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O BALANÇO GABA-GLUTAMATO NA SUSCETIBILIDADE AO TRANSTORNO DE DÉFICIT DE ATENÇÃO/HIPERATIVIDADE E IMPLICAÇÕES FARMACOGENÉTICAS

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Sumário

3'UTR – região 3' não traduzida

5HTT – gene do transportador de serotonina

ADRA2A – receptor adrenérgico alfa-2A

AMPA – α-amino-3-hidroxi-5-metil-4-isoxazolpropiônico

ANKK1 – gene da proteína quinase1

AREB6 – elemento regulatório ATP1A1 de ligação ao fator 6

BDNF – fator neurotrófico derivado do cérebro

CDH13 – gene da caderina 13

CES1 – gene carboxilesterase 1

CNV – variação de número de cópias

COMT- catecol-O-metiltransferase

CPF – córtex pré-frontal

CYP2C9 – gene do citocromo P450 família 2 subfamília C membro 9

CYP2D6 – gene do citocromo P450 família 2 subfamília D membro 6

DA – dopamina

DAT – proteína transportadora de dopamina

DAT1 – gene do transportador de dopamina

DC-VC – hipótese doença comum, variante comum

DC-VR – hipótese doença comum, variante rara

DNA – ácido desoxirribonucleico

DRD2– gene do receptor D2 de dopamina

DRD4 – gene do receptor D4 de dopamina

DRD5 – gene do receptor D5 de dopamina

DSM-5 – Manual de Diagnóstico e Estatística de Transtornos Mentais, quinta edição

DSM-IV – Manual de Diagnóstico e Estatística de Transtornos Mentais, quarta edição

E/I – excitatório/inibitório

ER – liberação estendida

FDA – Food Drug Administration

FLTR3 – gene da a proteína transmembrana rica em leucina fibronectina 3

GABA – ácido γ-aminobutírico

GABRA1 – gene do receptor $GABA_A$ sunidade α1

GABRA6 - gene do receptor $GABA_A$ sunidade α6

GABRB2 - gene do receptor $GABA_A$ sunidade β2

GABRP - gene do receptor $GABA_A$ sunidade ρ

GAD1 –gene do ácido glutâmico descarboxilase 1

GAT – transportador de glutamato

GKAP - proteína de ancoragem quinase G

GPCR – receptor ligado a proteína G

GRIN2A – gene da subunidade 2A do receptor glutamatérgico NMDA

GRIN2B – gene da subunidade 2B do receptor glutamatérgico NMDA

GRM5 – gene do receptor metabotrópico de glutamato 5

GRM7 – gene do receptor metabotrópico de glutamato 7

GWAS – estudo de associação por varredura genômica

H3K4me3 – trimetilação da lisina 4 na histona H3

HER2 – gene do fator de crescimento epidermal

HTR1B – gene do receptor 1B de serotonina

IC – intervalo de confiança

iGluR – receptor ionotrópico de glutamato

IR – liberação imediata

KA – cainato

Kb – quilobase

KDa – quilo Daltons

LPHN3 – gene da latrofilina 3

MAO – monoamina-oxidase

MFD – metilfenidato

mGluR – receptor metabotrópico de glutamato

MYOD – fator determinante de mioblasto

NA – noradrenalina

NAA/Cr - razão do N-acetil-aspartato pela creatina

NCAM1 – genes da molécula de adesão neural 1

NET – proteína transportadora de noradrenalina

NMDA – N-metil-D-aspartato

NOS1 – gene do óxido nítrico sintetase

OR – razão de chances

pb – pares de base

QI – quociente de inteligência

SHANK3- gene da proteína SHANK3

SICI – inibição intercortical curta

SLC1A3 – gene do transportador de glutamato de alta afinidade

SLC6A1 – gene do transportador de GABA

SLC6A3 – gene do transportador de dopamina

SLC6A4 – gene do transportador de serotonina

SLC9A9 – gene do soluto carregador família 9 membro A9

SNAP-25 – proteína 25kD associada ao sinaptossomo

SNP – polimorfismo de nucleotídeo único

SNV – variação de nucleotídeo único

TDAH – transtorno de déficit de atenção e hiperatividade

TEA – transtorno do espectro autista

TEN1- teneurina1

TF – fator de transcrição

TPH2 – gene triptofano hidroxilase 2

TTC12 – gene da proteína com domínio de repetição tetratricopeptideo 12

UNC-5 – receptor de guias axonais descoordenado-5

VKORC1 – gene da vitamina K óxido-redutase

VNTR – número variável de repetições em tandem

O Transtorno de Déficit de Atenção/Hiperatividade (TDAH) está entre as doenças psiquiátricas mais comuns na infância e adolescência, determinando prejuízo significativo na qualidade de vida dos pacientes. Os estudos moleculares nos últimos anos não foram suficientes para entender a base genética do transtorno nem esclarecer quais os genes envolvidos, devido ao pequeno efeito de cada um e da interação entre genes e destes com fatores ambientais. Estudos recentes sugerem que os sintomas de hiperatividade/impulsividade do TDAH podem ser devido à falha da inibição comportamental resultante de uma modulação inapropriada das sinalizações GABA e glutamato. É possível que os déficits de transmissão de catecolaminas presentes no TDAH sejam um efeito secundário do desbalanço excitatório/inibitório. Os estudos de variantes genéticas envolvidas na variabilidade de resposta ao tratamento com o metilfenidato, o fármaco mais utilizado para tratar o TDAH, poderiam fornecer dados que potencialmente poderiam auxiliar no aprimoramento do tratamento. Portanto o presente trabalho teve como objetivo geral investigar o papel de variantes nos genes *LPHN3, GAD1, GABRA1, GABRB2* e *GAT1* na suscetibilidade e na farmacogenética do TDAH. Ao longo da Tese, os resultados foram organizados em quatro artigos. No primeiro, o estudo de associação entre a *LPHN3* e o TDAH avaliou 523 crianças e adolescentes com TDAH e 132 controles, o estudo farmacogenético avaliou 172 crianças através da escala de sintomas SNAP-IV no momento da prescrição, no primeiro e terceiro mês de tratamento. Os resultados sugerem o haplótipo CGC derivado dos SNPs rs6813183, rs1355368 e rs734644 como sendo de risco para o TDAH (P = 0,02; OR = 1,46; IC95%: 1.07 – 1,97). A interação desse haplótipo com o SNP rs965560, localizado no cromossomo 11q, aumenta levemente o risco ao nível nominal (P = 0,03; OR = 1,55; IC95%: 1,02 - 2,36). Pacientes homozigotos para o haplótipo CGC mostraram uma resposta mais rápida ao tratamento, com interação significante entre o haplótipo e o tratamento ao longo do tempo ($P < 0,001$). Homozigotos para o haplótipo GT derivado dos SNPs rs6551665 e rs1947275 mostraram uma interação nominalmente significante com o tratamento ao longo do tempo (P = 0,04). Nossos resultados replicaram estudos prévios, demonstrando que a *LPHN3* confere suscetibilidade ao TDAH, interage com o cromossomo 11q e modula a resposta ao metilfenidato. No segundo trabalho, o estudo de associação de *GAD1* e o TDAH avaliou 547 crianças com TDAH e seus pais biológicos, e os sintomas de hiperatividade/impulsividade foram avaliados pela escala SNAP-IV em 323 crianças. Uma amostra composta de 2.000 crianças

que nasceram em Pelotas em 1993 e que foram avaliadas aos 10 e 15 anos para a escala de hiperatividade SDQ também foi analisada. Os resultados demonstram que o alelo C do SNP rs11542313 foi mais transmitido dos pais para crianças com TDAH (χ^2= 5,02; P = 0,03); os haplótipos de GAD1 foram associados com escores de sintomas de hiperatividade/impulsividade (χ^2= 8,98; P=0,01), sendo que o haplótipo CG derivado dos SNPs rs3749034 e rs11542313 confere os escores mais altos desses sintomas (IC95%: 0,02–0,43; P=0,03). Embora *GAD1* não tenha sido associado com os sintomas de hiperatividade na amostra populacional, nossos resultados na amostra clínica sugerem que o *GAD1* está associado à suscetibilidade do TDAH, contribuindo principalmente para os sintomas de hiperatividade/impulsividade. No terceiro trabalho, avaliamos SNPs nos genes GABRA1, GABRB2 e GAT1 através de análises caso-controle e baseadas em famílias. A amostra compreendeu 542 crianças com TDAH e seus pais biológicos e 132 controles. Não houve evidência de associação entre esses polimorfismos e a suscetibilidade ao TDAH (valores de *P* entre 0,108 e 0, 937). O quarto trabalho foi uma revisão sistemática entre os anos de 2010 e 2013 sobre os estudos farmacogenéticos do TDAH. Nós pudemos comprovar o aumento exponencial dos estudos marcados principalmente pela mudança de foco do sistema dopaminérgico para outros genes de neurotransmissão e de neurodesenvolvimento; e o aumento de estudos com novas abordagens. Contudo, a heterogeneidade metodológica ainda provavelmente determina as divergências encontradas na literatura. A presente Tese acrescenta dados de uma rota biológica pouco explorada que é o balanço excitatório/inibitório gerado por glutamato e GABA, e que pode estar envolvida na etiologia do TDAH. Mais estudos são necessários para reiterar a participação desses sistemas e aumentar a compreensão dos mecanismos moleculares na suscetibilidade ao TDAH.

Abstract

Attention-Deficit/Hyperactivity Disorder (ADHD) is one of the most common psychiatric disorders in children and adolescents, leading to significant impairments in the life quality of patients. At present, molecular studies still did not disclose the genetic basis of the disorder or clarified which genes are implicated in the disorder, due to the small effect of each variant and the interaction between genes and among them with environmental factors. Recent studies have suggested that ADHD hyperactive/impulsive symptoms are due to behavioral disinhibition resulting from inappropriate modulation of both glutamatergic and GABAergic signaling. It is possible that deficits in catecholaminergic transmission observed in ADHD may arise as a secondary effect of excitatory/inhibitory imbalance. Genetic variants studies involved in the variability of methylphenidate response, the most widely drug used to treat ADHD, could provide data that potentially help to improve the treatment management. The aim of the present study was to investigate the contribution of single nucleotide polymorphisms (SNPs) at *LPHN3, GAD1, GABRA1, GABRB2* e *GAT1* genes to ADHD susceptibility and pharmacogenetics. First, the association between *LPHN3* and ADHD was evaluated in 523 children and adolescents with ADHD and 132 controls. In the pharmacogenetics study, 172 children with ADHD were investigated. The primary outcome measure was the parent-rated Swanson, Nolan, and Pelham Scale - version IV (SNAP-IV) applied at baseline, first and third months of treatment with methylphenidate. The results suggest that the CGC haplotype derived from SNPs rs6813183, rs1355368, rs734644 as an ADHD risk haplotype (P = 0.02; OR = 1.46; CI95%: 1.07 – 1.97). Its interaction with the 11q chromosome SNP rs965560 slightly increases risk, at nominal level (P = 0.03; OR = 1.55; CI95%: 1.02 – 2.36). Homozygous individuals for the CGC haplotype showed a faster response to MPH treatment as a significant interaction effect between CGC haplotype and treatment over time ($P < 0.001$). Homozygous individuals for the GT haplotype derived from SNPs rs6551665 and rs1947275 showed a nominally significant interaction with treatment over time (P = 0.04). Our findings replicate previous findings reporting that *LPHN3* confers ADHD susceptibility, interacts with 11q region, and moderates MPH treatment response in children and adolescents with ADHD. In the second paper, the association study evaluated a clinical sample that consisted of 547 families with ADHD probands and hyperactive/impulsive symptoms which were evaluated based on SNAP-IV scores. Also, a population-based sample comprised of 2,000 individuals from the Pelotas

1993 Birth Cohort Study was evaluated for SDQ hyperactivity subscale collected at both 11 and 15 years assessments. The *C* allele of rs11542313 was significantly overtransmitted from parents to ADHD probands (χ^2= 5.02; P = 0.03). *GAD1* haplotypes were associated with higher hyperactive/impulsive scores in ADHD youths (χ^2= 8.98; P=0.01). In the specific haplotype test, the GC haplotype was the one with the highest hyperactive/impulsive scores (CI 95%: 0.02–0.43; P=0.03). Even though *GAD1* was not associated with ADHD symptoms in the general population sample, our results from the clinical sample suggest that the *GAD1* gene is associated with ADHD susceptibility, contributing particularly to the hyperactive/impulsive symptoms domain. The SNPs at GABRA1, GABRB2 e GAT1 genes were investigated by family-based and case-control approaches. The sample comprised 542 youths with ADHD and their parents, and 132 youths without ADHD as controls. There was no evidence of association between these polymorphisms and ADHD susceptibility (*P* values ranging from 0.108 to 0.937). The last paper was a systematic review of the literature on ADHD pharmacogenetics between 2010 and 2013. We observed that the number of studies continues to grow with a focus shift on ADHD pharmacogenetics studies from dopaminergic genes to other neurotransmitters and neurodevelopmental genes. An increasing number of studies using new approaches were also observed. However, the heterogeneity in methodological strategies of the studies likely explains the inconsistent results. All these findings add data about an underexplored pathway which is the excitatory/inhibitory balance induced by glutamate and GABA, and may be involved in ADHD etiology. Further studies are needed to elucidate the potential role of these systems and increase the understanding of molecular mechanisms to ADHD susceptibility.

CAPÍTULO I

Introdução

1. Considerações Gerais

O Transtorno de Déficit de Atenção/Hiperatividade (TDAH) é uma doença psiquiátrica comum que atinge cerca de 5,3 a 7,1% de crianças e adolescentes em idade escolar ao redor do mundo (Polanczyk et al. 2007; Willcutt 2012). Ele é caracterizado pelo desenvolvimento inapropriado e níveis prejudiciais de desatenção, hiperatividade e impulsividade de acordo com Manual de Diagnóstico e Estatística de Transtornos Mentais, versão 5 (DSM-5), onde são subdivididos em dois grupos comportamentais: desatenção e hiperatividade/impulsividade (American Psychiatric Association, 2013).

A frequência de meninos diagnosticados entre crianças e adolescentes é maior do que meninas e essa proporção pode variar de 2:1 a 9:1 (Bauermeister et al. 2007; Polanczyk and Jensen 2008). Essa diferença pode ser influenciada pela sub-representação de meninas que são encaminhadas para tratamento clínico. Enquanto meninos apresentam mais sintomas de hiperatividade/impulsividade e transtornos externalizantes, sintomas de desatenção são mais comuns em meninas, assim como transtornos internalizantes (Steinhausen 2009). Assim, é provável que haja um viés nessas estimativas, já que a probabilidade de que meninos sejam encaminhados para tratamento é maior (Rucklidge 2010). Já em adultos, a diferença entre os gêneros diminui e a distribuição tende a ser mais similar (Rasmussen and Levander 2009; Wilens et al. 2009).

O diagnóstico desse transtorno é fundamentalmente clínico, baseado nos critérios do DSM-5, a criança ou adolescente deve apresentar pelo menos seis sintomas de desatenção e/ou seis sintomas de hiperatividade/impulsividade por, no mínimo, seis meses, e com caracterização de prejuízo em atividades sociais, acadêmicas ou ocupacionais. Algumas mudanças nos critérios de diagnósticos foram estabelecidas pelo DSM-5 em relação à edição anterior, o DSM-IV (American Psychiatric Association, 1994). Entre as quais incluem a elevação da idade de início dos sintomas de sete para doze anos e a necessidade de cinco sintomas para o diagnóstico positivo de TDAH em adultos, e não seis, como estabelecido para crianças. Os sintomas reconhecidos pelo DSM-5 são apresentados na tabela 1 (American Psychiatric Association, 2013).

Tabela 1: Sintomas do TDAH de acordo com o DSM-5.

Desatenção	Hiperatividade/ Impulsividade
1. Dificuldade em organizar tarefas e atividades(;)	1. É inquieto com as mãos e os pés quando sentado;
2. Dificuldade em seguir instruções e finalizar tarefas;	2. Parece estar sempre com o "motor ligado" ;
3. Dificuldade em manter a atenção durante atividades ou brincadeiras;	3. Corre pelo ambiente e "escala" tudo, em momentos inapropriados;
4. Evita se engajar em tarefas que exijam esforço mental sustentado;	4. Dificuldade em brincar ou se engajar em atividades de lazer quieto;
5. Perda frequente de coisas necessárias à realização de tarefas;	5. Fala excessivamente;
6. Parece não estar ouvindo;	6. Dificuldade em ficar sentado, em sala de aula E outras situações ;
7. Fácil distração por estímulos externos;	7. Dá respostas impulsivas, sem esperar o final da pergunta;
8. Esquecimento em atividades diárias;	8. Dificuldade em esperar pela sua vez;
9. Não dá atenção a detalhes.	9. Interrompe os outros facilmente.

Os sintomas do TDAH frequentemente persistem ao longo da vida, sugerindo que o mesmo possa pode ser considerado uma doença crônica do desenvolvimento (Faraone et al. 2006). O curso clínico é bastante complexo e, embora as taxas de remissão sejam diversificadas, os fatores associados à persistência do TDAH até a vida adulta são bastante controversos na literatura. Diversos estudos sugerem que até 66% das crianças com TDAH apresentam sintomas após a adolescência (Barkley et al. 2002; Faraone et al. 2006; Lara et al. 2009). Além disso, os sintomas de hiperatividade/impulsividade tendem a diminuir com a idade, enquanto que os sintomas de desatenção, desorganização e distração são mais persistentes (Biederman et al. 2000).

Juntamente com o TDAH, altas taxas de comorbidades são observadas, sendo os transtornos disruptivos do comportamento, de ansiedade e de humor os mais frequentes (Spencer 2006). A presença de comorbidades parece influenciar na continuidade dos

sintomas, além de contribuir para o desenvolvimento de psicopatologias graves (Newcorn 2008). Os danos nas atividades pessoais e sociais devido ao TDAH são significativos ao longo da vida do indivíduo (Klein et al. 2012). Na infância, o transtorno está associado a um maior risco de baixo desempenho escolar, repetências e relações conturbadas com familiares e colegas. Na adolescência, indivíduos com TDAH são mais propensos a cometerem crimes, não finalizar o ensino médio e, além disso, fazer uso de drogas ilícitas. Adultos com TDAH sofrem com prejuízos em atividades profissionais, divórcios, prisões, internações psiquiátricas, maiores problemas na condução de veículos automotivos e possuem um risco maior de apresentar um comportamento antissocial associado ao abuso/dependência de drogas (Barkley 2004; Barkley et al. 2004; Wilens and Dodson 2004; Klein et al. 2012). Portanto, esses resultados destacam a importância para as famílias e a sociedade em geral de um acompanhamento e/ou tratamento prolongado de crianças com TDAH.

2. Tratamento farmacológico

Embora a intervenção psicossocial seja eficaz em muitos aspectos familiares e escolares, a terapia farmacológica tem um papel essencial no tratamento do TDAH (Kornfield et al. 2013). Muitos estudos documentaram a eficácia de estimulantes (por exemplo, o metilfenidato, anfetamina) e não-estimulantes (atomoxetina) na redução dos sintomas de TDAH, bem como na melhora do desempenho neuropsicológico em medidas de funções executivas (Greenhill et al. 2002; Blum et al. 2011).

O medicamento de primeira escolha e mais utilizado é o metilfenidato (MFD). Seu mecanismo de ação ocorre através do bloqueio do transportador de dopamina (DAT) (Faraone and Mick 2010). No entanto, sabe-se que o MFD também bloqueia com eficiência o transportador da norepinefrina (NET) (Hannestad et al. 2010). Alguns estudos têm demonstrado a melhora induzida pelo MFD sobre o funcionamento do córtex pré-frontal, onde a densidade de NET é maior (Madras et al. 2005; Hannestad et al. 2010). O fármaco também induz o aumento da excitabilidade das células corticais, que é mediada pela ativação dos receptores adrenérgicos alfa-2-A (ADRA2A) (Andrews and Lavin 2006). As formulações orais comumente prescritas são de liberação imediata (IR) ou de liberação

estendida (ER). Uma alternativa para a administração oral é o sistema transdérmico que proporciona a liberação de MFD através da pele (Anderson and Scott 2006). Todos eles apresentam boa resposta, reduzindo em cerca de 50% os sintomas em aproximadamente 70% de crianças, embora as farmacocinéticas sejam bem diferentes. (Faraone and Buitelaar 2010). Os efeitos adversos do metilfenidato não são graves, mas podem ter impacto nos domínios do funcionamento biológico, psicológico e social a curto e longo prazo (Taylor et al. 2004). Estudos randomizados controlados do MFD sugerem que a insônia, a redução do apetite e a cefaléia são particularmente comuns em curto prazo (Graham and Coghill 2008). Há também algumas evidências de aumento dos níveis de emotividade, no retraimento social, em náuseas e dores abdominais. Em ensaios clínicos de tratamento de pacientes nunca medicados, uma proporção relativamente pequena, mas significativa dos participantes, sofrem persistentes reações adversas ao MFD e que levam a retirada do medicamento, mesmo com os ajustes de dose. Estes efeitos adversos são mais acentuados entre crianças pré-escolares (Wigal et al. 2006), e crianças com déficit intelectual e social (Aman *et al.* 2005).

Uma opção alternativa de tratamento são as anfetaminas que aumentam concentrações sinápticas de neurotransmissores na fenda sináptica. Elas competem com as monoaminas endógenas para o transporte até os terminais nervosos e, uma vez dentro do terminal pré-sináptico, a anfetamina desloca monoaminas da vesícula citosólica, bombeando neurotransmissores dos neurônios para a sinapse. Neste mecanismo ocorre também a inibição da recaptação e, provavelmente, a inibição da monoamina-oxidase (MAO), aumentando sinergicamente as concentrações de neurotransmissores na sinapse (Heal et al. 2013). Assim como o MFD, as anfetaminas têm diferentes formulações como IR, ER e o pró-fármaco lisdexamfetamina.

Medicamentos não-estimulantes, como a atomoxetina, também são intervenções psicofarmacológicas alternativas para o tratamento do TDAH. A atomoxetina é um inibidor altamente específico do NET com elevada eficácia do tratamento (Michelson et al. 2001). Os agonistas α-2 (guanfacina e clonidina), que inicialmente eram agentes anti-hipertensivos, também demonstraram redução dos sintomas do TDAH.

Em 2011, o *Food and Drug Administration* (*FDA*), órgão regulamentador de medicamentos dos Estados Unidos, aprovou a prescrição da formulação de liberação

extendida de guanfacina e clonidina para o tratamento de crianças e adultos com o transtorno (Inutiv, 2011).

Embora os agentes farmacológicos sejam considerados eficazes e seguros, existe uma considerável variabilidade interindividual entre os pacientes sobre a dosagem, resposta à medicação e ocorrência de eventos adversos (Greenhill et al. 1996; Vaughan and Kratochvil 2006). Devido a esta variabilidade, o tratamento é, muitas vezes, determinado empiricamente na prática clínica através de uma titulação de dose gradual e uma abordagem de tentativa e erro.

3. Neurobiologia

O estudo pioneiro que propôs que os sintomas do transtorno resultariam de anormalidades nos sistemas de catecolaminas foi desenvolvido por Wender e cols. (1971). Pacientes com TDAH teriam níveis de dopamina (DA) e de noradrenalina (NA) diminuídos, sendo que a medicação estimulante usada para tratar os sintomas compensaria esses déficits (Wender et al. 1971). Apesar de ambos os neurotransmissores terem um papel importante na fisiopatologia do TDAH, o sistema dopaminérgico foi o foco principal das investigações (Genro et al. 2010). A hipótese dopaminérgica postulada por Levy (1991) sugere que déficits de DA em regiões cerebrais corticais e no estriado causariam os sintomas desse transtorno.

No cérebro há quatro vias dopaminérgicas bem definidas: a nigroestriatal que é parte do sistema extrapiramidal e controla os movimentos; a mesolímbica que, se projetando para o núcleo *accumbens*, se relaciona com o comportamento e a sensação de prazer; a via mesocortical, que ao se projetar na área tegmental ventral do mesencéfalo, atinge o córtex límbico onde atua sobre a função cognitiva e no controle motor frontal e a tuberoinfundibular que na hipófise controla a secreção de prolactina (van der Kooij and Glennon 2007). Entretanto, apenas as vias mesocortical e nigroestriatal estariam implicadas no TDAH; uma hipofunção nas áreas corticais seria responsável por déficits cognitivo e das funções executivas, ou seja, do conjunto de funções responsáveis pelo início e desenvolvimento de uma atividade com objetivo final determinado (Davids *et al*. 2003; Cardinal *et al*. 2004). Estudos com modelos animal, neuropsicologia e neuroimagem estrutural e funcional geraram evidências que corroboraram com a teoria dopaminérgica e

complementaram que o envolvimento desse sistema é mais complexo do que proposto originalmente, bem como sugerem a participação de outras redes neuronais e de neurotransmissores (Makris et al. 2009; Faraone and Mick 2010; Genro et al. 2010b).

Os estudos de neuroimagem contribuiram de forma significativa para o esclarecimento das bases neuronais do TDAH ao localizar anormalidades nas regiões cerebrais e redes neuronais associadas à cognição e comportamento consistentes com as características clínicas (Valera et al. 2007). Indivíduos com TDAH possuem volumes cerebral e cerebelar significantemente diminuídos em comparação a controles saudáveis. A redução geral que é observada pode ser devido a uma redução da espessura e um atraso na maturação do córtex pré-frontal (Sowell et al. 2003; Shaw et al. 2006). O córtex pré-frontal (CPF) é responsável pela atenção, planejamento, organização, além de regular respostas comportamentais através do controle inibitório (Arnsten 2009). Testes neuropsicológicos mostraram que crianças com TDAH têm desempenho prejudicado em funções cognitivas e executivas que exigem as características acima, reforçando o envolvimento do lobo frontal e áreas subcorticais (Swanson et al. 1998; Arnsten and Li 2005).

Outras anormalidades cerebrais encontradas em pacientes com TDAH estão nos lobos temporoparietais, gânglios da base (núcleo caudado e globo pálido) corpo caloso, cerebelo, amígdala, hipocampo e tálamo (Cortese 2012). Os sistemas corticoestrital e corticocerebelar consistem de circuitos paralelos que subservem comportamentos motores, cognitivos e emocionais. No sistema corticoestrial, o caudado e o putâmen são alvos complementares de projeções que saem da região neocortical para o estriado (Makris et al. 1999). As projeções corticopalidal são pré-motores e de córtices motor e somatosensorial primário (Künzle 1977). O circuito frontocerebelar conecta as regiões frontocortical com o cerebelo através de uma alça . O cerebelo contribui à organização de ordem superior das funções do cérebro e ele é topologicamente ligado a diferentes áreas sensório-motoras primárias através de ponte por meio da via de alimentação para a frente e, por meio do tálamo através da retroalimentação (Haines et al. 1997; Schmahmann and Sherman 1998). Déficits motores são características do TDAH como se movimentar ou falar excessivamente em situações inapropriadas, falta de habilidade motora fina, bem como, força de controle e inconveniência (Pitcher et al. 2002; Pitcher et al. 2003).

Disfunções no circuito de recompensa também são uma característica central do TDAH. Esse circuito consiste principalmente da amígdala, núcleo *accumbens*, estriado, tálamo, prosencéfalo basal , tronco cerebral límbico e áreas corticais e as disfunções nessas áreas levam a uma redução do controle exercido pelas recompensas futuras sobre o comportamento atual, onde há uma diminuição no seu " valor " para recompensas imediatas e um aumento à medida que é necessário esperar (Sonuga-Barke 2005). A neuroanatomia funcional dos circuitos envolvidos na patofisiologia do TDAH pode ser observada na Figura 1.

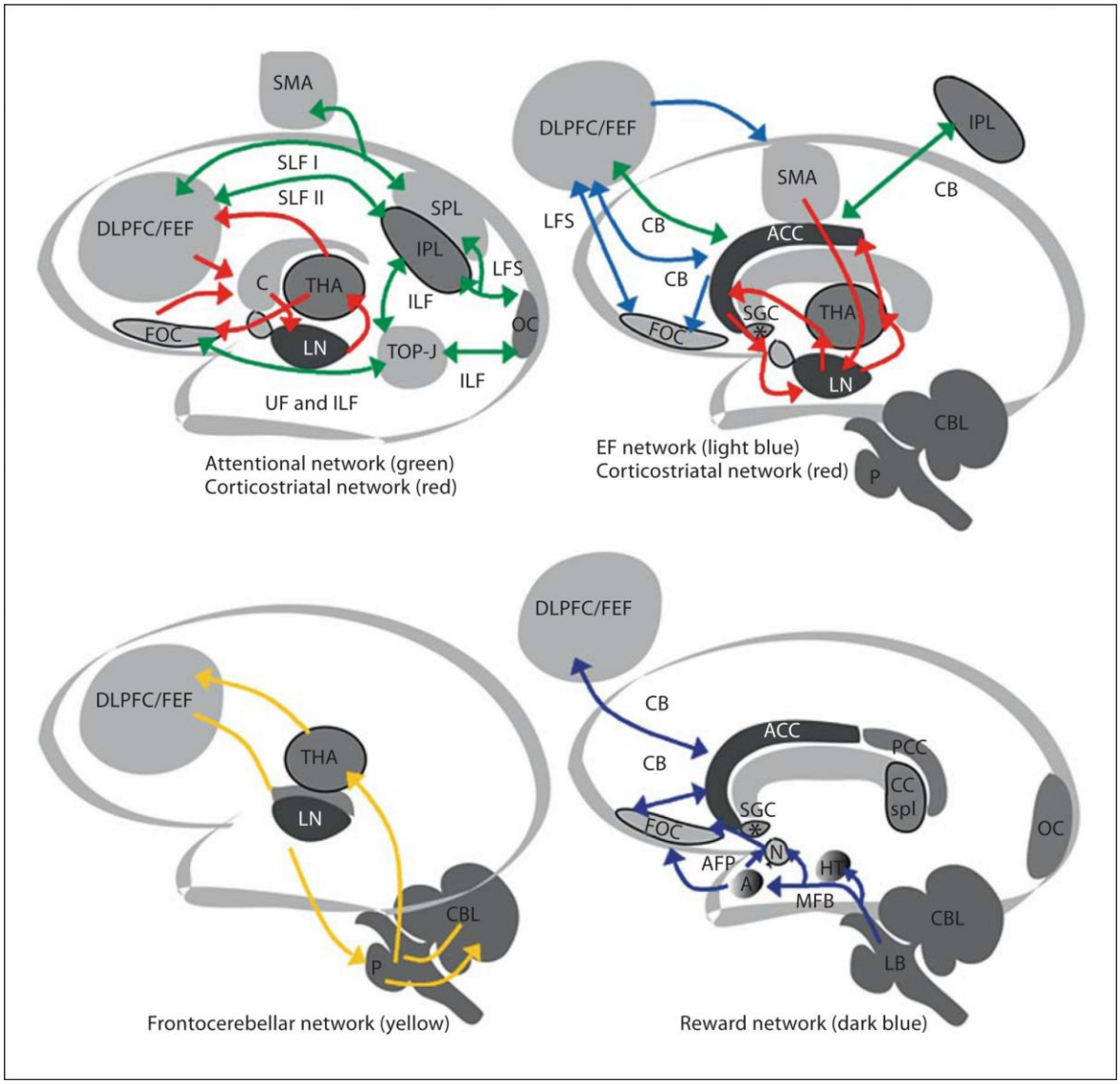

Figura 1. Anatomia baseada nas redes neuronais do TDAH. A =Amigdala; ACC = Cortex cingulado anterior; AFP = via amigdalofugal; C = núcleo caudato; CBL = cerebelo; Ccspl = esplênio do corpo caloso (incluindo istmo); FEF =campo frontal do olho; HT = hipotálamo; ILF = fascículo longitudinal inferior; IPL = lóbulo parietal inferior; LB =tronco cerebral límbico; LFS = sistema de fibra local; LN = núcleo lenticular; MFB = feixe do cérebro anterior medial; N = núcleo *accumbens*; OC = córtex occipital; P = ponte; PCC =

córtex cingulado posterior; SGC = córtex cingulado subgenual; SLF = fascículo longitudinal superior; SMA = área motor suplementar; SPL = lóbulo parietal superior; THA =tálamo; TOP = junção temporo-occipito-parietal; UF = fascículo uncinado. (Figura adaptada de Makris et al. 2009).

Diversos estudos em crianças com TDAH apontaram algumas anormalidades cerebrais vinculadas ao neurodesenvolvimento. Em um estudo longitudinal para verificar o desenvolvimento cortical de pacientes com TDAH, Shaw e cols. (2007) observaram um importante atraso de maturação cerebral em crianças com TDAH quando comparadas com crianças com padrão típico de desenvolvimento. Os autores concluíram que o padrão do desenvolvimento cerebral é paralelo entre os grupos, porém o desenvolvimento difere no tempo, ou seja, a doença parece estar mais relacionada a um atraso no desenvolvimento do que a um desvio neste processo. O atraso na maturação foi mais proeminente em regiões pré-frontais (Shaw *et al*. 2007). Além disso, outros resultados sugerem que a remissão dos sintomas de TDAH ao longo da vida pode ser caracterizada por uma normalização nesse atraso inicial de desenvolvimento, enquanto que a persistência dos sintomas pode ser caracterizada por uma trajetória de desenvolvimento neurológico mais desviante da normal (Shaw *et al*. 2010). Portanto, o TDAH pode ser considerado um transtorno do neurodesenvolvimento, no qual genes envolvidos no crescimento e plasticidade neural representam um importante conjunto de novos genes candidatos.

O nível de dopamina tônica (extrasináptica) é controlado de duas formas: baixas concentrações de dopamina que escapa da fenda sináptica e glutamato liberado a partir de aferentes corticais (principalmente pré-frontal) muito próximos ao terminal de dopamina. O glutamato estimula de perto heteroreceptores pré-sinápticos no terminal de dopamina para liberá-la diretamente para dentro do espaço extrasináptico. Normalmente, um nível baixo de dopamina tônica determina elevados potenciais de ação orientados para respostas de dopamina fásica (sinápticas). Um córtex subdesenvolvido, imaturo, ou pré-frontal hipoativo irá reduzir esta entrada glutamatérgica, resultando em níveis anormalmente baixos de dopamina tônica em TDAH (Solanto et al. 2001). Sagvolden e cols. (2005) sugeriram que o TDAH possa estar associado com uma desregulação do controle da dopamina tônica/fásica, causando redução das respostas de dopamina fásica, apesar dos baixos níveis de dopamina tônica. Isso pode ser causado por vários fatores, como por exemplo, mecanismos genéticos desacoplando essa regulação normalmente ajustada (Sagvolden *et al*. 2005).

4. Etiologia

A etiologia do TDAH tem sido alvo de vários estudos, embora isso, as causas precisas ainda não foram completamente elucidadas. O TDAH parece ser o resultado de uma combinação de fatores genéticos e ambientais que altera o desenvolvimento cerebral, resultando em anormalidades estruturais e funcionais (Thapar et al. 2013).

4.1 Fatores ambientais

Os fatores ambientais que são epidemiologicamente associadas com o TDAH incluem adversidades psicossociais, desordens mentais maternas, violência, estresse, fumo e ingestão de álcool materno durante o período pré-natal e pós-natal.

Um estudo longitudinal correlacionou o ambiente familiar e gravidez com o diagnóstico de TDAH em crianças e sintomas descritos por mães e professores. Os autores encontraram que conflitos familiares, falta de apoio social às mães, eventos adversos e desavenças no curso da gravidez foram associados com os relatos das mães com filhos diagnosticados (Pires et al. 2013). A exposição de mulheres a estresse no terceiro trimestre de gravidez aumenta o risco de TDAH (Class et al. 2014), assim como a violência doméstica (Webb 2013). O nascimento prematuro também aumenta a ocorrência do transtorno, muito provavelmente devido ao grau de imaturidade ao nascimento (Lindström et al. 2011). A exposição ao tabaco durante e após a gravidez é altamente prejudicial ao neurodesenvolvimento infantil, uma vez que induz mudanças na dinâmica celular, acionando uma cascata de fatores de risco neurotóxicos que afetam negativamente o processamento sensorial (Heath and Picciotto 2009). A exposição pré-natal ao álcool também pode determinar mudanças patológicas que aumentam a probabilidade do diagnóstico em decorrência do seu efeito na modulação da expressão dos transportadores de catecolaminas (Kim et al. 2013).

4.2 Fatores genéticos

As investigações genéticas iniciais designadas como clássicas, que envolveram amostras de famílias, gêmeos e crianças adotivas, mostraram que o TDAH possui uma contribuição genética substancial na sua etiologia.

Estudos familiares demonstraram um risco para a doença aumentado de duas a oito vezes em pais biológicos e irmãos de crianças com TDAH (Faraone et al. 2005). Em

relação aos estudos de adoção, eles evidenciaram que pais biológicos de crianças com esse transtorno apresentam três vezes mais chances de ter TDAH que pais adotivos, sendo que esses apresentam risco similar ao da população geral, confirmando a existência de um componente genético no TDAH (Sprich et al. 2000; Faraone et al. 2005).

O estudo de gêmeos fornecem evidências mais sólidas da herdabilidade ao avaliar separadamente fatores genéticos e ambientais. Através da observação de concordância do diagnóstico entre gêmeos monozigóticos e dizigóticos é calculada a fração da variabilidade fenotípica atribuída aos genes. Uma revisão de vinte estudos de gêmeos estimou a herdabilidade média do TDAH em 76%, uma das mais altas entre os transtornos psiquiátricos (Faraone et al. 2005). Um grande estudo de gêmeos sugeriu que o transtorno é melhor visualizado como o extremo quantitativo de fatores genéticos e ambientais que operam dimensionalmente ao longo da distribuição de sintomas do TDAH, indicando que os mesmos fatores etiológicos estariam envolvidos em toda a gama de sintomas de desatenção, hiperatividade e impulsividade (Larsson et al. 2012). Assim, estudos de escores poligênicos podem elucidar o papel de variância genética aditiva para traços comportamentais em amostras de TDAH e da população em geral (Hawi et al. 2015; Martin et al. 2015; Stergiakouli et al. 2015).

Devido à complexidade e heterogeneidade fenotípica, a hipótese mais aceita para explicar a contribuição genética no TDAH é a Doença Comum – Variante Comum (DC-VC), na qual variantes comuns de diversos genes com pequenos efeitos interagem entre si e com fatores ambientais. (Nigg et al. 2010). Entretanto, os estudos de variantes comuns até o momento explicam uma pequena fração da herdabilidade do TDAH, fazendo com que novas metodologias e hipóteses surjam. Variantes raras com efeitos maiores também foram reconhecidas como importantes para o desenvolvimento do transtorno (Hinney et al. 2011a; Elia et al. 2012). Estudos de genética molecular recentes demonstraram que variantes raras e comuns de genes associados à neurotransmissão e ao neurodesenvolvimento estão envolvidas na etiologia do TDAH (Akutagava-Martins et al. 2013).

5. Estudos genéticos moleculares

5.1 Genes candidatos

A abordagem de genes candidatos iniciaram os estudos genéticos moleculares com objetivos de identificar genes específicos envolvidos na etiologia do TDAH. Partindo de uma hipótese prévia, os estudos de associação foram realizados com genes envolvidos na neurotransmissão. Baseados na hipótese dopaminérgica (Genro et al. 2010a), o gene da proteína transportadora de dopamina (*DAT1*) e do receptor D4 de dopamina (*DRD4*) foram os principais investigados (Akutagava-Martins et al. 2013).

O gene do *DAT1* está localizado no cromossomo 5p15.3 (Vandenbergh et al. 1992). Ele codifica o transportador responsável pela recaptação da dopamina da fenda sináptica para o neurônio pré-sináptico. Além disso, ele é densamente distribuído pelo estriado e núcleo *accumbens*, representando o mecanismo primário de regulação da dopamina nessas regiões cerebrais (Ciliax et al. 1999). Esse transportador é o principal sítio de ação do metilfenidato, e o fato de que camundongos nocaute para esse gene apresentam fenótipos similares a algumas características do TDAH, determinou o gene *DAT1* como o primeiro alvo de estudos moleculares do TDAH (Faraone et al. 2014).

O polimorfismo mais estudado consiste em um número variável de repetições em tandem (VNTR) de 40 pares de base (pb) localizado na região 3' não traduzida (3'UTR). Os alelos de dez e nove repetições são os mais comuns (10R e 9R, respectivamente). Enquanto o alelo de 10R do VNTR 3'UTR é considerado de risco em crianças e adolescentes, o alelo 9R foi associado com a forma persistente do transtorno (Franke et al. 2010). Em uma metanálise que reuniu 34 estudos independentes, uma associação modesta, porém significativa, foi detectada entre o alelo de dez repetições (10R) e o TDAH em crianças e adolescentes. Outro VNTR, de 30 pb localizado no íntron 8 do gene, também foi associado ao TDAH, cujo o alelo de seis repetições (6R) foi considerado de risco. Para os dois VNTRs, verificou-se uma variabilidade substancial de tamanhos de efeito entre os estudos analisados. Essa variabilidade pode ser possível por causa da heterogeneidade alélica (Gizer et al. 2009). Outros estudos mostraram associações significativas com polimorfismos presentes na região promotora do gene *DAT1* em crianças e adolescentes e em adultos com TDAH (Brookes et al. 2008; Genro et al. 2008; de Azeredo et al. 2014)

O gene *DRD4* é um bom candidato para os estudos de psicopatologias devido a sua alta expressão em regiões cerebrais implicadas em atenção e inibição como o córtex cingulado anterior (Noaín et al. 2006). O gene foi primeiramente associado com a "busca por novidades", um traço de personalidade com altos níveis de impulsividade e excitabilidade que é comum no TDAH (Benjamin e cols., 1996; Ebstein e cols., 1996). Ele está localizado no cromossomo 11p15.5 (Gelernter et al. 1992), sendo a principal variante um VNTR de 48 pb localizado no éxon 3, altamente polimórfico e potencialmente funcional (Van Tol et al. 1992). O alelo contendo sete repetições (7R) foi consistentemente associado ao TDAH, embora com tamanho de efeito pequeno e heterogêneo entre amostras (Gizer et al. 2009). A análise das sequências de DNA do alelo 7R demonstrou um excesso de variantes raras em casos de TDAH quando comparados aos controles. Essas variantes parecem ser TDAH-específicas (Grady et al. 2003; Grady et al. 2005). Essa observação foi replicada em amostras clínicas e populacionais no Brasil (Tovo-Rodrigues et al. 2012; Tovo-Rodrigues et al. 2013).

Os genes do receptor de dopamina D5 (*DRD5*), do transportador de serotonina (*5HTT* ou *SLC6A4*) e do receptor 1B de serotonina (*HTR1B*), também foram associados à suscetibilidade ao TDAH em metanálise (Gizer et al. 2009). Entretanto, as associações encontradas nesses genes revelam tamanhos de efeito pequenos e explicam pouco da herdabilidade do Transtorno (Akutagava-Martins et al. 2013).

Genes relacionados ao neurodesenvolvimento também foram investigados. Um destes genes é o da proteína de 25kD associada a sinaptossomo (*SNAP-25*), localizada no cromossomo 20p11.2 (Maglott et al. 1996). Essa proteína está envolvida no crescimento axonal e plasticidade sináptica, bem como no acoplamento e fusão de vesículas sinápticas nos neurônios pré-sinápticos, regulando a exocitose de neurotransmissores (Sollner et al. 1993). Dois estudos de meta-análises evidenciaram associação modesta entre o SNP rs3746544 e suscetibilidade ao TDAH. (Gizer et al. 2009).

5.2 Estudos de associação de varredura genômica

Devido aos avanços tecnológicos, computacionais e estatísticos, surgiram os estudos genéticos de associação de varredura genômica (do inglês, *genome-wide association studies*, GWAS) que se tornaram a principal metodologia para testar a hipótese DC-VC.

Ao contrário da abordagem de genes candidatos, esses estudos não possuem hipóteses *a priori* e examinam milhões de SNPs que cobrem todo o genoma para identificar variantes causais ou que estão em desequilíbrio de ligação com a variante associada ao fenótipo. Porém, os GWAS por investigarem milhares de SNPs apresentam uma correção rigorosa para múltiplos testes, com o valor de *P* inferior a 5×10^{-8} para que um SNP seja associado ao desfecho, o que exige um tamanho amostral muito grande (Cichon et al. 2009).

Os estudos conduzidos em amostras de TDAH até o momento não evidenciaram nenhuma associação significativa ao nível genômico para amostras independentes em crianças e adolescentes ou adultos, e há pouca sobreposição de resultados. Contudo, as associações nominais observadas nesses estudos são em genes de rotas de neurodesenvolvimento potencialmente relevantes ao TDAH (Lesch et al. 2008; Neale et al. 2008; Mick et al. 2010; Neale et al. 2010a; Hinney et al. 2011a; Stergiakouli et al. 2012a; Ebejer et al. 2013; Yang et al. 2013a; Sánchez-Mora et al. 2015; Zayats et al. 2015).

Os dois estudos mais recentes com TDAH infantil relatam alguma sobreposição com os resultados de estudos anteriores. No estudo de Yang e cols. (2013) dois genes de proteína quinase (*PRKG1* e *CAMK1D*) e dois genes de integrina (*ITGAE* e *ITGA11*) estavam entre os principais resultados . Estes genes foram top hits de estudos anteriores do TDAH infantil e adulto (Lesch et al. 2008, Neale et al 2010a, Neale et al. 2010b) e são genes interessantes para estudos posteriores. Em outro estudo, uma análise que considerou apenas os genes candidatos previamente identificados no TDAH revelou fortes sinais para *SLC9A9*, envolvido nos processos de sinalização celular, e *TPH2*, envolvida na síntese de serotonina (Zayats et al. 2015). Ambos os genes foram associados com TDAH nominalmente num GWAS anterior (Lasky-Su et al. 2008).

Talvez a descoberta mais significativa de GWAS até agora seja a identificação do gene da caderina 13 (*CDH13*) como de suscetibilidade ao TDAH, o único que estava entre top hits de três estudos independentes. A caderina 13 está envolvida na adesão celular e implicada em processos de neurodesenvolvimento; esse gene foi primeiramente associado com um fenótipo quantitativo que abrange sintomas de desatenção e hiperatividade - impulsividade (Lasky-Su et al. 2008). Este gene também estava entre top hits de outros dois GWASs (Lesch et al. 2008, Mick et al. 2010), mas não alcançou significância estatística em uma meta-análise (Neale et al. 2010b). No entanto, os resultados de três

estudos de genes candidatos independentes apoiam *CDH13* como um gene de risco para o TDAH. O primeiro descreveu uma associação entre este gene e o desempenho em um teste de memória de trabalho verbal em uma amostra de crianças com TDAH (Arias-Vásquez et al. 2011), já o segundo, observou uma associação entre *CDH13* e sintomas de hiperatividade-impulsividade em jovens com o transtorno, mas não em adultos (Salatino-Oliveira et al. 2015a). No terceiro e mais recente, em modelo animal, os autores demonstraram que a proteína CDH13 se localiza no compartimento pré-sináptico de sinapses GABAérgicas hipocampais e assegura o balanço excitatório/inibitório na região CA1. A CDH13 é essencial para a flexibilidade cognitiva e a correta formação da memória, sendo que a sua deficiência resulta em déficits de aprendizado e memória, bem como hiperatividade locomotora. Essas alterações comportamentais estão associadas com sintomas observados no TDAH (Rivero et al. 2015).

Apesar dos estudos de GWAS não apresentarem resultados significantes ao nível genômico, eles levaram a identificação de vários genes candidatos novos e promissores. Assim, as conexões entre os processos biológicos importantes para o desenvolvimento cerebral e o TDAH são alvos de investigações genéticas desse complexo transtorno.

5.3 Variação no número de cópias

A variação de número de cópias (do inglês, *copy number variation*, CNVs) constitui uma classe de variantes genéticas, onde segmentos de DNA apresentam tamanhos grande (>100 kb ou >500 kb) e podem estar tanto deletados como duplicados. Embora SNPs sejam muito mais frequentes que CNVs no genoma, estima-se que a contribuição relativa destas duas classes de polimorfismo para a variação genômica seja similar (Malhotra and Sebat 2012). CNVs mais frequentes no genoma estão localizados em espaços intergênicos e íntrons, na maioria dos casos (Conrad et al. 2010), contribuindo significativamente para a heterogeneidade genética (Pinto et al. 2007). Os que estão localizados em regiões exônicas ocorrem em baixa frequência (menor que 1%), sendo raros em sua grande maioria e potencialmente deletérios (Chicon et al. 2009). Quando intragênicos, o processo de deleção ou duplicação que dá origem ao CNV pode resultar em danos a genes inteiros, interferindo nos padrões de expressão e função dos genes. Estas estruturas podem ser tanto herdadas quanto mutações *de novo* (Merikangas et al. 2009).

Alguns estudos relataram um enriquecimento global de CNVs grandes e raros em crianças e adolescentes com TDAH comparados aos controles (Williams et al. 2010; Stergiakouli et al. 2012b; Williams et al. 2012; Yang et al. 2013b), bem como em adultos (Ramos-Quiroga et al. 2014). O primeiro estudo de CNV não observou o enriquecimento global de CNVs, mas havia uma frequência maior de segmentos duplicados e deletados em genes ligados ao neurodesenvolvimento. Todos os CNVs detectados eram herdados, sendo que alguns já haviam sido descritos em amostras de esquizofrenia e autismo. Além disso, foram observadas deleções nos genes de receptores metabotrópicos de glutamato 5 e 7 (*GRM5* e *GRM7*) (Elia et al. 2010). O mesmo grupo replicou esses resultados em outro estudo e sugeriram que até 10% de casos de TDAH apresentavam CNVs nos genes glutamatérgicos ou em genes que interagem com eles (Elia et al. 2012). Akutagava-Martins et al. (2014) investigaram CNVs em genes de receptores metabotrópicos glutamatérgicos em três amostras brasileiras, porém não encontraram diferenças significativas no número total de CNVs entre indivíduos com TDAH e controles, mas a presença de CNVs foi associada com quociente de inteligência (QI) menor nos pacientes com TDAH. Além disso, CNVs no gene *GRM5* foram associados com a presença do transtorno de ansiedade comórbido com o TDAH. Esses pesquisadores sugeriram que CNVs nos genes glutamatérgicos poderiam influenciar a variabilidade clínica e cognitiva encontrada nos pacientes com o transtorno (Akutagava-Martins et al. 2014).

6. Estudos farmacogenéticos

A promessa da farmacogenética do TDAH é muito abrangente, com o potencial de desenvolver esquemas de medicação individualizada que melhorem a resposta, diminuam o risco de efeitos colaterais e melhorem a tolerabilidade, assim contribuindo para a adesão ao tratamento em longo prazo, melhorando a eficácia geral (Evans and Johnson 2001).

A farmacogenética converge em modelos que investigam o papel do genótipo na predição de resposta ao tratamento individual para os principais alvos do medicamento, representados por proteínas transportadoras e receptores. Dados demonstrando a eficácia do tratamento farmacológico com metilfenidato impulsionaram a pesquisa básica que focou no sistema catecolaminérgico para a identificação da base genética do TDAH.

Assim, os genes *DRD4* e *DAT1* foram os mais investigados nos estudos de farmacogenética também, mas os resultados atualmente disponíveis sobre eles são conflitantes e não permitem determinar se esses genes são preditores de resposta farmacológica (Polanczyk et al. 2010). Além disso, há pouca atenção na variabilidade genética do metabolismo do fármaco. As anfetaminas e a atomoxetina são metabolizadas pelo citocromo P450 2D6, cujo gene *CYP2D6* constitui uma variante genética bem conhecida em humanos (Sauer et al. 2003). Já o metilfenidato é convertido a ácido ritalínico inativo pela enzima carboxilesterase 1, cujo gene *CES1* foi pouco estudado (Nemoda et al. 2009; Bruxel et al. 2013)

De modo geral, os estudos na área de farmacogenética do tratamento do TDAH, tanto com o metilfenidato quanto a atomoxetina, são escassos. Os estudos de variantes genéticas envolvidas na variabilidade interindividual na resposta a tratamentos farmacológicos fornecem dados que podem auxiliar no aprimoramento do tratamento. Entretanto, dado o número limitado de estudos, geralmente conduzidos em amostras pequenas, a replicação em amostras independentes é de extrema importância para validação dos resultados.

7. Balanço GABA/Glutamato

A dopamina e outros neuromoduladores exercem distintas ações regulatórias na transferência de informações através dos circuitos neuronais que conectam, entre outras estruturas, áreas corticais frontais com o estriado (o núcleo *accumbens*, o núcleo caudato e o putamen), o pálido, o tálamo, a substância *nigra* e a área tegmental ventral (Alexander *et al*. 1986). Em geral, os neurônios nas áreas corticais frontais enviam projeções glutamatérgicas excitatórias para os neurônios médio-espinhal do estriado, incluindo o núcleo *accumbens* que geralmente estão silenciados. Estas estruturas enviam projeções GABAérgicas inibitórias para os neurônios normalmente ativos do pálido e substância *nigra* que inibem núcleos talâmicos através de ligações GABAérgicas. Finalmente, o tálamo completa o circuito através do envio de projeções glutamatérgicas excitatórias para os neurônios corticais e várias interações ocorrem entre as estruturas. (Haber et al. 2000) (Figura 2).

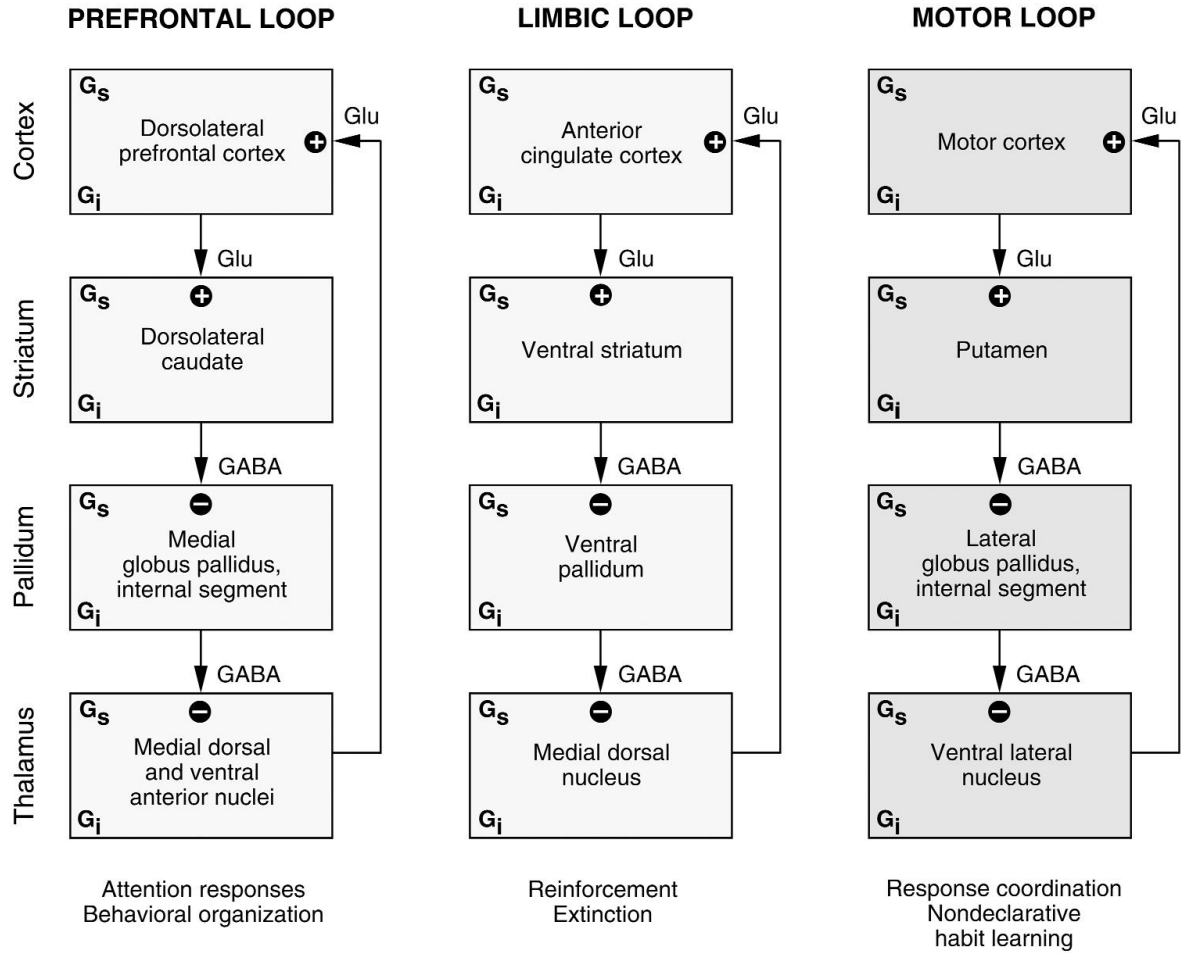

Figura 2. Projeções excitatórias e inibitórias nas entre as estruturas neuronais (Figura adaptada de Sagvolden et al. 2005)

Durante a maturação cerebral, o desenvolvimento de um equilíbro excitatório/inibitório (E/I) adequado ocorre com a mudança de ação do GABA - de despolarizante para hiperpolarizante (Ben-Ari *et al*. 2007). Perturbações no equilíbrio E/I também podem ocorrer quando a formação ou a manutenção de uma classe de sinapses prevalece sobre outras. A perda seletiva das sinapses excitatórias ou inibitórias pode ocorrer durante o período inicial de formação e consolidação de sinapses ou mais tarde, no desenvolvimento, durante o refinamento da atividade-dependente de circuitos neuronais que podem envolver mutações em genes codificadores de canais de íons ou subunidades de receptores $GABA_A$; estes levariam a circuitos com atividade anormal e propensos a crises convulsivas (Noebels 2003).

7.1 Glutamato

O glutamato é o componente essencial para que ocorra a neurotransmissão GABérgica e glutamatérgica. O glutamato é o principal neurotransmissor excitatório do sistema nervoso central e, durante o desenvolvimento, há uma alta concentração de glutamato em regiões neurogênicas do cérebro, onde estão localizadas células progenitoras neurais multipotentes, estando envolvido em processos de promoção e/ou inibição da proliferação, sobrevivência, migração e diferenciação destas células. Estas células-tronco apresentam grande potencial e diferenciam-se nos três principais tipos celulares: neurônios, astrócitos e oligodendrócitos. Acredita-se que o glutamato esteja envolvido na regulação da neurogênese através da mediação de secreção de fatores neurotróficos (Jansson and Åkerman 2014).

A ação do glutamato se dá através da ativação de dois tipos de receptores: Receptores ionotrópicos (iGluR), de ação rápida e classificados conforme o agonista ao qual se liga com alta afinidade, sendo eles o N-metil-D-aspartato (NMDA), α-amino-3-hidroxi-5-metil-4-isoxazolpropiônico (AMPA), Cainato (KA) (Dingledine et al. 1999); e os receptores metabotrópicos (mGluR), de ação lenta e duradoura. Há oito subtipos de receptores, classificados em três grupos distintos com base na homologia de sequências, mecanismo de transdução de sinal e propriedades farmacológicas: Grupo 1(mGluR1 e mGluR5), que aumenta a atividade do receptor NMDA (Wiśniewski and Car 2002); Grupo 2 (mGluR2 e mGluR3) que diminui a atividade do receptor NMDA(Harrison et al. 2008); e Grupo 3 (mGluR4, mGluR6, mGluR7 e mGluR8), que são importantes para o desenvolvimento do sistema nervoso e funcionamento normal do cérebro (Palazzo et al. 2008). Os dois tipos de receptores, iGluR e mGluR, estão amplamente distribuídos em todo o sistema nervoso central, incluindo áreas de interesse no TDAH (Lesch et al. 2013; Mukherjee and Manahan-Vaughan 2013).

Imagens de ressonância magnética demonstram um aumento do tônus glutamatérgico nas regiões frontais, córtex pré-frontal e do estriado em crianças com TDAH em comparação a crianças com desenvolvimento típico. Esta elevação é corrigida pela administração de MFD e atomoxetina (Carrey et al. 2003; MacMaster et al. 2003; Moore et al. 2006). Já em adultos com TDAH, alguns estudos observaram aumento do tônus glutamatérgico nos gânglios basais e no hemisfério cerebelar esquerdo (Ferreira et al. 2009; Perlov et al. 2010). Em contrapartida, outros estudos descreveram redução no córtex

cingulado anterior direito, regiões do mesencéfalo esquerdo e gânglios basais de adultos com TDAH quando comparados a controles (Perlov et al. 2007; Dramsdahl et al. 2011; Maltezos et al. 2014).

Camundongos noucate para o gene *Dat/Slc6a3* apresentam hiperatividade locomotora insensível à cocaína e MFD (Giros et al. 1996; Sora et al. 1998). Essa hiperatividade é aumentada por inibidores do receptor NMDA, no entanto pode ser suprimida quando drogas que aumentem a sinalização glutamatérgica são administradas (Gainetdinov et al. 2001). Em ratos lesionados com 6-OHDA, observou-se uma expressão aumentada dos genes *Dat/Slc6a3* e do gene do receptor de dopamina D4 (*Drd4*) no mesencéfalo. Há também aumento de expressão do gene de carregador de soluto família 1, membro 3 (*SLC1A3*) no estriado. Esse gene codifica uma proteína glial de transporte de glutamato de alta afinidade (Masuo et al. 2002). A expressão de genes envolvidos na rota de sinalização glutamatérgica está alterada em ratos espontaneamente hipertensos, o modelo animal mais frequentemente utilizado na pesquisa do TDAH (Sagvolden et al. 2009).

A associação entre o TDAH e genes do sistema glutamatérgico foram foi inicialmente observada em varreduras genômicas. Picos sugestivos de ligação em regiões cromossômicas nas quais se localizavam transportador e receptores de glutamato foram identificadas (Fisher et al. 2002; Smalley et al. 2002; Ogdie et al. 2004). Mais tarde, um estudo baseado em famílias encontrou associação entre um SNP no gene *SLC1A3* e suscetibilidade ao TDAH (Turic et al. 2005). Esse mesmo grupo encontrou associações entre SNPs no gene da subunidade 2A do receptor glutamatérgico NMDA (*GRIN2A*) e o TDAH (Turic et al. 2004). Entretanto essa associação não foi replicada em outros estudos (Adams et al. 2004; Park et al. 2013). Um SNP no gene da subunidade 2B do receptor glutamatérgico NMDA (*GRIN2B*) também foi associado ao TDAH inicialmente (Dorval et al. 2007), embora não tenha sido replicado (Park et al. 2013).

A *LPHN3* é um membro da subfamília das latrofilinas (LPHN) de receptores acoplados à proteína G (GPCRs) e várias vias de sinalização intracelular podem ser ativadas por elas em nível pré e pós-sináptico. Latrofilinas 1 e 2 servem como receptores para α-latrotoxina, um componente do veneno da aranha viúva negra (*Latrodectus mactans*), que ao ativar os GPCRs neuronais, estimulam a exocitose de vesículas pré-sinápticas contendo GABA e

glutamato. (Lelianova et al. 1997; Matsushita et al. 1999; Linets'ka et al. 2002; Mee et al. 2004).

Arcos-Burgos e cols. (2004) realizaram um estudo de ligação em uma população isolada da Colômbia, os Paisas, onde a prevalência de TDAH é alta. Os autores encontraram uma forte associação na região cromossômica 4q13.2 (Arcos-Burgos et al. 2004). O mapeamento dessa região levou a identificação do gene *LPHN3,* no qual os pesquisadores analisaram SNPs e identificaram haplótipos de risco que foram estatisticamente significantes para suscetibilidade ao TDAH e replicaram esses resultados em outras amostras. Verificaram também a expressão do gene em diversas áreas cerebrais, mostrando que as maiores ocorrem na amígdala, núcleo caudato, cerebelo e córtex cerebral; e menores expressões foram detectadas nos corpos calosos, hipocampo, extrato cerebral inteiro, polo occipital, putamen, lobo frontal e temporal. Eles ainda avaliaram a atividade neuronal de acordo com a presença do haplótipo de suscetibilidade (a partir de três SNPs) pela medida da razão do N-acetil-aspartato pela creatina (NAA/Cr). Todos os indivíduos portadores do haplótipo apresentaram atividade reduzida no tálamo lateral esquerdo, tálamo medial esquerdo e no estriado direito, e aumentada significativamente no *vermis* cerebelar inferior-posterior. (Arcos-Burgos et al. 2010).

O estudo de Ribasés e cols. (2011) replicou a associação entre *LPHN3* e TDAH em uma amostra de adultos, utilizando 43 SNPs e contribuindo para um fator de suscetibilidade comum para TDAH ao longo da vida. Jain e cols. (2011) demonstram que o SNP no gene *LPHN3* interage com outros SNPs nos genes da molécula de adesão neural 1 (*NCAM1)* e da proteína quinase (*ANKK1*), que são adjacentes ao gene do receptor da dopamina D2 (DRD2), localizados na região cromossômica 11q e aumentam substancialmente o risco de desenvolver TDAH (Jain *et al*. 2011). Essa interação também está associada com a severidade dos sintomas (Acosta et al. 2011). A caracterização da rede gênica (*LPHN3, NCAM1, TTC12, ANKK1, DRD2 e CDH13*) demonstrou associação ao TDAH, comportamentos disruptivos e transtorno de uso de substâncias (Arcos-Burgos *et al*. 2012). Pacientes com TDAH com dois haplótipos de risco no *LPHN3* cometiam mais erros de omissão em testes neuropsicológicos e apresentavam redução do funcionamento pré-frontal que indivíduos com ao menos um haplótipo de proteção (Fallgatter et al. 2013).

Latrofilinas tem sete regiões transmembranas, bem como uma longa sequência extracelular N-terminal contendo um peptídeo sinal de 19 aminoácidos (sítio proteolítico GPCR e domínio GPS) e uma região de glicosilação rica em serina e treonina (Ichtchenko *et al*. 1998; Sugita *et al*. 1998). Em neurônios pós-sinápticos, a porção C-terminal das latrofilinas interage com proteínas da família SHANK (Kreienkamp et al. 2000), que são proteínas que conectam receptores de neurotransmissores, canais iônicos e outras proteínas de membrana às vias de sinalização da actina citoesquelética e acoplada da proteína G. Ainda atua na formação de sinapses e maturação das espinhas dendríticas (Durand et al. 2012).

Recentemente, pesquisadores identificaram que a proteína transmembrana rica em leucina fibronectina 3 (FLTR3) é um ligante endógeno da LPHN3, regulando o número de sinapses excitatórias *in vitro* e *in vivo* (O'Sullivan et al. 2012). As FLTR são expressas em vários tecidos, incluindo o cérebro, onde atuam na migração celular e guias de axônios (Yamagishi et al. 2011). A adesão transsináptica mediada pela interação FLRT3-LPHN3 dispara eventos de transdução de sinal pré e pós-sináptico, que ativa e modula a eficácia e plasticidade sináptica (Lesch et al. 2013). A LPHN3 também está implicada no desenvolvimento sináptico e é importante na determinação das taxas de conectividade dos principais neurônios do córtex (O'Sullivan et al. 2014).

A perda da função do ortólogo *lphn3.1* durante o desenvolvimento do zebrafish provoca uma redução e deslocamento de neurônios dopaminérgicos no diencéfalo ventral e um fenótipo motor hiperativo / impulsivo . Esse comportamento é normalizado pelo uso de MFD e atomoxetina (Lange et al. 2012). Camundongos noucate para *LPHN3* também apresentam hiperatividade e níveis alterados de receptores e transportadores de dopamina e serotonina (Dat1, Drd4, 5HTT, 5Ht2a), mudanças na expressão dos genes relacionados ao metabolismo de neurotransmissores (Gad1 e Th), bem como, nos genes de desenvolvimento neuronal (Ncam e Nur1)(Wallis et al. 2012).

7.2 Ácido gama-aminobutírico (GABA)

Enquanto que no cérebro maduro o ácido gama-aminobutírico (GABA) atua como transmissor inibitório, durante período embrionário e perinatal, esse neurotransmissor despolariza células-alvo e dispara influxo de cálcio. A sinalização de cálcio mediado pelo

GABA regula uma variedade de diferentes processos de desenvolvimento a partir da migração, proliferação, diferenciação de células; e morte celular (Owens and Kriegstein 2002). Embora a geometria e a seletividade celular e subcelular dos axônios GABAérgicos sejam principalmente determinados geneticamente, ramificações e arborizações axonais são reguladas pela atividade e experiência, e exigem muitas vezes fatores neurotróficos derivados do cérebro (BDNF) (Nagappan and Lu 2005). Assim, a estimulação sensorial contribui para moldar os circuitos neuronais, enquanto que a privação sensorial retarda significativamente sua maturação (Chattopadhyaya *et al*. 2004; Di Cristo *et al*. 2004; Chattopadhyaya *et al*. 2007). Além disso, o aperfeiçoamento das redes imaturas precisa de processos adaptativos envolvendo mecanismo de experiência ou atividade-dependente, que levam à formação de novas sinapses e eliminação de outras. Usando técnicas de imagem e abordagens eletrofisiológicas, vários padrões de atividades coerentes foram caracterizados no início do desenvolvimento (Allene and Cossart 2010).

É importante notar que interneurônios GABA, que são extremamente heterogêneos, controlam a geração de oscilações de rede comportamentalmente relevantes e os padrões de atividade em adultos, por meio de modos inibitórios múltiplos (Bragin et al. 1995; Freund and Buzsáki 1996; Parra et al. 1998). Eles também têm desenvolvido padrões únicos de migração e vias de regulação, de modo que controlam a formação de uma rede desde o início. A montagem do cérebro, incluindo a formação de estruturas em camadas e vias de fibras, não necessita de liberação do transmissor e sim de um programa geneticamente determinado (Verhage *et al*. 2000). Entretanto, as etapas subsequentes, em particular, a formação de sinapses excitatórias GABA e a sequência de desenvolvimento GABA-glutamato, pode constituir um passo fundamental na construção de uma rede cortical funcional e o seu reforço por atividade neuronal.

O GABA é sintetizado através da descarboxilação do glutamato pelo ácido glutâmico descarboxilase (GAD), que possui duas isoformas, GAD_{65} e GAD_{67} que são codificados pelos genes *GAD2* e *GAD1* respectivamente. Seus pesos moleculares são diferentes (65 e 67 KDa), assim como suas propriedades catalíticas, cinéticas e localização celular, sendo GAD_{65} expresso predominantemente nas sinapses GABAérgicas e associado às vesículas, enquanto que o GAD_{67} é uniformemente distribuído em todo o citosol (Kaufman *et al*. 1991; Esclapez *et al*. 1994).

O *GAD1* está localizado no cromossomo 2q31, possuindo 46 kb e consiste de 17 éxons (Laprade and Soghomonian 1999). Considerando que a conversão de glutamato em GABA é um ponto crítico para a neurotransmissão inibitória, o gene *GAD1* é um candidato potencial na suscetibilidade de transtornos psiquiátricos. É importante ressaltar que em camundongos noucate para o gene *Lphn3*, recentemente associado ao TDAH, o gene *Gad1* apresenta expressão alterada (Wallis et al 2012),

A expressão de *GAD1* e níveis relacionados de GABA parecem ter uma papel específico no crescimento do axônio interneural e a formação de sinapses durante o desenvolvimento (Chattopadhyaya *et al*. 2007). O GAD_{67} também tem sido associado a doenças psiquiátricas. Estudos *post-mortem* demonstraram diminuição nos níveis protéicos e de mRNA de GAD_{67} em cérebros de esquizofrênicos (Guidotti et al. 2000; Huang and Akbarian 2007; Hashimoto et al. 2008). O alelo G no SNP rs3749034 foi associado a níveis reduzidos de mRNA nas regiões do hipocampo e córtex pré-frontal em cérebros de esquizofrênicos *post-mortem*. A predição funcional sugere que a mudança do alelo G para A no rs3749034 cria dois sítios de ligação a fatores transcrição putativos (TF), elemento regulatório ATP1A1 de ligação ao fator 6 (AREB6) e fator determinante de mioblasto (MYOD) (Addington et al. 2005; Straub et al. 2007). A redução da espessura cortical foi também significativamente associada em homozigotos do alelo G, reforçando que essa variante é de risco para TDAH (Brauns et al 2013). Marenco e cols (2010) avaliaram a modulação genética dos níveis de GABA por ressonância magnética em voluntários saudáveis. Os autores encontram uma associação significativa entre baixos níveis de GABA/creatina em portadores do alelo C no SNP rs11542313. Eles ainda evidenciaram sinergia entre o SNP em *GAD1* (rs11542313) e o polimorfismo funcional Val158Met do gene da Catecol-O-metil-transferase (*COMT*), que atua no catabolismo de DA (Straub et al. 2007; Marenco et al. 2010), uma associação já encontrada em estudo anterior (Straub et al. 2007). Outros estudos também mostraram associações em SNPs individuais e haplótipos em *GAD1* com esquizofrenia (Addington et al. 2005; Zhao et al. 2007; Du et al. 2008; Mellios et al. 2009).

Em uma amostra com 48 famílias em que os probandos apresentavam transtorno bipolar, o alelo A do polimorfismo rs2241165 em *GAD1* foi preferencialmente transmitido a eles (Geller *et al*. 2008). A mesma associação já havia sido relatada em crianças esquizofrênicas (Addington *et al*. 2005) Esse SNP apresentava forte desequilíbrio de

ligação (D'93) com o SNP rs3749034. Associações de autismo com SNPs e haplótipos em *GAD1* também já foram descritas (Geller et al. 2008; Chang et al. 2011).

O GABA exerce seus efeitos através de três principais receptores denominados $GABA_A$, $GABA_B$ e $GABA_C$ (Macdonald and Olsen 1994). Os receptores $GABA_A$ são os portais ligantes dos canais de íons, os quais são principalmente compostos de várias subunidades homólogas que podem ser agrupadas de acordo com a similaridade da sequência: α (α1-6), β (β1-4), γ(γ1-3) e δ (δ1-2) (Ishikawa *et al*. 2004). Os receptores $GABA_B$ são constituídos de 7 domínios espalhados na membrana de aminoácidos, ligados via uma proteína G pela sua via de sinalização. Dois genes para o receptor $GABA_B$ são conhecidos, *GABBR1* e *GABBR2* (Goei *et al*. 1998). Os receptores $GABA_C$ também são portais ligantes de canais de íons que são compostos de múltiplas subunidades do mesmo tipo nomeados como ρ1, ρ2 e ρ3 (Bailey *et al*. 1999). O GABA atua em sinapses inibitórias no cérebro através da ligação aos receptores específicos transmembrana na membrana plasmática de ambos os neurônios, pré e pós-sináptico, em processos neuronais. Essa ligação provoca a abertura de canais iônicos para permitir o influxo de íons de carga negativa como o íon cloreto na célula ou íon potássio carregados positivamente para fora da célula. Esta ação resulta numa mudança negativa no potencial transmembrana, normalmente causando hiperpolarização.

Uma investigação ampla de genes das subunidades dos receptores $GABA_A$ detectou associações entre SNPs e haplótipos em *GABRA1*, *GABRP* e *GABRA6* e esquizofrenia em uma amostra de pacientes portugueses (Petryshen et al. 2005). Em um segundo momento, Petryshen e cols. (2005) replicaram os resultados de *GABRA1* e *GABRP* em uma amostra de base familiar independente alemã. Em conjunto, estes resultados apóiam o envolvimento do *cluster* gênico do receptor $GABA_A$ do cromossomo 5q na esquizofrenia, e sugerem que a os haplótipos possam alterar a expressão de genes relacionados ao GABA (Petryshen *et al*. 2005). Outros estudos também observam alterações no receptor $GABA_A$ com níveis diminuídos de mRNA α1 e α5 e os níveis de proteína de subunidade α2 aumentada no córtex pré-frontal de pacientes esquizofrênicos (Volk *et al*. 2002; Hashimoto *et al*. 2008; Duncan *et al*. 2010). Além disso, o mRNA da subunidade γ2 estava diminuído em pacientes com esquizofrenia com uma regulação positiva de β2 mRNA (Akbarian *et al*. 1995; Huntsman *et al*. 1998). A subunidade β2 de $GABA_A$ também foi associada à esquizofrenia. A frequência do alelo C do SNP rs187269 era significantemente maior nos

pacientes esquizofrênicos do que nos controles. Os autores também encontraram uma interação entre esse SNP, outro no gene *GAD2* e outros dois SNPs em *GAD1* associado à esquizofrenia (Zhao et al. 2007). Esse mesmo alelo estava em um haplótipo preferencialmente transmitido aos probandos de uma amostra alemã (Petryshen et al. 2005), dando suporte para a associação encontrada previamente.

A perda em camundongos do gene *gabrb3*, que codifica subunidades β3 de receptores $GABA_A$, altamente expressos durante o desenvolvimento, é suficiente para determinar características fenotípicas como a desinibição do tálamo e crises associadas a déficits de aprendizagem e de memória, habilidades motoras deficientes em tarefa repetitiva, hiperatividade e um perturbado ciclo de repouso-atividade (DeLorey *et al*. 1998). Receptores extrasinápticos $GABA_A$, que são capazes que detectar concentrações baixas de GABA no ambiente presente no espaço extracelular a fim de gerar uma forma de inibição tônica, podem estar envolvidos na desregulação dessa rede associada com esquizofrenia, epilepsia, depressão e doença de Parkinson (Hines et al. 2011; Brickley and Mody 2012).

Uma diminuição significativa nos níveis de proteína e transcrição das subunidades α4, α5 e β1 no córtex frontal de indivíduos autistas também foi descrita (Fatemi *et al*. 2010). Estes resultados sugerem que os receptores de $GABA_A$ podem estar alterados em distúrbios neuropsiquiátricos do desenvolvimento. Em um estudo de caso, os autores observaram que uma duplicação entre as regiões cromossômicas 4p13 e 4p12, onde está localizado um cluster gênico de subunidades dos receptores $GABA_A$, estava sendo segregado em duas famílias cujos membros apresentavam autismo, TDAH, transtorno bipolar, dificuldades de aprendizagem e intelectual, entre outros fenótipos (Polan et al. 2014).

O responsável por fazer a recaptação do GABA é o transportador do GABA (GAT). Quatro subtipos de GATs foram identificados: GAT-1, GAT-2, GAT-3 e o Betaina/GABA transportador 1 (BGT-1). GAT-1 é o transportador neuronal principal e está expresso abundantemente nos terminais dos neurônios pré-sinápticos GABAérgicos e nas células gliais (Dalby 2003; Sarup *et al*. 2003). O gene *SLC6A1* está localizado no cromossomo 3p25.3, compreendendo 46,5 kb e possuindo 16 éxons , é o responsável por codificar o GAT-1. Polimorfismos em *SLC6A1* foram fortemente associados com transtornos de ansiedade quando se considera a gravidade dos ataques de pânico sindrômico como

covariável (Thoeringer *et al*. 2009). Foi demonstrado também o redução de mRNA de GAT-1 em um subgrupo de neurônios no córtex pré-frontal de pacientes esquizofrênicos (Volk et al. 2001). Uma meta-análise de quatro grandes estudos de associação por varredura genômica apresentou sete SNPs candidatos localizados no gene *SLC6A1* que podem estar associados com o TDAH (Neale et al. 2010b). Os camundongos noucate para o gene do transportador do GABA subtipo 1 (*Gat1*) são hiperativos e exibem memória prejudicada, aumento de impulsividade e baixos níveis de atenção, além de ataxia devido a defeitos na coordenação motora e equilíbrio, sendo considerado um novo modelo animal para o TDAH por causa desses fenótipos (Yang et al 2013).

Investigações do envolvimento do GABA no TDAH são limitadas. Um estudo usando estimulação magnética cranial verificou uma redução da inibição intercortical curta (SICI) em crianças com TDAH quando comparadas com controles. Essa redução era correlacionada com a severidade dos sintomas e habilidades motoras (Gilbert et al. 2011). A SICI é mediada pela inibição dos receptores $GABA_A$ no córtex motor primário e é relevante para a inibição de tarefas motoras e expectativa de recompensa, como dificuldade de controle e com seleção de resposta, que são sintomas do TDAH. O decréscimo de SICI pode refletir um mecanismo crítico da patofisiologia do comportamento hiperativo/impulsivo. Corroborando com esses achados, outro estudo revelou diminuição de concentrações de GABA no córtex motor e somatosensorial primário de crianças com o transtorno comparadas com crianças controles (Edden et al. 2012). Em ratos espontaneamente hipertensivos ocorre uma redução significante do nível tônico de GABA no hipocampo, podendo ser a causa da disfunção na transmissão das catecolaminas encontradas nesse modelo animal (Sterley et al. 2013).

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CAPÍTULO II

Justificativas e Objetivos

O TDAH é um dos transtornos mentais mais prevalentes entre crianças e adolescentes e os prejuízos nas atividades pessoais e sociais são significativos ao longo da vida do indivíduo e de seus familiares. Considerando a alta herdabilidade e etiologia multifatorial, o TDAH tem sido o foco de muitos estudos moleculares nos últimos anos. Contudo, os resultados publicados não foram suficientes para determinar a base genética do transtorno e nem esclarecer os mecanismos envolvidos na heterogeneidade fenotípica encontrada.

O metilfenidato (MFD) é o fármaco estimulante mais utilizado no Brasil para o tratamento do TDAH, possuindo grande efetividade no tratamento dos sintomas e com os efeitos adversos menos graves. Embora o MFD seja considerado seguro e efetivo, há uma considerável variabilidade interindividual entre os pacientes com relação à resposta ao medicamento, dosagem de efeito e ocorrência de efeitos adversos. Devida a essa variabilidade, o tratamento ainda é determinado empiricamente na prática clínica.

Genes que contribuem para o metabolismo e atividade de neurônios glutamatérgicos e GABAérgicos são críticos na sua função, pois afetam o desenvolvimento neural e a normalidade cerebral, podendo determinar predisposição aos distúrbios neuropsiquiátricos. Estudos recentes sugerem que modulação inapropriada das sinalizações de GABA e glutamato resultam em uma falha na inibição comportamental e pode originar os sintomas de hiperatividade/impulsividade vistos no TDAH.

O gene *LPHN3,* que codifica um receptor de membrana envolvido no desenvolvimento sináptico glutamatérgico, já foi identificado como um possível gene candidato para o TDAH, no entanto outros genes do sistema GABAérgico não foram estudados neste transtorno.

O presente trabalho teve como objetivo geral investigar o papel de variantes nos genes envolvidos no balanço GABA-Glutamato na suscetibilidade e na farmacogenética do TDAH.

Os objetivos específicos foram:

1- Testar a associação entre o TDAH e 5 polimorfismos do gene *LPHN3* e sua possível interação com dois polimorfismos localizados no cromossomo 11q;

2- Verificar se os polimorfismos no gene LPHN3 têm algum efeito na resposta ao tratamento com metilfenidato em crianças e adolescentes com TDAH;

3- Investigar a possível associação de polimorfismos nos genes *GAD1, GABRA1, GABRB2* e *GAT1* com a suscetibilidade ao TDAH e especificamente com os sintomas de hiperatividade/impulsividade;

4- Realizar uma revisão sistemática sobre os estudos de farmacogenéticas no TDAH.

CAPÍTULO III

LPHN3 and Attention-Deficit/Hyperactivity Disorder: a susceptibility and pharmacogenetics study

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LPHN3 and attention-deficit/hyperactivity disorder: a susceptibility and pharmacogenetic study

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Latrophilin 3 (LPHN3) is a brain-specific member of the G-protein coupled receptor family associated to both attention-deficit/hyperactivity disorder (ADHD) genetic susceptibility and methylphenidate (MPH) pharmacogenetics. Interactions of *LPHN3* variants with variants harbored in the 11q chromosome improve the prediction of ADHD development and medication response. The aim of this study was to evaluate the role of *LPHN3* variants in childhood ADHD susceptibility and treatment response in a naturalistic clinical cohort. The association between *LPHN3* and ADHD was evaluated in 523 children and adolescents with ADHD and 132 controls. In the pharmacogenetic study, 172 children with ADHD were investigated. The primary outcome measure was the parent-rated Swanson, Nolan and Pelham Scale – version IV applied at baseline, first and third months of treatment with MPH. The results reported herein suggest the CGC haplotype derived from single nucleotide polymorphisms (SNPs) rs6813183, rs1355368 and rs734644 as an ADHD risk haplotype ($P = 0.02$, OR = 1.46). Although non-significant after multiple testing correction, its interaction with the 11q chromosome SNP rs965560 slightly increases risk ($P = 0.03$, OR = 1.55). Homozygous individuals for the CGC haplotype showed faster response to MPH treatment as a significant interaction effect between CGC haplotype and treatment over time was observed ($P < 0.001$). Homozygous individuals for the GT haplotype derived from SNPs rs6551665 and rs1947275 showed a nominally significant interaction with treatment over time ($P = 0.04$). Our findings replicate previous findings reporting that *LPHN3* confers ADHD susceptibility, and moderates MPH treatment response in children and adolescents with ADHD.

Keywords: Attention-deficit/hyperactivity disorder, genetic susceptibility, *LPHN3*, medication response, pharmacogenetics

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Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by persistent and age-inappropriate patterns of inattention, hyperactivity and impulsivity (American Psychiatric Association 2013). The prevalence estimated for school-aged children and adolescents ranges from 5.3% to 7.1% (Polanczyk *et al.* 2007a; Willcutt 2012). Attention-deficit/hyperactivity disorder is a complex and heterogeneous disorder and its etiology is still unclear. Although environment plays a relevant role, genetic factors contribute substantially for ADHD development with an average heritability of 76% (Faraone *et al.* 2005).

Numerous ADHD susceptibility molecular genetics studies have been conducted using several approaches. Candidate gene studies focused primarily on genes involved in monoamine systems (Gizer *et al.* 2009). Altogether, these studies have identified many variants, generally of small effect, which do not explain the great ADHD heritability (Akutagava-Martins *et al.* 2013).

Methylphenidate (MPH) is the first line treatment for ADHD based on an effectiveness rate of around 70% (Wolraich *et al.* 2007). Although MPH is considered effective and safe, there is considerable interindividual variability among patients regarding treatment outcome (Vaughan & Kratochvil 2006).

Early ADHD pharmacogenetic investigations were conducted based on MPH action on the dopaminergic system (Faraone & Mick 2010; Hannestad *et al.* 2010). Although *DAT1* and *DRD4* have been extensively studied, their role in MPH pharmacogenetics remains unclear (Bruxel *et al.* 2014; Polanczyk *et al.* 2010). Several other pharmacogenetic surveys with genes involved in ADHD susceptibility and drug metabolism have been conducted but no evidences that they could reliably predict medication response have been reported (Bruxel *et al.* 2014; Kieling *et al.* 2010). Thus, the search for candidate genes associated with ADHD susceptibility and response to medication remains an active area of investigation.

Recently, a new gene has been associated with both ADHD susceptibility and MPH pharmacogenetics, the latrophilin 3 gene (*LPHN3*). Latrophilin 3 gene is a brain-specific member of the LPHN subfamily of G-protein coupled receptors (GPCRs), which have been shown to be important for the regulation of neurotransmitters exocytosis (Linets'ka *et al.* 2002; Rahman *et al.* 1999) and synaptic development (O'Sullivan

et al. 2014). Arcos-Burgos *et al.* (2004) conducted a linkage analysis in an isolated population from Colombia (the Paisa) and found an association between 4q13.2 (near *LPHN3*) and ADHD. The association between *LPHN3* single nucleotide polymorphisms (SNPs) was confirmed in the Paisa population as well as in other independent populations by fine mapping approaches (Arcos-Burgos *et al.* 2010). In a case–control study of Spanish adults with ADHD, an association between different *LPHN3* SNPs and ADHD was reported (Ribasés *et al.* 2011). The presence of *LPHN3* genetic variants related to ADHD was also described in patients from the USA. In this last study, signature SNPs that distinguish between protective and susceptibility haplotypes were observed (Domené *et al.* 2011).

Additional susceptibility studies reported an interaction between *LPHN3* and the neural cell adhesion molecule 1 gene (*NCAM1*), tetratricopeptide repeat domain 12 gene (*TTC12*) and ankyrin repeat and kinase domain containing 1 gene (*ANKK1*) gene cluster, which is adjacent to the dopamine D2 receptor gene (*DRD2*) and maps to 11q region. Two studies showed that the interaction between *LPHN3* and the 11q cluster doubles the risk for ADHD and predicts both severity and outcome (Acosta *et al.* 2011; Jain *et al.* 2012).

Arcos-Burgos *et al.* (2010) reported that the rs6551665 SNP was associated with both ADHD susceptibility and MPH treatment response efficacy. The G allele carriers presented a better response to medication in the inattentive symptom domain (Arcos-Burgos *et al.* 2010). Labbe *et al.* (2012) reported that the rs6551665 G allele was associated with a poor response to treatment regarding hyperactive symptoms. These investigators also reported that the rs6858066 G allele confers both risk to ADHD and better treatment response (Labbe *et al.* 2012). Choudhry *et al.* (2012) examined the role of SNPs in *LPHN3* in relation to MPH response, taking into account maternal smoking and stress during pregnancy. Analyses of rs6551665, rs1947274 and rs6858066 SNPs showed that the GCA haplotype was associated with symptom improvement (Choudhry *et al.* 2012).

Considering these evidences, the aim of this study was to evaluate the association of ADHD with five SNPs at the *LPHN3* gene, their possible interaction with two SNPs at 11q chromosome, as well as to investigate if these *LPHN3* SNPs have an effect on response to MPH treatment in children with ADHD.

Materials and methods

Subjects and diagnostic procedures

The investigated sample included 655 unrelated Brazilian children and adolescents (523 children with ADHD and 132 unaffected controls) who were consecutively evaluated in the ADHD Outpatient Program at Hospital de Clínicas de Porto Alegre. The diagnoses of ADHD and comorbidities were achieved through a three-step process: application of semi-structured interviews (Schedule for Affective Disorders and Schizophrenia for School-Age Children – Present and Lifetime Version), diagnostic discussion in a clinical committee and clinical evaluations with the child and his/her parents. Experienced child psychiatrists confirmed all generated diagnoses (Polanczyk *et al.* 2007b; Zeni *et al.* 2007). A control group of 132 children and adolescents without evidence of any current or past mental illness as defined by the DSM-IV was selected from 12 public schools from Porto Alegre, Brazil. Inclusion and diagnostic criteria have been fully described elsewhere (Schmitz *et al.* 2006). The control sample inclusion criteria were age between 6 and 18 years and contact with the biological mother. All control children had at most three inattentive and three hyperactivity symptoms in the Swanson, Nolan and Pelham Scale – version IV (SNAP-IV) (Swanson *et al.* 2001) that was completed by the teacher. The Ethical Committee of Hospital de Clínicas de Porto Alegre approved the study protocol. Parents provided written informed consent and children and adolescents provided verbal assent to participate.

Clinical assessments and pharmacological intervention

The SNAP-IV scale was applied in 241 patients. It is composed of subscales that provide scores for nine items both in the inattention and hyperactive/impulsivity symptom domains and eight items in the oppositional symptom domain. Each SNAP-IV item is rated on a scale from absence (score = 0) to severe symptoms (score = 3). This measure has been frequently used in ADHD investigations, including those designed to assess clinical interventions (Polanczyk *et al.* 2007b; Salatino-Oliveira *et al.* 2011; Zeni *et al.* 2007). Inclusion criteria for the pharmacogenetic study were: ADHD diagnosis according to DSM-IV criteria, age between 4 and 17 years, being drug-naïve for MPH and MPH dose prescription equal to or higher than 0.3 mg/kg/day.

Dosages of short-acting MPH were augmented until no further clinical improvement was detected or until there were significant adverse events (Rohde 2002). Clinical assessments were performed at baseline before medication and after 1 and 3 months of MPH treatment by child psychiatrists. At baseline, psychiatrists prescribed MPH doses closer to 0.3 mg/kg/day. After 1 month, subjects received doses between 0.5 and 0.7 mg/kg/day and at 3 months, they received doses of 1.0 mg/kg/day according to clinical improvement and adverse effects occurrence. If adverse effects emerged during titration, doses were reduced to previously tolerated levels. Methylphenidate was administered preferentially twice daily (at 0800 and 1200 h), but an extra dose between 1700 and 1800 h was allowed for children needing continuous coverage during evenings. Psychiatrists were blind to patients' genotypes.

SNP selection

LPHN3 rs6551665, rs1947275, rs6813183, rs1355368 and rs734644 SNPs were selected based on different protective and susceptibility alleles for ADHD as described by Domené *et al.* (2011). The first two SNPs were also associated with medication effectiveness (Arcos-Burgos *et al.* 2010). Single nucleotide polymorphisms rs6813183 and rs734644 were associated with ADHD susceptibility in a Spanish population sample (Ribasés *et al.* 2011). The 11q rs754672 and rs965560 SNPs were selected based on Jain *et al.* (2012) results.

Genotyping

DNA was extracted from whole blood lymphocytes as previously described (Lahiri & Nurnberger 1991). All SNPs were genotyped using Taqman SNP genotyping assays (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommended protocol for the allelic discrimination system (7500 Real Time PCR System; Applied Biosystems).

In silico functionality prediction

In order to investigate if the SNPs investigated were potentially functional, two SNP effect databases were accessed: F-SNP (http://compbio.cs.queensu.ca/F-SNP/) (Lee & Shatkay 2008) and SNPinfo–FuncPred (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm/) (Xu & Taylor 2009). The consensus result from both databases was used to investigate the putative effect of SNPs on gene function. Score values for SR proteins higher than the default threshold of the bioinformatic tool ESEFINDER mean that a sequence was found as putative exonic splicing enhancer (ESE) to which these proteins

bind. Default values are currently set as follows: SF2/ASF = 1.956; SC35 = 2.383; SRp40 = 2.670; SRp55 = 2.676. They are defined as the median of the highest scores for each sequence in a set of 30 randomly chosen 20 nucleotides sequences (from the starting pool used for functional systematic evolution of ligands by exponential enrichment) (Cartegni *et al.* 2003).

Statistical analyses

Susceptibility analyses

Linkage disequilibrium (LD) analyses were performed between *LPHN3* SNPs using the Multiple Locus Haplotype Analysis (MLOCUS 3.0). The same method was employed to test LD between 11q loci. *LPHN3* haplotypes were derived using HAPLOVIEW version 4.0.

The association of *LPHN3* markers with ADHD susceptibility and afterward *LPHN3* and 11q interaction were tested in a case–control design by haplotype-based analyses. Haplotype frequencies and ORs were estimated using the haplotype main effect model implemented in the UNPHASED software (version 3.1.7) (Dudbridge 2008), controlling for covariates. The null hypothesis is that none of the test haplotypes make any contribution, i.e. they have no main effect on the overall OR by using a likelihood-ratio test. To obtain an estimate of a haplotype risk relative to all others together, the 'Specific test haplotype' option was selected. As the expectation–maximization algorithm does not accurately estimate low haplotype frequencies, haplotypes with frequencies <0.05 were excluded. Potential confounders evaluated were IQ, ethnicity, comorbid conditions (mood disorders, anxiety disorders and disruptive behavior disorders), age and sex using the command 'Test Confounder'. Those variables associated with both the study factor and outcome for a $P \leq 0.10$ were included in the models. Significance was estimated using 10 000 permutations. The permutation command in UNPHASED corrects the global *P*-value for each particular haplotype block to account for multiple testing correction.

Pharmacogenetic analyses

The susceptibility risk alleles according to the literature (Arcos-Burgos *et al.* 2010; Domené *et al.* 2011; Ribasés *et al.* 2011) were taken as reference for these analyses. Categorical variables were tested by χ^2 test or Fisher exact test, whereas continuous variables with and without normal distribution were tested, respectively, by analysis of variance (ANOVA) and Kruskal–Wallis tests. Potential confounders evaluated were IQ, ADHD subtype, comorbid conditions (mood disorders, anxiety disorders and disruptive behavior disorders), ethnicity, age and sex. Potential confounders to be entered in the models were defined based on conceptual analyses of the literature and by means of a statistical definition (association with the study factor and with the outcome at $P \leq 0.10$).

Analyses of the effects of different genotypes on treatment response over time were performed using a mixed-effects model, which produces a flexible framework for the analysis of repeated measures while accounting for missing data (e.g. loss of follow-up) (Gibbons *et al.* 1993; Gueorguieva & Krystal 2004; Mallinckrodt *et al.* 2001). Each haplotype block was analyzed separately to observe its effects on treatment over time. The best covariance structure fitting the data was selected based on the one with the lowest Akaike information criterion (AIC) value among those in which convergence has been achieved. Independent factors included in the model were treatment over time, haplotype blocks and the interaction between these factors. The total SNAP-IV baseline scores and the mean MPH daily doses prescribed at baseline and at the first month assessment were included in the model as covariates for conceptual reasons. Efficacy effect size estimates were based on Cohen f^2, the ratio of variance explained to unexplained variance for the main effect (Cohen *et al.* 2003; Lindenau & Guimarães 2012; McGough *et al.* 2009). The analyses were restricted to those patients with total SNAP-IV baseline scores higher than 1 to allow sufficient room for improvement, as previously described (Salatino-Oliveira *et al.* 2011).

For all analyses, the significance level was set at 0.05. Bonferroni correction was performed for all independent tests performed. SPSS version 18.0 was used for these analyses (SPSS Inc., Chicago, IL, USA).

Results

Association study

The estimated genotype frequencies for all polymorphisms investigated herein are shown in Table 1. Allele frequencies were similar to those described for the European population found in the HapMap database (http://www.ncbi.nlm.nih.gov/). The genotype distribution did not deviate significantly from Hardy–Weinberg equilibrium.

Demographic and clinical characteristics of ADHD and control patients are presented in Table 2. Although ethnicity evaluated by skin color was not significant between cases and controls, it was included in the model as a confounder given its effect at 'Test Confounder' test in the UNPHASED software, and to avoid hidden population stratification. An independent analysis was performed for African Brazilians cases and controls, essentially with the same characteristics (Table S1)

Pairwise LD was assessed for all combinations of the five polymorphisms spanning the *LPHN3* gene region. A haplotype block was formed by rs6551665 and 1947275 (Block 1). The remaining SNPs rs6813183, rs1355368 and rs734644 formed the second block (Block 2). 11q SNPs rs965560 and rs754672 were not in LD (Fig. S1). Based on these results, four haplotypes were derived for *LPHN3* Block 1 and seven haplotypes for *LPHN3* Block 2 (Table 1); however, only haplotypes with frequencies higher than 5% that account for 90% of the investigated chromosomes were included in the association analyses and are shown in Table 3. Attention-deficit/hyperactivity disorder risk haplotypes according to previous studies (Arcos-Burgos *et al.* 2010; Domené *et al.* 2011; Jain *et al.* 2012; Ribasés *et al.* 2011) were GT in Block 1 and CGC in Block 2.

LPHN3 Block 2 haplotypes were associated with ADHD (global *P*-value = 0.02). In the specific test haplotype including only the most frequent haplotypes, the CGC haplotype was the one of risk, as in previous studies [$P = 0.02$, OR = 1.46 (1.07–1.97)] (Table 3). *LPHN3* Block 1 haplotype analyses showed a *P*-value close to the statistical significance threshold with ADHD (global $P = 0.09$). This borderline value is due to the GT haplotype ($P = 0.06$).

As the 11q SNPs rs754672 and rs965560 were not in LD, they were tested separately and their interactions with *LPHN3* haplotype blocks were assessed independently. An interaction between *LPHN3* Block 2 and rs965560 was observed. The interaction CGC*A showed a slightly greater effect on ADHD susceptibility [$P = 0.03$, OR = 1.55 (95% CI = 1.02–2.36)] than CGC haplotype alone. However, this interaction is no longer significant after Bonferroni correction

Secondary analyses were performed with patients stratified by ADHD subtype. Similar results to those observed in the whole sample were observed for the combined subtype, but not for the inattentive subtype (Table S2).

Pharmacogenetic study

A total of 172 ADHD patients fulfilled inclusion criteria for the pharmacogenetic study. Demographic and clinical characteristics from these patients are similar to those described for the whole sample (Table 2). The initial mean MPH dose was 0.47 ± 0.15 mg/kg/day and after first month of

Table 1: Genotype and haplotype frequencies for ADHD cases and controls

	Genotype		Case	MAF	Control	MAF
LPHN3 SNPs	rs6551665	AA	0.377	0.40 (G)	0.470	0.34 (G)
		AG	0.446		0.379	
		GG	0.177		0.151	
	rs1947275	CC	0.621	0.21 (T)	0.727	0.16 (T)
		CT	0.341		0.228	
		TT	0.038		0.045	
	rs6813183	CC	0.495	0.30 (G)	0.477	0.29 (G)
		CG	0.402		0.462	
		GG	0.103		0.061	
	rs1355368	AA	0.128	0.35 (A)	0.144	0.40 (A)
		AG	0.434		0.515	
		GG	0.438		0.341	
	rs734644	CC	0.566	0.25 (T)	0.492	0.30 (T)
		CT	0.365		0.424	
		TT	0.069		0.084	
11q SNPs	rs965560	AA	0.113	0.32 (A)	0.091	0.30 (A)
		AG	0.407		0.424	
		GG	0.480		0.485	
	rs754672	CC	0.300	0.46 (T)	0.364	0.42 (T)
		CT	0.472		0.431	
		TT	0.228		0.205	
		Haplotypes*				
	Block 1	AC	0.595		0.659	
		AT	0.005		0	
		GT	0.197		0.182	
		GC	0.203		0.159	
LPHN3	Block 2	CGC	0.623		0.566	
		GAT	0.231		0.253	
		CAC	0.055		0.112	
		GAC	0.048		0.016	
		GGC	0.025		0.017	
		CAT	0.014		0.024	
		CGT	0.004		0.012	

MAF, minor allele frequency.
*Haplotypes derived from HAPLOVIEW analysis.

treatment, mean dose was 0.60 ± 0.17 mg/kg/day. Only 4.7% of patients received concomitant medication. No potential confounders were associated with both the presence of risk haplotypes and total scores in SNAP-IV (data not shown but available upon request).

Each *LPHN3* haplotype block was analyzed separately. The haplotype blocks were analyzed according to the presence of none, one or two risk haplotypes (in Block 1, the reference was GT haplotype and in Block 2, CGC haplotype). In Block 1 analysis (rs6551665 and rs1947265), the model included treatment over time and the presence of the GT haplotype as main effects, total SNAP-IV baseline scores and the mean daily MPH dose as covariates (conceptual confounders) and interactions between treatment over time and presence of GT haplotype were also included (Table S3). The covariance structure with the lowest AIC value was the Toeplitz one. Independent effects of treatment over time ($F_{(2,260)} = 69.58$, $P < 0.001$) and of the total SNAP-IV baseline scores ($F_{(1,155)} = 111.21$, $P < 0.001$) were found. No significant effects of the mean daily MPH dose ($F_{(1,161)} = 1.93$, $P = 0.17$) and the presence of GT haplotype ($F_{(2,225)} = 1.08$, $P = 0.34$) were observed. Moreover, a nominally significant interaction effect between the GT haplotype and treatment over time ($F_{(4,262)} = 2.58$, $P = 0.04$, $f^2 = 0.31$) was observed. Although non-significant after Bonferroni correction, the trajectory of ADHD symptoms during MPH treatment based on findings from the mixed-effects model suggested that homozygous individuals for the GT haplotype ($n = 10$) have a faster response to MPH treatment, with a major effect during the first month (Fig. 1).

In *LPHN3* Block 2 analyses (rs6813183, rs1355368 and rs734644), the model included treatment over time and the presence of CGC haplotype as main effects, total SNAP-IV baseline scores and the mean daily MPH dose as covariates. Interactions between treatment over time and presence of CGC haplotype were also included (Table S4). As in the first model, independent effects of treatment over time ($F_{(2,257)} = 83.17$, $P < 0.001$) and of the total SNAP-IV baseline scores ($F_{(1,157)} = 108.54$, $P < 0.001$) were found. A significant effect of the presence of Block 2 CGC haplotype

Table 2: Demographic and clinical characteristics of the sample

Characteristics*	ADHD ($n=523$)	Control ($n=132$)	*P*-value
Age	10.56 (3.16)	11.77 (3.26)	<0.001
IQ	92.51 (13.75)	96.51 (13.15)	0.003
Male	403 (77.1%)	83 (62.9%)	0.002
Ethnicity			0.229
European-Brazilian	412 (77.6%)	100 (75.8%)	
African-Brazilian	111 (22.4%)	32 (24.2%)	
ADHD subtypes			
Inattentive	206 (39.4%)		
Hyperactive	26 (5.0%)		
Combined	275 (52.6%)		
Subthreshold	13 (2.5%)		
Comorbid condition			
CD	63 (12.0%)	1 (0.8%)	<0.001
ODD	196 (37.5%)	13 (9.8%)	<0.001
Anxiety disorder	152 (29.1%)	23 (17.4%)	0.004
Mood disorder	79 (15.1%)	2 (1.5%)	0.001

CD, conduct disorder; IQ, intelligence quotient; ODD, oppositional defiant disorder.
*Data are given as number (percentage) or mean (±SD).

Table 3: Haplotype analyses of *LPHN3* in case–control sample (a). Interaction analysis between LPHN3 Block 2 and 11q rs965560 (b)

	Case *n* (%)	Controls *n* (%)	Haplotype-specific *P*-value; OR (CI)
(a) Marker haplotype (Block 1)†			
GT	213 (20.3)	42 (15.9)	0.06; 1.40 (0.97–2.01)
AC	622 (59.5)	174 (65.9)	—
GC	206 (19.7)	48 (18.2)	—
Global *P*-value $\chi^2=4.74$, df = 2, $P=0.09$			
(a) Marker haplotype (Block 2)‡			
CGC	603 (68.5)	139 (60.4)	0.02; 1.46 (1.07–1.97)
GAT	224 (25.4)	65 (28.3)	—
CAC	53 (6.0)	26 (11.3)	—
Global *P*-value $\chi^2=7.79$, df = 2, $P=0.02$			
(b) Marker haplotype (Block 2*11q rs965560)			
CAC*G	46 (5.3)	25 (11.0)	—
CGC*A	194 (22.4)	38 (16.6)	0.03; 1.55 (1.02–2.36)
CGC*G	403 (46.6)	101(44.4)	—
GAT*A	79 (9.2)	24 (10.6)	—
GAT*G	144 (16.6)	40 (17.5)	—
Global *P*-value $\chi^2=10.24$, df = 4, $P=0.04$			

The UNPHASED program re-estimates haplotype frequencies and includes only haplotypes with frequencies ≥5% in the association analyses.
† rs6551665 and rs1947275.
‡ rs6813183, rs1355368 and rs734644.

($F_{(2,218)}=4.74$, $P=0.01$) and a significant interaction effect between the CGC haplotype and treatment over time ($F_{(4,259)}=5.30$, $P<0.001$, $f^2=0.09$) were observed. As in Block 1, homozygous individuals for the CGC haplotype ($n=70$) showed a faster response to MPH treatment, mainly in the first month (Fig. 2).

In silico prediction

Only the rs734644 SNP from Block 2 presented an *in silico* suggestion of being potentially functional. Both databases accessed suggested a role for this SNP on splicing regulation. A putative role on ESE was identified when C and T alleles were compared. While the C allele presented four putative motifs for SR protein family interacting proteins (involved in RNA splicing) SF2/ASF ($S=2.0$), SRp40 ($S=2.9$), SC35 ($S=3.6$), and SRp55 ($S=2.9$), the T allele presented just two potential motifs: SC35 ($S=3.4$), and SRp55 ($S=3.7$).

Discussion

This study investigated the role of *LPHN3* variants in ADHD susceptibility and their possible interaction with 11q SNPs. The effect of *LPHN3* SNP on response to MPH treatment

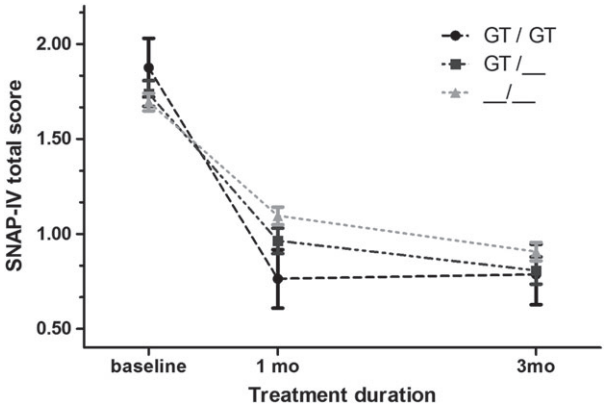

Figure 1: Mean SNAP-IV total scores during MPH treatment according to Block 1 haplotypes in a mixed-effects model ($n = 172$). There was an effect of treatment over time [$F_{(2,260)} = 69.58$, $P < 0.001$], but not for presence of GT haplotype [$F_{(2,225)} = 1.08$, $P = 0.34$]. However, there was a nominally significant interaction between these factors [$F_{(4,262)} = 2.58$, $P = 0.04$, $f^2 = 0.31$] on improvement of SNAP-IV total scores in children and adolescents during the treatment with MPH for 3 months.

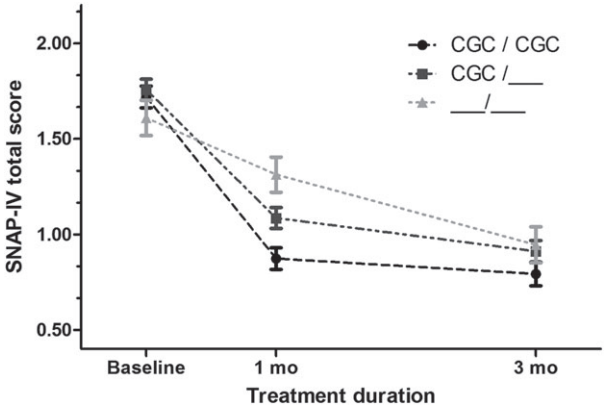

Figure 2: Mean SNAP-IV total scores during MPH treatment according to Block 2 haplotypes in a mixed-effects model ($n = 172$). There were effects of treatment over time [$F_{(2,257)} = 83.17$, $P < 0.001$] for presence of CGC haplotype [$F_{(2,218)} = 4.74$, $P = 0.01$]. A significant interaction between these factors [$F_{(4,259)} = 5.30$, $P < 0.001$, $f^2 = 0.09$] on improvement of SNAP-IV total scores in children and adolescents during the treatment with MPH for 3 months was observed.

in children with ADHD was also evaluated. The main results suggested that *LPHN3* Block 2 CGC haplotype confers risk for ADHD. Although non-significant after correction for multiple testing, the present results suggest an interaction between this haplotype and 11q rs965560 A allele on ADHD risk Moreover, ADHD symptoms improvement due to MPH treatment was associated with the CGC haplotype.

Although the role of these *LPHN3* haplotypes on ADHD susceptibility remains unclear, these SNPs were associated with ADHD risk in several studies. The GT haplotype (rs6551665 and rs1947275) was considered as an ADHD risk haplotype by Domené *et al.* (2011). The G allele at rs6551665, which is included in the GT haplotype, was associated with ADHD in four different populations even after heterogeneity tests and multiple testing corrections were performed (Arcos-Burgos *et al.* 2010). Nevertheless, the A allele of this same SNP (rs6551665) was significantly overtransmitted to ADHD probands in a group with mild or minimal maternal stress during pregnancy (Choudhry *et al.* 2012).

The *LPHN3* Block 2 CGC haplotype was transmitted to all ADHD individuals from Paisa families, showing linkage between 4q13.12 region and ADHD by Arcos-Burgos *et al.* (2010) study. A case–control study showed a strong association between TCAC haplotype (rs1868790, rs6813183, rs12503398 and rs734644) and ADHD combined subtype in an adult ADHD sample (Ribasés *et al.* 2011). Two SNPs from that study (rs6813183-C and rs734644-C) were similarly associated with ADHD as shown in this study. The CGC haplotype was considered as a risk haplotype in several populations (Domené *et al.* 2011). Moreover, the C allele at rs734644 was included in an *LPHN3* risk haplotype in a study that evaluated cognitive response control (Fallgatter *et al.* 2013). Individuals with two risk haplotypes made more omission errors and had a more anterior location in the brain area during inhibitory control processing than patients carrying at least one *LPHN3* non-risk haplotype. Moreover, these individuals presented reduced NoGo-anteriorization, an electrophysiological marker of prefrontal functioning (Fallgatter *et al.* 2013).

The interaction analysis between *LPHN3* CGC haplotype and 11q rs965560 A-allele of the *NCAM1* gene showed a slightly greater effect for ADHD susceptibility than when the CGC haplotype is considered alone. However, this result should be viewed cautiously because it was not maintained after statistical correction for multiple testing. The present findings differ somewhat from a previous study (Jain *et al.* 2012). First, in the sample investigated herein, rs965560 and rs754672 polymorphisms were not in LD, therefore they were not analyzed as haplotype. Second, the interaction was not observed with the same SNPs, probably because Block 1 (in which rs6551665 is located) was not significant in this study. Third, the interaction observed did not double risk for ADHD as described by Jain *et al.* (2012). It is possible that this interaction might occur with different magnitude of effects in different populations.

NCAM1 is expressed in neurons and promotes cell adhesion, cell migration as well as synaptic plasticity, neurite outgrowth and axon fasciculation (Maness & Schachner 2007). It has an important regulatory role during pre-frontal cortex development (Cox *et al.* 2009). LPHN3 also contributes to the cortex development, controlling the number of synapses formed (O'Sullivan *et al.* 2014). As LPHNs have adhesion-like N-terminal domains, it might be possible to interact with ligands on adjacent cells or in the extracellular matrix (Silva & Ushkaryov 2010). The knowledge of the physiological mechanism of the interaction is necessary to determinate how this interaction might contribute to the development of ADHD symptoms.

The pharmacogenetic study suggested that the presence of the GT haplotype at Block 1 was associated with different trajectory of improvement on ADHD symptoms, presenting

faster medication response with a moderate effect. Although the present results did not survive multiple test correction, and the number of carriers of this haplotype is small, they are in line with those previously reported by Arcos-Burgos *et al.* (2010) and Choudhry *et al.* (2012). Arcos-Burgos *et al.* (2010) was the first to report a pharmacogenetic effect of *LPHN3* with both SNPs investigated herein in Block 1. They reported an association of the G allele at rs6551665 with a more efficient MPH treatment response. Some conflicting results were also described. Labbe *et al.* (2012) reported a worse response in hyperactive symptoms associated with the same G allele.

Moreover, our findings also suggest that the presence of the CGC (rs6813183, rs1355368 and rs734644) haplotype in Block 2 was associated with a different trajectory of ADHD symptoms' improvement with a small but highly significant effect. Homozygous carriers of this haplotype showed faster symptoms' improvement.

The rs734644 SNP at Block 2 CGC haplotype is located at exon 10 in a minimal critical region, between hormone receptor and GPCR proteolysis site, and its role is yet unknown. However, this region is contained in the Stalk domain needed to bind its ligand, α-latrotoxin (Krasnoperov *et al.* 1999; Silva & Ushkaryov 2010). The *in silico* analysis suggested a role of this region as ESEs, which are discrete sequences within exons that promote both constitutive and alternative splicing. SNPs that are located within these sites might disrupt mRNA splicing and affect protein function (Yuan *et al.* 2006). Exonic splicing enhancers are located at approximately 30–40% at exon ends and are evolutionary conserved (Cáceres & Hurst 2013). Different alleles at rs734644 might have different roles concerning gene transcription efficiency and may affect LPHN3 function. Nevertheless, *in vitro* functional studies are needed to confirm these *in silico* predictions and to test whether these haplotypes affect LPHN3 function.

The present findings should be understood in light of some limitations. For the association study, there is a disproportion between the number of cases and controls. An equivalent number of case and control subjects would be preferable. However, the present results replicate previous published findings, as discussed. Although a study with 48 ancestry informative marker did not show significant population structure in this population (Santos *et al.* 2010), no genomic control was performed to this specific sample. Therefore, our findings could have been biased by hidden genetic heterogeneity present in our specific sample of the southern Brazilian population; therefore, we included ethnicity as conceptual confounder in all analyses. For the pharmacogenetic study, the sample size is moderate and we do not have information about MPH plasma level in our patients. As our study design is observational naturalistic, we did not have a placebo arm in this trial, so we had no internal control to correct for any effect of time (e.g. regression to the mean) or expectancy bias. This design may be valuable to better appreciate the role of genetic factors in routine clinical practice beyond the realm of controlled clinical trials. The improvement of ADHD symptoms in our sample was similar with those previously reported in randomized clinical trials (MTA Cooperative Group 1999). Although a placebo response was likely present in our study and decreased the power by reducing the measurement precision of drug efficacy, it is unlikely that a placebo response was systematically related to the polymorphisms assessed. In addition, we minimized the chance that the higher reduction in SNAP-IV total scores with MPH treatment detected in homozygous individuals for GT and CGC haplotypes might be attributed to other events, because we performed an extensive assessment of potential confounders between groups with none, one or two risk haplotypes for each block, a strategy not usually performed in previous pharmacogenomic investigations of ADHD. Due to small number of individuals with both risk haplotypes, we did not perform the analysis with both haplotypes to observe the joint effect on treatment. Moreover, MPH was administered with no control of adherence by investigators. We cannot rule out that lack of adherence occurred to some extent in the remaining sample. Nevertheless, there was an important overall decrease in SNAP-IV total scores according to the parents during follow-up. The fact that MPH doses showed no association in this protocol did not discard this possibility, as the study design is not adequate to investigate the association between genotype and MPH dose. Most studies that used a fixed-dose range found that medication response is dose dependent (Froehlich *et al.* 2011). As usual, the challenge faced by research into the genetic basis of psychopharmacological drug responses is to identify genes of relatively small and/or moderate effects against a background of substantial genetic and environmental variation. Additionally, selection bias and factors, such as socioeconomic status or health care, may differ between individuals. Although we restricted our functionality prediction analyses to the consensus results from databases aiming to reduce the potential false-positive results, it is not known if rs734644 may affect *LPHN3*. *In vitro* experiments are needed to evaluate the function of this Block 2 polymorphism and its role. Moreover, we are unaware of any previous studies that investigated the role of the second haplotype block in response to MPH treatment.

In conclusion, our findings replicate previous studies that show *LPHN3* confers ADHD susceptibility, and moderates response to MPH treatment in children and adolescents with ADHD.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1: LD plots showing *D*′ values between *LPHN3* SNPs (a) and 11q SNPs (b).

Table S1: Demographic and clinical characteristics of the African-Brazilian cases and controls

Table S2: Haplotype analyses of *LPHN3* in case–control sample according to subtype (a). Interaction analysis between LPHN3 Block 2 and 11q rs965560 (b).

Table S3: Mixed-effects model to evaluate the association between *LPHN3* Block 1 and SNAP-IV total score in children and adolescent treated with MPH

Table S4: Mixed-effects model to evaluate the association between *LPHN3* Block 2 and SNAP-IV total score in children and adolescent treated with MPH

CAPÍTULO IV

The involvement of the *GAD1* gene in Attention-Deficit/Hyperactivity Disorder

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The involvement of the *GAD1* gene in Attention-Deficit/Hyperactivity Disorder

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ABSTRACT

Attention-Deficit/Hyperactivity Disorder (ADHD) is one of the most common neurodevelopmental disorders of childhood. Recent studies suggest a role for γ-aminobutyric acid (GABA) on ADHD hyperactive/impulsive symptoms due to behavioral disinhibition resulting from inappropriate modulation of both glutamatergic and GABAergic signaling. The glutamic acid decarboxylase (*GAD1*) gene encodes a key enzyme of GABA biosynthesis. The aim of the present study was to investigate the possible influence of *GAD1* SNPs on ADHD susceptibility in both clinical and general population samples. The clinical sample consisted of 547 families with ADHD probands recruited at the ADHD Outpatient Clinics from Hospital de Clínicas de Porto Alegre. Hyperactive/impulsive symptoms were evaluated based on parent reports from the Swanson, Nolan, and Pelham Scale - version IV (SNAP-IV). The *C* allele of rs11542313 was significantly overtransmitted from parents to ADHD probands (*P*=0.03). *GAD1* haplotypes were associated with higher hyperactive/impulsive scores in ADHD youths (global *P-value* = 0.01). In the specific haplotype test, the GC haplotype was the one with the highest hyperactive/impulsive scores (*P* = 0.03). Even though *GAD1* was not associated with ADHD symptoms in the general population sample, our results from the clinical sample suggest that *GAD1* gene is associated with ADHD susceptibility, contributing particularly to the hyperactive/impulsive symptom domain.

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is one of the most common and early onset psychiatric disorders, affecting 5.3 to 7.1% of school-aged children [Polanczyk and others 2007; Willcutt 2012]. According to Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM -5) criteria, ADHD is characterized by two basic groups of symptoms: inattention and hyperactivity/impulsivity [American Psychiatric Association, 2013]. However, these symptoms may be expressed to different extent among children with ADHD. Findings from families, twin and neurocognitive studies suggest that ADHD may best viewed as the extreme from a normal behavioral trait present in the general population opposed to the usual categorical diagnosis [Asherson and Trzaskowski 2015; Larsson and others 2012; Levy and others 1997; Salum and others 2014].

ADHD is a very heterogeneous neurodevelopmental disorder, and its etiology is not yet completely understood. Functional and structural neuroimaging studies support that executive dysfunctions in ADHD result from deficits in inhibitory control [Durston and others 2011; Swanson and others 1998]. Reward dysfunctions are also a core feature of ADHD. Reward signaling and executive functions share thalamocortical–basal ganglia circuits [Alexander and others 1990; Bowirrat and Oscar-Berman 2005]. ADHD is one of the disorders with the strongest genetic component in psychiatry with estimated heritability of 76% [Faraone and others 2005]. So far, candidate gene and GWAS studies have identified many variants, generally of small effect, which do not explain the great heritability of ADHD [Akutagava-Martins and others 2013]. This difficulty has been mainly attributed to ADHD clinical heterogeneity. Genetics studies of ADHD symptom domains may explain a specific clinical variable and a particular neuronal system dysfunction, decreasing the phenotypic variability [Bralten and others 2013], given that

hyperactive/impulsive and inattentive domains demonstrated some genetic specificities and different neuronal networks [Makris and others 2009; Greven and others 2011].

The γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the mammalian central nervous system. GABA cells form key projections within and between relevant cortical and subcortical regions involved in behavioral inhibition and self-control [Boy and others 2011; Silveri and others 2013]. The study of GABAergic system in ADHD has been limited. Recent studies suggest a role for GABA on ADHD hyperactive/impulsive symptoms due to behavioral desinhibition resulting from inappropriate modulation of glutamatergic and GABAergic signaling. Short intercortical inhibition (SICI), which is modulated by (GABA)A-mediated inhibition at the level of the primary motor cortex, was reduced in ADHD children and correlated with ADHD symptom severity and motor skills [Gilbert and others 2011]. In addition, GABA concentration was reduced in children with ADHD compared to typically developing youths [Edden and others 2012]. The cortical inhibition produced by GABAergic interneurons and modulated by dopamine is believed to provide an increased signal-to-noise ratio in the frontal cortex (Winterer and Weinberger, 2004).

Glutamic acid decarboxylase (GAD) is the key enzyme in GABA synthesis in inhibitory interneurons. GAD_{67} is one of two major isoforms of GAD, an enzyme that converts glutamate to GABA. GAD_{67} is encoded by *GAD1* gene located at chromosome 2q31 [Pinal and Tobin 1998]. GAD_{67} occurs as both homo- and heterodimers in both soluble and membrane-bound forms, primarily within the cell soma but also to a limited degree in terminals [Kanaani and others 1999]. *GAD1* SNPs were associated with schizophrenia susceptibility and demonstrated a possible functional role in gene expression, thus influencing brain function [Straub and others 2007]. Given these findings,

the aim of present study was to investigate the possible influence of *GAD1* SNPs on ADHD susceptibility in a clinical sample of Brazilian youths and in a general population sample.

MATERIALS AND METHODS

Subjects and Diagnostic Procedures

The clinical sample included 547 ADHD children and adolescents and their biological parents recruited at the ADHD Outpatient Clinics (ProDAH) from Hospital de Clínicas de Porto Alegre. ADHD and comorbidities were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria [American Psychiatric Association, 1994], following a previously reported three-stage protocol [Bruxel and others 2013]. It included application of semi-structured interviews (Schedule for Affective Disorders and Schizophrenia for School-Age Children - Present and Lifetime Version), diagnostic discussion in a clinical committee, and clinical evaluations with the child and his/her parents. Experienced child psychiatrists confirmed all generated diagnoses The Swanson, Nolan, and Pelham Scale-Version IV (SNAP-IV) was applied to 323 of those patients by child psychiatrists blind to genotype. The Ethics Committee of Hospital de Clínicas de Porto Alegre approved the study protocol. Parents provided written informed consent and children and adolescents provided verbal assent to participate.

The population-based sample comprised individuals from the Pelotas 1993 Birth Cohort Study, as described in detail elsewhere [Victora and others 2006]. We randomly selected 2,000 subjects from a total of 5,265 children born alive in the city of Pelotas during the year of 1993. Follow-up visits were scheduled at multiple times (in 2004 and

2008) and included assessments of mental health problems and risk factors. Child mental health data were collected using the validated Brazilian Portuguese version of the Strengths and Difficulties Questionnaire (SDQ) [Fleitlich-Bilyk and Goodman 2004; Goodman 1997]. The SDQ hyperactivity subscale (ranging from 0 to 10), collected with the primary caregiver, was used as outcome measures for the present study. The data used in the present study were collected at both 11 and 15 years of age assessments.

Genotyping

Blood samples were collected from patients enrolled at ProDAH and their parents whenever possible. DNA was extracted from whole blood lymphocytes as previously described [Lahiri and Nurnberger 1991]. DNA samples from the 1993 Pelotas Birth Cohort were obtained from saliva, using Oragene® OG-250 DNA Self-Collection kit, following the manufacturer's recommended protocol (DNA Genotek Inc., Kanata, Ontario, CA).

The polymorphisms rs3749034 and rs11542313 were genotyped using Taqman SNP genotyping assays (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommended protocol for the allelic discrimination system (7500 Real Time PCR System, Applied Biosystems, Foster City, CA, USA). These SNPs were selected based on data from the literature and putative function [Addington and others 2005; Marenco and others 2010; Straub and others 2007].

Statistical Analyses

Allele frequencies were estimated by counting. Deviations from Hardy–Weinberg Equilibrium were assessed by the χ^2 test. The *D'* was estimated as a measure of pairwise linkage disequilibrium (LD) between SNPs at *GAD1* gene. The association of the SNPs

with ADHD susceptibility was tested in a family-based approach. Hyperactivity/impulsivity SNAP-IV scores and SDQ scores were compared among alleles and haplotypes by likelihood-ratio test. The default full model tests the null hypothesis of no difference in estimated additive genetic value (AddVal) between haplotypes, controlling for covariates. AddVal gives the change in the expected trait value due to a haplotype, relative to the reference haplotype. All analyses were carried out with UNPHASED software (version 3.1.7) [Dudbridge 2008].

As the expectation-maximization algorithm does not accurately estimate low haplotype frequencies, haplotypes with frequencies < 0.05 were excluded. Potential confounders evaluated were IQ, ethnicity, comorbid conditions (mood disorders, anxiety disorders, and disruptive behavior disorders), age, and gender using the command "Test Confounder". Those variables associated with outcome at $P \leq 0.10$ were included in the models. Significance was estimated using 10,000 permutations. The permutation command in UNPHASED corrects the global *P*-value for each particular haplotype block to account for multiple testing.

RESULTS

Demographic and clinical characteristics of the ADHD and population-based samples are presented in Table 1. The most common comorbid disorders observed in ADHD patients were anxiety, disruptive behavior, and mood disorders.

The estimated genotype frequencies for all polymorphisms investigated herein are shown in Table 2. Allele frequencies were similar to those described at the NCBI database (http://www.ncbi.nlm.nih.gov) for CEU sample. The genotype distribution did not deviate from Hardy-Weinberg Equilibrium. Despite the SNPs rs3749034 and rs11542313 were in

promoter region and exon 3, respectively, LD among them was strong both in the ADHD sample (D'=97) and in the general population sample (D'=92).

Family-based association analysis for each *GAD1* SNP and haplotypes were carried out in the clinical sample. The rs11542313 *C* allele was significantly overtransmitted from parents to ADHD probands (*P*=0.03). No preferential transmission of rs3749034 nor haplotypes from rs3749034/rs11542313 were observed (P= 0.27 and *P*=0.08, respectively) (Table 3).

Significant differences in hyperactivity/impulsivity scores in the SNAP-IV mean scores between *GAD1* SNPs in children/adolescents with ADHD were observed, with gender as a covariate (Table 4). Additive genetic value for hyperactive/impulsive score is higher in carriers of rs3749034 *G* allele (*P*=0.005, Cohen's D = 0.19) and rs11542313 *C* allele (*P*= 0.03; Cohen's D = 0.16). Therefore haplotype analysis was performed. Haplotypes also demonstrated statistical differences in hyperactivity/impulsivity scores (*P*=0.01). In the specific haplotype test, the GC (rs3749034/rs11542313) haplotype was the one with highest hyperactive/impulsive mean scores (*P* = 0.03). In the general population sample, the analyses included gender and ethnicity as covariates. No statistical differences were observed at 11 and 15 years.

DISCUSSION

This study provides evidence of an association between *GAD1* and ADHD susceptibility, contributing particularly to the hyperactive/impulsive domain. The family-based tests demonstrated an overtransmission of rs11542313 C allele from parents to

youths with ADHD. Transmission disequilibrium tests are based on marker allele transmissions from heterozygous parents to the affected offspring [Spielman and Ewens 1996]. The lower frequency of rs3749034 heterozygous in comparison to rs11542313 along with the small effect size of a single SNP might be the reasons for rs3749034 negative finding at the family-based approach. Although these no significant results, the quantitative analyses using SNAP-IV hyperactive/impulsive mean scores showed an association between hyperactive/impulsive symptoms and both GAD1 polymorphisms and their derived haplotype. These results were not replicated in the general population sample. However, the results presented herein suggest a role for GABA in clinical ADHD.

While in the mature brain GABA acts as inhibitory neurotransmitter, during the central nervous system development, it is initially excitatory contributing to neurons proliferation, migration, differentiation, and establishment of proper networks [Owens and Kriegstein 2002]. It is reasonable to suggest that an abnormal GABA action could lead to pathological changes in neuronal activities and contribute to neurodevelopmental disorders such as ADHD. This is consistent with some reviews demonstrating that ADHD subjects have smaller brains when compared to controls [Castellanos and Tannock 2002; Kieling and others 2008]. Moreover, GABA has been implicated in dopaminergic neurotransmission in the striatum [Tritsch and others 2012].

GAD1 expression and related GABA levels have also been found to have a specific role in sculpting interneuron axon growth and synapse formation during development [Chattopadhyaya and others 2007] Single nucleotide polymorphisms (SNPs) of this gene have been associated with mRNA levels of the GAD_{67} isoform. . The G allele at rs3749034 was associated with reduced transcript levels in hippocampal and prefrontal regions of schizophrenic postmortem brains. Functional prediction suggests that the change of G for

A allele at rs3749034 creates two additional putative transcription factor (TF) binding sites - namely ATP1a1 regulatory element binding factor 6 (AREB6) and myoblast determining factor (MYOD) [Straub and others 2007].

Marenco and others [2010] assessed genetic modulation of GABA levels by proton magnetic resonance spectroscopy (MRS) in healthy volunteers. The authors showed significant low GABA/Creatine levels in rs11542313 C allele carriers (P=0.001) and a trend for interaction between this SNP and *COMT* rs4680 (Val^{158}Met) (P=0.05) [Marenco and others 2010]. In the case of rs11542313 (a synonymous coding SNP), changes in the efficiency of protein production due to alteration of the mRNA secondary structure may account for these effects [Nackley and others 2006]. These MRS findings support our results by demonstrating that rs11542313 C allele was associated with decreased GABA levels, which may be implicated in ADHD susceptibility.

Typically developing children with no psychiatric diagnoses that present hyperactive/impulsive symptoms demonstrate neurodevelopmental changes resembling those found in youth with ADHD. However, the rate of change in cortical thickness in the bilateral superior and middle frontal gyri is greater for the typically developing youths than for ADHD at all assessed ages [Shaw and others 2011]. Moreover, high impulsivity on the 5-Choice Serial Reaction Time Task (5-CSRTT) is accompanied by a significant reduction in grey-matter density and linked to a reduced expression of dendrite spine markers and both GAD enzyme isoforms [Caprioli and others 2014]. Another study demonstrated that reduced cortical GABA synthesis in the prefrontal cortex of an animal model increases locomotor activity but not attention in the 5-CSRTT [Asinof and Paine 2013].

The idea that ADHD is an extreme of behavioral traits comes, at some extent, from the observation that hyperactivity/impulsivity and inattention symptoms are present in the

general population. The results reported herein, however, did not associate hyperactive/symptoms in the population-based sample probably due to a developmental alteration in subcortical GABA levels in ADHD patients which may be not so evident at the population level. Moreover the small effect size of a gene can disappear into the wide variance of symptoms in the population.

The present findings should be understood in the light of certain limitations. First, this is a cross-sectional study whereas it would be better to investigate the involvement GABA and glutamate genes related to receptors and transporters in a longitudinal approach, given that hyperactivity/impulsivity symptoms tend to decline over time and also that GABA and glutamate levels change throughout development [Bollmann and others 2015; Larsson and others 2011]. Second, results observed herein demonstrated a small effect of *GAD1* gene in hyperactive/impulsive symptoms, which is expected for single genes in multifactorial diseases. Studies of polygenic risk scores may elucidate the role of additive genetic variance to behavioral traits in both general population and ADHD clinical samples [Hawi and others 2015; Martin and others 2015; Stergiakouli and others 2015]. Finally, this was the first study to associate *GAD1* gene and ADHD susceptibility, thus present results should be understood as preliminary and must be replicated in larger and independent samples.

Taken all together, the present results suggest that *GAD1* gene, a critical enzyme in GABA biosynthesis, is associated with ADHD susceptibility, contributing particularly to the hyperactive/impulsive symptom domain in ADHD children and adolescents.

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Table 1. Clinical and Demographic Characteristics of samples

	ADHD youths (N = 323)[a]	1993 Pelotas Birth Cohort (N = 2,000)[a]
Age	10.04 (2.88)	*
Gender	250 (77.4)	1916 (47,7)
Ethnicity	257 (84.0)	2578 (64,7)
Anxiety disorders [b]	98(30.8)	488 (13.1)
Disruptive Behavior Disorder	127(39.8)	-
Mood disorders [c]	35 (11.0)	148 (4,0)

* Assessments were made at age 11 and 15 years.

[a] Data are given as number (percentage) or mean (± standard deviations).

[b] Only generalized anxiety disorder and social phobia were evaluated in general population sample.

[c] Only major depressive disorder and bipolar disorders were evaluated in general population

Table 2. Genotype and haplotype frequencies for ADHD youths and population-based sample.

Polymorphims	**Genotype**	**ADHD youths**	**MAF**	**1993 Pelotas Birth Cohort**	**MAF**
rs3749034	AA	15 (4.6)	0.21 (A)	71 (3.6)	0.18 (A)
	AG	108 (33.4)		558 (27.9)	
	GG	200 (61.9)		1371 (68.6)	
rs11542313	CC	45 (13.9)	0.41 (C)	294 (14.7)	0.39 (C)
	CT	173 (53.6)		959 (48.0)	
	TT	105 (32.5)		747 (37.4)	
	Haplotypes				
	AT	126 (19.6)	D' 97	676 (16.9)	D' 92
	AC	1 (0.002)		22 (0.005)	
	GT	251 (38.8)		1772 (44.3)	
	GC	268 (41.4)		1528 (38.2)	

MAF: Minor Allele Frequency

Table 3.Family-based analysis of *GAD1* gene

SNP	Allele	Transmited (frequency)	Untransmited (frequency)	χ^2	*P Value*
rs3749034	A	138 (0.19)	155 (0.22)	1.18	0.27
	G	580 (0.81)	563 (0.78)		
rs11542313	C	304 (0.43)	262 (0.37)	5.02	0.02
	T	408 (0.57)	450 (0.63)		
Haplotypes (rs3749034, rs11542313)	AT	138 (0.19)	155 (0.22)	5.03	0.08
	GC	308 (0.43)	262 (0.36)		
	GT	272 (0.38)	301 (0.42)		

Table 4. Differences between hyperactive/impulsive score according to GAD1 SNPs and haplotypes across the samples

ADHD sample

Alleles	AddVal[+]	CI 95%		P-value
		Lower bound	Upper bound	
A[*]	–	–	–	–
G	0.360	0.107	0.612	0.005
C	0.224	0.019	0.430	0.03
T[*]	–	–	–	–

Haplotypes	AddVal[+]	CI 95%		Haplotype-specific P-value; AddVal (CI)
		Lower bound	Upper bound	
AT[*]	–	–	–	–
GC	0.419	0.060	0.778	0.03; 0.22 (0.02– 0.43)
GT	0.296	0.018	0.574	–

Global P-value $\chi^2 = 8.98$; df = 2; $P = 0.01$

1993 Birth Cohort - 11 years of age assessment

Alleles	AddVal[+]	CI 95%		P-value
		Lower bound	Upper bound	
A[*]	–	–	–	–
G	0.011	-0.016	0.039	0.42
C	-0.003	-0.025	0.017	0.73
T[*]	–	–	–	–

Haplotypes	AddVal[+]	CI 95%		Haplotype-specific P-value; AddVal (CI)
		Lower bound	Upper bound	
AT[*]	–	–	–	–
GC	0.003	-0.026	0.033	0.81; -0.002 (-0.02– 0.02)
GT	0.008	-0.021	0.038	–

Global P-value $\chi^2 = 0.35$; df = 2; $P = 0.84$

1993 Birth Cohort - 15 years of age assessment

Alleles	AddVal+	CI 95%		P-value
		Lower bound	Upper bound	
A*	–	–	–	–
G	0.010	-0.018	0.037	0.49
C	0.0002	-0.020	0.021	0.98
T*	–	–	–	–

Haplotypes	AddVal+	CI 95%		Haplotype-specific P-value; AddVal (CI)
		Lower bound	Upper bound	
AT*	–	–	–	–
GC	0.008	-0.022	0.038	0.91; -0.001 (-0.02– 0.02)
GT	0.013	-0.017	0.042	–

Global P-value $\chi^2 = 0.69$; df = 2; $P = 0.71$

*Reference haplotype

+ AddVal shows estimative additive genetic value for hyperactive/impulsive score for each haplotype based on reference haplotype score.

CAPÍTULO V

Association study of GABAergic genes and attention-deficit/hyperactivity disorder

Manuscrito em preparação a ser submetido ao *Psychiatric Genetics*

Association study of GABAergic genes and attention-deficit/hyperactivity disorder

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Attention-Deficit/Hyperactivity Disorder (ADHD) is a neurodevelopmental disorder characterized by age-inappropriate symptoms of inattention, hyperactivity and impulsivity. ADHD has a strong genetic component, and the estimated heritability is among the highest in psychiatric disorders. Nevertheless, molecular studies only explained a small part of ADHD heritability so far. Recent evidences support a role for γ-aminobutyric acid (GABA) in ADHD etiology. A study found reduced short intercortical inhibition (SICI) in ADHD children compared to controls. SICI is mediated by inhibition of $GABA_A$ receptors and is relevant to process such as motor task inhibition and reward expectation (Gilbert et al. 2011). In addition, GABA transporter subtype 1 (*GAT1*) gene knockout mouse displays a behavior similar to ADHD and has been suggested as a new animal model for the disorder (Yang et al. 2013).

An association between rs1037715 at $GABA_A$ receptor subunit α1 (*GABRA1*) gene and schizophrenia was found in a Portuguese sample and replicated in an independent family-based German sample. Also, it was observed that rs187269 G allele of the $GABA_A$ receptor subunit β2 (*GABRB2*) gene was over-transmitted in a haplotype to probands in Germany sample (Petryshen et al. 2005). Another study reported an association between rs2930152 of *GAT1* gene and anxiety disorder (Thoeringer et al. 2009). Based on these evidences, the aim of present study was to investigate the possible association between these polymorphisms and ADHD genetic susceptibility in youth patients.

The sample comprised 542 youths with ADHD and their parents, and 132 youths without ADHD. Diagnostic criteria, and clinical and demographic characteristics are described elsewhere (Salatino-Oliveira et al. 2012). All polymorphisms (rs1037715, rs187269, and rs2930152) were genotyped using the TaqMan allelic discrimination system (Applied Biosystems Inc., Foster City, California, USA). The association hypotheses were

tested by both family-based and case–control approaches using UNPHASED 3.1.7 software (sites.google.com/site/fdudbridge/software). The Ethical Committee of Hospital de Clínicas de Porto Alegre approved the study protocol. Parents provided written informed consent and children and adolescents provided verbal assent to participate.

Allele frequencies in cases were 0.880 (C) and 0.120 (T) for rs1037715; 0.668 (A) and 0.332 (G) for rs187269; 0.656 (G) and 0.344 (A) for rs2930152. For controls, the frequencies were 0.864 (C) and 0.136 (T) for rs1037715; 0.671 (A) and 0.329 (G) for rs187269; 0.648 (G) and 0.352 (A) for rs2930152. Genotype frequencies were in Hardy–Weinberg Equilibrium. No evidence of association was observed in either family-based (P=0.803, P=0.108, and P=0.492 for rs1037715, rs187269, and rs2930152, respectively) or case–control approaches (P=0.477, P=0.937, and P=0.802 for rs1037715, rs187269, and rs2930152, respectively).

This is the first association study of GABRA1, GABRB2, and GAT1 genes in ADHD, which found no evidence of association between these genes and ADHD. Nonetheless, a role for GABA in ADHD genetic susceptibility cannot be ruled out since components of the GABAergic system are encoded by several different genes that could be involved in ADHD etiology.

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CONFLICT OF INTEREST

Dr Luis Augusto Rohde was on the speakers' bureau and/or acted as consultant for Eli-Lilly, Janssen-Cilag, Novartis and Shire in the last three years. The ADHD and Juvenile Bipolar Disorder Outpatient Programs chaired by him received unrestricted educational and research support from the following pharmaceutical companies in the last three years: Eli-Lilly, Janssen-Cilag, Novartis, and Shire. He also receives research support from Brazilian government institutions (CNPq, FAPERGS, HCPA and CAPES), authorship royalties from Oxford Press and ArtMed and received travel awards for taking part of 2014 APA meeting from Shire. Dr Mara H. Hutz receives support from Brazilian government institutions (CNPq, FINEP and CAPES). Dr. Guilherme V. Polanczyk is employed by the University of São Paulo.He receives research support from the National Council for Scientific and Technological Development (CNPq) [Bolsa de Produtividade em Pesquisa], the São Paulo Research Foundation (FAPESP), and the University of São Paulo. He has served as a consultant to Shire. He has served on the speakers' bureau of Shire. He has received royalties from Editora Manole. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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CAPÍTULO VI

ADHD Pharmacogenetics across the life cycle: New Findings and Perspectives

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REVIEW ARTICLE

AMERICAN JOURNAL OF medical genetics PART B
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ADHD Pharmacogenetics Across The Life Cycle: New Findings and Perspectives

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Attention-deficit/hyperactivity disorder (ADHD) is a complex and heterogeneous disorder, affecting individuals across the life cycle. Although its etiology is not yet completely understood, genetics plays a substantial role. Pharmacological treatment is considered effective and safe for children and adults, but there is considerable inter-individual variability among patients regarding response to medication, required doses, and adverse events. We present here a systematic review of the literature on ADHD pharmacogenetics to provide a critical discussion of the existent findings, new approaches, limitations, and recommendations for future research. Our main findings are: first, the number of studies continues to grow, making ADHD one of the mental health areas with more pharmacogenetic studies. Second, there has been a focus shift on ADHD pharmacogenetic studies in the last years. There is an increasing number of studies assessing gene–gene and gene–environment interactions, using genome-wide association approaches, neuroimaging, and assessing pharmacokinetic properties. Third and most importantly, the heterogeneity in methodological strategies employed by different studies remains impressive. The question whether pharmacogenetics studies of ADHD will improve clinical management by shifting from trial-and-error approach to a pharmacological regimen that takes into account the individual variability remains unanswered. © 2014 Wiley Periodicals, Inc.

Key words: ADHD; pharmacogenetics; medication response; stimulants; atomoxetine

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INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is characterized by persistent and age-inappropriate patterns of inattention, hyperactivity, and impulsivity [American Psychiatric Association, 2013]. It is one of the most common neurodevelopmental disorders affecting individuals across the life cycle. While the reported prevalence for school-aged children and adolescents is around 5.3–8.7% [Polanczyk et al., 2007a; Merikangas et al., 2010; Kessler et al., 2012; Willcutt, 2012], rates from 2.5% to 4.4% are described for adults [Kessler et al., 2006] reflecting late brain maturation in a

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proportion of cases [Giedd and Rapoport, 2010]. ADHD is frequently comorbid with other neuropsychiatric and neurodevelopmental disorders, including oppositional defiant disorder, conduct disorder, anxiety disorders, mood disorders, and substance abuse disorders [Souza et al., 2004].

ADHD is a complex and heterogeneous disorder and its etiology is not yet completely understood. Despite evidences that environmental factors play an important role in its etiology, classical genetics studies support a strong genetic contribution for ADHD [Biederman and Faraone, 2005; Schmitt and Romanos, 2012]. Neurobiological studies suggest that ADHD is a frontal-striatum-cerebellum disorder, since these regions present lower maturation, volume, and activity in these patients [Castellanos et al., 2002; Cortese, 2012]. Also, neuropsychological data showed that ADHD children have behavioral inhibition failures and a poorer performance in cognitive and executive functions. These neuropsychological processes are related to frontostriatal circuits [Swanson et al., 1998]. Recent hypotheses have proposed that ADHD might also be the result from core deficits in inhibitory control to reward processes, which would lead to executive dysfunctions and impaired signaling of delayed reward, respectively [Nigg and Casey, 2005; Sonuga-Barke, 2005]. Functional magnetic resonance imaging (fMRI) studies have found decreased activation in ventral striatal area during reward anticipation [Scheres et al., 2007; Ströhle et al., 2008; Plichta et al., 2009]. It is possible that multiple neural pathways contribute to ADHD, so a dysfunction in any of these circuits could lead to symptoms and determine the heterogeneity seen in ADHD [Sonuga-Barke, 2005; Durston, 2003].

Pharmacotherapy has an essential role in the treatment of ADHD [Kornfield et al., 2013]. Many studies have documented the efficacy of stimulants (e.g., methylphenidate—MPH, amphetamines) and non-stimulants (atomoxetine) in reducing ADHD symptoms, as well as improving neuropsychological performance on measure of executive functions [Greenhill et al., 2002; Blum et al., 2011]. The alpha-2 agonists clonidine and guanfacine, which were developed and initially utilized as antihypertensive agents, also showed improvement in ADHD symptoms [Sallee et al., 2013]. Recently, the United States Food and Drug Administration (FDA) approved the extended-release formulations of clonidine and guanfacine for ADHD children and adolescents [Intuniv, 2011].

The most recognized brain effect of MPH and a potential mechanism of action for ADHD symptom improvement is the dopamine transporter (DAT) blockade [Faraone and Mick, 2010]. However, it is known that MPH also blocks efficiently the norepinephrine transporter (NET) [Faraone and Mick, 2010; Hannestad et al., 2010]. Some studies have demonstrated MPH-induced improvement on prefrontal cortex functioning where NET density is higher. It was postulated that NET could transport dopamine in the prefrontal cortex because dopamine has a greater affinity for NET as compared with its affinity for DAT [Madras et al., 2005; Hannestad et al., 2010]. MPH also induces the increase in cortical cell excitability, which is mediated by activation of adrenergic alpha-2-A receptors (ADRA2A) [Andrews and Lavin, 2006]. MPH is currently prescribed either as immediate-release (IR) or extended-release (ER) formulations. An alternative to oral MPH administration is the MPH transdermal system that delivers racemic MPH, D-L-threo-MPH, through the skin [Anderson and Scott, 2006]. All of them determine good response in decreasing ADHD symptoms, although their pharmacokinetics are quite different [Faraone and Buitelaar, 2010].

An alternative treatment option is amphetamines. Their primary action is to increase synaptic concentration of neurotransmitters in the synaptic cleft. Amphetamines compete with the endogenous monoamines for transport into nerve terminals. Once inside the presynaptic terminal, amphetamines displace monoamines from the cytosolic pool, pumping neurotransmitter out of neurons into the synapse. The reuptake inhibition and probably inhibition of monoamine oxidase (MAO) also occur with this mechanism increasing synergically neurotransmitter concentrations in the synapse [Heal et al., 2013]. Like MPH, amphetamines have different formulations as IR, ER, and the prodrug lisdexamfetamine [Heal et al., 2013].

Non-stimulant medications, such as atomoxetine, are alternative psychopharmacological interventions for ADHD treatment. Atomoxetine is a highly specific NET inhibitor with adequate treatment efficacy [Michelson et al., 2001]. Although pharmacological agents are considered as effective and safe, there is considerable inter-individual variability among patients regarding response to medication, dosing effects, and occurrence of adverse events [Greenhill et al., 1996; Vaughan and Kratochvil, 2006]. Due to this variability, the treatment is often determined empirically in clinical practice through a gradual dosage titration and a trial-and-error approach.

Pharmacogenetic studies try to explain how individual genetic variability influences the pharmacokinetics and pharmacodynamics of the drug [Weinshilboum, 2003]. The term pharmacogenomics has been used to encompass studies investigating variations in network of genes and their relationship to medication response and to emphasize these insights to discover new therapeutic targets and optimize drug therapy [Evans and Johnson, 2001]. The great potential of ADHD pharmacogenetics (here used interchangeably with pharmacogenomics) for clinical application lies in predicting better medication choices, avoiding adverse effects, maximizing individual treatment outcomes and determining the most appropriate drug dosage. Early investigations on ADHD susceptibility genes were conducted based on data describing MPH action. Therefore, it has been hypothesized that polymorphisms in these same genes may also influence medication response in individual patients. Numerous initial studies focusing on dopaminergic genes have described evidence of genetic factors influencing MPH and atomoxetine response, as summarized in several reviews [Stein and McGough, 2008; Genro et al., 2010; Froehlich et al., 2010; Kieling et al., 2010; Polanczyk et al., 2010]. It is important to bear in mind that many factors (e.g., phenotypic presentation including ADHD types and comorbidities, neurocognitive processes, environment, and genetics determinants) combined in different ways contribute to ADHD phenotypic heterogeneity. Unfortunately, how this heterogeneity interferes on treatment response prediction and medication efficacy is not very clear yet. Moreover, the variance of the treatment response explained by pharmacogenetic data does not seem to be substantial [Polanczyk et al., 2010].

In this context, our aim is to present an updated review of the literature and to provide a critical discussion of the findings, new approaches, limitations, and recommendations for future research.

METHODS

Since our group has produced previous systematic reviews on ADHD pharmacogenomics in children [Kieling et al., 2010; Polanczyk et al., 2010] and adults [Contini et al., 2012], we aimed to update this previous work providing a more integrative lifespan perspective. Thus, we implemented a similar methodological procedure in this systematic review than the one in the previous studies, offering a sense of continuity for the readers. A systematic review of the literature was performed in MEDLINE via PubMed database (http://www.ncbi.nlm.nih.gov) and PsychINFO (http://www.apa.org/pubs/databases/psycinfo/index.aspx). We looked for original studies that have ADHD pharmacogenetics information published between January 2010 and September 2013 for children and adolescents and between February 2012 and September 2013 for adults. Relevant studies in children and adolescents published earlier than 2010 and in adults earlier than 2012 covered in our previous reviews are also mentioned here.

The search was limited to articles published in English. The following key-words were employed: attention-deficit/hyperactivity disorder or ADHD combined with the following terms: pharmacogen*, gene*, response to medication, stimulant response, MPH, atomoxetine, amphetamine, lisdexamfetamine. In addition, a blanket search was conducted with only the term "Attention-Deficit/Hyperactivity Disorder" to ensure no relevant studies were missed. Next, an extensive review of the references of pertinent articles was carried out.

We selected articles that met the following inclusion criteria: (1) assessment of any medication indicated for ADHD treatment across the life cycle; (2) ADHD diagnosis according to the Diagnostic and Statistical Manual of Mental Disorders—version IV (DSM-IV) or the International Classification of Diseases—10th Revision (ICD-10); (3) studies that used a candidate-gene or genome-wide association approach; (4) studies that defined response to medication in terms of symptoms improvement, evaluation of dose–response relationship, occurrence of adverse events, or objective parameters that reflect the central nervous system functioning (e.g., neuropsychological tests, cerebral blood flow, etc.).

The literature mining identified 90 publications. After reviewing the abstracts, we selected 33 articles and the full text of these publications and their reference lists were reviewed. Twenty-nine studies fulfilled the inclusion criteria and were included in this review. The lisdexamfetamine search did not result in any pharmacogenetic study.

RESULTS

Candidate Genes and Response to Medication

The majority of candidate genes investigated in pharmacogenetics studies are related to the catecholamine pathway, but neurodevelopmental genes and new genes involved in ADHD risk have been studied as well. Only one study evaluates atomoxetine as an ADHD medication. Investigations with amphetamine preparations or guanfacine were not found in the literature, and the remaining studies evaluate MPH treatment.

DAT1. The majority of studies on ADHD pharmacogenetics have focused on MPH response and the dopaminergic system. DAT inhibition by MPH in presynaptic neurons increases synaptic dopamine and neuronal signaling. This protein is distributed mainly in the *striatum* and *nucleus accumbens*, but also in the globus pallidus, cingulate cortex, thalamus, and the midbrain [Sasaki et al., 2012]. DAT represents the primary mechanism of dopamine regulation [Ciliax et al., 1999]. The gene that encodes DAT has been considered a good candidate for pharmacogenetics studies due to the major role of the transporter in stimulant's action. The most studied polymorphism is the 40 base pair (bp) variable number of tandem repeats (VNTR) in the 3′ untranslated region at the dopamine transporter gene (*DAT1* or *SLC6A3*). In vitro studies suggested that DAT VNTR has a functional role, with the 10R being associated with higher expression levels of the transporter [Mill et al., 2002; Purper-Ouakil et al., 2008].

Previous reports have described decreased MPH responses in individuals homozygous with either 9-repeat allele (9R) [Stein et al., 2005; Joober et al., 2007] and 10-repeat allele (10R) [Winsberg and Comings, 1999; Roman et al., 2002b; Purper-Ouakil et al., 2008]. In agreement with these last studies, a recent investigation reported the association between *DAT1* VNTR and MPH response in 89 stimulant-naïve children with ADHD. Outcomes were assessed using the Vanderbilt ADHD Parent Rating Scales (VADPRS) and the Vanderbilt ADHD Teacher Rating Scales (VADTRS). The investigators reported that patients lacking the 10R allele showed a greater improvement across MPH doses compared to 10R carriers with effect sizes around 0.59–0.64. A reduction of 56% on hyperactive-impulsive scores at MPH dose of 2 mg/kg/day was observed in the patients without 10R compared to placebo ($P = 0.008$) [Froehlich et al., 2011]. The main finding of this study was a gene–dose interaction. However, this finding should be interpreted with caution because the sample is small and the significance level of the tests was adjusted for the number of outcomes assessed but not for the number of SNPs investigated. Nevertheless similar findings were previously described in an adult sample [Kooij et al., 2008].

Better responses were also reported for the homozygous 10R on MPH treatment [Kirley et al., 2003]. Recently, a longitudinal open label trial evaluated neurocognitive fuctions as response inhibition, planning and working memory in 108 stimulant-naïve boys with ADHD according to *DAT1* genotypes after 4, 8, and 24 weeks of treatment with 0.5 mg/kg/day dose, and 8 weeks after MPH withdrawal. Response inhibition was measured by the Continuous Performance Test II (CPT II), planning ability by the Tower of London (ToL) test, and working memory by N-back test. At 4 weeks, no difference on response inhibition improvement according to genotype was observed; planning ability was better in 10R homozygous subjects as compared to 9R carriers. At the working memory test, all children improved regardless of genotypes. After 8 weeks of treatment, 10R carriers had better response inhibition compared to 9R homozygous; the planning ability continued better in 10R homozygous compared to 9R carriers; these results did not change when working memory was considered. At 24 weeks, 10R carriers continued with better response inhibition; no difference among genotypes in planning ability was observed, and working memory was better in 10R homozygous. After MPH interruption, response inhibition worsened in all patients, but planning ability and working memory improvements were maintained. These results

presented a high power analysis (>0.80) [Pasini et al., 2013]. However, the interpretation of these results is difficult, because the authors analyzed dominant and recessive effects of the 10R allele according to the improvement trajectory between tasks.

No pharmacogenetic effect of the *DAT1* VNTR was also reported in children [Langley et al., 2005; van der Meulen et al., 2005; Zeni et al., 2007; McGough et al., 2009] and in adults [Mick et al., 2006; Contini et al., 2010]. A recent meta-analysis demonstrated that there was no significant effect for the *DAT1* VNTR on both MPH treatment response ($P > 0.5$) and specific symptom dimensions ($P > 0.2$). However, a subanalysis of naturalistic trials detected that 10R homozygous patients exhibited less improvement than individuals that are not 10R homozygous. In summary, *DAT1* VNTR seems to be a non-reliable predictor of MPH treatment [Kambeitz et al., 2014]. Although *DAT1* is an obvious candidate for MPH pharmacogenetics studies, findings available in the literature did not consistently suggest a role for this gene in the ADHD pharmacogenetics. Possible methodological explanations for spurious and conflicting findings like those found with DAT gene are explored in Table II. Moreover considering that the VNTR is probably non-functional and the complex architecture of *DAT1* gene [Genro et al., 2008], other polymorphisms in this gene could account for these conflicting results.

DRD4. The dopamine receptor D4 (*DRD4*) gene is also frequently studied in ADHD pharmacogenetics, mainly a 48-bp VNTR polymorphism in the exon 3. The common variants are 2R, 4R, and 7R alleles. The 7R seems to be less responsive to dopamine, reducing the intracellular concentration of second messenger cyclic AMP [Asghari et al., 1995]. It is considered a risk allele for ADHD susceptibility [Gizer et al., 2009]. Froehlich et al. [2011] showed that 4R carriers have larger reductions on hyperactive-impulsive scores across MPH doses compared to other VNTR length allele carriers with effect sizes in the range of 0.40–0.61. Patients without the 4R showed 23% reductions in their hyperactive-impulsive scores compared to placebo, while 4R carriers reduced 40–49% ($P = 0.02$). Correcting findings for the number of polymorphisms evaluated, turned results not significant. However, there was a correction by the number of outcomes assessed ($\alpha = 0.025$) and the dose–gene interaction remained significant. This result is congruent with the expression of receptor enhanced by 4R and increased sensitivity to dopamine [Asghari et al., 1995; Schoots and Van Tol, 2003] and corroborates findings from a previous study [Cheon et al., 2007]. A recent study evaluated 114 Korean children with ADHD according to the presence of 4R homozygosity. The efficacy measure was defined by changes at Korean ADHD Ranting Scale (ADHD-RS), Clinical Global Impression Improvement (CGI-I) and Severity (CGI-S) from baseline to 8 weeks of treatment. The results were non-significant between genotypes [Ji et al., 2013]. Moreover, no effect was found in adult samples [Kooij et al., 2008; Contini et al., 2012]. Although there are some evidence to suggest a MPH greater responsiveness for 4R carriers, the available data are scarce and limited to children samples.

NET1. The noradrenergic system is implicated on the pathophysiology of ADHD based on the mechanism of MPH and on the fact that atomoxetine is a highly specific inhibitor of NET. Kim et al. [2010] conducted a study to evaluate the association between A-3081T (rs28386840) and G1287A (rs5569) single nucleotide polymorphisms (SNPs) at the norepinephrine transporter gene (*NET1* or *SLC6A2*) and response to MPH treatment in Korean children with ADHD. The study enrolled 112 children and employed the Clinical Global Impression-Improvement (CGI-I) score and ADHD Rating Scale (ADHD-RS) as outcome measurement. The significance level was adjusted for outcomes and SNPs investigated at $\alpha = 0.01$. A trend for good response to MPH treatment in CGI-I was observed among 61.4% of T allele carriers compared to 37.9% of A allele homozygous for the A-3081T SNP ($P = 0.03$). However, authors did not find a significant gene over dose interaction effect for this SNP on MPH response. No significant association was found between response to medication and G1287A polymorphism. The power to detect differences at $\alpha = 0.01$ was low for this sample size [Kim et al., 2010]. These results may be explained by the presence of T allele being correlated with decreased promoter activity and, consequently, reduced levels of NET within the brain [Kim et al., 2006]. On the other hand, patients who are T allele carriers may present a specific ADHD subtype, where the etiology is closely related to NET action. This might explain the better pharmacological response in this group [Kim et al., 2010]. In the following year, the same group investigated five *NET1* SNPs in a larger sample but they did not replicate the association between MPH response and *NET1* polymorphisms [Lee et al., 2011]. No effect was found in an adult sample [Kooij et al., 2008]. All these results taken together suggest that *NET1* influence on response to MPH is small. On the other hand, as most studies are from the same group, independent replication studies are clearly needed.

Yang et al. [2012] investigated whether *NET1* gene influences atomoxetine response in 111 Chinese ADHD children. The response was defined as at least a decrease of 25% from baseline on the ADHD-RS total score. Remission was defined as each ADHD-RS item score ≤ 1 at the end of the treatment. Their findings showed that rs3785143 in *NET1* had a nominally significant association with responder status; the C allele was present in 77.1% of responders, whereas T allele was observed in 55.8% ($P = 0.005$). The significance was maintained after 5,000 permutations performed for multiple test correction ($P = 0.04$). Since this SNP is located within intron 1, it is possible that its function is responsible for *NET1* transcription level or it is in linkage disequilibrium (LD) with a functional SNP. The rs2279805 at *NET1* was significantly associated with the status of remission: 29.8% of individuals who were C allele carriers achieved remission versus 15.5% of T allele carriers ($P = 0.02$). However, the significance disappeared after multiple test correction [Yang et al., 2012]. This SNP is located in the sixth intron of *NET1* and among the region of exons 4–9, which was associated with treatment response in another study [Ramoz et al., 2009]. Effect sizes for these SNPs were not estimated in that study.

Park et al. [2012b] examined the CPT in 53 children before and after MPH treatment. This test has been proposed as a potential ADHD endophenotype [Almasy and Blangero, 2001]. In that study, differences in baseline CPT measures and CPT post-treatment changes were assessed based on G1287A and A-3081T SNPs at *NET1* gene. The corrected significance level was set at $P = 0.006$. Although no significant differences in SNP frequencies were observed according to baseline CPT measures, children with the G/G

genotype at G1287A showed a greater decrease in the mean omission error scores after MPH administration compared to A allele carriers ($P = 0.006$). The effect size of the gene was not evaluated. These results suggest improvement of attention after medication that is consistent with other studies showing that the G allele induced greater MPH response [Yang et al., 2004]. In addition, the T allele carriers at A-3081T showed a greater decrease in the mean commission errors scores compared to those A/A homozygous ($P = 0.007$), meaning improved impulsive behavior [Park et al., 2012b]. As mentioned before, T allele carriers may present lower NET levels within the brain, which might explain the better MPH response by having to block fewer NET to achieve response. Kim et al. [2013a] analyzed the association between neuropsychological measurements and G1287A and A-3081T SNPs at *NET1* gene after MPH treatment. The study enrolled 101 ADHD children and employed Comprehensive Attention Test (CAT), which are visual and auditory selective attention task, sustained attention task, and flanker interference task; which measure the changes in response time variability. The corrected significance level was set at $\alpha = 0.0034$. No significant difference between the genotypes was found, but there was an additive effect of A allele at G1287A SNP in the auditory selective attention task at an uncorrected level ($P = 0.02$) [Kim et al., 2013a].

It is important to notice that these *NET1* findings were derived from Asian samples. Different ethnic samples can present different genotype frequencies and this diversity may result in different medication response.

ADRA2A. The ADRA2A receptor is a central component of the noradrenergic system with a putative role on MPH action demonstrated in studies with animal models [Arnsten and Dudley, 2005; Andrews and Lavin, 2006]. Previous pharmacogenetics studies with the adrenergic alpha 2A gene (*ADRA2A*) have demonstrated that G allele at SNP C-1291G (rs1800544) is associated with greater reduction of inattentive symptoms over time [Polanczyk et al., 2007b; da Silva et al., 2008]. Froehlich et al. [2011] also found a main effect of this *ADRA2A* genetic variant (C-1291G) on MPH response ($P = 0.003$). However, the G allele was associated with significantly higher rates of hyperactive-impulsive symptoms on placebo and across doses. The gene–dose interaction was below the threshold of statistical significance ($P = 0.03$) No association with inattentive improvements was found in that study. Yang et al. [2012] investigated a haplotype with two SNPs at *ADRA2A* (rs1800544 and rs553668) and described an association between GG haplotype and non-remission of ADHD symptoms with atomoxetine treatment, but the significance disappeared after multiple test correction. It is possible that GG haplotype may determine inadequate functioning of $\alpha 2$ receptor, which could lead to less improvement [Yang et al., 2012]. Kim et al. [2013a] showed the changes in mean response time variability increased additively with presence of G allele at *Msp*I SNP for flanker interference task. The effect size was 0.09 based on Cohen's f^2. For the sustained attention task, the additive effect of G allele was only detected at an uncorrected level [Kim et al., 2013a]. No effect of ADRA2A was found in an adult sample [Contini et al., 2011]. As mentioned earlier the potential reasons for this diversity of findings are described in Table II.

COMT. The catechol-*O*-methyltransferase enzyme catabolizes both dopamine and norepinephrine, especially in the prefrontal cortex. The most studied SNP at the catechol-*O*-methyltranferase gene (*COMT*) is a functional polymorphism, which leads to amino acid substitution valine to methionine (Val158Met—rs4680). The Met allele results in a threefold to fourfold reduction in enzyme activity [Lotta et al., 1995]. A study from our group tested the effect of the *COMT* gene in response to MPH on oppositional symptoms in 251 ADHD children and adolescents. In that investigation ADHD boys with at least one Met allele have a significantly higher improvement on oppositional defiant disorder symptoms over time than Val homozygous when they are treated with MPH [Salatino-Oliveira et al., 2011]. It has been hypothesized that comorbidity between ADHD and oppositional defiant disorder is due to shared genetic liability either operating directly or indirectly through gene–environment interactions [Nadder et al., 2002]. There are evidences that the high-activity *COMT* genotype in ADHD may play a role in the manifestation of antisocial behavior [Langley et al., 2010]. Moreover, COMT may play a regulator role in catecholamine balance created by MPH action in the prefrontal cortex [Chen et al., 2004]. Froehlich et al. [2011] evaluated the same SNP and showed that Val homozygous had greater improvement in hyperactive-impulsive symptoms with increasing doses when compared to other groups, but this finding did not achieve statistical significance. However, this pattern is consistent with previous pharmacogenetics studies that have suggested a positive association between Val allele and response to MPH [Cheon et al., 2008; Kereszturi et al., 2008]. No effect was found in an adult sample [Contini et al., 2012]. COMT association with medication response may be restricted to some symptoms and further studies in children as well as in adults are warranted.

TPH2 and DBH. The tryptophan hidroxylase-2 (TPH2) is a brain-specific enzyme involved in serotonin biosynthesis, converting the amino-acid tryptophan to 5-hydroxy tryptophan, which is further decarboxylated into serotonin [Walther et al., 2003] Serotonin dysregulation has been related to impulsive and aggressive behaviors in children, and has thus been hypothesized to play a causal role in ADHD [Lucki, 1998]. The dopamine beta hydroxylase enzyme (DBH) catalyzes the conversion of dopamine (DA) into norepinephrine (NE) and is expressed within the secretory vesicle [Gaspar et al., 1989]. Since changes in both these catecholamine networks could be underlying ADHD pathophysiology, the genes that encode these two enzymes were considered candidate genes for ADHD susceptibility [Roman et al., 2002a]. These genes were investigated in an adult sample by Contini et al. [2012]. No significant association of either gene with response to MPH was reported. Given their function, these enzymes might interfere on responsiveness, but this was not demonstrated in that single study.

5-HTT. The potential role of serotonin in ADHD was addressed by Gainetdinov et al. [1999]. These investigators observed reduced hyperactivity after a selective serotonin reuptake blocker (fluoxetine) administration to mice whose DAT gene was knocked out. The serotonin transporter (5-HTT) is responsible for the presynaptic re-uptake of serotonin. A 44 bp insertion/deletion functional polymorphism in the 5-HTT gene promoter region (HTTLPR) leads to changes in expression of this transporter in humans. The basal activity of the long variant (L) is about threefold higher than the short (S) variant [Heils et al., 1996]. A functional SNP (A > G—rs25531) has also been identified within the LPR

region, located in the L allele, resulting in its division into two distinct alleles (L_A and L_G); the L_A variant is associated with 5-HTT mRNA higher levels while the L_G and S variants are associated with lower transcriptional activity [Lipsky et al., 2009].

Thakur et al. [2010] conducted a study to evaluate the association between 5-HTTLPR and behavior response to MPH. A total of 157 children with ADHD were assessed using Conners' Global Index for parents and teachers (CGI-Parents and CGI-Teachers) during treatment with placebo and MPH. They identified an association between the triallelic 5-HTTLPR polymorphism and behavioral response to MPH assessed by CGI-Parents. L_AL_A individuals presented significant improvement with MPH and responded minimally to placebo; homozygous children for the low expressing alleles ($S + L_G = S'$) responded significantly better to placebo and did not have additional improvement with MPH, whereas $S'L_A$ genotype had an intermediate profile. No association between 5-HTTLPR polymorphism and therapeutic response as assessed by teachers was reported [Thakur et al., 2010]. These results showed that patients had significant behavioral response independently from the treatment with the active drug or placebo. These findings are difficult to integrate to previous results because it started as a comparative investigation (MPH vs. placebo), but it was decomposed in two naturalistic investigations in which the genetic effects were assessed independently. No effect was found in an adult sample with both 5-HTTLPR and serotonin HTR1B receptor gene (*HTR1B*) (rs11568817, rs6296, and 13212041) [Contini et al., 2012]. These negative results suggest that serotoninergic genes may not be involved with response to MPH either in children or adults, but more studies with different SNPS are needed.

BDNF. Given that ADHD is often regarded as a neurodevelopmental disorder, genes affecting neuroplasticity and neuronal development are a new set of candidate genes. One such gene is the brain-derived neurotrophic factor (*BDNF*), which is involved in several processes including differentiation and survival of dopaminergic and serotonergic neurons [Hyman et al., 1991; Henningsson et al., 2009]. The most studied *BDNF* SNP is Val66Met (rs6265). This polymorphism may impact intracellular trafficking and BDNF activity-dependent secretion, and might be related to ADHD pathophysiology [Kent et al., 2005]. MPH response analyses according to *BDNF* Val66Met polymorphism were reported in a prospective study. The significance level was set at $P = 0.0017$, because they evaluated seven SNPs (one in the BDNF and six from other genes) and four outcomes. The total sample comprised 102 ADHD children and response status was assessed by CGI-I scores and ADHD-RS. The results pointed to a higher proportion of symptom remission (95.2%) in Val/Val homozygous than in Met-carrier children (74.1%) ($P = 0.013$). Val allele homozygous were more frequently (81%) assessed as "not ill" or "very mild" according to CGI-I compared to 37% of Met allele carriers ($P = 0.0002$). Moreover, more than 50% reduction in ADHD-RS scores was observed in 95.2% of the Val allele homozygous patients and in 74.1% of Met allele carriers ($P = 0.018$) [Kim et al., 2011]. The Val/Val genotype has been related to increased activity-dependent secretion of BDNF [Flanagin et al., 2006]. Met allele carriers show decreased volume in the dorsolateral prefrontal cortex and subcortical regions [Pezawas et al., 2004]. An explanation for better MPH response in ADHD children who are Val/Val homozygous might be due to a lower degree of brain anatomical deficit and functional impairment in these individuals [Kim et al., 2011].

SNAP25. Another neurodevelopmental gene is synaptosomal-associated protein 25 (SNAP-25), a neuron-specific protein implicated in exocytotic catecholamine release. Contini et al. [2012] evaluated two SNPs (rs3746544 and rs363020) on MPH response in 164 adults with ADHD and no effect was found. Although several evidences support a role for these genes is ADHD susceptibility, their role on response to medication remains to be determined.

LPHN3. Recently a new gene has been associated with ADHD pathogenesis, the latrophilin 3 gene (*LPHN3*). The association between markers at chromosome 4q13.2 (near *LPHN3*) and ADHD was first observed in a linkage study of a large multigenerational families in a population isolate, the Paisa from Colombia, where the prevalence of ADHD is high [Arcos-Burgos et al., 2002]. LPHN3 is a member of the LPHN subfamily of G-protein coupled receptors (GPCRs), which have been shown to be important for exocytosis of neurotransmitter regulation [Rahman et al., 1999; Linets'ka et al., 2002]. A subsequent study by Arcos-Burgos et al. [2010] examined extensively *LPNH3*; stimulant response was assessed in 240 children from a US sample. ADHD symptoms were rated on and off stimulant medication on the Strengths and Weaknesses of ADHD-Symptoms and Normal-Behavior (SWAN) scale. A significant association between response to stimulant medication and SNP marker rs6551665 was found both at the marker and haplotype levels. Individuals with either one or two G alleles had a better response to medication in three out of nine questions which compose the inattentive dimension and one out of nine questions that integrated the hyperactive/impulsivity dimension of SWAN scale. When the authors analyzed scale full score, they found an association for the inattentive dimension, but not for the hyperactive/impulsivity dimension [Arcos-Burgos et al., 2010].

The effect of six SNPs within the *LPHN3* gene on MPH response was evaluated in an independent sample of 416 children with ADHD. The main outcomes were evaluated with the Restricted Academic Situation Scale Task (RAST) and motor activity as measured by a hand held automatic device (Actiwatch) for hyperactivity dimension. Four SNPs (rs1947274, rs2345039, rs6551655, and rs6858066) have a significant effect in discriminating good responders from non-responders [Labbe et al., 2012]. These authors reported that the rs6858066 G allele confers both risk to ADHD and better treatment response. Similar findings were reported in an adult sample [Ribasés et al., 2011]. Labbe et al. [2012] also reported that the G allele of SNP rs6551665 is associated with poor response to treatment, whereas Arcos-Burgos et al. [2010] found an association with a better response with this same SNP. These authors do neither hypothesize a functional role for these SNPs nor for LPHN3 in MPH response. Since the first study found an association with inattentive dimension and the second one with the hyperactive dimension, it is possible that differences in sample subtype composition in terms of symptoms might be responsible for divergent results in *LPHN3* pharmacogenetics studies. Inconsistent findings in many genes appear again as in previous reviews. The heterogeneity of outcomes measures and population stratification could explain some of the conflicting results. However, several studies corrected the analysis and demonstrated that most positive associations showed small effect sizes

for the studied genes. Executive functions analyzed above are potentially useful as endophenotypes. They are hypothesized to be more suitable for detecting risk genes because they are genetically less complex by being etiologically closer to biological pathway leading from gene to behavior, diminishing phenotypic heterogeneity [Castellanos and Tannock, 2002].

Gene–Gene Interactions Studies

Investigations on individual genetic polymorphisms may not be the best approach for pharmacogenetic studies, since genetic factors might work primarily through a complex mechanism that involves multiple genes and, possibly, environmental factors. Thus, the effect of the gene/polymorphism might be missed if the single gene is examined without allowing for its potential interactions with these other unknown factors. One way to assess this relationship is conducting gene–gene interaction studies [Cordell, 2009].

Hong et al. [2012] investigated independent and interaction effects of *DAT1*, *DRD4*, *ADRA2A*, and *NET1* genes on MPH response in 103 children with ADHD, using ADHD-RS and CGI-I scores as outcome measures of treatment response. The results suggest some independent positive findings for SNPs in *NET1*. For the G1287A polymorphism, the proportion of good responders was 30.2% for G/G homozygous children and 16.0% for A-allele carriers children ($P = 0.08$). Considering the A-3081T SNP, 28.9% of T-allele carriers were good responders when compared to 7.4% with the A/A genotype ($P = 0.02$). Considering the significance level set for the analyses after correction for multiple comparisons ($\alpha = 0.0083$), multivariate logistic regression demonstrated effects for interactions between the *DRD4* VNTR genotypes and those of either the *ADRA2A Dra*I (rs553668) ($P = 0.0004$) or the *NET1* A-3081T polymorphisms on MPH response ($P = 0.01$) (trend); significant interaction effects were also detected between the *ADRA2A Dra*I polymorphisms genotypes and those of either the *NET1* G1287A or the A-3081T polymorphisms ($P = 0.0066$ and 0.0003, respectively). In addition, trends for interaction effects were detected between the *DAT1* VNTR genotypes and the *NET1* A-3081T polymorphisms ($P = 0.06$); and between *DRD4* VNTR genotypes and the *ADRA2A* C-1291G polymorphisms ($P = 0.06$). However, given the small group sizes produced by dividing the sample according to genotypes and response status, the chance of type II errors probably increased, negatively influencing the power of these analyses (Nagelkerke $R^2 = 0.40$) [Hong et al., 2012].

Jain et al. [2012] screened possible interacting regions within *LPHN3* gene and performed a correlation subset analysis. The rs6551665 *LPHN3* associated SNP showed an interaction effect with a haplotype harbored on 11q. This haplotype (rs666642–rs877137) encompasses the neuronal cell adhesion molecule 1 (*NCAM1*) and ankyrin repeat and kinase domain containing 1 (*ANKK1*) genes. This pharmacogenetic study evaluated 82 individuals with ADHD. The effect of *LPHN3*–11q interaction was examined using SWAN scores (questions 1–9 indicate inattentive symptoms and questions 10–18 indicate hyperactivity symptoms) during treatment. The results demonstrated a significant two locus model effect compared with a single locus effect for question 18 ($P = 0.0036$), when additive effects at *LPHN3* and dominant effect at 11q are considered. The GG genotype at rs6551665 together with two copies of the susceptibility haplotype on 11q (G-G) were correlated with significant improvement of symptoms in question 18 after treatment, whereas the AA genotype at rs6551665 and fewer than two copies of 11q haplotype were correlated with poorer response. This result should be interpreted cautiously, as the analysis of one question cannot be extended to the full hyperactive dimension. Further investigations are necessary to elucidate the biological mechanism of gene products on 11q and *LPHN3* to determinate how this interaction plays [Jain et al., 2012].

Gene–gene interactions have been increasingly explored in ADHD susceptibility studies, and they are becoming more attractive and studies investigating these interactions have emerged in recent years in the pharmacogenetics field. It is important to notice that the small effect size of candidate genes studies reviewed here suggests that response to MPH is influenced by several different genes and they can interact with each other. However, in the studies addressed here no a priori hypotheses were clearly established or biological pathways were determined for these interactions.

Genome-Wide Association Studies

By scanning the whole genome and identifying promising areas, the genome-wide approach might be relevant to ADHD pharmacogenetic investigations. One of the first genome-wide association studies in pharmacogenetics evaluated response to transdermal MPH in 187 children with ADHD. No association at genome-wide statistical significance was observed. However, among the top findings, the glutamate receptor, metabotropic 7 gene (*GRM7*) and two SNPs within *NET1* showed potential involvement in MPH response [Mick et al., 2008]. This possible association with *NET1* is consistent with results previously described in the candidate gene studies detailed above.

Increase in blood pressure is a potential adverse effect of MPH treatment [Cortese et al., 2012]. Recently, Mick et al. [2011] conducted a genome-wide association analysis of blood pressure response to MPH in 140 children. SNPs (316,934) were available for analysis. Due to the small sample size for GWAS and a heterogeneous sample, they did not identify a genome-wide statistically significant association between MPH treatment and changes in blood pressure. Suggestive findings involved genes functionally related to blood pressure regulation and other cardiovascular phenotypes, as a SNP in K^+-dependent Na^+/Ca^{2+} exchanger (*SLC24A3*). A genetic enrichment analysis implicated five biological processes: FERM domains, immunoglobulin domains, the transmembrane region, channel activity and type-III fibronectins [Mick et al., 2011]. This set of genes may be involved in adverse effects and should be further explored.

To date, only two GWAS in ADHD pharmacogenetics have been applied to identify genetic variants associated with medication response or adverse effect. Both have failed to identify genes at the stringent genome-wide significance level. GWAS with larger samples are required to detect moderate or small effect of genes involved with treatment response and may contribute to discover

other molecular targets in less obvious pathways relate to ADHD pathophysiology.

Gene–Environment Interaction Studies

In addition to genetic factors, environmental contributions might influence phenotypic variability and also clinical aspects of the disorder, as severity and comorbid profile. Chazan et al. [2011] demonstrated that an adverse environment can predict a worse MPH response in ADHD children. In gene–environment studies, it has been hypothesized that genotypes modulate response to environmental risk factors and may play a pivotal role in the disorder [Wermter et al., 2010]. Grizenko et al. [2010] assessed differences between ADHD subtypes (combined/hyperactive vs. inattentive) taking into account genetics factors (*DAT1* VNTR, *DRD4* VNTR, and 5-HTTLPR), comorbidities, environments factors and response to MPH treatment in 371 children with ADHD. Treatment response was evaluated by Conners' Global Impression Scale for parents and teacher (CGI-P and CGI-T, respectively). Comorbidity and stress during pregnancy were also assessed for each participant. Children with ADHD combined/hyperactive subtypes presented higher comorbid rate of conduct disorder, higher frequency of L/L genotype for the 5-HTTLPR, good response to treatment and were exposed to moderate stress during their mothers' pregnancy when compared to children with the inattentive subtype. No differences were found across subtypes for the *DAT1* and *DRD4* genes. Moreover, a combination of the L/L genotype and stress during pregnancy lead to an eight times higher risk of having combined/hyperactivity subtype, and this may be one of many possible mechanisms linking stress to genotype in ADHD development [Grizenko et al., 2010]. Notice that this study did not evaluate if genes influence medication response by itself. However, it demonstrated that ADHD subtypes might lead to different disorder outcomes concerning treatment response. Effects of genotypes, comorbidities, and environmental factors on disease may also differ among ADHD subtypes. These features must be considered in future pharmacogenetics studies. It has been demonstrated that hyperactive children respond more to pharmacotherapy, but the non-hyperactive ones need lower MPH doses to improve symptoms [Barkley et al., 1991; Stein et al., 2003]. The study of pooled samples comprising all subtypes could lead to heterogeneous results.

A study examined the role of SNPs in *LPHN3* in MPH response, taking into account maternal smoking and stress during pregnancy in 132 nuclear families. Main outcomes were Conners' Global Index for parents and teachers, and the clinical staff completed the Clinical Global Impression scale. Several association tests were conducted using each SNP separately. Since most of them were associated with the three tag SNPs (rs6551665–rs1947274–rs6858066), haplotype analysis was also performed. Considering the CGI-overall improvement scale, the risk haplotype AAG was associated with poor treatment response in the mild or minimal maternal stress group ($P = 0.01$), while the GCA haplotype have a better improvement with treatment ($P = 0.03$). However, considering Conners' Global Index for parents and teacher, this statistical significance was not maintained. It is interesting to notice different association between CGI for parents and teachers as well as clinical response, possibly due to reference bias [Choudhry et al., 2012]. This finding is in agreement with the one from the first *LPHN3* study where the G allele from the rs6551665 leads to a good response to MPH treatment [Arcos-Burgos et al., 2010]. However, it differs from Labbe et al. study [2012] results where the G allele at rs6858066 confers better response and G allele at rs6551665, poor response. Despite the small number of studies with *LPHN3*, it is possible that this gene influences clinical response only in favorable environments.

Thakur et al. [2012] conducted a study to evaluate the association between 30 tag SNPs within *NET1* and response to MPH treatment in children with ADHD, with stratification based on maternal smoking during pregnancy. The study enrolled 475 children evaluated by Conners' Global Index, Clinical Global Impression, and the Restricted Academic Situation Scale (RASS). The significance level after Bonferroni correction was set at $\alpha = 0.002$. The authors observed that the T allele at rs36021 was associated with more behavioral and cognitive deficits in the subsample which mothers smoked during pregnancy. In response to MPH, the T allele was associated with greater improvement on the CGI ($P = 0001$), Conners' ($P = 0.009$), and RASS ($P = 0.0003$). On the other hand, in the sample in which mothers did not smoke during pregnancy, the C allele at rs3785152 was associated with significant improvement on CGI ($P = 0.0005$) and RASS task disengagement ($P = 0.0003$). As both SNPs are located within introns, they might be involved in gene regulation or be in LD with functional variants [Thakur et al., 2012]. It is interesting to note that the same rs36021 allele seems to interact with exposure to maternal smoking and leads to both worst behavioral and cognitive functions and better response to MPH treatment. It is possible to speculate that a severe case may present greater room for improvement than other cases, so the effects of pharmacotherapy may be easier to be detected in this group.

Environmental factors are involved in ADHD etiology and evidence demonstrates that these factors also influence gene associations. The studies reported here show that environment is an important feature to be considered and evaluated in pharmacogenetic studies as they may alter response to medication and gene effects. Also, studies considering G×E interactions may contribute to explore the controversial results from various genes.

Neuroimaging Studies

Neuroimaging measurements constitute endophenotypes of great interest and have already been applied in several studies to investigate neurobiological effects of risk genes in ADHD [Tost et al., 2012]. However, this methodology was employed in few pharmacogenomic studies. Szobot et al. [2011] evaluated ADHD risk alleles at *DRD4* and *DAT1* genes and striatal DAT occupancy after treatment with MPH. A total of 17 children with ADHD and substance use comorbidity underwent a single-photon emission computed tomography (SPECT) at baseline and after 3 weeks on MPH; the results showed that the combination of *DRD4* 7R allele and 10R homozygosity at *DAT1* was significantly associated with a smaller DAT occupancy in caudate and putamen, bilaterally ($P = 0.02$–0.006). The R^2 of these analyses ranges from 0.50 to 0.56. These associations are lower if genotypes are not included. Striatal DAT occupancy was not significant for each genotype considered separately ($P \geq 0.08$). Drug use had an independent

effect in all brain areas (except left *putamen*) and there was no significant MPH dose effect. It is possible to speculate that there is an additional effect of both *DRD4* risk allele and risk genotype at *DAT1*, leading to a less efficient MPH occupancy [Szobot et al., 2011].

One study investigated MPH response-related hemodynamic changes according to SNAP-25 polymorphisms. The functional near-infrared spectroscopy (fNIRS) was evaluated in 16 children right-handed with ADHD on or off MPH with an interval of 24 hr. Outcomes assessed were the difference of oxyhemoglobin (HbO_2) and deoxyhemoglobin (HHb) levels recorded during incongruent stimuli and neutral stimuli. They showed that *Mnl*I (rs3746544) genotype at the *SNAP-25* gene was significantly associated with HbO_2 levels change in right prefrontal ($P = 0.015$) and HHb levels in left prefrontal ($P = 0.033$) regions with MPH treatment; mean left prefrontal HHb concentration increased during MPH use in patients with *Mnl*I G-allele carriers, whereas it decreased in patients with T/T genotype. The right prefrontal HbO_2 concentration increased in the T/T and decreased in the T/G or G/G group. The SNAP-25 *Dde*I polymorphism (rs1051312) was significantly associated with change of right prefrontal HHb concentration with MPH use ($P < 0.001$). HHb levels in right prefrontal increased with MPH treatment in the *Dde*I C-allele carriers and decreased in the T/T group. When both *Dde*I and *Mnl*I genotypes were taken into account, the genotype status was significantly associated with right HHb concentration change ($P = 0.003$). Pairwise comparisons revealed that children with *Dde*I C-allele carriers and *Mnl*I G-allele carriers had significantly different right HHb concentration changes when compared with children with *Dde*I T homozygous and *Mnl*I T homozygous or *Dde*I T homozygous and *Mnl*I G-allele carriers genotypes ($P = 0.05$ and < 0.001, respectively). They did not find association between genotype and treatment with behavioral performance. The blood flow cerebral increases during brain activation, but not all of oxygenized blood is used, so HbO_2 increases and HHb decreases during sustained activation. Higher HbO_2 levels and lower HHb levels might be related with neurovascular coupling and increased blood flow with HHb levels from activated brain region. Individual T homozygous in both SNPs did not increase HHb in right prefrontal, suggesting genotypes differently affect neurovascular coupling [Öner et al., 2011]. It is difficult to compare this study with other previous studies because they did not evaluate symptomatic response and comorbidity as well as did not split groups between responders or non-responders.

In another study, SPECT was performed in 37 drug-naïve ADHD children before and after treatment with MPH according to genotype for *NET1* G1287A and A-3081T polymorphisms. The clinical evaluation relied on ADHD-RS and CGI-I scores. Before MPH treatment, no significant differences in cerebral perfusion were observed between children with different *NET1* genotypes. After treatment, they found that children with G1287A G/G genotype showed more symptoms improvement as compared to A-allele carriers ($P = 0.022$). Hyperperfusion was observed in the right inferior temporal gyrus and left middle temporal gyrus of children with G/G genotypes compared to those without this genotype ($P < 0.001$ for both). No significant perfusion differences were observed in association with the A-308T SNP. This finding should be interpreted with caution, as it was significant only at the uncorrected threshold, but it suggests that G1287A may contribute to an intermediate phenotype [Park et al., 2012a].

Although promising, the number of neuroimaging studies currently available is limited. The lack of uniformity of methodologies, outcomes investigated, and replication makes the interpretation of these results a difficult task.

Drug Metabolism Genes and Adverse Events

Besides the study of response to medication itself, adverse events have attracted great interest lately. The carboxylesterase 1 (*CES1*) gene encodes the main enzyme involved in MPH metabolism. In a naturalistic study, Bruxel et al. [2013] assessed 213 Brazilian children with ADHD based on −75 T > G *CES1* (rs3815583) polymorphism. The primary outcome was appetite reduction, the most reported MPH adverse effect, measured by the Barkley Stimulant Side Effect Rating Scale. The G-allele carriers had worse appetite reduction scores than T/T homozygous over time of treatment ($P = 0.03$); G-allele carriers presented a 3.5 times higher risk to have the highest appetite reduction scores when compared to T allele homozygous ($P = 0.009$) with effect size based on Cohen's f^2 of 0.02. A trend effect of the daily mean MPH dose was also observed ($P = 0.08$). The SNP functionality is unknown, but since it is located at the 5′ UTR it could have an effect on gene regulation [Bruxel et al., 2013].

Cardiovascular adverse effect due to MPH treatment has also been reported. SNPs at norepinephrine genes (*NET1* G1287A, *NET1* A-3081T, *ADRA2A Dra*I, *ADRA2A* C-1291G) were investigated in 101 Korean children with ADHD. Electrocardiographic parameters (PR, QRS, AT intervals), heart rate (HR) resting, seated pulse, and blood pressure (BP) were evaluated. The data revealed that children with the *ADRA2A* C-1291G C/C genotype showed an 18.5% increase in diastolic blood pressure (DBP) when compared to baseline, but children with the G/G or G/C genotype showed only a 0.2% decrease after MPH administration. Individuals with a *NET1* A-3081T T/T genotype showed 12.5% increase in HR compared to baseline; whereas children with the A/T or A/A genotype showed a 3.5% and 2.5% increase after treatment, respectively [Cho et al., 2012]. Two previous studies reported that the sympathomimetic effects of norepinephrine activation by MPH along with activation of dopamine systems might lead to increases in systolic blood pressure (SBP), DBP, and HR at therapeutic doses [Volkow et al., 2003; Negrao et al., 2009].

Recently, the relationship of stimulant side effects and dopamine receptor genes (*DRD1* rs4532, *DRD2* rs6277, *DRD3* rs6280 *DRD4* 7932167, and *COMT* rs4680) was evaluated in 90 Caucasian children. The outcome was the modified Barkley Side-effect scale, which was reduced to three factors (nausea, social withdrawal, and irritability) using principal components analysis, which accounted for 48.2% of the variance. The allelic status was coded by presence of minor, heterozygous, and major homozygous. The main findings demonstrated a significant effect of the minor allele homozygous for *DRD1* polymorphism (rs4532) with more severe side effect for withdrawal and a trend for nausea after Bonferroni correction. This SNP is located in the 5′ UTR and it may affect gene expression [Levy et al., 2013].

Another study examined the association between adverse effects due to MPH treatment and *ABCB1* gene. It encoded efflux

transporter P-glycoprotein, a member of the membrane transport protein families belonging to adenosine triphosphate-binding cassette and expressed in brain tissues [Sakaeda et al., 2003]. A study proposed that ABCB1 limits MPH transport into the brain [Zhu et al., 2006]. A total of 134 ADHD children were assessed according to G2677A/T (rs2032582) SNP, using Barkley Stimulant Side Effect Rating Scale at 1, 2, and 4 weeks of MPH treatment. A logistic regression analysis showed that TT homozygous have nine times higher risk of adverse effects when compared to others genotypes ($P = 0.005$). A functional assay was also performed for to determine if SNPs variability differ in MPH transport. It was observed that the T allele reduced ABCB1 MPH-transporting activity across the cell membrane when compared to A or G allele. These results suggest that T homozygous children could have reduced P-glycoprotein function, leading to more absorption due to diminished efflux activity and therefore more MPH bioavailability, resulting in severe adverse effects [Kim et al., 2013b].

Park et al. [2014] conducted a study to evaluate the association between adverse events of MPH and Neurotrophin 3 (*NTF3*) SNPs in 96 ADHD children. *NTF3* is a neurotrophic factor that involved in the differentiation, development, maintenance, and survival of several kinds of neurons [Maness et al., 1994]. Two SNPs were selected, rs6332 and rs1805149, from tag SNPs of *NTF3*. The polymorphism rs6332 located in exon 3 was associated with ADHD susceptibility, intelligence, and selective attention deficit [Syed et al., 2007; Cho et al., 2010]. The gene seems to have a role in the pathophysiology of mood disorders [Duman, 2002]. Adverse events were assessed using the Barkley Stimulant Side Effect Rating Scale during 2 weeks of treatment. Principal components analysis was conducted to reduce the number of variables. Six factors were selected (emotionally, disengagement, aches/tics, over-focus/euphoria, sleep/appetite, dizziness/drowsiness), which accounted for 63.1% of variance. Each factor had more than one item of Barkley Rating Scale. The results demonstrated the A/A homozygous at rs6332 presented the highest emotionally and over-focus/euphoria factor scores ($P = 0.042$ and $P = 0.045$, respectively). When the subjects were divided according to rare allele, individuals with the A allele had statistically higher emotionally factor score compared to individuals without this allele. Also, they divided according to common allele and found that patients with the G allele had lower over-focus/euphoria factor score compared to patients without. The authors showed the emotional adverse event was associated with rs6332. So, they analyzed each item of emotional factor. Subjects A/A homozygous showed the highest proneness to crying and nail biting scores compared to others genotypes. Subjects with A allele had statistically higher proneness to cry and subjects with G allele showed lower nail biting compared to subjects without, respectively, allele. The rs1805149 SNP did not presented any significant association. The results demonstrated that *NTF3* SNP is associated with specific emotional side effects, corroborating with the possible role the *NTF3* gene in emotional problems [Hock et al., 2000; Fernandes et al., 2010]. However, a limitation of this study is that the analyses were not corrected for multiple comparisons; no effect size of the SNP was presented [Park et al., 2014].

Atomoxetine is metabolized mainly by CYP2D6 enzyme, converting to 4-hydroxyatomoxetine, the major oxidative metabolite, which is equipotent to atomoxetine, but circulates in plasma with lower concentrations [Sauer et al., 2003]. People with reduced activity of the enzyme are defined as poor metabolizers (PM) and their drug plasma concentration is higher compared to people with normal activity. Extensive metabolizers (EM) require lower doses to reach onset of response. Ultrarapid metabolizers (UM) show higher CYP2D6 activity [Gonzalez and Idle, 1994]. *CYP2D6* is a well-known highly polymorphic gene. ter Laak et al. [2010] investigated ten out of a hundred children treated for ADHD with standard doses of atomoxetine according to genotype status for CYP2D6 and CYP2C19, a minor pathway in atomoxetine metabolism. These children were selected based on late response and adverse effects development during treatment. These investigators showed that 8 of 10 genotyped patients were CYP2D6 poor metabolizers. Five patients exhibited low CYP2D6 activity with normal CYP2C19 activity, three patients have low CYP2D6 and CYP2C19 activity, one exhibited normal CYP2D6 activity but low CYP2C19 activity, and one patient exhibited both normal CYP2D6 and CYP2C19 activity. Dose adjustments were performed before and after CYPs genotyping, as well as clinical outcomes assessments. Before CYP2D6 genotyping, the initial maintenance dose was increased for three of the eight patients with low CYP2D6 activity, which led to more adverse effects in all three patients and to the refusal of further treatment by the patient with the lowest CYP2D6 activity. After genotyping, the dose of atomoxetine was reduced in the other two patients, which led to a better response in both cases. Three other patients stopped atomoxetine treatment before dose adjustment, and refused to restart atomoxetine at a lower dose after receiving therapeutic advice based on their genotype. In total, four of eight patients with low CYP2D6 activity stopped atomoxetine treatment because of initial adverse effects. In four other patients, doses were reduced after genotyping, leading to better tolerability and efficacy. The patient exhibiting only low CYP2C19 activity responded better after a change of atomoxetine intake from evening to morning. The patient with normal CYP2D6 and CYP2C19 activity responded better after dose increase [ter Laak et al., 2010]. This study corroborates with others that poor metabolizers had more adverse effects than EM [Michelson et al., 2001]. The study of adverse effects is of great importance, since they may impact directly on pharmacotherapy adherence.

INTEGRATIVE COMMENTS

In recent years, two phenomena can be identified in ADHD pharmacogenetic field. First, the number of studies continues to grow, making ADHD one of the mental health areas with more pharmacogenetics studies. This was clearly exemplified in a previous review on pharmacogenetics of child psychiatric disorders. It identified only one study in autism pharmacogenetics and another one in major depressive disorder or anxiety disorder, in comparison to the 33 studies in ADHD pharmacogenetics [Polanczyk et al., 2010]. Second, and probably most important, there has been a change of focus concerning ADHD pharmacogenetic studies. There are a decreasing number of published studies targeting dopaminergic genes while more attention has been given to noradrenergic genes (see Table I). This could be explained by the large amount of *DAT1* and *DRD4* studies without consistent genetic effects in clinical response. Moreover, as we stressed in our previous

TABLE I. Pharmacogenetics Studies Assessing Candidate Genes

Refs.	Gene	Polymorphism	Design	Sample characteristics	Medication, dose	Primary outcome measure	Primary results
Froehlich et al. [2011]	DAT1	3′ UTR 40-bp VNTR	RCT, double-blind, multiple dose, placebo, 4w (n = 89)	Mean age: 8.13y, 73% boys, 79% Caucasian	MPH	Vanderbilt ADHD rating scales for parents and teachers	Children without 10R presented great improvement at hyperactivity symptoms
	DRD4	Exon 3 48-bp VNTR			Mean maximum dose: 1.57 mg/kg/day		Children without 4R presented less improvement hyperactivity symptoms;
	COMT	rs4680 (Val158Met)					No association
	ADRA2A	C-1291G					No association
Contini et al. [2010]	DAT1	3′ UTR 40-bp VNTR	Naturalistic, 1mo (n = 171)	Mean age: 35y	MPH-IR	Good response: reduction ≥30% at Swanson, Nolan, and Pelham Rating Scale version IV (SNAP-IV) and Clinical Global Impression-Severity (CGI-S): 1–2 points	No association
		Intron 8 240-bp VNTR		52% male European–Brazilians	Minimal dose: 0.3 mg/kg/day		
		C-839T					
Pasini et al. [2013]	DAT1	3′ UTR 40-bp VNTR	Clinical trial, 24w (n = 108)	Mean age: 9.9y	Short acting-MPH	Executive functions	10R carrier showed great improvement in response inhibition
				Male	Mean dose: 0.5 mg/kg/day		10R homozygous presented great improvement in planning ability
							10R homozygous showed great improvement in working memory
Ji et al. [2013]	DRD4	Exon 3 48-bp VNTR	Case-control, 8w	Mean age: 9.6y, 71.2% male Korean	OROS-MPH	Clinical Global Impression-Improvement (CGI-I)	No association
			n = 114 cases and 84 controls		Minimal dose: 18 mg/day	Clinical Global Impression-Severity (CGI-S)	
						ADHD-RS	
Kim et al. [2010]	NET1	A-3081T (rs28386840)	Naturalistic, 8w (n= 112)	Mean age: 9.1y, 82% boys Korean	MPH	good response: 1–2 points at Clinical Global Impression-Improvement (CGI-I)	Children T allele carriers presented better response;
		G1287A (rs5569)				ADHD-RS	No association

(*Continued*)

TABLE I. (*Continued*)

Refs.	Gene	Polymorphism	Design	Sample characteristics	Medication, dose	Primary outcome measure	Primary results
Lee et al. [2011]	NET1	rs2242446	Clinical trial, 8w (n = 112)	Mean age: 10.2, 83% boys Korean	OROS-MPH	Good response: 1–2 points at CGI-I and CGI-S; reduction ≥50% at ADHD-RS	No association
		rs5568					
		rs5569					
		rs998424					
		rs1616905					
Yang et al. [2012]	NET1	rs3785143	Prospective, clinical trial, 8–12w (n = 111)	Mean age: 9.6y, 82.9% male Chinese	Atomoxetine	Response: reduction ≥25% ADHD-RS	C allele was the most presented in responders
	NET1	rs2279805			Dose range 0.5–1.4 mg/kg/day	Remission: score ≤1 ADHD-RS	Individuals C allele carriers achieved remission
	ADRA2A	rs1800544					These SNPs in GG haplotype were associated with non-remission of symptoms
	ADRA2A	rs553668					
Park et al. [2012b]	NET1	A-3081T (rs28386840)	Clinical trial, 8w (n = 53)	Mean age: 8.9y, 84.9% male Korean	MPH	CPT	T-allele carriers showed a greater decrease in the mean commission errors
		G1287A (rs5569)			Dose range 0.35 −1.77 mg/kg/day		Children G/G showed a greater decrease in the mean omission error scores
Contini et al. [2011]	ADRA2A	C −1291G	Naturalistic, 1mo (n = 165)	Mean age: 35y, 54.5% male European–Brazilians	MPH-IR	Good response: reduction ≥30% at SNAP-IV and CGI-S: 1–2 points	No association
		G −262A			Minimal dose: 0.3 mg/kg/day		
		C1780T					
Kim et al. [2013a]	NET1	G1287A (rs5569)	Prospective study, 12w (n = 101)	Mean age: 8.7y, 80% male Korean	OROS-MPH	Comprehensive Attention Test (CAT)	Additive effect of A allele in auditory selective attention task (uncorrected) on better response
	ADRA2A	A-3081T (rs28386840)			Initial mean dose: 0.24 mg/kg/day		No association
		rs553668					No association
		C-1291G					Additive effect of G allele in flanker interference task on better response
Contini et al.	5-HTT	5-HTTLPR	Naturalistic, 1mo	Mean age: 35y, 54.3%	MPH-IR	Good response: reduction	No association

(*Continued*)

TABLE I. (*Continued*)

Refs.	Gene	Polymorphism	Design	Sample characteristics	Medication, dose	Primary outcome measure	Primary results
[2012]	HTR1B	rs6296	(n = 164)	male European-Brazilians	Minimal dose: 0.3 mg/kg/day	≥30% at SNAP-IV and CGI-S: 1–2 points	
	TPH2	rs11568817					
	DBH	rs13212041					
	DRD4	rs1843809					
	COMT	rs4570625					
	SNAP25	Exon 3 48-bp					
		rs4680					
		rs363020					
Salatino-Oliveira et al. [2011]	COMT	rs4680 (Val158Met)	Naturalistic, 3mo (n = 251)	Mean age: 9.5y, boys European–Brazilian	MPH	SNAP-IV	Met carriers showed greater improvements at ODD symptoms than Val homozygous
					Minimal dose: 0.3 mg/kg/day		
Thakur et al. [2010]	5HTT	5-HTTLPR	Placebo-controlled, double blind, 2w (n = 157)	Mean age: 9.0y, 83.4% male	MPH	Conner's rating scale for parents and teachers	L_AL_A showed improvement with MPH
				87.3% Caucasian	0.5 mg/kg/day		S′ S′ responded better to placebo
							S′L_A genotype had an intermediate profile
Kim et al. [2011]	BDNF	rs6265 (Val66Met)	Prospective study, 12w (n = 102)	Mean age: 8.7y, 80.4% male	OROS-MPH	Good response: 1–2 points at CGI-I and CGI-S; reduction ≥50% at ADHD-RS	Val homozygous showed greater improvement than Met carriers
				87.3% Caucasian	Minimal dose: 0.21 mg/kg/day		
Arcos-Burgos et al. [2010]	LPHN3	rs6551665	ON/OFF medication (n = 240)	Mean age: 12.0y, 70.8% male United States	Stimulant medication	SWAN	G allele carriers presented a greater response to medication
Labbe et al. [2012]	LPHN3	rs1947274	Placebo-controlled, double blind, 3w (n = 416)	Mean age: 9.0y, 76.7% male	MPH	RAST	G allele is associated with a poor response to treatment
		rs2345039		85% Caucasian	0.5 mg/kg/day	Actiwatch	G allele was associated with greater response to treatment
		rs6551655					
		rs6858066					

TABLE II. Suggested Guidelines for Future ADHD Pharmacogenetics Studies

Study methodology	Methodological requirements
Randomized clinical trials	Clinically sound outcome measures with cut-off point defined previously from analyses
A priori definition of the hypotheses tested	Study design deposited in appropriate open access repositories (e.g., clinical trials.gov)
Standard range doses	Power assessment before analyses
Genetic targets with biological plausibility	Presentation of the effect sizes
Adjustments for the effects of comorbidity	Presentation of nominal significance and correction for multiple comparisons
Adjustment for potential confounders	Make available all other genetic targets already explored with the same sample (published and unpublished)[a]

[a]Despite correction for multiple comparisons might not be implemented considering these targets, it is also important to inform the number of previous associations analyses performed.

revisions, ADHD pharmacogenetics needs to move to other directions. Future studies should address gene–gene and G×E interactions, and the discovery of new candidate genes through GWAS. Also, other outcome measures should be further explored, such as endophenotypes and neuroimaging data. Besides response to treatment, more studies should assess the pharmacokinetic properties of the drug in study and adverse events. There is a clear process of shift in this direction (Fig. 1). Since the amount of variance in treatment response explained by genes does not seem to be substantial based on findings available, this shift may open more promising paths for the ADHD pharmacogenetic field.

The genetic etiology of ADHD involves interactions among multiple genetic variants and environmental conditions [Nigg et al., 2010]. In this review, it was possible to note that many articles evaluate more than one gene. Two studies employed a gene–gene interaction approach and found an effect of the interaction in MPH response, but they did not provide clear biological plausibility for their findings. However, several new investigations continue to perform analyses for each SNP separately although assessing several SNPs, but without performing synergy or epistatic interactions tests among them. It is important to note that gene–gene interaction analyses need caution in the way of interpreting the findings. When all possible interactions are accounted for, the number of potential tests is very large and splitting samples according to many SNPs leads to a diminished power for analyses. Future studies will require larger samples sizes and a priori plausible biological hypothesis to address gene–gene interactions. Another very relevant question that remains open and should be further explored is whether genes involved in ADHD would be differentially expressed across the life cycle (childhood and adulthood) and how it could moderate treatment response, the results already published did not provide data that could explain the inconsistent findings in different developmental stages.

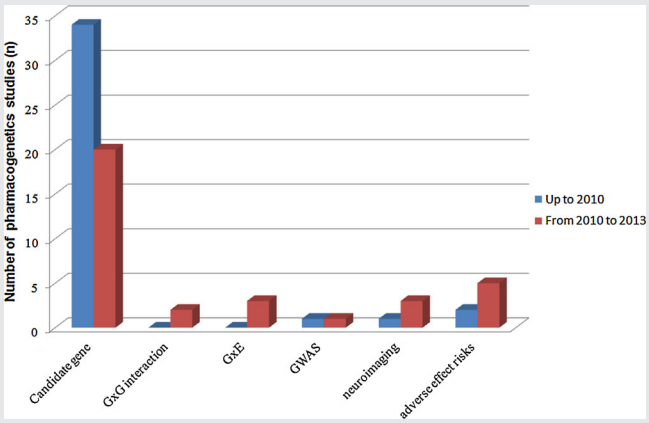

FIG. 1. Number of pharmacogenetics studies according to different outcomes in attention-deficit/hyperactivity disorder in two reviews. Data for the period up to 2010 were taken from Kieling et al. [2010].

Environmental factors implicated in ADHD often involve exposure to adverse circumstances early in life. For example, the consequences of fetus exposure to the toxic effects of nicotine are well documented [Ernst et al., 2001; Blood-Siegfried and Rende, 2010]. Thus, it is not a surprise that the environment factors reported in the three gene–environment interaction studies described in this review were maternal smoking and maternal stress during pregnancy. The focus in ADHD pharmacogenetics is still how genes and environmental factors interact with one another in relation to drug response.

There has also been increasing interest in cognitive and neuroimaging endophenotypes. The first investigations produced encouraging results as the refinement of phenotypes that can be explained by genotypic differences in treatment outcome. In addition, this kind of study may provide means to investigate the biological mechanisms involved in the action of the medication used for the disorder. Neuroimaging methods are being applied to investigate neurobiological effects of risk genes in ADHD and they may provide an explanation on how gene variants and environmental influences can affect brain activities before and after stimulant administration.

A large portion of ADHD patients present comorbidities. It has been reported that comorbidity may moderate ADHD treatment response. For example, two studies demonstrated that ADHD children with comorbid oppositional defiant disorder have reduced or lack of response to treatment [Ghuman et al., 2007; Goez et al., 2007]. Therefore, it is essentially important that there are

some clinical strategies to manage frequently comorbid disorders with ADHD and to optimize outcomes [Shier et al., 2013]. On the other hand, the prevalence of these conditions leads to samples without statistical power to detect the influence of comorbidities in ADHD treatment [Polanczyk et al., 2008].

In this review, almost all studies that investigated MPH pharmacogenetics focused on genetic variants of drug targets, such as transporters and receptors and have emphasized symptom reduction. Five studies and one GWAS addressed tolerability during MPH treatment. These investigations suggest that the prediction of adverse events and medication tolerability would be a clinically relevant area of research, since adverse events are major impediments to long-term treatment adherence [McGough et al., 2006, 2009]. Surprisingly, little attention has been paid to the genetic variability of drug metabolism in the area of ADHD pharmacogenomics. Atomoxetine metabolism by CYP2D6 is well established but more pharmacogenetics studies are necessary to understand if there is room for CYP2D6 testing when using atomoxetine in the context of personalized medicine.

The fact that MPH doses were not associated with genotype in some studies cited in this review does not discard the possibility of association. In naturalistic studies, clinicians have incremented doses in each follow-up visit according to response and adverse events with initial and follow-up doses that, although not fixed, were supposed to target respectively around 0.3 and 0.7 mg/kg/day at a routine clinical practice. Hence, some studies designs are not adequate to investigate the association between genotype and MPH dose. Froehlich et al. [2011] investigated fixed-dose range and found that MPH response was dose dependent according to *DAT1* VNTR.

Effect size in pharmacogenetics studies can provide the gene magnitude to predict a degree of variability in treatment response. According to data structure, the studies in this review described different effect size types. All of them showed that SNPs have a small effect size and therefore they support the prediction that response to MPH is influenced by several different polymorphisms, each one exerting a small effect [Faraone and Mick, 2010], given that medications have large effect sizes in general [Spencer et al., 2000].

The heterogeneity in methodological strategies employed by different studies remains impressive. Differences encompass the design of the study (retrospective, naturalistic, open-label, placebo-controlled), response of treatment definition (25%, 30%, or 50% reduction of symptoms), scales used to assess response, information source (clinician, parents, and/or teachers reports), dose titration, duration of treatment, statistical analysis (multiple comparisons without correction), identification and control for relevant confounding factors, sample sizes, among others. However, one thing calls even more attention regarding methodological issues: very few if any of the investigations in the field clearly disclose if their research questions were set a priori of analyses and the number of other genes/SNPs comparisons made besides the one with positive results or those presented in the article (e.g., authors ran uncountable association and only present those that are related to the biological plausibility of the manuscript). This lack of information opens a large avenue for Type I error. Thus, these methodological differences in the studies taken all together are likely to explain the inconsistent results and constitute a significant barrier to interpret non-convergent findings as failure to replicate findings [Polanczyk et al., 2008]. Investigations should employ more standardized study designs while examining wider dose ranges of both MPH and atomoxetine, as well as standardized outcome measures, because environmental factors, differences in personal styles in perceiving and reporting symptoms among teachers and parents, as well as the interaction between the observer and the child have an important impact on the child's behaviors and the way of reporting them [Stein, 2004]. On the other hand, power assessment before beginning new studies will provide reliable findings, even though methodologies are different between several studies.

As ADHD diagnosis is based on operational criteria—DSM-5—standardized outcome measures of medication response are also required. Because the studies identified in this review used several different outcomes, it becomes difficult to compare them. In addition, the different study designs also account for the finding heterogeneity. Consistent results from different methodological strategies have been found in randomized clinical trials. The approach used to define genotype groups for analysis also represents a limitation in many studies, since rare genotypes are grouped together and the analyses take into account a dominant effect. When other ways of grouping genotypes, based on the presence of one or more alleles, were implemented, different results have emerged. The dosage of medication should be evaluated by standard range dose, which deduce better the pharmacogenetics effect than flexible dosage. Moreover, it would be interesting to study novel molecular targets as glutamate-related genes, which mediate intracellular signaling neuron pathway [Lesch et al., 2013]; or neurodevelopmental genes during medication treatment, since medicated children with ADHD did not differ from control patients in white matter volume of brain and unmedicated children with ADHD differ from control group [Castellanos et al., 2002]. Medication effect might influence these genes responsible for synaptic plasticity or brain development. Metabolism genes would be better understood if the medication plasma concentration levels could be monitored. It would help to understand how the medication clearance undergoes according to genotype and its influence on dosage medication.

Based on everything exposed above, it is possible that a proportion of the findings presented up to now are based on convenient and unsubstantiated strategies, focusing on significant statistical results. To improve the quality of research in the field, we suggest some recommendations for future studies which are listed in Table II. Since power is always a matter of attention in pharmacogenetic investigations, multi-sites studies with comparable methodology, allowing joint analyses, are extremely important [see Polanczyk et al., 2008].

To date, several studies have been dedicated to understand the genetic variants underlying interindividual variability in pharmacological parameters, as well as adverse effects and efficacy. Such information could improve treatment, by shifting from trial-and-error approach to a pharmacological regimen that takes into account the individual variability. Given the small effect of genetics variants studied so far, it is an open question whether, how, or when results from ADHD pharmacogenetics studies will be useful in clinical management.

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CAPÍTULO VII

Discussão

A discussão mais específica referente aos resultados obtidos nesta Tese encontra-se nos capítulos anteriores de III a VI. No presente capítulo, serão mencionados alguns aspectos mais gerais deste trabalho com a finalidade de agregar os resultados e suas implicações no futuro dos estudos genéticos e farmacogenéticos do TDAH.

O trabalho iniciou com a tentativa de replicar dados que até então apresentavam evidências de um gene pouco conhecido e que estaria envolvido na suscetibilidade ao TDAH e que poderia predizer melhora do tratamento com estimulante (Arcos-Burgos et al. 2010). Nossos resultados foram similares aos estudos prévios e mais recentes em diferentes populações que demonstraram a importância do gene *LPHN3* na etiologia do transtorno. Contudo, não há o esclarecimento do processo biológico de como a *LPHN3* desempenha um papel para o desenvolvimento do TDAH. Sabe-se que a ausência do gene causa sintomas comportamentais presentes no transtorno (Wallis et al. 2012). Porém, a região mapeada no cromossomo 4q13.2 está localizada entre os éxons 4 e 19 do gene *LPHN3* e a maioria dos SNPs analisados encontram-se em regiões intrônicas (Arcos-Burgos et al. 2010; Domené et al. 2011; Ribasés et al. 2011; Fallgatter et al. 2013). Assim, nenhum dos SNPs associados até o momento modifica drasticamente a estrutura da proteína para ser considerada a variante causal. A reconstrução filogenética de haplótipos sugere que os alelos de proteção para o transtorno evoluíram dos alelos de suscetibilidade, o que seria condizente com uma mudança genética sutil na função da LPHN3 que predisporia o TDAH e a falta de mutações drásticas não seria inesperada (Domené et al. 2011).

A compreensão do mecanismo estrutural da interação LPHN3-FLRT3 está agregando novas informações e direcionando os próximos estudos para a elucidação do funcionamento molecular da LPHN3 nas sinapses excitatórias. Os experimentos de adesão celular e densidade sináptica através da deleção dos domínios protéicos da LPHN3 sugerem que para a interação ocorrer com FLRT3, é necessário apenas o domínio OLF. Por outro lado, a presença de ambos os domínios OLF e Lectina é crucial para interagir com teneurina 1 (TEN1), uma proteína de superfície celular envolvida no neurodesenvolvimento. Esses dados indicam que o domínio extracelular da LPHN3 contém um sítio de ligação distante da membrana celular, situado em uma haste alongada e potencialmente flexível o suficiente para abranger a fenda sináptica, com diferenciáveis sítios de ligação pra FLRT3 e TEN1 (O'Sullivan et al. 2014). Além disso, o FLRT3 pode dimerizar, bem como interagir com receptores de guias axonais descoordenados-5 (UNC5,

do inglês, Uncoordenated-5), que atuam como sinais repulsivos na migração neuronal. Ainda, pode ocorrer a formação um complexo trimérico LPHN3-FLRT3-UNC5, na qual FLRT3 possui sítios específicos para cada um, mas LPHN3 e UNC5 não interagem entre si (Lu et al. 2015). Isso mostra o quão complexas são as interações entre as moléculas de adesão. Se a LPHN3 atua como molécula de adesão com FLTR3 no desenvolvimento das sinapses glutamatérgicas, seria plausível sugerir a realização de estudos estruturais e dinâmicos da possível interação biológica entre LPHN3 e NCAM1 e que reforçariam o resultado obtido na presente Tese e em trabalhos prévios que demonstraram a interação estatística significativa entre os SNPs nos seus respectivos genes (Acosta et al. 2011; Jain et al. 2011).

A partir do conhecimento dos domínios protéicos responsáveis pela interação entre LPHN3 e FLRT3, houve uma busca por mutações que pudessem interferir na ligação e servir de modelo para os estudos de interação *in vivo*. A mudança de animoácidos em alguns resíduos do domínio OLF da LPHN3 realmente causam a perda de ligação com FLRT3 (Jackson et al. 2015; Lu et al. 2015). Mas ao fazer uma busca no banco de dados do NCBI (http://www.ncbi.nlm.nih.gov), apenas duas variações de nucleotídeo único (do inglês, single nucleotide variation, SNV), rs376388884 e rs769983094, cuja frequência alélica é invariável foram identificadas. Por outro lado, o estudo de uma mutação em *FLRT3* que já havia sido observada (Seiradake et al. 2014) e denominada de "mutante de dimerização FLRT3-FF" demonstrou que ela diminui a dimerização de FLRT3 e elimina completamente a ligação com LPHN3. Consequentemente, isso afetaria a migração tangencial de neurônios (Lu et al. 2015). Com essas evidências, o foco dos próximos estudos deverá ser a busca de variantes raras e comuns no domínio OLF do gene *LPHN3* que potencialmente possam implicar na patofisiologia do TDAH enquanto que o gene *FLRT3* torna-se um gene candidato promissor para futuros estudos moleculares.

Considerando que a LPHN3 também pode estar presente nos neurônios pós-sinápticos e desencadeiam a sinalização via proteína G através da interação com proteínas SHANK, o estudo do gene dessa proteína se torna mais um candidato para estudos genéticos. Mutações em *SHANK3* conferem risco para o transtorno do espectro autista (TEA) que são caracterizados por prejuízos na interação social e comunicação, bem como interesses e padrões comportamentais restritos que podem sobrepor com TDAH (Moessner et al. 2007). As mutações em *SHANK3* podem modificar a morfologia das espinhas dendríticas e

que resultaria nos defeitos nas sinapses estriatais e circuitos cortico-estriatais (Peca et al. 2011). SHANK3 também acopla a proteína de ancoragem quinase G (GKAP) e interage com o complexo formado pelo receptor NMDA, proteína de densidade pós-sináptica 95 (PSD-95) e o óxido nítrico sintetase 1 (NOS1) ativando a cascata de ativação via segundo mensageiro. O gene *NOS1* também é associado ao TDAH, principalmente a comportamentos impulsivos (Reif et al. 2009; Hoogman et al. 2011; Salatino-Oliveira et al. 2015b). SHANK ainda podem se ligar a proteínas HOMER, que também são proteínas de densidade pós-sinápticas que interagem com mGluR5 (Xiao et al. 2000) e tanto CNVs como SNPs em GRM5 já foram associados com TDAH (Elia et al. 2010; Hinney et al. 2011b)

O estudo do sistema GABAérgico foi uma abordagem inovadora e que resultou em artigos inéditos. Nós partimos da hipótese *a priori* que o GABA influencia os sintomas de hiperatividade/impulsividade do TDAH (Gilbert et al. 2011; Edden et al. 2012). Entretanto, a falta de estudos de genes GABAérgicos com TDAH nos levou a busca de possíveis variantes em estudos de associação com outros transtornos do neurodesenvolvimento que parecem ter sobreposição de mecanismos genéticos, como por exemplo: TEA, transtorno bipolar, transtorno de ansiedade e esquizofrenia (Ramamoorthi and Lin 2011). A escolha de SNPs em *GAD1* foi baseada na possível funcionalidade que pudesse predizer que um dos alelos aumentasse ou diminuísse a atividade da enzima. Nossos resultados demonstraram que esse gene está associado a suscetibilidade ao TDAH, principalmente com os sintomas de hiperatividade/impulsividade em crianças. Por esses dados serem originais, nós tentamos replicar os resultados em uma amostra populacional, através da abordagem que considera o TDAH melhor compreendido dimensionalmente como o extremo de uma distribuição normal de sintomas de atenção, hiperatividade e impulsividade, na qual fatores genéticos e ambientais contribuam para isso (Larsson et al. 2012; Bralten et al. 2013). Mas não obtivemos sucesso, uma vez que o GABA pode estar diferentemente expresso ao longo do neurodesenvolvimento de pacientes com TDAH (Bollmann et al. 2015). Pelos resultados apresentados, é importante considerar a homogeneização dos fenótipos como estratégia no intuito de analisar rotas genéticas que possam explicar essas características, pois os sintomas de hiperatividade/impulsividade e desatenção apresentam algumas especificações genéticas e redes neuronais distintas, como

também se expressam diferentemente ao longo da vida dos indivíduos (Greven et al. 2011; Shaw et al. 2012; Bralten et al. 2013).

Em uma perspectiva de continuidade desse estudo, seria necessário verificar se mecanismos epigenéticos podem controlar a expressão do gene *GAD1* diferentemente em indivíduos com TDAH e controles, pois durante a maturação do córtex pré-frontal, que inicia no período pré-natal e continua até a puberdade, ocorre um aumento da transcrição de mRNA de *GAD1* que está correlacionado com o remodelamento da cromatina, através do aumento da trimetilação da lisina 4 na histona H3 (H3K4me3). Em pacientes esquizofrênicos encontra-se uma redução de mRNA de *GAD1* e de metilação em H3K4. Redução similar é encontrada em pacientes que são bialélicos para os SNPs no *GAD1* identificados como de risco para a doença (Huang and Akbarian 2007).

Outra questão relevante para os estudos posteriores é relação da expressão de GAD_{67} aos receptores NMDA. Em culturas celulares de neurônios corticais, antagonistas para esses receptores reduzem a expressão de GAD_{67}, reduzindo as sinapses inibitórias (Kinney et al. 2006; Zhang et al. 2008). Danos genéticos nos receptores NMDA reduzem GAD_{67} no córtex e hipocampo de camundongos, um déficit que é associado a hiperlocomoção e inibição de pré-pulso prejudicada (Belforte et al. 2010). Estudos post-mortem demonstraram que em neurônios GABAérgicos com redução de GAD_{67} há uma diminuição na densidade da subunidade NR2A (Woo et al. 2004). Assim, perduram algumas dúvidas: A diminuição de mRNA de *GAD1* encontrada em cérebros esquizofrênicos pode ser causada por alguma ou mais variantes genéticas deletérias nas subunidades dos receptores NMDA? A associação encontrada com SNPs em *GAD1* pode ser na verdade uma associação em SNPs nas subunidades de NMDA? Variantes genéticas em GAD1 e NMDA poderiam ser sinérgicas e causar fenótipos mais graves?

De acordo com a literatura, um marcador do córtex motor encontrado reduzido em pacientes com TDAH é mediado pela inibição dos receptores $GABA_A$ (Gilbert et al. 2011). A partir da hipótese de que algum SNP em alguma subunidade desses receptores pudesse estar associado a uma atividade comprometida contribuindo para esse fenótipo, nós selecionamos um SNP em *GABRA1* e outro em *GABRB2* que já haviam sido associados com esquizofrenia em estudos anteriores (Petryshen et al. 2005; Zhao et al. 2007). Os resultados obtidos no presente trabalho não apoiaram um papel desses genes na

suscetibilidade ao TDAH, mas também não exclui que outras subunidades possam desempenhar alguma função.

Primeiramente, a estratégia a ser adotada para selecionar novas subunidades a serem estudadas é verificar se ocorre expressão diferenciada entre crianças e adultos. Pois enquanto que em crianças os receptores $GABA_A$ são mais prováveis de serem compostos das subunidade α2 ou α5, β1 ou β3 e γ1, em adultos as subunidades mais predominantes são α1, β2 e γ2 (Fillman et al. 2010). Essa expressão diferenciada também poderia explicar nossos resultados negativos, em razão da nossa amostra ser composta de crianças e adolescentes e estudamos os receptores mais predominantes em adultos.

As propriedades farmacológicas de cada subunidade de $GABA_A$ também devem ser consideradas. A ação sedativa dos benzodiazepínicos é mediada pela ligação do receptor $GABA_A$ contendo a subunidade α1 , demonstrando por camundongos nocaute α1(Rudolph et al. 1999). Já a atividade ansiolítica desses fármacos é mediada pelas subunidades α2, α3 e α5 dos receptores $GABA_A$. Essa atividade parece ocorrer com baixa ocupação da subunidade α2 , enquanto que em α3 ocorre alta ocupação (Atack et al. 2005; Dias et al. 2005). A utilização de agonistas inversos parciais que atuam seletivamente nas subunidade α5 melhoram a aprendizagem e a memória (Crestani et al. 2002; Yee et al. 2004).

Por último, procurar variantes nas porções mais divergentes das sequências das subunidades dos receptores $GABA_A$, que se localiza na alça citoplasmática entre as proteínas transmembranas 3 e 4. Nessas porções divergentes estão os sítios de modificações pós-traducionais que intervêm na função do canal e nas interações entre as subunidades com as proteínas envolvidas na regulação do tráfego de íons e localização (Hines et al. 2011).

A respeito de dados farmacogenéticos, os resultados encontrados com o gene *LPHN3* corroboraram com a maioria dos estudos anteriores, no qual os mesmos alelos que predispões risco ao TDAH também predizem uma rápida resposta ao tratamento, principalmente no primeiro mês. Contudo, o tamanho do efeito do gene na resposta ao medicamento foi pequeno para ambos os haplótipos, o que é condizente com as doenças multifatoriais.

Nós realizamos uma revisão sistemática dos estudos farmacogenéticos em crianças em adultos durante o período de três anos, desde a última revisão publicada sobre o assunto (Kieling et al. 2010). Nós pudemos comprovar o aumento exponencial dos estudos

marcado principalmente pela mudança de focos de genes do sistema dopaminérgicos para genes do sistema noradrenérgicos e de neurodesenvolvimento, provavelmente devido grande número de estudos com efeitos inconsistentes na resposta clínica dos pacientes ao tratamento com os genes *DAT1* e *DRD4*. Pudemos notar o aumento das investigações que buscam identificar efeitos das interações gene-gene e gene-ambiente, bem como endofenótipos neuropsicológicos e neuroimagens e a predição do desenvolvimento de efeitos adversos. Contudo, a heterogeneidade metodológica dos estudos e a conveniência dessas metodologias em focar nos resultados significativos, sem a devida relevância biológica, leva aos dados divergentes encontrados na literatura.

Devido ao pequeno efeito de variantes genéticas estudadas até então, os dados farmacogenéticos não parecem ser úteis para predizer eficácia e tolerabilidade do tratamento com metilfenidato. A farmacogenética é mais útil na resposta ao medicamento que dependa de poucas variantes genéticas como ocorre no tratamento da varfarina para desordens tromboembolíticas, no qual dois genes envolvidos na metabolização e farmacodinâmica (*CYP2C9* e *VKORC1*, respectivamente) explicam cerca de 30% da variação de dose (Botton et al. 2011); e também o fármaco trastuzumab (Herceptina®) que interfere na superexpressão do gene *HER2* que está presente nas formas mais agressivas de câncer de mama (Boekhout et al. 2011). A grande questão que fica é: Devemos manter os esforços nos estudos farmacogenéticos do TDAH? Os resultados obtidos até agora sugerem que a farmacogenética não beneficiará os pacientes até que possamos elucidar os mecanismos etiológicos e neurobiológicos do TDAH e encontrarmos a maioria dos marcadores genéticos que expliquem a grande herdabilidade desse transtorno. Ainda assim, será difícil estabelecer as relações de causalidade dos fatores ambientais e genéticos e como eles interagem, pois além de diferentes combinações alélicas, podem existir diferentes combinações gênicas e diferentes fatores ambientais que atuam no desenvolvimento da ampla gama de fenótipos vistos no TDAH.

A presente Tese acrescenta dados de uma rota biológica pouco explorada que é o balanço excitatório/inibitório gerado por glutamato e GABA e que pode estar envolvido na etiologia do TDAH. Mais estudos são necessários para reiterar a participação desses sistemas e aumentar a compreensão dos mecanismos moleculares. As estratégias das futuras investigações devem integrar os estudos de varredura genômica de variantes comuns e raras, expressão gênica em vários estágios do desenvolvimento dos indivíduos,

as interações gene-gene e gene-ambiente. Diante de todas essas dificuldades, a busca pelo entendimento desse transtorno não pode parar, pois só assim poderemos aprimorar o diagnóstico, otimizar o tratamento e desenvolver novos alvos terapêuticos.

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