposidade.

Em modelo animal, descrevemos que as dietas restritiva e hipercalórica aumentam a atividade locomotora das genitoras no campo aberto, e que a dieta hipercalórica reduz o medo inato dessas fêmeas. Ao observarmos o comportamento das genitoras no labirinto em cruz elevado, descrevemos que as fêmeas que receberam a dieta hipercalórica exploravam mais o ambiente, o que nos sugere uma redução no comportamento do tipo ansioso. No teste de memória de curto e longo prazo não observamos nenhuma alteração na memória dessas fêmeas. No entanto, quando analisamos os mesmos testes comportamentais nos machos das proles dessas genitoras, nem a dieta materna, nem a dieta da prole influenciaram na atividade locomotora, no medo inato e no comportamento do tipo ansioso. Em contrapartida, a memória de curto prazo estava melhorada naqueles que receberam a dieta restritiva, e na de longo prazo observou-se interação no efeito da dieta materna e do filhote, que melhoraram os índices de reconhecimento nos filhotes.

A expressão de genes do sistema dopaminérgico foi alterada pelas dietas tanto nas genitoras quanto nos filhotes, na ATV e no HPT desses animais. Na ATV, pode-se observar uma elevação nos níveis de mRNA dos genes *Th, Drd1, Drd2* e *Drd3* nas genitoras que receberam dieta restritiva e hipercalórica. O gene *Slc6a3/Dat1* teve sua expressão aumentada apenas nas fêmeas que receberam a dieta hipercalórica. Na prole dessas fêmeas, observamos, pelo contrário, uma redução generalizada na expressão dos genes relacionados ao sistema dopaminérgico na ATV. Nos genes *Th, Drd2* e *Slc6a3/Dat1* a interação entre a dieta materna e a dieta da prole foi significativa para alterar a expressão

gênica na prole. A dieta materna alterou a expressão de *Ddc* e *Drd3*, e a dieta da prole também influenciou a expressão de *Ddc* na ATV.

Já no HPT, demonstramos que a alteração na expressão gênica ocasionada pela dieta foi mais expressiva nas genitoras. Os genes *Drd1, Drd3, Maoa* e *Th* estavam com sua expressão elevada nas genitoras que receberam tanto a dieta restritiva quanto a dieta hipercalórica. Mas apenas as fêmeas que receberam dieta hipercalórica tiveram aumento dos níveis de mRNA de *Comt* e *Ddc* no HPT. Já o gene *Drd4* estava com expressão reduzida nas fêmeas que receberam as dietas restritiva e hipercalórica. A expressão gênica da prole foi menos alterada pela dieta nessa área. Apenas o gene *Maob* foi influenciado tanto pela dieta materna quanto pela dieta da prole, que reduziram os níveis de mRNA desse gene em todos os grupos. Ao analisarmos apenas o efeito da dieta da prole na expressão dos genes selecionados, observamos que as dietas influenciaram na expressão dos genes *Drd3* e *Comt*.

É possível observar, portanto, através dos dados apresentados, a importância do sistema dopaminérgico e sua relação com a alimentação, tanto em humanos quanto em camundongos. Variantes em genes relacionados ao sistema dopaminérgico podem modificar a transmissão dopaminérgica, provocando maior ou menor sensação de recompensa após a ingestão alimentar. Ao intensificar a sensação de recompensa, mais alimentos palatáveis serão ingeridos, o que pode ocasionar desfechos como sobrepeso e obesidade. Além disso, alterações na expressão de genes do sistema dopaminérgico podem ser ocasionadas tanto pela alimentação materna durante a gestação e a lactação, quanto pela alimentação na vida adulta. Fica claro, portanto, que esse sistema é de extrema importância, e deve ser mais estudado, com o objetivo de possibilitar uma maior compreensão dos mecanismos envolvendo a recompensa alimentar para possivelmente auxiliar, no futuro, o tratamento individualizado de pacientes obesos, de acordo com seus padrões de expressão de genes do sistema dopaminérgico.

6. PERSPECTIVAS

Após os resultados obtidos nessa tese, intensificaram-se nossos objetivos de estudarmos de maneira mais aprofundada, os mecanismos epigenéticos envolvidos na expressão gênica da prole. Os genes cuja expressão foi alterada pela dieta materna serão futuramente estudados quanto à metilação da região promotora, para confirmar se esse fator epigenético está elevando ou reduzindo a expressão dos genes do sistema dopaminérgico. Além disso, pretende-se analisar também os miRNAs que podem estar interferindo pós-transcricionalmente na expressão gênica.

O estudo de outras áreas coletadas (bulbo olfatório e hipocampo), bem como, o estudo de outros sistemas relacionados à sensação de recompensa pela ingestão alimentar, também poderão ser realizados. O sistema serotoninérgico já foi muito estudado em humanos pelo grupo de pesquisa, portanto, há a possibilidade de fazer novamente uma comparação entre humanos e animais, como a desenvolvida na presente tese.

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8. ANEXOS

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO

UFCSPA
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 171/15

2) DATA DO PARECER: 09/03/2016 Parecer 388/15

3) TÍTULO DO PROJETO:

Análise comportamental, bioquímica e epigenética do efeito da nutrição materna em modelo animal.

4) PESQUISADOR RESPONSÁVEL:

Márcia Giovenardi

5) RESUMO DO PROJETO:

O projeto visa analisar parâmetros comportamentais, bioquímicos e epigenéticos dos sistemas dopaminérgico e serotonérgico em camundongos expostos a diferentes dietas (restrição calórica-DR, normal-DC e hipercalórica-DHC). Protocolo 1: camundongas serão acasaladas na proporção de 3:1 e iniciarão a dieta quando confirmada a prenhez (esfregaço vaginal), sendo avaliadas no 1º e 10º dia pós-natal, quanto a massa corporal e no 7º dia pós-natal quanto ao comportamento maternal, 8º dia – ansiedade (labirinto em cruz) e 9º dia, quanto a locomoção (campo aberto). Estas serão avaliadas também, quanto a massa corporal nos dias 3, 9, 15 e 21 pós-natal e eutanasiadas por decaptação no 22º dia, para coleta de sangue troncular para avaliação bioquímica (glicemia, colesterol total, triglicerídeos, HDL, LDL e colesterol) e regiões cerebrais para análise da expressão de genes envolvidos nos sistemas dopaminérgico e serotonérgico. Protocolo 2: as ninhadas serão padronizadas ao nascimento, em oito filhotes cada e, 3 machos e 3 fêmeas de cada ninhada serão submetidos as respectivas dietas (independentemente da dieta materna). Será verificado na prole: massa corporal nos dias

3, 9, 15 e 21 pós-natal, 55º dia - ansiedade (labirinto em cruz) e 60º dia - locomoção (campo aberto), sendo as fêmeas acompanhadas quanto ao ciclo estral (3 ciclos regulares) e testadas em diestro. A eutanásia será realizada em torno de 65 dias pós-natal, da mesma forma e com avaliação das mesmas variáveis descritas para as mães.

6) OBJETIVOS DO PROJETO:

Geral: Analisar parâmetros comportamentais, bioquímicos e epigenéticos dos sistemas dopaminérgico e serotonérgico em camundongos expostos a diferentes dietas (restrição calórica-DR, normal-DC e hipercalórica-DHC).

Específicos:

- analisar o efeito das diferentes dietas nos comportamentos maternal, de ansiedade e de locomoção das genitoras;

- analisar o efeito das diferentes dietas na expressão de genes dos sistemas dopaminérgico e serotoninérgico das genitoras;

- analisar o efeito das diferentes dietas nos comportamentos de ansiedade e locomoção da prole;

- analisar o efeito das diferentes dietas na expressão de genes dos sistemas dopaminérgico e serotonérgico da prole;

Identificar padrão de metilação nos genes que possuam modificação da expressão de genes do sistema dopaminérgico da prole;

- identificar padrão de metilação nos genes que possuam modificação da expressão de genes do sistema serotoninérgico da prole;

- analisar o efeito de diferentes dietas no perfil lipídico da genitora e da prole.

7) FINALIDADE DO PROJETO:

[ ] Ensino [X] Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

Título	[X]	Adequado	[ ]	Comentários
Introdução	[X]	Adequada	[ ]	Comentários
Objetivos	[X]	Adequados	[ ]	Comentários
Relevância e Justificativa	[X]	Adequados	[ ]	Comentários
Materiais e Métodos	[X]	Adequados	[ ]	Comentários

Detalhar na metodologia:

- o cálculo do tamanho da amostra foi apresentado e referenciado;

- o procedimento de acasalamento e adequado a espécie em consonância com os objetivos da pesquisa.

- a justificativa para a decaptação foi apresentada e referenciada.

Cronograma para execução da pesquisa [X] Adequado [ ] Comentários

Orçamento e fonte financiadora [X] Adequados [ ] Comentários

Referências Bibliográficas [X] Adequadas [ ] Comentários

9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE:

[x] Sim [ ] Não

10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:

Grau de dor/estresse: **B** [ ] **C** [X] **D** [ ] **E** [ ]

Justifique:

Espécie: Camundongo **Número Amostral:** 15/grupo

Redução Amostral: [ ] Sim [x] Não

Justifique:

Substituição de Metodologia: [ ] Sim [X] Não

Se achar necessário, justifique e sugira uma nova metodologia:

Aprimoramento da Metodologia: [ ] Sim [X] Não

Se achar necessário, justifique e sugira aprimoramentos da metodologia:

Acomodação e manutenção dos animais: [x] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Manipulação dos animais: [X] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Analgesia dos animais (se aplicável): [X] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com analgésico substituto

Anestesia dos animais (se aplicável): [X] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com anestésico substituto:

Eutanásia dos animais (se aplicável): [X] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com metodologia substituta:

Local de Realização (Biotério/Labotarório): Biotério da UFCSPA, Laboratório de Fisiologia comportamental e metabólica e o Laboratório de Biologia Molecular.

Outra instituição. Qual?

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade
Não referida	Camundongos	Macho	15
Não referida	Camundongas	Fêmeas	45

12) RECOMENDAÇÃO:

[X] Aprovado

[ ] Com Pendência

[ ] Não aprovado

Data de início 10/03/2016 Data de Término 30/12/2019

Comentários gerais sobre o projeto:

O tema é relevante, o uso dos animais é justificado por necessitar avaliação genética, metabólica e comportamental *in vivo*. O projeto está adequado para ser desenvolvido na forma apresentada.

ARTIGO CIENTÍFICO 4

Study of gene expression in the ventral tegmental area and hypothalamus of mice dams and male offspring: which internal control gene should be used?

A ser submetido ao periódico Einstein, fator de impacto 0.104.

Study of gene expression in the ventral tegmental area and hypothalamus of mice dams and male offspring submitted to a diet intervention: which internal control gene should be used?

Estudo de expressão gênica na área tegmentar ventral e hipotálamo de camundongos gestantes e prole de machos submetidos à intervenção dietética: qual gene de controle interno deve ser utilizado?

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ABSTRACT

INTRODUCTION: The quantitative reverse transcription polymerase chain reaction (RT-qPCR) is one of the most reliable and accurate techniques for gene expression analysis. The levels of messenger RNA transcripts are submitted to analysis after normalization with an internal control or a reference gene (housekeeping genes, HKG). However, the selection of the HKG needs to consider the physiological conditions and area of the brain studied, because these conditions may alter the expression of these genes. Therefore, this study evaluated three HKG (beta-actin, peptidyl prolyl isomerase A, glyceraldehyde-3-phosphate dehydrogenase) in ventral tegmental area (VTA) and hypothalamus (HPT) of pregnant mice and male offspring.

METODOLOGY: Female mice were randomized in control, restrictive and hypercaloric diet groups, and mated with isogenic male mice. After weaning, the offspring received the same diets provided to the dams. VTA and HPT were macroscopically dissected, the total RNA extracted and cDNA synthesis performed by reverse transcription. Amplification was performed using specific primers for each of the HKG, by reverse transcription real-time polymerase chain reaction with *SYBR™ green*. Stability of internal control genes was calculated by NormFinder software.

RESULTS: *Ppia* gene was the most stable gene in VTA of mice dams and in HPT of male offspring. *Actb* gene presented greater stability in HPT of mice dams and in VTA of male offspring.

CONCLUSION: In this study, we contribute with posterior genic expression studies, that also used the animal model of female pregnant mice and male offspring submitted to restriction and hypercaloric diets.

Key-words: Housekeeping genes, diet, pregnancy, mice, gene expression

RESUMO

INTRODUÇÃO: Transcrição reversa seguida de reação em cadeia da polimerase (RT-qPCR) é uma das técnicas mais confiáveis e precisas para análise da expressão gênica. Os níveis de transcritos de RNA mensageiro são submetidos à análise após normalização com controles internos ou genes de referência (genes *housekeeping*, GHK). No entanto, a seleção do GHK precisa considerar as condições fisiológicas e a área do cérebro estudada, porque essas condições podem alterar a expressão desses genes. Portanto, este estudo avaliou três GHK (beta-actina, peptidilprolil isomerase A, gliceraldeído-3-fosfato desidrogenase) na área tegmentar ventral (ATV) e hipotálamo (HPT) de camundongos prenhes e filhotes machos.

METODOLOGIA: Fêmeas foram randomizadas em grupos de dietas controle, restritiva e hipercalórica, e acasaladas com camundongos machos isogênicos. Após o desmame, a prole recebeu as mesmas dietas fornecidas às mães. ATV e HPT foram macroscopicamente dissecados, o RNA total extraído e o cDNA sintetizado por transcrição reversa. A amplificação foi realizada utilizando primers específicos para cada um dos HKG, através de reação em cadeia da polimerase em tempo real por transcrição reversa com *SYBR™ green*. A estabilidade dos genes de controle interno foi calculada pelo software NormFinder.

RESULTADOS: *Ppia* foi o gene mais estável em ATV de camundongos e em HPT de filhotes machos. O gene *Actb* apresentou maior estabilidade no HPT das fêmeas de camundongos e no ATV dos filhotes machos.

CONCLUSÃO: Neste estudo contribuímos com estudos de expressão gênica posterior, que também utilizaram o modelo animal de camundongos fêmeas prenhes e filhotes machos submetidos a dietas restritiva e hipercalórica.

Descritores: Genes *housekeeping*, dieta, gravidez, camundongos, expressão gênica

INTRODUCTION

Ventral tegmental area (VTA) is a central nervous system (CNS) region with a remarkable concentration of dopaminergic neurons [1]. VTA projects its axons to various brain areas, such as amygdala, nucleus accumbens and prefrontal cortex (PFC), acting on the limbic system, appetite control, sleep and motivation [2]. In the same way, the hypothalamus (HPT), which also receives and send projections to the VTA, presents a remarkable importance in feeding regulation and metabolism (see review in [3]). Appropriate nutrition during the pre and postnatal period is crucial for proper fetal growth [4]. The most exposed organ during pregnancy is the brain, due to its extensive growth and the development of new neuronal pathways [5]. Diets are environmental factors that can trigger metabolic diseases, by altering epigenetic factors that modify gene expression without altering genomic DNA sequence.

Understanding the mechanisms of gene expression and how it is influenced by the behavioral pattern may support many studies with this approach. Thus, gene expression assays have been shown to be efficient in analyzing specific transcripts and in solidifying the understanding of gene expression patterns, as well as the recognition of new ways of interaction of the regulatory process, aiding in the identification of several biological processes and also their implications in some diseases [6].

Reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR) is a widely used technique for gene expression analysis, as it is an experimental method with high reproducibility, sensitivity and accuracy [7]. However, each cell has a variable gene expression, dependent of many inhibitory factors, such as cell cycle, exposure to drugs, cytokines, hormones, diet and others [8]. It may also undergo interference from the technique extrinsic factors, such as extraction of messenger RNA (mRNA) differences between samples, performance of RT-qPCR, pipetting errors and RNA impurity [9]. Thus, the use of standardization parameters is required to obtain reliable quantitative expression measures in the analysis of target genes and to correct the differences between the analyzed samples, and other intrinsic variations [10].

The use of an internal control gene, also called housekeeping gene (HKG), is one of the most commonly used techniques to avoid the cited variation in gene expression studies. These genes are constitutive, present in all cells, since they are enzymes or essential molecules to the proper functioning of the organism and have a constant synthesis rate independently of the cell cycle. However, caution is required when

choosing HKG, since the transcription levels of these can vary according to the experimental conditions and tissues analyzed [11-13]. Therefore, the objective of this study was to analyze the expression in order to select the most stable internal control genes in VTA and HPT of mice dams and their offspring, when submitted to hypercaloric and restrictive diets during pregnancy and lactation, and throughout adulthood.

METHODOLOGY

Animals

All procedures were performed according to standards proposed by National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Protocols were previously approved by the Institutional Animal Care and Use Committee of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA, protocol number 388/15). All efforts were made to minimize animal suffering and to use only the necessary number of animals to produce reliable scientific data.

Female BALB/c mice (n = 15) with approximately 70 days-old, obtained from the animal house of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) were used in the study. All animals were housed under controlled temperature (23 ± 1ºC), humidity (55 ± 5 %) and light-dark cycle of 12 hours (lights off at 18:00). Females were individually maintained in polypropylene cages (30x20x13cm of dimension), with *ad libitum* access to water and the food access depended on the dietary group they were randomly assigned. The three dietary groups were: control diet group (CONT; n=12) received standard chow diet (Nuvital, Curitiba, Brazil) with 3.4 Kcal/g of which 63% of calories from carbohydrate, 26% of protein, 11% of fat; restriction diet group (RD; n=14) fed a 30% caloric restriction, adjusted according to CONT group consumption; and a hypercaloric diet group (HD; n=13) provided a custom made chow (Pragsoluções Biociências, Jaú, Brazil) consisting of 5.0 kcal/g of which 42.4% carbohydrate, 9.9% protein, 47.7% fat.

After 25 days of diet adaptation, females were placed with isogenic male mice for approximately 7 days for reproduction in a maximum proportion of 3:1. During this period, all animals received standard chow. Pregnancy confirmation was obtained with the analysis of vaginal smears for the detection of sperm. During pregnancy and lactation, dams received the previously assigned diet. When offspring weaned, they

were randomly distributed into the three dietary groups, in a way that each dam had at least one pup in each diet. Fifteen male mice were used in this study.

Tissue collection was performed on the 22nd postpartum day (PPD) on mice dams, and on the 100th PPD on the offspring. VTA and HPT were macroscopically dissected with sterile materials on an ice-cold surface in a stereo microscope, and stored in RNA later™ stabilization solution (Invitrogen, São Paulo, Brazil) for subsequent molecular analysis.

Molecular analysis

For total RNA extraction, PureLink™ RNA Mini Kit (Invitrogen, São Paulo, Brazil) was used according to manufacturer's instructions. In summary, brain tissues homogenization was performed with 0.3 mL of Lysis Buffer containing 1% 2-mercaptoethanol. Next, 70% ethanol was added and vigorously vortexed to disperse any precipitate. To bind RNA to the membrane, homogenized was transferred to a spin cartridge and centrifuged (12000xg, 15 seconds, at room temperature). The membrane was washed three times, and then dried by centrifugation (12000xg, 1 minute, room temperature). RNase-free water (30 µL) was used for RNA elution. Finally, RNA concentration as well as the quality of samples (A260/A280) were evaluated by spectrophotometry using the BioSpec-nano equipment (Shimazu, Kyoto, Japan).

Complementary DNA (cDNA) synthesis was performed using the M-MLV Reverse Transcriptase kit (Invitrogen, São Paulo, Brazil), according to manufacturer's instructions. Initially, total RNA (0.1-4 µg, up to a maximum volume of 10 µL) was incubated at 65ºC with a mix containing 1µL (0.5µg/µL) of oligo(dT)$_{12\text{-}18}$, 1µL to 10mM deoxynucleotide triphosphates (dNTPs; Invitrogen, São Paulo, Brazil) and water treated with diethyl pyrocarbonate (DEPC) until the volume of 12µL. After 5 minutes, the mixture was refrigerated on ice, briefly centrifuged and incubated again for 2 minutes at 37 ° C with 4µl of 5X first-strand buffer, 2µl 0.1M dithiothreitol buffer (DTT) and 1µL (40 units/µL) of RNaseOUT™. Finally, 1 µl (200 units) of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) was added to the final volume and incubated at 37°C for 50 minutes. The reaction was heat inactivated at 70°C for 15 minutes.

Reference genes amplification was performed in a StepOnePlus™ thermocycler (Applied Biosystems™ Foster City, CA, USA), with 7.5 µl SYBR™ Select Master Mix (Applied Biosystems™, São Paulo, Brazil), 0.33 µM of forward and reverse primers, 5 ng of cDNA and nuclease-free water to a total volume of 15 µL per well. Product specificity

was obtained by melting curve analysis. All samples run in duplicates. Primer sequences are listed on table 1. Primer3 software was used to design primers based on mice sequences in GenBank database and Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, NCBI, Bethesda, USA) was used to confirm primer specificity.

Determination of expression stability

NormFinder (the Visual Basic application for Microsoft Excel) was used to calculate the reference genes stability. The expression of each HKG was transformed from logarithmic Ct values to linear values with the 2^{-Ct} formula. NormFinder then uses a model-based approach to rank reference genes according to their expression stability, which is calculated by an estimation of intra- and inter-group expression variation [12, 14].

RESULTS

In mice dams' VTA, both *Actb* and *Ppia* genes were the most stable. However, Normfinder select the lower stability value, and *Ppia*, with a stability level of 0,605 was the selected gene (see figure 1a). In HPT the most stable HKG was *Actb*, with a stability value of 3.260 (see figure 2b).

Accordingly to the mice dams findings, in the male offspring the most stable genes in VTA were also Actb and *Ppia.* Nonetheless, Normfinder detected the lowest stability level of 0.150 in *Actb* gene (see figure 1b). With a stability value of 1.350, the software suggested *Ppia* as the most stable gene in HPT of the male offspring, although *Actb* also had a very adequate stability value of 1.435 (see figure 2b).

Gapdh was the less stable gene in VTA and HPT of mice dams, with stability values of, respectively, 3.665 and 8.831 (see figures 1a and 2a). The same gene also had the highest stability value in the male offspring studied areas, with stability values of 3.445 in VTA, and 4.916 in HPT (see figures 1b and 2b).

DISCUSSION

HKG are genes that remain active in almost all cells and have a constant expression rate. Actin filaments are highly conserved proteins involved in cell motility,

structure, integrity and intercellular signaling, and is one of the three types of cytoskeletal elements in the cytosol of eukaryotes (see review in [15]). There are different isoforms of this protein, however β-actin (ACTB) is the one of interest for this study, as it is found mainly in the CNS [15]. *Actb* has been used as an internal control gene in distinct brain areas such as the PFC, striatum, hypothalamus and cerebellum in animal model [16, 17]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), encoded by *Gapdh* gene, is an important enzyme in the glycolytic pathway, therefore present in all tissue cells [18]. Peptidyl-prolyl cis-trans isomerase (PPIase) catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides and accelerate the folding of proteins. PPIase is a cyclosporin binding-protein and may play a role in cyclosporin A-mediated immunosuppression, thus the previous gene identification *CypA* [19]. A review of previously published data by Sharan, Vaiphei [20] in human studies, showed *PPIA,* followed by *ACTB* and *GAPDH* as the most suitable in several gene expression analysis. In animal studies [21], the same genes are equally the most used as HKGs, therefore we selected them for the stability analysis.

Females and males have very different physiologies, especially due to hormonal changes. The maternal brain undergo many complex behavioral and neurobiological adaptations to help the mother to adapt to the demands of pregnancy and the subsequent lactation, including the expression of maternal behaviors after birth, suppression of aversion responses to pups, and reduction in anxiety (see review in [22]). Therefore, it is very likely that female dams and male offspring have different gene expression that may influence even the constitutive genes of the brain. In the VTA, the best HKG of the selected genes for this study was *Ppia* in mice dams and *Actb* in the offspring groups. To the best of our knowledge, this is the first study to describe the best HKG in VTA of mice dams and their offspring that were both submitted to a diet intervention. The methodology used to select the HKG is rarely described in studies of gene expression. Lindblom, Johansson [23] analyzed the best HKG in the VTA of male rats submitted to food restriction. *Actb, Ppia* and *Tubb5* (beta 5-tubulin) genes were chosen between 5 other candidate HKG. Other studies describe the HKG used in the analysis, but do not indicate the methodology used in the selection. *Actb* was used as HKG for the gene expression analysis in the VTA of male rodents fed a restriction [24] or a cafeteria [25] diet. Few studies used *Gapdh* as HKG for gene expression analysis in this area, in male rodents fed a high-fat [26] and restriction [27] diets, and also nicotine exposure [28].

Normfinder also identify the best HKG in HPT, and described *Actb* as the most stable in the dams groups, and *Ppia* as the best selection in the offspring sample. Other studies, despite not indicating the methodology in which they selected the HKG, also used the same genes identified in the present analysis. Two studies, one with male and female rats submitted to cafeteria diet [29], and the other with 3 generations of female rats that received a high-fat diet throughout pregnancy and lactation [30], also investigated dopaminergic expression in HPT and used *Actb* as the HKG. *Ppia* was also the selected HKG in a study with male mice submitted to high- and low-fat diets [31]. However, a study pregnant female rats [32] and another study with male mice [26], both submitted the animals to a high-fat diet and used *Gapdh* as the HKG. Nonetheless, Sellayah, Sek [33] realized a study with offspring mice exposed to different diets in the pre- and postnatal period and concluded that *Gapdh* presents variation in the stability of mRNA in HPT. de Moura, Lazzari [34] indicated *Actb* as the most stable HKG in the olfactory bulb, PFC, hippocampus and striatum using virgin females wistar rats during the diestrum period. Another study, conducted with male wistar rats, concluded that *Actb*, as well as *Gapdh*, were the most stable internal control genes in the cortex and hippocampus of rats maintained on a food regiment of dietary restriction until age of 12 and 18 months [35].

Several criteria must be taken into consideration for the HKG selection, such as constant mRNA expression in all samples analyzed regardless of experimental conditions. Besides, the amplification needs to be specific for RNA (primers must be design to anneal at exon-exon junctions, to prevent DNA amplification) and this expression must be comparable to that of the target genes [12]. However, it has become clear in recent years that no HKG, even constitutively expressed in all cells, exhibits stable gene expression and thus requires a more detailed literary analysis to choose the ideal HKG gene for the experimental conditions in question. To date, no study has systematically validated which constitutive genes are most stable in HPT and VTA for application in an animal model that evaluates the effects of nutrition in the dopaminergic system.

According to a systematic literature review by Chapman and Waldenstrom [11], the most commonly used HKGs in RNA quantification and gene expression studies are *GAPDH* and/or *ACTB*. They concluded this from the analysis of 128 articles in which the following criteria met: the study was conducted with vertebrates; complete accessibility to the article; gene expression results were reported. Yet, few studies (15%)

performed a panel of potential internal control genes with the aim of normalizing the values of gene expression. However, their stability of expression depends on the tissue and experimental conditions, so the importance of selecting the best HKGs to generate reliable and reproducible results.

However, there is a lack of studies that have proposed to evaluate the stability of *Ppia* in brain areas, especially those related to food reward, such as VTA and HPT. What is noteworthy is that *Ppia* is shown to be a stable internal control gene in different studies of gene expression, nonetheless some studies with different models that analyzed different areas indicate that this gene do not fit as an internal control. Gholami, Loh [36] did an experiment with the objective of selecting suitable endogenous reference genes for qPCR in kidney and hypothalamus under testosterone influence. They concluded that *Ppia* was the most stable HKG in HPT, among *Gapdh*, *Actb,* fosoribosyltransferase 1 (*Hprt)* and beta-2-microglobulin (*B2m)*, which is in line with our results in mice dams, where *Ppia* presented stability value of 4.671 while *Gapdh* 8.831.

The influence of diet on gene expression begins from intrauterine life. Ruegsegger, Grigsby [37] demonstrated through a mouse experiment that a hypercaloric diet during pregnancy has an influence on the risk of obesity and interferes in the development of the offspring. Likewise, Giraudo, Della-Fera [30] evaluated the influence of maternal diet under hypothalamic gene expression over three generations in mice. However, we did not find in the literature studies that proposed to analyze the most stable HKGs in brain areas related to reward and hunger in an animal model in the gestational period. The present study described for the first time the adequacy of three HKGs for RT-qPCR experiments in HPT and VTA in females during gestation and lactation and their male offspring.

CONCLUSION

The validation of the reference genes is a fundamental step to obtain reliable and reproducible data in the RT-qPCR, considering the possible stability variation of particular HKGs according to the tissue analyzed and the applied methodology. In this study, we conclude that the best reference gene for female mice during pregnancy and lactation periods with a diet intervention in VTA is *Actb*; while in HPT it is *Ppia*. We also described the best HKG for the male offspring submitted to the same diets, and

detected *Ppia* in VTA and *Actb* in HPT. Therefore these results contribute to subsequent studies of gene expression that use a model similar to the described in this manuscript.

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29. Plut, C., et al., *Gender differences in hypothalamic tyrosine hydroxylase and alpha(2)-adrenoceptor subtype gene expression in cafeteria diet-induced hypertension and consequences of neonatal androgenization.* J Pharmacol Exp Ther, 2002. **302**(2): p. 525-31.
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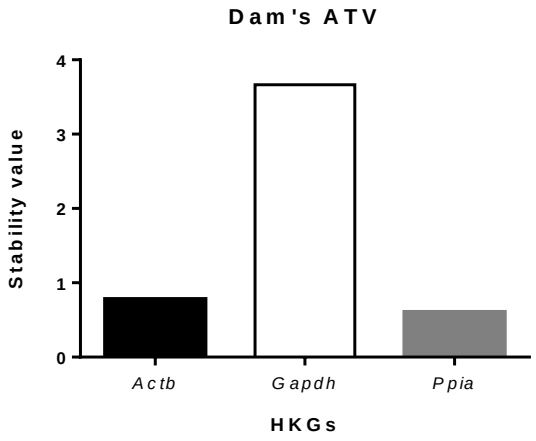
Figure 1: Housekeeping genes in the ventral tegmentar area (VTA) of mice dams and their offspring.

a)



b)

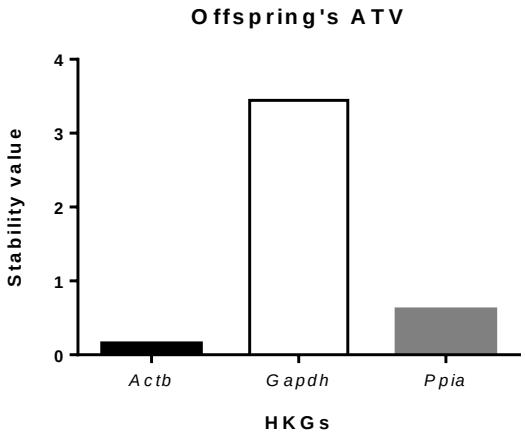
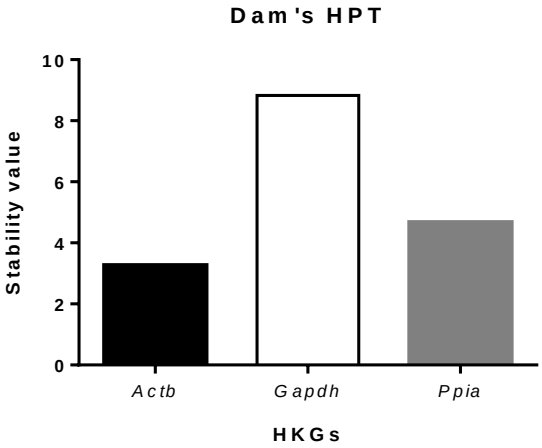
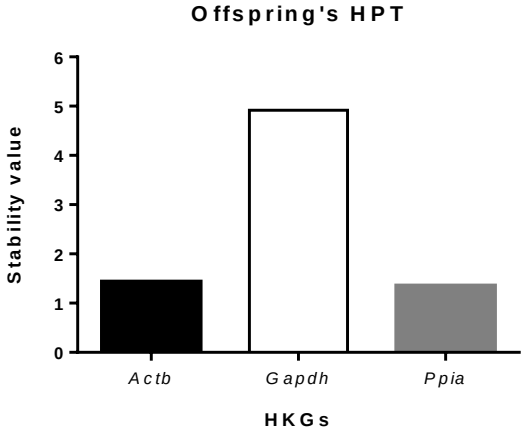


Figure 2: Housekeeping genes in the hypothalamus (HPT) of mice dams and their offspring.

a)



b)



9. CURRÍCULO LATTES

Vanessa Feistauer

Endereço para acessar este CV: http://lattes.cnpq.br/8194248147711296
Última atualização do currículo em 07/08/2019

Bacharel em Biomedicina (2012) pela Fundação Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), com habilitações em Análises Clínicas, Biologia Molecular e Microbiologia. Mestre em Ciências da Saúde, área de Biologia Celular e Molecular (2015), pela UFCSPA. Doutora em Biociências (2019), pela UFCSPA. **(Texto informado pelo autor)**

Identificação

Nome Vanessa Feistauer
Nome em citações bibliográficas FEISTAUER, V.;FEISTAUER, VANESSA

Endereço

Formação acadêmica/titulação

2015 - 2019 Doutorado em Biociências.
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.
Título: Sistema dopaminérgico e dieta: uma investigação de variantes genéticas em humanos e expressão gênica em roedores, Ano de obtenção: 2019.
Orientador: Silvana de Almeida.
Coorientador: Márcia Giovenardi.
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil.
Palavras-chave: sistema dopaminérgico; dieta; variantes genéticas; expressão gênica.
Grande área: Ciências Biológicas
Grande Área: Ciências Biológicas / Área: Fisiologia / Subárea: fisiologia.

2013 - 2015 Mestrado em Ciências da Saúde (Conceito CAPES 5).
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.
Título: VARIANTES EM GENES RELACIONADOS AO SISTEMA DOPAMINÉRGICO VERSUS PADRÃO DE INGESTÃO ALIMENTAR E PARÂMETROS DE ADIPOSIDADE EM CRIANÇAS: VALIDAÇÃO DE UM ESTUDO DE NUTRIGENÉTICA,Ano de Obtenção: 2015.
Orientador: Silvana de Almeida.
Coorientador: Vanessa Suñé Mattevi.
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil.

2009 - 2012 Graduação em Biomedicina.
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.

2006 - 2009 Curso técnico/profissionalizante.
Centro Sinodal de Ensino Médio Dorothea Schäfke, CSEMDS, Brasil.

Formação Complementar

2011 - 2011 Extensão universitária em Curso de Extensão em Uroanálise do Curso de Biomed. (Carga horária: 12h).
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.

Atuação Profissional

Hospital Materno Infantil Presidente Vargas, HMIPV, Brasil.
Vínculo institucional
2012 - 2012 Vínculo: Estágio Curricular, Enquadramento Funcional: Estagiária, Carga horária: 30

Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.
Vínculo institucional
2013 - 2019 Vínculo: Bolsista, Enquadramento Funcional: Pesquisa e Desenvolvimento, Carga horária: 40, Regime: Dedicação exclusiva.
Vínculo institucional
2012 - 2012 Vínculo: Monitoria Voluntária UFCSPA, Enquadramento Funcional: Monitora voluntária, Carga horária: 2
Vínculo institucional
2012 - 2012 Vínculo: Estágio Curricular, Enquadramento Funcional: Estagiária, Carga horária: 30
Vínculo institucional
2011 - 2012 Vínculo: Bolsista, Enquadramento Funcional: Monitoria, Carga horária: 8
Outras informações Programa de Educação pelo Trabalho para a Saúde (PET-SAÚDE), instituído pela Portaria Interministerial MS/MEC nº 1.802/2008.
Vínculo institucional
2010 - 2012 Vínculo: Iniciação Científica, Enquadramento Funcional: Estágiária voluntária, Carga horária: 20
Outras informações Laboratório de Cocos Gram-Positivos UCSPA
Vínculo institucional
2010 - 2010 Vínculo: Monitoria Voluntária, Enquadramento Funcional: Monitora voluntária, Carga horária: 4
Outras informações Monitoria disciplina de Informática I do curso de Biomedicina

Faculdade Cenecista de Osório, FACOS, Brasil.
Vínculo institucional
2016 - 2016 Vínculo: Professor Visitante, Enquadramento Funcional: Professora Colaboradora

Áreas de atuação

1. Grande área: Ciências Biológicas / Área: Genética / Subárea: genética.
2. Grande área: Ciências Biológicas / Área: Fisiologia / Subárea: fisiologia.

Idiomas

Inglês Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.
Português Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.

Prêmios e títulos

2018 Trabalho destaque no III Encontro do PPG Biociências & Encontro de Pesquisa em Biologia Celular, PPG Biociências - UFCSPA.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por
Ordem Cronológica

1. FISCH, JOANA ; **FEISTAUER, VANESSA** ; DE MOURA, ANA CAROLINA ; SILVA, ANDREW OLIVEIRA ; BOLLIS, VANESSA ; PORAWSKI, MARILENE ; ALMEIDA, SILVANA ; GUEDES, RENATA PADILHA ; BARSCHAK, ALETHEA GATTO ; GIOVENARDI, MÁRCIA . Maternal feeding associated to post-weaning diet affects metabolic and behavioral parameters in female offspring. PHYSIOLOGY & BEHAVIOR JCR, v. 204, p. 162-167, 2019.

2. **FEISTAUER, VANESSA**; CAMPAGNOLO, PAULA D.B. ; MATTEVI, VANESSA S. ; ALMEIDA, SILVANA . Evaluation of association of DRD2 TaqIA and -141C InsDel polymorphisms with food intake and anthropometric data in children at the first stages of development. GENETICS AND MOLECULAR BIOLOGY (ONLINE VERSION) JCR, v. XX, p. XX-XX, 2018.

Produção técnica

Redes sociais, websites e blogs

1. ALMEIDA, SILVANA ; **FEISTAUER, V.** ; FIEGENBAUM, M. ; GENRO, J. ; ALVES, A. ; SZCZESNY, L. ; RODRIGUES, M. ; LEOPOLDINO, I. ; COMARU, L. ; MOUSQUER, G. T. . DNA Explica. 2019; Tema: Promoção e divulgação científica de assuntos relacionados à genética e à biologia molecular. (Rede social).

Demais tipos de produção técnica

Bancas

Participação em bancas de trabalhos de conclusão

Trabalhos de conclusão de curso de graduação

1. **FEISTAUER, V.**. Participação em banca de Pâmela Victoria Von Burg.AVALIAÇÃO DA EXPRESSÃO DO GENE GLUTATIONA PEROXIDASE 1 EM CARCINOMA HEPATOCELULAR. 2018. Trabalho de Conclusão de Curso (Graduação em Biomedicina) - Fundação Universidade Federal de Ciências da Saúde de Porto Alegre.
2. **FEISTAUER, V.**. Participação em banca de Gabriela Barros Pires.INVESTIGAÇÃO DA ASSOCIAÇÃO DE POLIMORFISMOS NO GENE ESR1 COM SUSCETIBILIDADE AO CARCINOMA HEPATOCELULAR. 2016. Trabalho de Conclusão de Curso (Graduação em Biomedicina) - Fundação Universidade Federal de Ciências da Saúde de Porto Alegre.
3. **FEISTAUER, V.**. Participação em banca de Mirelen Moura de Oliveira Rodrigues.ANÁLISE DO POLIMORFISMO 2561C>T NO GENE SLC4A1 EM DOADORES DE SANGUE. 2015. Trabalho de Conclusão de Curso (Graduação em Biomedicina) - Fundação Universidade Federal de Ciências da Saúde de Porto Alegre.
4. **FEISTAUER, V.**. Participação em banca de GABRIELA WASKOW.SISTEMA SANGUÍNEO KELL: AVALIAÇÃO DO POLIMORFISMO 1910>T NO GENE KELL EM DOADORES DE SANGUE. 2015. Trabalho de Conclusão de Curso (Graduação em Biomedicina) - Fundação Universidade Federal de Ciências da Saúde de Porto Alegre.

Eventos

Participação em eventos, congressos, exposições e feiras

1. III Encontro do PPG Biociências & Encontro de Pesquisa em Biologia Celular.AVALIAÇÃO DO EFEITO DE DIETA HIPERCALÓRICA E DE RESTRIÇÃO DURANTE A GESTAÇÃO E LACTAÇÃO NA EXPRESSÃO DE mRNA DE Drd2 E Th EM CAMUNDONGOS GENITORAS. 2018. (Encontro).
2. Minicurso Sequenciamento de nova geração (next generation sequencing) no III Encontro do PPG Biociências & Encontro de Pesquisa em Biologia Celular. 2018. (Outra).
3. XLI Reunião Anual da SBNeC. AVALIAÇÃO DE COMPORTAMENTO DE ANSIEDADE, ATIVIDADE LOCOMOTORA E MEMÓRIA DE CAMUNDONGOS LACTANTES SUBMETIDAS A DIETAS HIPERCALÓRICA E DE RESTRIÇÃO. 2018. (Congresso).
4. 37ª Semana Científica HCPA.AVALIAÇÃO DE COMPORTAMENTO DE ANSIEDADE, ATIVIDADE LOCOMOTORA E MEMÓRIA DE CAMUNDONGOS LACTANTES SUBMETIDAS A DIETAS HIPERCALÓRICA E DE RESTRIÇÃO. 2017. (Outra).
5. 38th World Congress of the International Union of Physiological Sciences at the Riocentro Convention Center. MOTOR ACTIVITY, ANXIETY-LIKE AND MEMORY BEHAVIORS EVALUATION OF LACTATING SUBMITTED TO HYPERCALORIC AND RESTRICTION DIETS. 2017. (Congresso).
6. II Encontro do PPG Biociências da UFCSPA & Econtro de Pesquisa em Fisiologia do RS.AVALIAÇÃO DE COMPORTAMENTO DE ANSIEDADE, ATIVIDADE LOCOMOTORA E MEMÓRIA DE CAMUNDONGOS LACTANTES SUBMETIDAS A DIETAS HIPERCALÓRICA E DE RESTRIÇÃO. 2017. (Encontro).
7. Seminars in Neurosciences. 2017. (Seminário).
8. I Encontro do Programa de Pós Graduação em Biociências, XIX Encontro de Geneticistas do Rio Grande do Sul e I Encontro Regional Sul da Sociedade Brasileira de Genética Médica.ANÁLISE DOS EFEITOS EPIGENÉTICOS DA NUTRIÇÃO MATERNA NO SISTEMA DOPAMINÉRGICO DA PROLE EM MODELO ANIMAL. 2016. (Encontro).
9. II Congresso Latino-Americano de Resistência Microbiana e IX Sul Encontro de Controle de Infecção. Avaliação de formação de biofilme em amostras de Staphylococcus coagulase-negativo isoladas na cidade de Porto Alegre. 2011. (Congresso).
10. II Congresso Latino-Americano de Resistência Microbiana e IX Sul Encontro de Controle de Infecção. 2011. (Congresso).
11. IV Congresso Internacional de Bioanálises, VII Congresso Sul-Brasileiro de Biomedicina e XI Semana Gaúcha de Biomedicina. 2011. (Congresso).
12. IV Mini-Curso de Capacitação de Jovens Cientistas da UFCSPA. 2011. (Outra).
13. IV Semana Científica da UFCSPA e I Semana de Tecnologia e Inovação. 2011. (Outra).
14. IV Semana Científica da UFCSPA e I Semana de Tecnologia e Inovação.Avaliação da Atividade Antimicrobiana da Tigeciclina em Diferentes Meios de Ágar Mueller-Hinton. 2011. (Outra).
15. VII Jornada Acadêmica de Biomedicina UFCSPA e III Simpósio Habilitações da Biomedicina. 2011. (Simpósio).
16. V Simpósio Brasileiro de Microbiologia Aplicada. 2011. (Simpósio).
17. I I Simpósio Internacional de Microbiologia Clínica:.Avaliação da Atividade Antimicrobiana da Tigeciclina em Diferentes Meios de Ágar Mueller-Hinton. 2010. (Simpósio).
18. V Congresso Brasileiro de Células-Tronco e Terapia Celular. 2010. (Congresso).
19. I Ciclo de Palestras Sobre Saúde e Meio Ambiente da UCSPA. 2009. (Outra).
20. I Curso Teórico-Prático de Colheita de Sangue Venoso da UFCSPA. 2009. (Outra).
21. Simpósio de Terapias Inovadoras. 2009. (Simpósio).

Orientações

Orientações e supervisões concluídas

Trabalho de conclusão de curso de graduação

1. Bruna Eliza Nordio da Silva. ANÁLISE DA ESTABILIDADE DE HOUSEKEEPING GENES SOBRE A EXPRESSÃO GÊNICA EM DIFERENTES ÁREAS CEREBRAIS EM MODELO ANIMAL. 2018. Trabalho de Conclusão de Curso. (Graduação em Biomedicina) - Fundação Universidade Federal de Ciências da Saúde de Porto Alegre. Orientador: Vanessa Feistauer.
2. Carolina Kalkmann da Silva Oliveira. ANÁLISE DA EXPRESSÃO GÊNICA DO RECEPTOR DE DOPAMINA D2 NA ÁREA TEGMENTAR VENTRAL DE CAMUNDONGOS FÊMEAS LACTANTES SUBMETIDAS A TRÊS DIFERENTES DIETAS. 2018. Trabalho de Conclusão de Curso. (Graduação em Biomedicina) - Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul. Orientador: Vanessa Feistauer.

Educação e Popularização de C & T

Redes sociais, websites e blogs

1. ALMEIDA, SILVANA ; **FEISTAUER, V.** ; FIEGENBAUM, M. ; GENRO, J. ; ALVES, A. ; SZCZESNY, L. ; RODRIGUES, M. ; LEOPOLDINO, I. ; COMARU, L. ; MOUSQUER, G. T. . DNA Explica. 2019; Tema: Promoção e divulgação científica de assuntos relacionados à genética e à biologia molecular. (Rede social).

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