Factors genètics de susceptibilitat al Trastorn per dèficit d'atenció amb hiperactivitat (TDAH)

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FACTORS GENÈTICS DE SUSCEPTIBILITAT AL TRASTORN PER DÈFICIT D'ATENCIÓ AMB HIPERACTIVITAT (TDAH)

Memòria presentada per
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Tesi dirigida pel **Dr. Bru Cormand Rifà** i la **Dra. Marta Ribasés Haro** al servei de Psiquiatria de l'Hospital Universitari Vall d'Hebron de Barcelona i al Departament de Genètica de la Universitat de Barcelona.

Dr. Bru Cormand Rifà

Dra. Marta Ribasés Haro

Cristina Sánchez Mora
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Al meu avi

"Si el cerebro humano fuese tan sencillo que lo pudiésemos entender entonces seríamos tan estúpidos que tampoco lo entenderíamos"

Jostein Gaarder

"La naturaleza humana es el centro capital de las ciencias y es fundamental desarrollar una ciencia del hombre. Esto se ha de hacer aplicando el método experimental, el único fundamento sólido que podemos dar a esta ciencia, ha de radicar en la experiencia y la observación. Debemos comenzar por una rigurosa investigación de los procesos psicológicos humanos y de su comportamiento moral e intentar a continuación averiguar sus principios y causas. Debemos partir de datos empíricos y no de una pretendida intuición de la esencia de la mente humana, que es algo que se escapa a nuestra comprensión. Nuestro método debe ser inductivo, más que deductivo, y si los experimentos de este tipo son juiciosamente reunidos y comparados, podemos esperar establecer una ciencia, no inferior en certeza, aunque superior en utilidad a cualquier otra"

Tratado de la Naturaleza Humana, Hume

"Mi punto de vista fue que existen dos caminos al considerar la selección natural, la aproximación desde el punto de vista del gen y la aproximación desde el individuo. Entendidos apropiadamente son equivalentes, son dos visiones de la misma verdad. Podemos saltar de uno a otro y será todavía el mismo neodarwinismo"

El gen egoísta, Richard Dawkins

Abreviatures

5HIAA	**Àcid 5-hidroxiindolacètic**
5HT	**Serotonina o 5-hidroxitriptamina**
5HT1	**Receptors serotoninèrgics 1**
AADC o DDC	**DOPA-decarboxilasa**
AMPc	**Adenosín monofosfato cíclico**
BDNF	**Factor neurotròfic derivat de cervell *Brain-derived neurotrophic factor***
CNR1	**Receptor cannabinoid 1**
CNVs	**Variants de número de copia.**
COMT	**Catecol- O- metil transferasa**
DA	**Dopamina**
DBH	**Dopamina-β-hidroxilasa**
DRD1	**Receptor D1 dopamina.**
DRD2	**Receptor D2 dopamina.**
DRD3	**Receptor D3 dopamina.**
DRD4	**Receptor D4 dopamina.**
DRD5	**Receptor D5 dopamina.**
FE	**Funcions executives**
GABA	**Àcid gamma-aminobutíric**
GWAS	**Estudi d'associació a escala genòmica**
HRR	**Risc relatiu d' haplotip (Haplotype Relative RisK)**
HTR1B	**Receptor de serotonina 1B**
Indels	**Insercions o duplicacions**
LD	**Desequilibri de lligament**
L-DOPA	**L-dihidrofenilalanina**
MAF	**Freqüència de l'al·lel minoritari (*Minor Allele Frequency*)**
MAO	**Monoamina Oxidasa**
NGF	**Factor de creixement neuronal**
NT3, 4 o 5	**Neurotrofina 3, 4 o 5**
p75NGFR	**Receptor de neurotrofines de baixa afinitat no selectiu**
PAH	**Feninalanina hidroxilasa**
PET	**Tomografia per emissió de positrons**
RM	**Ressonància magnètica**
SCL6A4	**Transportadors de serotonina**
SLC6A3	**Transportador de Dopamina.**
SNC	**Sistema nerviós central**
SNP	**Polimorfisme de nucleòtid simple (Single Nucleotide Ploymorphisme)**
SPECT	**Tomografia Computarizada per Emissió de Fotons Individuals**
TDAH	**Trastorn per dèficit d'atenció amb hiperactivitat.**
TDT	**Test de desequilibri en la transmissió (Transmission Disequilibrium Test)**
TH	**Tirosina hidroxilasa**
TPH	**Triptòfan hidroxilasa**
TPH1	**Gen triptòfan hidroxilasa**
Trk A,B i C	**Receptors neurotròfics tirosina cinases**
VNTR	**Repeticions en tàndem**

Introducció

Hipòtesi i Objectius

Resultats

Discussió

Introducció

1. GENERALITATS

1.1 Clínica del TDAH

1.1.1 Introducció històrica

La primera descripció científica sobre l'existència d'una síndrome clínica que afecta la població infantil basada en la inatenció, la conducta hiperactiva i la impulsivitat data de començaments del segle XX. L'any 1902 Still publicà una descripció clínica de 43 casos d'allò que actualment es coneix com a Trastorn per Dèficit d'Atenció amb Hiperactivitat (TDAH) (Still, 1902). Quan ens endinsem en la història del TDAH, un dels fets més sorprenents són els successius canvis en la manera d'anomenar el trastorn al llarg dels anys: "trastorn post-encefàlic de la conducta" els anys 1917 i 1918 als Estats Units, "trastorn de l'impuls hipercinètic" on es considerava que les disfuncions cerebrals es localitzaven a la zona talàmica i produien dèficits en la filtració d'estímuls [Denhoff et al., 1957] o "disfunció cerebral mínima" on s'introduien els factors ambientals en l'etiologia del trastorn i es pretenia donar a entendre que tota la simptomatologia del TDAH depenia d'una alteració del sistema nerviós central (SNC) però no constituïa en si mateixa un dany cerebral [Clements and Peters, 1962]. No és fins els anys 70 quan es va demostrar que la falta d'atenció és un símptoma més del trastorn [Douglas et al., 1976; Sykes et al., 1972; Sykes et al., 1973] i l'any 1980 es publicà la tercera edició del Manual Diagnòstic i Estadístic dels Trastorns Mentals (DSM-III), que situava el dèficit d'atenció com el símptoma principal del trastorn a les classificacions internacionals i es va adoptar la denominació de "trastorn per dèficit d'atenció amb o sense hiperactivitat". L'any 1987, a la versió revisada del DSM-III (DSM-III-R) es torna a situar la hiperactivitat com a símptoma central del trastorn i es fixa el nom de "trastorn per dèficit d'atenció amb hiperactivitat" vigent actualment. A partir del DSM-IV (1994) es descriu l'existència de tres subtipus clínics de TDAH: combinat, inatent i hiperactiu-impulsiu, que posteriorment es mantenen al DSM-IV TR (2000).

Inicialment el TDAH fou considerat un trastorn específic de l'edat infantil que remetia en l'adolescència (DSM-II). No obstant, els anys 70 va augmentar el número de publicacions centrades en la clínica del TDAH i el seu tractament farmacològic en pacients adults [Borland and Heckman, 1976]; [Hechtman, 1996]Huessy, 1974; [Mann and Greenspan, 1976]; [Wood et al., 1976]. Aquesta simptomatologia en individus adults es va relacionar amb una disfunció dels lòbuls frontal i caudat i, més concretament, amb alteracions en les funcions executives com la

memòria de treball [Pontius, 1973]. L'any 1990 es va publicar un estudi en què mitjançant tomografia per emissió de positrons (PET) es posava de manifest que els adults amb TDAH presentaven alteracions en el metabolisme cerebral de la glucosa a l'escorça prefrontal [Ernst et al., 1999]. Aquest estudi va permetre per primera vegada validar el diagnòstic del TDAH a l'edat adulta i la seva disfunció biològica amb una prova de neuroimatge. Estudis de neuroimatge posteriors, estudis transversals, l'eficàcia de diferents tractaments, els avenços en el coneixement de les bases biològiques del TDAH en adults i les repercussions socials corroboren la persistència del trastorn a l'edat adulta [Biederman, 1993; Biederman, 1994; Wilens, 1995].

En els últims anys s'han desenvolupat un conjunt de programes d'integració del TDAH especialitzats en el diagnòstic i tractament del TDAH en adults que han conduït a la creació l'any 2003 de la *European Adult ADHD Network*, integrada per especialistes en el TDAH adult de 18 països europeus, Líban i Israel (www.adult-adhd.net). Posteriorment s'han creat altres consorcis d'investigació europeus i a nivell mundial centrats en l'estudi del TDAH en adults, tant a nivell genètic (*International Multicentre Persistent ADHD CollaboraTion,* IMpACT) com a nivell clínic (*International Collaboration on ADHD and Substance Abuse*, ICASA; www.trimbos.nl).

1.1.2 Manifestacions clíniques i classificació del TDAH-Diagnòstic.

La simptomatologia principal del TDAH consisteix en un patró persistent d'excessiva inatenció, hiperactivitat i impulsivitat que s'inicia en la infantesa i que genera dificultats d'adaptació social i de conducta. Els pacients reclutats per a la realització d'aquest treball han estat avaluats i diagnosticats amb els criteris DSM-IV-TR (Caixa 1).

Caixa 1. Criteris DSM-IV per al diagnòstic del TDAH.

A. Es compleixi 1 o 2:

1. Sis o més dels següents símptomes de desatenció que persisteixen com a mínim durant sis mesos amb una intensitat que és desadaptativa i incoherent en relació al nivell de desenvolupament:

Inatenció

(a) Sovint no presta atenció suficient als detalls o comet errors per distracció en les tasques escolars, el treball o d'altres activitats.
(b) Sovint té dificultats per mantenir l'atenció en les tasques o activitats lúdiques
(c) Sovint sembla que no escolta quan se li parla directament
(d) Sovint no segueix instruccions i no finalitza les tasques escolars, encàrrecs o obligacions del lloc de treball (no és a causa d'un comportament negativista o a una incapacitat per a comprendre les instruccions)
(e) Sovint presenta dificultats per organitzar tasques i activitats
(f) Sovint evita, li desagrada o és reticent pel que fa a la dedicació a tasques que requereixen un esforç mental sostingut (com treballs escolars o domèstics)
(g) Sovint extravia objectes necessaris per a les tasques o activitats (p. ex. joguines, deures escolars, llapis, llibres o eines)
(h) Sovint es distreu fàcilment per estímuls irrellevants
(i) Sovint és descuidat en activitats diàries

2. Sis o més dels següents símptomes d'hiperactivitat-impulsivitat que persisteixen com a mínim durant sis mesos amb una intensitat que és desadaptativa i incoherent en relació all nivell de desenvolupament

Hiperactivitat

(a) Sovint mou en excés mans i peus o es mou a la cadira
(b) Sovint abandona la seva cadira a classe o en altres situacions en què s'espera que romangui assegut
(c) Sovint corre o salta excessivament en situacions en què és inapropiat fer-ho (en adolescents o en adults pot limitar-se a sentiments objectius d'inquietud)
(d) Sovint té dificultats per jugar i dedicar-se tranquil·lament a activitats de lleure
(e) Sovint "es troba en marxa" o actua com si tingués un motor
(f) Sovint parla en excés

Impulsivitat

(g) Sovint precipita les respostes abans d 'haver-se completat les preguntes
(h) Sovint té dificultats per guardar el torn
(i) Sovint interromp o es fica en les activitats dels altres (p.ex. en converses o en jocs)

B. Alguns símptomes d'hiperactivitat - impulsivitat o desatenció que provoquen alteracions ja estaven presents abans dels set anys d'edat.

C. Algunes alteracions provocades pels símptomes es manifesten en dos o més ambients (p.ex. escola (o treball) i casa)

D. Hi ha d'haver proves clares d'un deteriorament clínicament significatiu de l'activitat social, acadèmica i laboral.

E. Els símptomes no apareixen exclusivament en el transcurs d'un trastorn generalitzat del desenvolupament, esquizofrènia o un altre trastorn psicòtic, i no s'expliquen més bé per la presència d'un altre trastorn mental (p.ex. trastorn de l'estat d'ànim, trastorn d'ansietat, trastorn dissociatiu o trastorn de personalitat).

Els criteris del DSM-IV-TR defineixen tres subtipus de TDAH que són, per ordre de major a menor freqüència tant en població infantil com en població adulta: subtipus combinat (51-65%), subtipus inatent (28-40%) i subtipus hiperactiu/impulsiu (6-21%) [Grevet et al., 2006; Jacob et al., 2007; Wilens et al., 2004; Young and Gudjonsson, 2008]. El subtipus clínic combinat presenta sis o més símptomes d'inatenció i d'hiperactivitat/impulsivitat, i una major gravetat clínica que els altres subtipus clínics [Faraone and Biederman, 1998; McGough et al., 2005; Sprafkin et al., 2007]. El subtipus clínic inatent compleix sis o més símptomes únicament d'inatenció i, finalment, el subtipus clínic hiperactiu/impulsiu presenta únicament sis o més símptomes d'hiperactivitat/inatenció (APA2002). El DSM-IV-TR recull la possibilitat de remissió de la simptomatologia del TDAH a l'edat adulta i es parla de TDAH residual quan un pacient amb TDAH des de la infància no compleix a l'edat adulta el criteri de presentar sis o més símptomes d'inatenció o hiperactivitat/impulsivitat, però la simptomatologia que presenta produeix un malestar significatiu. (APA2002).

1.1.3 Evolució del TDAH al llarg de la vida

Els estudis longitudinals realitzats en individus diagnosticats amb TDAH a la infantesa han permès observar l'evolució del trastorn al llarg de la vida i demostren que el TDAH és un trastorn que s'inicia a l'edat infantil i que persisteix a l'edat adulta en alguns casos [Barkley et al., 2002; Borland and Heckman, 1976; Gittelman et al., 1985; Lambert, 1988; Mannuzza et al., 1998; Rasmussen and Gillberg, 2000]. Hi ha encara un cert debat respecte a la proporció de pacients en què el TDAH persisteix fins l'edat adulta a causa, probablement, de la diversificació entre els criteris de persistència o de remissió. En una meta-anàlisi publicadas l'any 2006, en què s'inclouen 23 estudis longitudinals, es demostra com el fenomen de remissió és un concepte clau per tal d'explicar la variabilitat observada entre els diferents estudis [Faraone et al., 2006]. Aquest estudi posa en evidència com la persistència sindròmica, quan es compleixen tots els criteris diagnòstics, és una classificació molt més restrictiva que la definició simptomàtica, en què els criteris de diagnòstic es compleixen de forma parcial. D'altra banda, es creu que la tendència a la remissió del TDAH amb l'edat pot ser un artefacte causat per la inestabilitat evolutiva del DSM-IV-TR i no degut a l'evolució natural del trastorn [Faraone et al., 2006; Faraone et al., 2000; McGough i Barkley, 2004]. És a dir, els criteris del DSM-IV-TR no tindrien en compte de forma adequada els canvis que es produeixen en el trastorn al llarg de la vida del pacient.

Els símptomes principals del TDAH, com la inatenció, la hiperactivitat i la impulsivitat, també experimenten canvis longitudinals [Young and Gudjonsson, 2008]. La inatenció és el grup de símptomes que presenten una major persistència temporal que arriba al 80% dels individus estudiats segons criteris de persistència sindròmica [Biederman et al., 2000]. Sovint es manifesta tant en població adulta com en població infantil com una disfunció en la capacitat de concentració amb una elevada facilitat per a la distracció, desorganització, avorriment i necessitat de fer coses noves contínuament, així com amb dificultats per prendre decisions, falta de visió general i elevada sensibilitat a l'estrès. Per altra banda, el símptomes que defineixen la impulsivitat presenten una continuïtat intermitja, de manera que el 60% dels individus estudiats mantenen criteris de persistència sindròmica a l'edat adulta. Aquesta es manifesta en adults com a impaciència, necessitat d'actuació abans de pensar, i impulsivitat en les relacions humanes i a l'hora d'iniciar una nova feina. En canvi, els símptomes que defineixen la hiperactivitat són els que perduren menys al llarg del temps, de tal manera que aproximadament un 50% dels individus estudiats mantenen criteris diagnòstics a l'edat adulta segons la definició de persistència sindròmica. El grup de símptomes que defineixen la hiperactivitat en població infantil inclou un excés de moviment continu i, en canvi, en individus adults la simptomatologia evoluciona com una incapacitat per relaxar-se, amb sensació d'una gran inquietud interna, incapacitat per estar durant un llarg període de temps assegut sense realitzar una activitat o mantenir-se en silenci. L'any 2008 Young i Gudjonsson [Young and Gudjonsson, 2008] van presentar un estudi cas-control en què s'avaluava el perfil neuropsicològic, les característiques clíniques i l'adaptació social en individus diagnosticats amb TDAH i en població control. En funció de la persistència o no del trastorn a l'edat adulta l'estudi permetia diferenciar tres grups d'individus amb TDAH: TDAH persistent, TDAH en remissió parcial i TDAH en remissió total. Actualment s'accepta que aproximadament un 30% dels casos amb TDAH a l'infància presentaran el trastorn a l'edat adulta.

Independentment de la persistència o no del trastorn a l'edat adulta, diversos estudis demostren que els individus amb TDAH a l'edat infantil presenten a l'edat adulta una major freqüència de trastorns psiquiàtrics tals com ansietat, depressió, consum de drogues i trastorns antisocials, que els individus que no han patit TDAH a la infància [Barkley et al., 2004; Biederman i Faraone, 2006; Mannuzza et al., 1993].

1.1.4 Comorbiditat

Una de les característiques clíniques que presenta el TDAH és la comorbiditat amb altres trastorns psiquiàtrics, tant a la població infantil com a l'adulta (Figura 1). A la població de TDAH infantil aproximadament el 65% dels casos presenten una o més comorbiditats, que inclouen trastorn negativista desafiant, trastorn per ansietat i de l'estat d'ànim, tics o síndrome de Tourette, trastorns de l'aprenentatge i trastorns generalitzats del desenvolupament com l'autisme [Biederman et al., 1991; Goldman et al., 1998; Pliszka, 1998]. També hi ha comorbiditat en la població adulta amb TDAH, en què el 75% del casos presenten comorbiditat amb, com a mínim, un altre trastorn psiquiàtric [Biederman et al., 1993; Kooij et al., 2004]. Trastorns d'humor, trastorns d'ansietat, trastorns de la son, trastorn de personalitat, trastorn d'aprenentatge i trastorn per abús de drogues són alguns dels trastorns comòrbids amb el TDAH [Biederman, 2004; Biederman et al., 1993; Kooij et al., 2001; Kooij et al., 2004; Mannuzza et al., 1998; Murphy i Barkley, 1996; Murphy et al., 2002; Rasmussen et al., 2001; Shekim et al., 1990].

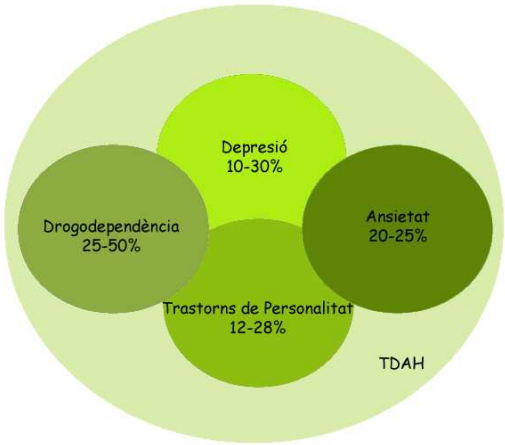


Figura 1. Comorbiditats generals en el TDAH.

S'han realitzat diversos estudis que demostren que hi ha un patró diferencial de comorbiditat en els diferents subtipus clínics de TDAH. Així, els pacients amb TDAH combinat mostren més comorbiditat amb el trastorn negativista desafiant (39,9%), el trastorn per ansietat (33,5%), el trastorn disocial (14,3%), el trastorn per tics motors (10,9%), els trastorns afectius (3,8%) i el trastorn bipolar (2,2%) (MTA 1999) [Biederman et al., 1991; Hurtig et al., 2007; Jensen et al., 1997; Smalley et al., 2007]. El perfil dels trastorns comòrbids es manté pràcticament constant al llarg de la vida amb l'excepció del trastorn de personalitat i el trastorn per abús de drogues, la freqüència dels quals augmenta a l'edat adulta [Biederman et al., 1993].

Les manifestacions pròpies del TDAH no depenen del gènere del pacient, de manera que no hi ha diferències significatives entre homes i dones amb TDAH. Tot i que les dades són controvertides, la diferència principal que s'observa entre sexes és en el patró de comorbiditat amb d'altres trastorns psiquiàtrics, de tal manera que els homes presenten una major comorbiditat amb trastorns per abús de drogues, trastorns de la conducta a la infantesa, com és l'oposicionista negativista disocial, i el trastorn de personalitat antisocial a l'edat adulta [Biederman et al., 2004; Biederman et al., 2005; Sprafkin et al., 2007].

1.2 Epidemiologia del TDAH

1.2.1 Prevalença del TDAH

En l'estimació de la prevalença del TDAH influeixen diversos factors que inclouen l'edat i el gènere, factors culturals i geogràfics, el subtipus clínic, la font d'informació de la simptomatologia en el diagnòstic, l'avaluació i la definició del trastorn. La bibliografia actual presenta una gran variabilitat quant als valors de prevalença del TDAH, que oscil.len entre el 2,2 i el 17,8% [Skounti et al., 2007]. Hi ha nombrosos estudis sobre la prevalença del TDAH en nens i adolescents que demostren que el TDAH és un dels trastorns psiquiàtrics més freqüents en la infantesa i que afecta entre l'1 i el 20% dels nens en edat escolar [Faraone et al., 2003]. En una revisió en què es consideren 102 treballs d'Europa i Nord Amèrica es mostra que la prevalença del TDAH a la població infantil és al voltant del 5,29% (5,01-5,56) i que la variabilitat d'aquesta prevalença està associada a les diferències en la metodologia de diagnòstic i no és específica de regió geogràfica [Polanczyk et al., 2007].

D'altra banda, no disposem de tantes dades sobre la prevalença del TDAH en població adulta com en el cas infantil. Tot i així, hi ha estudis que estimen que el 4,4% de la població adulta compleix criteris de diagnòstic de TDAH [Kessler et al., 2005; Kessler et al., 2006]. No obstant, l'any 2009 una meta-anàlisi realitzada en població general va estimar que la prevalença mitjana del TDAH en població adulta era del 2,5%, valor molt conservador atribuït principalment al fet que els criteris de diagnòstic del DSM-IV tendeixen a infraestimar el diagnòstic de TDAH en individus adults [Simon et al., 2009].

1.2.2 Distribució del TDAH per edat i gènere

Diversos estudis demostren que hi ha diferències en la prevalença del trastorn en funció del gènere. En població adulta la relació home:dona oscil·la entre 1:1 i 3:1 [Fayyad et al., 2007; Kessler et al., 2006] i en població infantil els valors van entre 3:1 i 9:1 [Bener et al., 2006; Benjasuwantep et al., 2002; Breton et al., 1999; Cohen et al., 1993; Cuffe et al., 2005; Ersan et al., 2004].

1.2.3 Prevalença del TDAH en funció de les diferents categories diagnòstiques

La prevalença del TDAH depèn també del subtipus clínic, i a la vegada està sota la influència de l'edat i el gènere. Així doncs, la freqüència del subtipus clínic hiperactiu/impulsiu decreix proporcionalment amb l'increment de l'edat, i la freqüència del subtipus clínic inatent incrementa amb l'edat del pacient i es presenta amb major freqüència en dones que en homes [Gomez et al., 1999; Hudziak et al., 1998].

1.3 Impacte socioeconòmic

Hi ha nombrosos estudis sobre l'impacte funcional del TDAH a nivell socioeconòmic al llarg de la vida centrats principalment en el rendiment acadèmic, l'adaptació al món laboral, les relacions interpersonals, la conducta sexual, la delinqüència i els accidents de trànsit [de Graaf et al., 2008; [Goodman, 2007]. Aquests estudis posen de manifest que el TDAH genera disfunció a diferents nivells de la vida dels pacients i té repercussions socials i econòmiques. Així, tant els individus TDAH infantils com adults mostren un rendiment acadèmic inferior al normal, fins i tot en individus amb nivells d'intel·ligència normals [Biederman et al., 2006]. A l'edat adulta presenten taxes d'atur més elevades, canvien de feina contínuament i presenten un increment en la freqüència de conductes delictives i antisocials així com en el nombre d'accidents de trànsit [Goodman, 2007].

1.4 Tractament farmacològic

En els últims anys s'ha produït un increment en l'experimentació farmacològica i en la comercialització de nous fàrmacs per al tractament del TDAH. Generalment podem classificar els fàrmacs utilitzats en el tractament del TDAH en dos grans grups: fàrmacs psicoestimulants i fàrmacs no psicoestimulants (Caixa 2) [Wilens, 2003]. Tots ells tenen en comú que produeixen

un increment dels nivells de neurotransmissors del tipus catecolamines (dopamina i noradrenalina) a l'espai sinàptic [Spencer et al., 2004].

Caixa 2. Classificació dels fàrmacs utilitzats en el tractament del TDAH.

Estimulants	Antidepressius tricíclics	Inhibidors de MAO	Agonistes α2-adrenèrgics	Altres
Metilfenidat	Amitriptilina	Fenelzina	Clonidina	Atomoxetina
Amfetamina	Desimipramina	Seligilina	Guanfacina	
Pemolina	Imipramina			
Bupropió	Clomipramina			
Modafinil	Nortriptilina			

MAO: Monoamina Oxidasa A i B

1.4.1 Fàrmacs psicoestimulants

Els fàrmacs psicoestimulants es van introduir com a tractament pel TDAH a finals dels anys 30 a partir de l'observació de la seva eficàcia en pacients infantils amb trastorns mentals [Spencer et al., 2004]. En l'actualitat s'ha confirmat la seva eficàcia i es consideren els fàrmacs de primera elecció en el tractament del TDAH [Nutt et al., 2007]. Entre els fàrmacs psicoestimulants hi ha el metilfenidat, les amfetamines, la pemolina, el bupropió i el modafinil, que formen un grup molt heterogeni a nivell farmacocinètic. El metilfenidat inhibeix principalment la recaptació de la dopamina i de la noradrenalina actuant sobre els seus transportadors, tot i que també actua sobre el receptor de dopamina D1 (DRD1) i afecta d'altres sistemes de neurotransmisió com el serotoninèrgic, el colinèrgic i l'histaminèrgic [Faraone et al., 2004; Wilens, 2003; [Peterson et al., 2009]. Les amfetamines inhibeixen la recaptació de dopamina i noradrenalina, i a la vegada promouen l'alliberació de dopamina a l'espai sinàptic i inhibeixen la seva degradació actuant sobre l'enzim monoamina oxidasa (MAO) [Fleckenstein et al., 2007]Weiss i Hechtman, 2006]. De la mateixa manera, la pemolina inhibeix la recaptació de la dopamina tot incrementant la seva concentració a l'espai sinàptic, però és un fàrmac menys utilitzat a causa dels seus efectes hepatotòxics. Finalment, el bupropió, o amfebutadona, actua bloquejant el transportador SLC6A3/5HTT [Dwoskin et al., 2006; Jefferson et al., 2005; Learned-Coughlin et al., 2003], mentre que es desconeix el mecanisme d'acció exacte del modafinil [Minzenberg and Carter, 2008].

1.4.2 Fàrmacs no psicoestimulants

El fet que molts pacients amb TDAH presentin una especial vulnerabilitat a desenvolupar comorbiditat amb un trastorn per abús de drogues psicoestimulants ha estimulat la investigació en nous fàrmacs no psicoestimulants sense potencial addictiu. S'han fet assatjos clínics amb fàrmacs com l'atomoxetina, antidepressius tricíclics, agonistes α2-adrenèrgics i fàrmacs nicotínics [Spencer et al., 2004; Verbeeck et al., 2009]. Els antidepressius tricíclics inhibeixen la recaptació de serotonina i noradrenalina, mentre que l'atomoxetina és un inhibidor selectiu de la recaptació d'aquest últim neurotransmissor [Jasinski et al., 2008; Lile et al., 2006].

1.5 Fisiopatologia

Inicialment les hipòtesis sobre l'etiologia del TDAH es van centrar en l'existència d'un possible dany cerebral, tot considerant-ne com a causes principals les lesions produïdes en el moment del part (hipòxia) o una encefalopatia prenatal [Barkley et al., 2006], però actualment el TDAH es considera un trastorn multifactorial i complex en què intervenen tant factors ambientals com genètics.

Els estudis de neuroimatge cerebral i de neurofisiologia realitzats fins ara han aportat dades importants per al coneixement de la neurobiologia del TDAH i recolzen la hipòtesi que el trastorn és una síndrome fronto-subcortical [Biederman, 2005; Bush et al., 2005]. Tot i així, els mecanismes neurobiològics subjacents al trastorn encara no es coneixen amb exactitud.

1.5.1 Models fisiopatològics del TDAH

La fisiopatologia del TDAH pot explicar-se mitjançant un model dual de les funcions executives (FE) cognitives i motivacionals [Sonuga-Barke, 2005] (Figura 2).

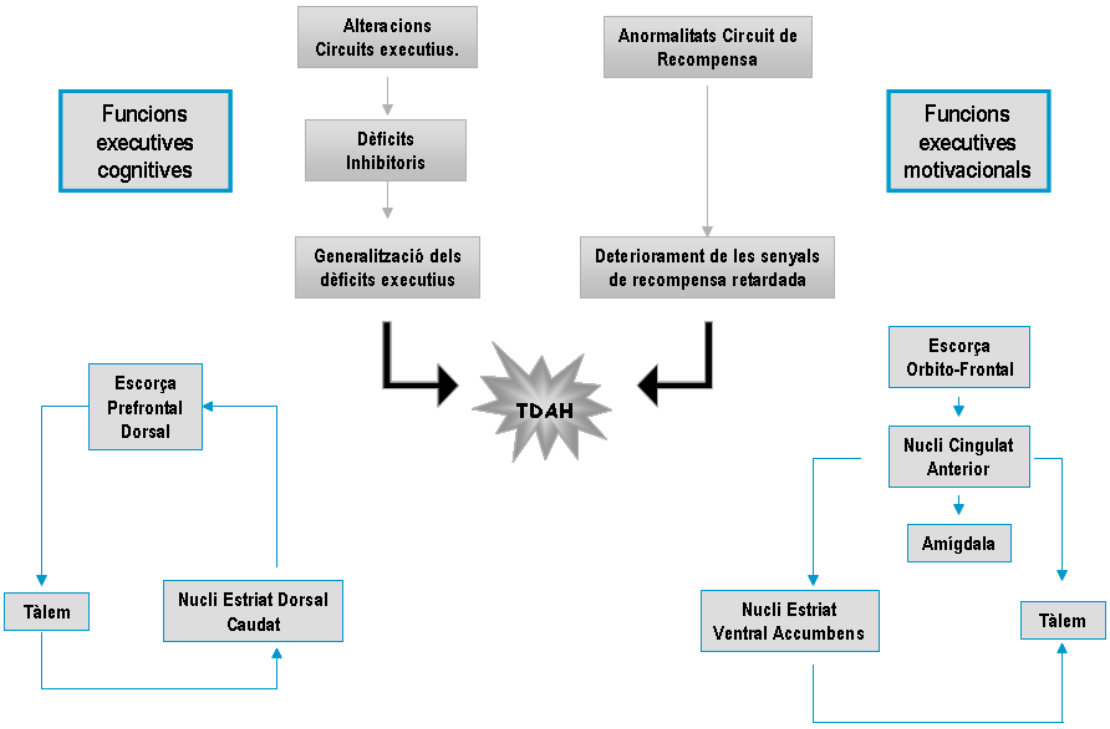


Figura 2. Model dual de les funcions executives cognitives i motivacionals.

Les FE cognitives fan referència als processos cognitius implicats en la conducta, en la planificació i en l'execució de tasques, mentre que les FE motivacionals fan referència els processos de recompensa i a l'impuls en la realització d'accions (Caixa 3).

El model dual proposa alteracions en circuits que clàssicament s'havien considerat funcionalment i anatòmicament diferents. En l'actualitat sabem que la informació pot viatjar d'un circuit a un altre a través de connexions talàmico–córtico–talàmiques [Zahm, 1999] i estriat–nigro–estriades [Haber et al., 2000], oferint així una explicació anatòmica a la influència que exerceixen les FE motivacionals sobre les cognitives i viceversa [Sonuga-Barke, 2005]. Així, se suposa que funcionalment els processos de les FE motivacionals i de les FE cognitives s'influeixen mútuament.

Caixa 3. Definicions

Funcions Executives cognitives:

Es refereixen als processos cognitius que s'encarreguen de la conducta dirigida a objectius, de la planificació i de l'execució de tasques, de l'anticipació. S'han relacionat amb el circuit cerebral dopaminèrgic dorsal.

Memòria de treball:

És la capacitat de mantenir activa la informació necessària per realitzar tasques complexes com el raonament, la comprensió i l'aprenentatge. És a dir, la memòria de treball permet emmagatzemar temporalment quantitats limitades d'informació per guiar la conducta de l'individu, de forma similar al paper que desenvolupa la memòria RAM en un ordinador. Es tracta d'un concepte teòric central tant en la psicologia cognitiva com en la neurociència.

Test de Stroop:

El test de Stroop és un test neuropsicologic que s'utilitza per avaluar el dèficit d'atenció i consisteix en anomenar el color imprès de paraules que signifiquen els colors i que no concorden amb el color de la tinta impressa (Ex: **blau**, **vermell**, **verd**...). L' alteració de l'atenció selectiva validaria la hipòtesi d'una sobrecàrrega d'informació no pertinent y no filtrada, deguda a una disminució de las funciones frontales.

Funcions Executives motivacionals:

Es relacionen amb els processos de recompensa i amb l'impuls en la realització de les accions. S'han relacionat amb el circuit ventral mesolímbic, també dopaminèrgic, que connectaria l'escorça prefrontal amb el nucli estriat.

Sistema de recompensa:

El Sistema de Recompensa és un circuit cerebral del sistema límbic implicat en el desenvolupament i el manteniment de patologies addictives. L'experiència de recompensa associada al consum de drogues va acompanyada de l'activitat d'aquest circuit dopaminèrgic mesolímbic, induint sentiments subjectius de plaer i emocions positives. Les recompenses actuen com a reforçants positius tot augmentant la freqüència i la intensitat del comportament que provoca la recompensa, així com mantenint aquest comportament després evitant que desaparegui.

Tot i així, sobretot els estudis neuropsicològics i de neuroimatge en TDAH, tant d'infantils com d'adults, s'han centrat en l'estudi de les FE cognitives perquè es consideren la causa principal de la simptomatologia: dèficit en la memòria de treball (Caixa 3), manteniment de l'atenció i control inhibitori [Frazier et al., 2004; [Schoechlin and Engel, 2005; Tucha et al., 2008].

1.5.2 Neuroimatge del TDAH

Els estudis de neuroimatge estructural en pacients amb TDAH s'han centrat en regions cerebrals i circuits prèviament relacionats amb el trastorn (Caixa 4). Aquests estudis demostren que els pacients infantils amb TDAH presenten una disminució del voltant del 4,7–5% del volum cerebral total en relació a individus control [Carmona et al., 2005] Castellanos et al., 1996; Castellanos et al., 2002]. Les àrees que estan més afectades són el nucli caudat, el cerebel, el cos callós i els lòbuls frontals [Durston et al., 2004; Tremols et al., 2008] (Figura 3).

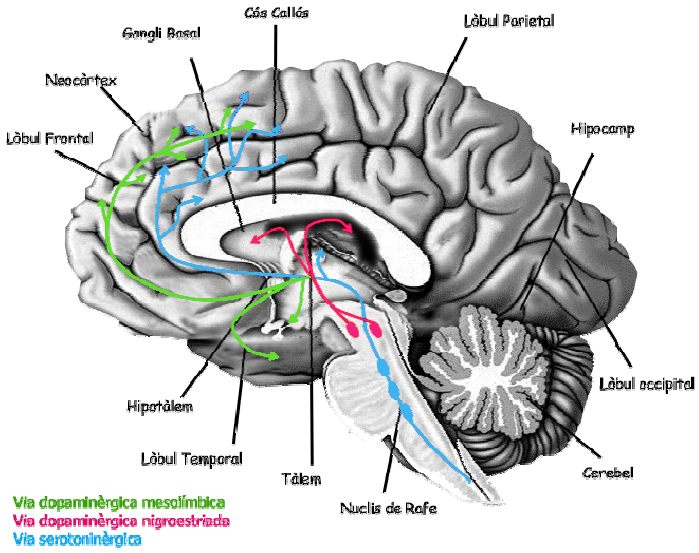


Figura 3. Esquema general del SNC.

Aquestes diferències cerebrals es confirmen en els estudis familiars, en què s'observa que només els individus amb TDAH presenten una disminució del volum cerebral i no els seus familiars sans [Castellanos et al., 2003; Durston et al., 2004].

Caixa 4. Estructures cerebrals implicades en el TDAH mitjançant estudis de neuroimatge estructural.

Cerebel:

Forma part de l'encèfal i una de les seves funcions més representatives és la coordinació dels moviments voluntaris del cos humà. A la vegada, el cerebel està implicat en algunes de les funcions cognitives com ara l'atenció, el llenguatge, la música i el processament d'altres estímuls sensorials temporals. S'ha identificat una alteració en la mida dels lòbuls inferoposteriors del cerebel en individus amb TDAH [Castellanos et al., 1996]

Cos Callós:

Es considera un element essencial en la transmissió de la informació inter-hemisfèrica. Els pacients TDAH presenten canvis en la zona anterior i posterior del cos callós. S'ha observat que els pacients que presenten puntuacions més elevades en les escales d'hiperactivitat i d'impulsivitat presenten una menor àrea rostral del cós callós [Giedd et al., 1994; Hynd et al., 1991].

Ganglis basals (Putamen i Globus Pallidus):

Els resultats dels estudis realitzats amb ressonància magnètica en pacients amb TDAH mostren una asimetria en els dos nuclis [Hynd et al., 1993].

Escorça prefrontal:

És la part anterior dels lòbuls frontals del cervell. Implicada en el plantejament de comportaments cognitius complexes, l'expressió de la personalitat, la presa de decisions i modulació del comportament social correcte. Els pacients amb TDAH presenten una disminució de la mida de l'escorça.

Lòbul temporal:

És la part del cervell encarregada de la percepció auditiva, memòria no verbal, de la categorització d'objectes, de la comprensió del llenguatge i del coneixement musical. També participa en la motivació i en la regulació de les emocions com ara l'ansietat, el plaer i la ira. Tot i ser una àrea candidata a contribuir al TDAH, s'hi han realitzat pocs estudis de neuroimatge. S'ha observat una reducció significativa del volum del lòbuls temporals en pacients amb TDAH [Castellanos et al., 2002].

Lòbul parietal:

És la zona cerebral encarregada de rebre les sensacions del tacte, calor i fred, pressió i dolor. Hi ha dos estudis amb resultats poc consistents en relació a la mida dels lòbuls parietals en el TDAH [Castellanos et al., 2002; Sowell et al., 2003].

Lòbul occipital:

S'encarrega del processament visual. S'ha observat una disminució en la mida del lòbul occipital en pacients amb TDAH [Castellanos et al., 2002].

Per altra banda, els paràmetres més estudiats en el TDAH mitjançant neuroimatge funcional fan referència a la memòria de treball, a l'atenció sostinguda i al control inhibitori [Bush et al., 1999; Bush et al., 2005; Bush et al., 2008; Makris et al., 2008; Rubia et al., 2000; Valera et al., 2005]. Així, mitjançant la tècnica de Tomografia Computarizada per Emissió de Fotons Individuals (SPECT) s'han identificat alteracions en la funció del nucli estriat en pacients amb TDAH [Lou, 1996] i utilitzant la tècnica de Tomografia per Emissió de Positrons (PET) (Caixa 5) s'ha demostrat que hi ha diferències en el metabolisme de la glucosa a l'escorça prefrontal entre individus adults amb TDAH i controls [Ernst et al., 1999]. Finalment, mitjançant la combinació de ressonància magnètica (RM) i el test de Stroop (veure Caixa 3), que s'utilitza per a la identificació i localització de danys cerebrals, s'han detectat disfuncions del nucli cingulat anterior relacionades amb el control de la inhibició en pacients adults amb TDAH [Bush et al., 1999; Bush et al., 2005].Tot i que els resultats obtinguts tant en TDAH adult com en TDAH infantil es repliquen, els estudis longitudinals demostren que les alteracions neuroanatòmiques relacionades amb el TDAH es normalitzen amb l'edat [Castellanos et al., 2002; Shaw et al., 2007].

Un altre aspecte important en la fisiopatologia del TDAH és la resposta a l'administració de psicoestimulants com el metilfenidat i la millora de la simptomatologia [O'Gorman et al., 2008]. Diversos estudis demostren que els efectes del metilfenidat no són homogenis entre individus, que aquestes diferències són degudes principalment al condicionament del sistema dopaminèrgic i que l'amplitud i la direcció dels efectes del metilfenidat sobre el metabolisme de la dopamina són dependents de l'estat dels circuits cerebrals implicats en les funcions executives [Ludolph et al., 2008; Volkow et al., 1997; Volkow et al., 2007a; Volkow et al., 2007b].

Caixa 5. Estudis de neuroimatge funcional

PET o Tomografia per Emissió de Positrons:

És una eina de diagnòstic no invasiva que permet mesurar l'activitat metabòlica del cos humà. De la mateixa manera que la tècnica SPECT, es basa en la detecció de la distribució tridimensional d'un metabòlit determinat, que s'introdueix a l'interior del cos mitjançant la injecció intravenosa d'un radiofàrmac. La imatge s'obté a través d'un aparell anomenat tomògraf que detecta els raigs gamma emesos pels pacients.

SPECT o Tomografia Computaritzada per Emissió de Fotons individuals:

És una eina de diagnòstic mèdic que utilitza els raigs gamma emesos per isòtops radioactius administrats al pacient. Les imatges que s'obtenen són bidimensionals però poden combinar-se diferents imatges per obtenir una imatge tridimensional. El procediment és similar al del PET , però en el SPECT és l'isòtop qui produeix directament el raig gamma, mentre que en el PET l'isòtop produeix un positró que després es destrueix amb un electró per produir els ratjos gamma.

fMRI o Imatge per Ressonància Magnètica Funcional:

És una tècnica d'imatge funcional que permet mostrar imatges de les regions cerebrals que executen una tasca determinada. Es basa en tres fets: (i) especificació cortical: cada funció cerebral es executada per una o més àrees definides del cervell, (ii) vasodilatació cerebral local de les àrees que estan executant una determinada funció; en conseqüència es produeix un increment d'oxigen i desoxihemoglobina en els teixits i, (iii) la desoxihemoglobina té un efecte magnètic que es pot detectar.

MEG o Magnetoencefalografia:

Tècnica no invasiva que registra l'activitat de les funcions cerebrals mitjançant la captació de camps magnètics i que permet investigar les relacions entre les estructures cerebrals i les seves funcions. L'activitat postsinàptica neuronal i l'activació de milions de neurones genera una activitat cerebral uniforme diferenciada i localitzada, que es pot registrar mitjançant un magnetòmetre situat al voltant del crani.

1.5.3 Neurofisiologia del TDAH

El principal problema que ens trobem amb la fisiopatologia del TDAH és que una sola teoria neurofisiològica no pot explicar totes les característiques que presenten els individus amb el trastorn [Castellanos et al., 2002]. Així, hi ha dues hipòtesis neurofisiològiques que podrien explicar la desregulació cognitiva de les funcions executives en els individus amb TDAH: (i) hipòtesi hipodopaminèrgica a l'escorça prefrontal i (ii) hipòtesi hiperdopaminèrgica al nucli estriat [Solanto, 2002]. Avui dia s'accepta que una disfunció fronto-subcortical explicaria la major part de la simptomatologia del TDAH, fet recolzat pels estudis de neuroimatge estructural i funcional que demostren una reducció del volum cerebral d'aquestes àrees i que localitzen les alteracions

cerebrals implicades en el trastorn als circuits fronto-subcortical-cerebel.lars [Durston et al., 2003]. Entre les estructures subcorticals associades al TDAH, el nucli estriat tindria una gran importància a causa de l'elevada quantitat de dopamina que vehiculitza [Lou, 1996]. Estudis en models animals indiquen que aquesta regió és vulnerable en situacions d'hipòxia perinatal, tot provocant hiperactivitat i una disminució del control inhibitori [Alexander et al., 1986], i estudis de neuroimatge mostren que el metilfenidat, fàrmac utilitzat en el tractament del TDAH, exerceix el seu efecte farmacològic a través de la seva unió al transportador de dopamina SLC6A3/DAT1 localitzat majoritàriament al nucli estriat [Volkow i Swanson, 2003; Volkow et al., 1998].

2. BASES GENÈTIQUES DEL TDAH

2.1 Heretabilitat del TDAH

Tot i l'elevat nombre d'estudis realitzats, actualment encara es desconeixen les causes exactes del TDAH. No obstant, el TDAH es considera un trastorn complex en què intervenen factors genètics i ambientals tant de risc com protectors. Així, l'acció combinada de diverses variants genètiques donaria lloc a un determinat grau de susceptibilitat genètica que s'expressaria de forma diferencial segons l'ambient. Per tal de determinar la importància de la contribució genètica al TDAH s'han dut a terme nombrosos estudis familiars, de bessons i d'adopció.

2.1.1 Estudis familiars

Els estudis familiars demostren que hi ha un major risc de patir TDAH entre els familiars d'individus afectats pel trastorn que en famílies sense cap membre afectat. A més, els familiars amb un parentesc en primer grau amb l'individu afectat tenen un risc de 4 a 5 vegades superior de presentar el trastorn que els familiars de segon o tercer grau [Biederman et al., 1991; Biederman et al., 1992, Cantwell, 1972]. Altres estudis mostren que els germans o pares de pacients amb TDAH tenen un risc de patir el trastorn entre 2 i 8 vegades superior que els individus sense familiars amb TDAH. En aquest sentit s'ha demostrat que els germans d'individus afectats pel trastorn que comparteixen un sol progenitor tenen un risc inferior de patir TDAH que el germans que comparteixen els dos progenitors, dades que recolzen l'existència de factors genètics de risc en el TDAH [Goodman, 2007].

2.1.2 Estudis de bessons

Els estudis de bessons ofereixen valors de concordança d'entre el 50-80% en bessons monozigòtics i del 30-40% en bessons dizigòtics,[Thapar et al., 2007] dades que han permès fer una estimació de l'heretabilitat del trastorn al voltant del 76% [Biederman i Faraone, 2005] (Figura 4). A més, aquests mateixos estudis descriuen una major heretabilitat dels símptomes d'hiperactivitat-impulsivitat, al voltant del 88%, que dels símptomes d'inatenció, amb una heretabilitat estimada del 79% [McLoughlin et al., 2007].

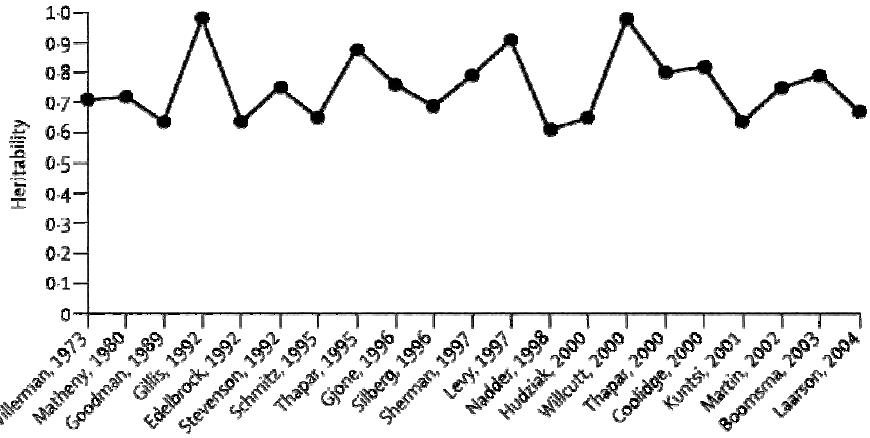


Figura 4. Estimació de l'heretabilitat del TDAH en diferents estudis. Adaptada de [Biederman i Faraone, 2005].

2.1.3 Estudis d'adopció

Els estudis d'adopció han permès demostrar que els factors ambientals tenen un pes inferior a la càrrega genètica amb resultats que indiquen que la freqüència del TDAH és superior en familiars biològics de pacients amb TDAH que en familiars adoptius [Cantwell et al., 1975; Sprich et al., 2000].

2.2 Factors ambientals

Es considera que els factors ambientals expliquen entre el 20 i el 30% de la variància del TDAH. La contaminació per plom, el consum de tabac o d'alcohol durant l'embaràs, les complicacions durant el part, com ara una llarga duració del part o l'eclampsia, i el baix pes corporal del nadó al naixement, s'han considerat factors ambientals biològics relacionats amb el trastorn [Langley et al., 2005; Strang-Karlsson et al., 2008]. A més, els factors psicosocials desfavorables també es consideren factors de risc pel TDAH [Biederman et al., 1995; Spencer et al., 2007] .

2.3 Factors genètics

La cerca de variants genètiques implicades en les malalties complexes és difícil principalment perquè en general no hi ha una única causa genètica capaç d'explicar el fenotip complet. Cadascuna de les variants de predisposició és tan sols un dels múltiples factors que participen en el desenvolupament de la patologia. Tot i que aquestes variants d'efecte menor poden suposar tan sols un petit augment del risc global, és important identificar-les per determinar finalment el panorama genètic complet de predisposició al trastorn. Per tal d'identificar els factors

genètics implicats en l'etiologia del TDAH s'han dut a terme diferents estratègies que inclouen estudis d'associació, de lligament i estudis en models animals. En l'actualitat els estudis d'associació són l'aproximació més utilitzada.

2.3.1 Estudis d'associació

Els estudis d'associació utilitzen enfocs no paramètrics per identificar gens de susceptibilitat a malalties complexes com el TDAH. Els estudis parteixen de l'anàlisi de variants polimòrfiques situades habitualment en gens funcionalment candidats, variants que cobreixen regions posicionalment candidates obtingudes a partir de projectes de mapatge per lligament genètic en famílies, o simplement variants que cobreixen tot el genoma. Finalment, mitjançant taules de contingència es comparen les freqüències al·lèliques o genotípiques dels marcadors d'interès entre un grup d'individus control i un grup d'individus afectats pel trastorn en estudi. En funció del grup control utilitzat per a l'anàlisi, els estudis d'associació es classifiquen en ***estudis d'associació cas-control poblacionals*** *i* ***estudis d'associació cas-control de tipus familiar*****.**

Els estudis d'associació cas-control poblacionals utilitzen individus no relacionats entre ells ni amb els pacients com a control poblacional. Aquesta aproximació pot donar lloc a vegades a resultats positius erronis (falsos positius o errors de tipus 1) i detectar diferències entre les poblacions comparades per causes independents del fenotip estudiat, a conseqüència principalment de l'estratificació poblacional (Figura 5). L'elecció de la població control, per tant, és fonamental en aquests estudis i ha d'estar aparellada per edat, sexe i ètnia amb el grup de pacients, excloent tots aquells individus que presenten un historial previ de la malaltia que s'analitza.

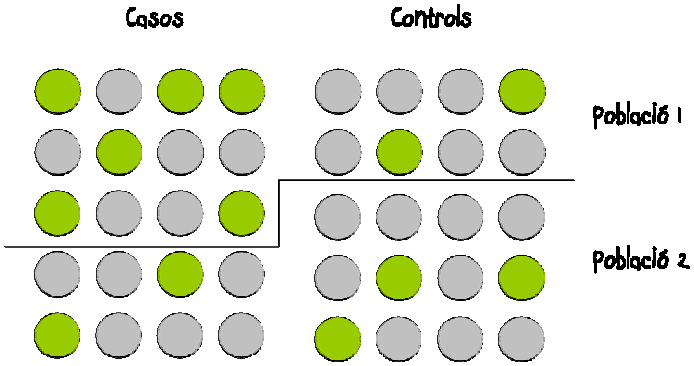
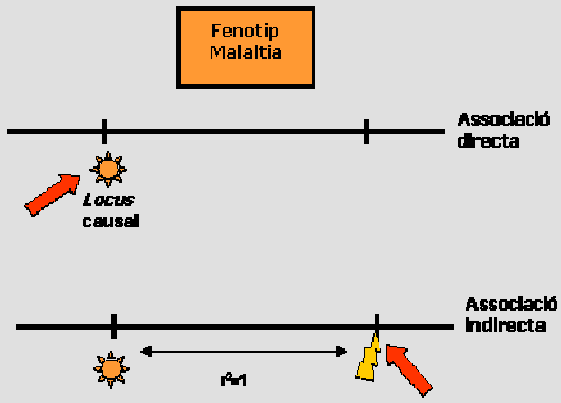


Figura 5. Estratificació poblacional. Els cercles representen individus genotipats per un determinat polimorfisme, on el color verd i el gris són les dues variants al·lèliques. A la població 1 observem com l'al·lel verd està sobrerepresentat en el grup de casos, mentre que a la població 2 es presenta amb una freqüència igual en casos i controls. La raó podria estar en una diferent etnicitat de les dues poblacions escollides per a l'estudi. Adaptada de [Balding, 2006] .

Els estudis d'associació cas-control familiars, en què s'utilitza informació genètica dels progenitors dels pacients com a control intern, són una alternativa per evitar l'estratificació poblacional. Entre els estudis familiars s'utilitzen majoritàriament dos mètodes: el risc relatiu d'haplotip (*Haplotype Relative Risk*; HRR [Schaid, 1998]) que utilitza els al·lels no transmesos dels progenitors a la descendència afectada per crear el grup control, i el test de desequilibri en la transmissió (*Transmission Disequilibrium Test*, TDT, [Spielman et al., 1993]), més utilitzat perquè presenta un algoritme més simplificat que utilitza trios familiars formats per un individu afectat i els seus progenitors heterozigots i compara els al·lels transmesos a la descendència amb els no transmesos mitjançant un test de Chi-quadrat. L'avantatge d'aquest últim test és la combinació d'associació i lligament però, en canvi, l'accessibilitat als trios i el fet que sovint els progenitors no són heterozigots fa que sigui difícil d'implementar [Cardon i Bell, 2001].

La selecció dels marcadors genètics a estudiar mitjançant estudis d'associació es basa en la hipòtesi que el marcador és funcionalment rellevant (associació directa amb el trastorn) o bé que està en desequilibri de lligament (*Linkage Disequilibrium*, LD) amb la variant funcional rellevant (associació indirecta) (veure Caixa 6 per més detalls) [Cardon and Bell, 2001] Zondervan i Cardon, 2007]

Caixa 6. Associació directa versus associació indirecta



Associació directa:
Anàlisidirecte de la variant genètica causant d'un fenotip determinat.

Associació indirecta:
Anàlisi d'una variant genètica que està en desequilibri de lligament amb la veritable variant causal del fenotip, basant-se en patrons coneguts de LD entre els polimorfismes d'una determinada regió cromosòmica.

Hi ha diferents tipus de variants genètiques que es poden estudiar, com microsatèl.lits, número variable de repeticions en tàndem (*Variable Number of Tandem Repats*, VNTRs), insercions/delecions (indels) o variants del número de còpies (*Copy Number Variants*, CNVs), entre d'altres. Però actualment els més estudiats són els polimorfismes d'un únic nucleòtid o *Single Nucleotide Polymorphisms* (SNPs) que conformen el 90% de la variabilitat genètica present al genoma humà, i que a més es poden genotipar de forma altament automatizada (Figura 6).

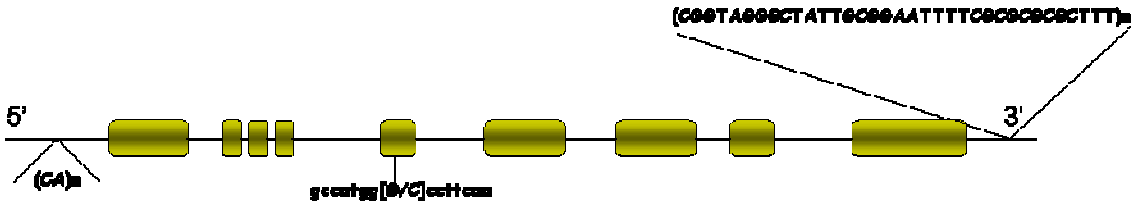


Figura 6. Esquema de l'estructura d' un gen. Les caixes verdes simbolitzen els exons. A la regió 5' del gen s'esquematitza un polimorfisme del tipus microsatèl·lit (C:citosina, A:adenina), caracteritzat per una variació (n) en el número de repeticions d'una unitat curta. A l'exó 5 s'observa un polimorfisme de tipus SNP, un canvi d' un únic nucleòtid, una guanina (G) per una citosina (C). A la regió 3' es representa un polimorfisme del tipus VNTR caracteritzat per la repetició, n vegades, d'una determinada seqüència d'entre 10 i 65 nucleòtids.

El coneixement que tenim sobre els patrons de LD del genoma en diferents poblacions ens permet seleccionar col·leccions de SNPs que capturin la major part de la variabilitat genètica de la regió del genoma que hom vol estudiar, evitant així recollir informació redundant [Balding, 2006]. El valor de LD es quantifica, entre d'altres mètodes, amb els paràmetres D'i r^2, que prenen valors entre 0 i 1. Valors de 0 impliquen independència o equilibri entre les variants que es comparen, mentre que valors de 1 impliquen l'existència d'una relació màxima de desequilibri. Si considerem dos polimorfismes (A i B) bial·lèlics (a1 i a2, b1 i b2) i D'=1, llavors hi ha una relació de dependència entre un al·lel del marcador A i un altre al·lel del marcador B, mentre que si r^2=1 a més els dos marcadors considerats tenen idèntiques freqüències al·lèliques. En aquest últim cas, el genotip d'un marcador es prediu perfectament a partir del genotip de l'altre de forma bidireccional i, per tant, la redundància genètica és total [Zondervan i Cardon, 2004]. En la fase III del projecte internacional HapMap (www.hapmap.org) s'ha posat a disposició pública la informació detallada dels genotips d'1,6 millions d'SNPs en 1184 individus d'11 poblacions diferents [Altshuler et al.]. Aquestes dades permeten selecionar de forma acurada marcadors no redundants que identifiquen grups de SNPs.

Els estudis d'associació es poden classificar també en funció de les regions genòmiques estudiades: (i) estudis d'associació de gens o regions candidates i (ii) estudis d'associació a escala genòmica (*Genome-Wide Association Studies* o GWAS), en què s'utilitzen sèries molt àmplies de marcadors distribuïts per tot el genoma per cobrir la major part de la variabilitat existent [Eberle et al., 2007]. L'avantatge de la utilització dels GWAS en relació als estudis d'associació tradicionals es que permeten la identificació de factors de risc genètic sense necessitat d'una hipòtesis prèvia sobre els mecanismes patogènics del trastorn, de forma similar als estudis de lligament genètic a escala genòmica en malalties monogèniques [Albayrak et al., 2008]; Neale et al., 2008].

2.3.1.1 Estudis d'associació de gens o regions candidates

Tot i que els resultats no sempre han estat consistents, des de la publicació l'any 1995 del primer estudi d'associació en TDAH [Cook et al., 1995] s'han dut a terme una gran quantitat d'estudis d'associació en diversos gens candidats a estar implicats en la fisiopatologia del TDAH. Inicialment, els estudis es van centrar en el sistema dopaminèrgic perquè és diana pels principals fàrmacs psicoestimulants utilitzats en el tractament del TDAH. Així, els dos gens més estudiats han estat *SLC6A3* i *DRD4,* que codifiquen el transportador i el receptor D4 de dopamina, respectivament [Cook et al., 1995; LaHoste et al., 1996]. Posteriorment s'han associat amb el TDAH altres gens relacionats amb els sistemes de neurotransmissió dopaminèrgica, noradrenèrgica o serotoninèrgica, gens relacionats amb factors neurotròfics o gens que codifiquen proteïnes del complexe SNARE (*Soluble NSF Attachment protein REceptor*), entre d'altres.

El gran nombre d'estudis d'associació en TDAH que s'han dut a terme fins a l'actualitat, sovint inconsistents o amb una mida mostral limitada, fan necessari recórrer a estratègies meta-analítiques, en què s'utilitzen les dades de diversos estudis d'associació realitzats en poblacions diferents amb l'objectiu d'incrementar el poder estadístic i obtenir resultats més consistents. Diverses meta-anàlisis realitzades en TDAH han permès identificar alguns gens que podrien contribuir a la susceptibilitat al TDAH, entre els quals hi ha els receptors dopaminèrgics D4 i D5 (*DRD4* i *DRD5)*, el receptor de serotonina 1B (*HTR1B*), els transportadors de dopamina i serotonina (*SCL6A3 i SCL6A4)*, la dopamina-β-hidroxilasa (*DBH*), la catecol-O-metiltransferasa (*COMT)* i la proteïna associada al sinaptosoma de *25 kDa* (*SNAP25*) [Cheuk i Wong, 2006; Faraone et al., 2004;[Faraone et al., 2005]Li et al., 2006; Purper-Ouakil et al., 2005; Yang et al.,

2007]. Altres gens, com ara el receptor adrenèrgic-α_{2A} (*ADRA2A*) o el transportador d'adrenalina (*SCL6A2*), també s'han relacionat amb el TDAH [Bobb et al., 2005; Eisenberg et al., 1999; Kim et al., 2006; Qian et al., 2003; Roman et al., 2003; Roman et al., 2006; Schmitz et al., 2006]. Finalment, un dels estudis d'associació més significatius, publicat l'any 2006 pel consorci "*International Multicenter ADHD Genetics*" (IMAGE) es va centrar en l'anàlisi de 51 gens implicats en les vies de neurotransmissió dopaminèrgica, serotoninèrgica i noradrenèrgica en un total de 5674 families amb un nen amb TDAH combinat. Així, asegurant una elevada cobertura genètica en termes de LD basada en la selecció de tagSNPs però també de SNPs potencialment funcionals, es van identificar 18 gens candidats associats al TDAH [Brookes et al., 2006]. A la Taula 1 es mostra un resum dels resultats de la major part dels estudis d'associació en TDAH publicats fins ara.

Taula 1. Estudis d'associació a gens candidats realitzats en pacients amb TDAH.

Gen	Polimorfisme	Disseny estudi	Fenotip	Genotip o al·lel associat	Significació	Referència
Sistema Dopaminèrgic						
DRD4	VNTR-48bp	CC	TDAHi-C	7R	-	[Swanson et al., 2000]
		BF	TDAHi	-	-	[Barr et al., 2001]
		BF	TDAHi	7R	-	[Rowe et al., 2001]
		BF	TDAHi	-	-	[Todd et al., 2001]
		CC i BF	TDAHi-I	2R-5R	p= 0,032	[Manor et al., 2002]
		BF	TDAHi	7R	p= 0,05	[Arcos-Burgos et al., 2004]
		BF	TDAHi	7R	-	[Kustanovich et al., 2004]
		BF	TDAHi-I	7R	p= 0,01	[Levitan et al., 2004]
		CC	TDAHi-I	7R	protector	[Bellgrove et al., 2005]
		BF	TDAHi	-	-	[Brookes et al., 2005]
		Meta-anàlisi-CC	TDAHi	7R	p< 0,05	[Faraone et al., 2005]
		Meta-anàlisi-BF	TDAHi	7R	p< 0,05	[Faraone et al., 2005]
		BF	TDAHi	-	-	[Kim et al., 2005b]
		CC	TDAHa (homes)	7R	-	[Mill et al., 2005]
		Meta-anàlisi-CC	TDAHi-C	7R	p< 0,05	[Todd et al., 2005]
		BF	TDAHi	(6R-7R)	p= 0,03	[Bhaduri et al., 2006]
		Cas-Control	TDAHi		p=0,05	[Brookes et al., 2006b]
		Meta-anàlisi-CC+BF	TDAHi	7R	p< 0,05	[Li et al., 2006a]
	Dup120 bp	BF	TDAHi	240 bp	p< 0,05	[McCracken et al., 2000]
		BF	TDAHi	-	-	[Barr et al., 2001]
		BF	TDAHi	-	-	[Todd et al., 2001]
		BF	TDAHi	240bp	p= 0,006	[Kustanovich et al., 2004]
		BF	TDAHi	-	-	[Lowe et al., 2004b]
		BF	TDAHi	-	-	[Brookes et al., 2005]
		BF	TDAHi	-	-	[Bhaduri et al., 2006]
	VNTR-Dup	BF	TDAHi	7R-240	p= 0,046	[Arcos-Burgos et al., 2004]
	C521T	BF	TDAHi	A	-	[Barr et al., 2001]
		BF	TDAHi	NS	-	[Payton et al., 2001]
		BF	TDAHi	A	-	[Lowe et al., 2004b]
		CC	TDAHi-I	A	p<0,05	[Bellgrove et al., 2005]
	C616G	BF	TDAHi	-	-	[Barr et al., 2001]
		BF	TDAHi	C	p= 0,008	[Lowe et al., 2004b]
		CC	TDAHi-I	-	-	[Bellgrove et al., 2005]
	rs9195457	BF	TDAHi	-	p<0,05	[Brookes et al., 2006a]
	Dup 12bp	BF	TDAHi	-	-	[Bhaduri et al., 2006]
	C376T	BF	TDAHi	C	-	[Lowe et al., 2004b]
DRD5	DRD5·PCR1	Meta-anàlisi- BF	TDAHi	148bp	p< 0,05	[Lowe et al., 2004a]
		Meta-anàlisi-CC+BF	TDAHi	148bp	p< 0,05	[Li et al., 2006d]
		BF	TDAHa (homes)	148bp	p = 0,003	[Mill et al., 2005]
		BF	TDAHi	148bp	P =0,00005	[Hawi et al., 2003]
	D4S2928	BF	TDAHi	-	-	[Hawi et al., 2003]
	D4S1582	BF	TDAHi	Al·lel 4	p=0,03	[Hawi et al., 2003]
	T1481C	BF	TDAHi	C	p=0,00093	[Hawi et al., 2003]
SLC6A3	VNTR $_{3'UTR}$	CC	TDAHa (homes)	10R	p=0,0009	[Mill et al., 2005]
		BF	TDAHi	10R	p=0,0016	[Cook et al., 1995]
		Meta-anàlisi-BF	TDAHi	10R	p< 0,05	[Faraone et al., 2005]
		Meta-anàlisi-	TDAHi	9R, 10R, 11R	-	[Li et al., 2006c]

		CC+BF				
		Meta-anàlisi-BF	TDAHi	10R	p=0,004	[Yang et al., 2007]
		Meta-anàlisi-CC		10R	-	
	VNTR $_{3'UTR}$	Meta-anàlisi-CC	TDAHi-C	440bp	p= 0,01	[Todd et al., 2005]
		BF	TDAHi	-	-	[Kim et al., 2005b]
		BF (2 poblacions)	TDAHi	10R	p=0,003 / 0,001	[Brookes et al., 2006a]
	VNTR $_{Intró\ 8}$	BF (2 poblacions)	TDAHi	6R	p=0,006	
	VNTR $_{3'UTR}$	Meta-anàlisi-CC	TDAHa	9R-9R	p=0,005	[Franke et al.]
	VNTR $_{3'UTR}$-VNTR $_{Intró\ 8}$			9R-6R	p<0,001	
		CC	TDAHi-C	10R-3R	<0,001	[Brookes et al., 2006b]
		CC	TDAHi	10R-6R	p= 0,002	[Asherson et al., 2007]
	rs2652511, rs10070282, rs2550946 i rs11564750	CC	TDAHi	-	p< 0,05	[Brookes et al., 2006a]
DBH	SNP en Intró 5	CC	TDAHi –S, Tourette	A1	p< 0,05	[Comings et al., 1996b]
		CC	TDAHi	A1	p<0,01	[Smith et al., 2003]
		BF	TDAHi-C	A2	p=0,0027	[Daly et al., 1999]
		BF	TDAHi-C	A2	p=0,03	[Roman et al., 2002]
		BF	TDAHi	A2	-	[Wigg et al., 2002]
		BF i CC	TDAHi	-	-	[Inkster et al., 2004]
	Intró 5, C1021T i G4444A	BF	TDAHi	-	-	[Bhaduri et al., 2006]
	Intró5, (CA)n i InsDel 19bp	BF	TDAHi	-	-	[Wigg et al., 2002]
	SNP G/T Exó5	BF	TDAHi	-	-	[Payton et al., 2001]
	InsDel 4bp- D7S2422	CC	TDAHi	Al1-A2	p=0,025	[Hawi et al., 2001]
	C1021T	BF	TDAHi-C	T	p< 0,05	[Zhang et al., 2005]
	33SNPs	CC	TDAHi-C	-	-	[Brookes et al., 2006a]
MAO-A	VNTR 30bp en 5'UTR	CC	TDAHi	4R i 5R	p< 0,05	[Lawson et al., 2003]
	(CA)n Intró 2	BF	TDAHi	-	-	[Huang et al., 2003],
		BF	TDAHi	-	-	[Payton et al., 2001]
	VNTR$_{5'UTR}$, (CA)n, G941T, A/G SNP	BF	TDAHi	G_{941}	p= 0,03	[Domschke et al., 2005],
				$3R_{(VNTR)}$-$6R_{((CA)n)}$ - G_{941}	p=0,01	
	G941T	BF	TDAHi	G_{941}	p <0,05	[Xu et al., 2007a]
	VNTR$_{5'UTR}$- G941T			3R-G	p <0,05	
	5 taggSNPs	BF	TDAHi-C	-	-	[Brookes et al., 2006b]
DRD2	Polimorfisme Taq1	CC	TDAHi	al·lel A1	p< 0,0009	[Comings et al., 1991]
		CC	TDAHi	al·lel A1	p =0,002	[Comings et al., 1996a]
		CC	TDAHi	al·lel A1	p =0,002	[Sery et al., 2006]
		CC	TDAHi	al·lel A1	-	[Kim et al., 2006b]
		BF	TDAHi	al·lel A1	-	[Rowe et al., 1999]
		BF	TDAHi	al·lel A1	-	[Huang et al., 2003]
	2 Polimorfismes	BF	TDAHi	-	-	[Kirley et al., 2002]
	23 taggSNPs	CC	TDAHi-C	-	-	[Brookes et al., 2006a]
DRD3	Ser9Gly i Intron 5 MspI	BF	TDAHi	-	-	[Barr et al., 2000]
		BF	TDAHi	-	-	[Payton et al., 2001]
		BF	TDAHa	-	-	[Muglia et al., 2002]
		CC	TDAHi	-	-	[Comings et al., 2000]
	28 taggSNPs	CC	TDAHi-C		-	[Brookes et al., 2006a]
COMT	Val108Met	Meta-anàlisi - CC+BF	TDAHi	-	-	[Cheuk and Wong, 2006]
		CC	TDAHa	Met/Met	p= 0,006	[Reuter et al., 2006]
		CC	TDAHi Comorbid	Met	p=0,005	[Gothelf et al., 2007]
ADRA2A	rs1800544	CC	S, Tourette+TDAH	G	p<10-7	[Comings et al., 2003]
		BF	TDAHi	-	-	[Xu et al., 2001]

		BF	TDAHi	-	-	[Roman et al., 2003]
		BF	TDAHi	G	p =0,03	[Park et al., 2005]
		CC	TDAHi-I	G	p= 0,02	[Schmitz et al., 2006]
		BF	TDAHi	-	-	[Wang et al., 2006]
		BF	TDAHi	-	-	[Deupree et al., 2006]
	rs1800545, rs553668, rs1800544	BF	TDAHi-I	rs1800544 (G)	p= 0,017	[Roman et al., 2006]
		BF	TDAHi	-	p<0,05	[Waldman et al., 2006]
	tagg SNPs	BF	TDAHi-C	-	-	[Brookes et al., 2006a]
ADRDA1C	Cys492Arg	BF	TDAHi	-	-	[Barr et al., 2001]
ADRDA 2C	(GT)n	BF	TDAHi	-	-	[Barr et al., 2001]
		BF	TDAHa	-	-	[De Luca et al., 2004]
SLC6A2	3 SNPs	BF	TDAHi	-	-	[Barr et al., 2002]
		BF	TDAHa	-	-	[De Luca et al., 2004]
	21 SNPs	CC	TDAHi	rs3785157	p< 0,05	[Xu et al., 2005a]
	taggSNPs	CC+BF	TDAHi	rs3785157, rs998424	p< 0,05	[Bobb et al., 2005]
	43 taggs SNPs	BF	TDAHi-C	rs3785143 rs11568324	p< 0,05	[Brookes et al., 2006a]
	-3081(A/T)	CC	TDAHi	T	p=0,008	[Kim et al., 2006a]
Sistema Serotoninèrgic						
HTR1B	861G>C	BF+CC	TDAHi	-	-	[Bobb et al., 2005]
		Multicèntric BF	TDAHi	G861	HRR: p=0,0065 TDTp=0,01	[Hawi et al., 2002]
		BF	TDAHi	G861(transmissió paterna)	p=0,03	[Quist et al., 2003]
		BF	TDAHi	-	-	[Heiser et al., 2007]
	102T>C	BF	TDAHi	-	-	[Heiser et al., 2007]
	21SNPs (861G>C)	BF	TDAHi-I	Bloc Haplotip 8 SNPS	p<0,01	[Smoller et al., 2006]
HTR2A		BF+CC	TDAHi	-	-	[Bobb et al., 2005]
	His452Tyr	Multicèntric BF	TDAHi	452His	p=0,026	[Hawi et al., 2002]
		BF	TDAHi	452His	p=0,03	[Quist et al., 2000]
		BF	TDAHi	452His (Nens)	p=0,04	[Guimaraes et al., 2007]
		BF	TDAHi	-	-	[Heiser et al., 2007]
	1438G>A	BF	TDAHi	-	-	
HTR1C		BF+CC	TDAHi	-	-	[Bobb et al., 2005]
5HTT	LPR,VNTR Intro2, SNP en 3'UTR	BF	TDAHi	-	-	[Heiser et al., 2007]
	LPR	BF	TDAHi	5HTT·LPR	p= 0,036	[Kim et al., 2005b]
	VNTR	BF	TDAHi	12R	p= 0,031	[Kim et al., 2005a]
	LPR, VNTR, 10 SNPs	BF	TDAHi	L	p= 0,019	[Curran et al., 2005]
	32 SNPs	CC	TDAHi-C	rs3785157	p<0,05	[Xu et al., 2005b]
	LPR, VNTR	BF	TDAHi	12R	HRR:p=0,01 TDT:p= 0,005	[Banerjee et al., 2006]
		"	"	12R-L	HRR:p=0,027 TDT:p= 0,008	
	LPR, rs140700	Meta-anàlisi-CC	TDAHa	rs140700	p = 0,01	[Landaas et al.]
		CC	TDAHa	S	p= 0,06	
	LPR	BF	TDAHi	-	-	[Wigg et al., 2006]
	LPR i VNTR	BF	TDAHi	S	p=0,016	[Li et al., 2007]
		BF	TDAHi	L-10R	p= 0,013	
	LPR i VNTR	CC	TDAHi	SS	protector p=0,018	[Zoroglu et al., 2002]
		CC	TDAHi	12R12R	protector p=0,001	
		BF	TDAHi-C	-	-	[Brookes et al., 2006a]
5HT1B	G861C SNP	CC	TDAHa (homes)	-	-	[Mill et al., 2005]

TPH , TPH-2	A218C	BF	TDAHi	-	-	[Tang et al., 2001]
	A218C i A6526G	BF	TDAHi	218A-6526G	protector p=0,002	[Li et al., 2003]
	A218C i A6526G	BF	TDAHi	218A-6526G	protector p=0,034	[Li et al., 2006a]
	rs4570625, rs11178997 i 4565946	BF	TDAHi	rs4570625 rs11178997	p=0,049 p=0,034	[Walitza et al., 2005]
	8SNPs	BF	TDAHi	rs1843809 T	p=0,0006	[Sheehan et al., 2005]
	taggSNPs	BF	TDAHi-C	-	-	[Brookes et al., 2006a]
19 gens serotoninèrgics		CC	TDAHi/a	DDC MAOB 5HT2A	TDAHa:p=0,00053 TDAHi:p=0,0017 TDAHa:p=0,0029 TDAHa-C:p=0,0036 TDAHi-C:p=0,0084	[Ribases et al., 2009b]
Neurotrofines						
10 gens Neurotrofines		CC	TDAHi/a	CNTFR NTF3 NTRK2	TDAHa:p =0,0077 TDAHi:p=9,1e-04 TDAHi:p=3,0e– 04 TDAHi :p =0 ,0084	[Ribases et al., 2008]
BDNF	Val66Met	BF	TDAHi	Val66	p=0,0005	[Kent et al., 2005]
	Val66Met i C270T	BF	TDAHi	C270	p=0,007	[Xu et al., 2007b]
	Val66Met	Meta-anàlisi-CC	TDAHa	-	-	[Sanchez-Mora et al.]
Altres gens candidats						
SNAP 25	(TAAA)n	BF	TDAHi	-	-	[Mill et al., 2002]
	(TAAA)n, (TG)n i SNPs rs6077690 (promoter) i rs 363006 (intron7)	BF	TDAHi	2015A/T-(TAAA)n-80609G/A	p<0,05	[Mill et al., 2004]
	1065T-G i 1069 T-C	BF	TDAHi	1065T-G i 1069 T-C	HRR:p=0,01 TDT:p=0,015	[Brophy et al., 2002]
	SNAP-25, 1065 T-G, 1069 T-C, 3'UTR	BF	TDAHi	TC haplotip	p=0,027	[Kustanovich et al., 2003]
	rs1051312 i rs3746544	Meta-anàlisi-CC	TDAHi	rs3746544	p=0,028	[Forero et al., 2009]
	1065 T-G, 1069 T-C	lligament	TDAHi	1065 T-G, 1069 T-C	p<0,05	[Barr et al., 2000]
	SNPs	BF	TDAHi-C	rs6039806 rs362549 rs362987 rs362998	p=0,005 p=0,012 p=0,039 p=0,019	[Feng et al., 2005]
	rs3746544, rs 1051312	CC	TDAHi	rs3746544	p=0,008	[Choi et al., 2007]
	rs6077690, rs6039769 i rs 363006	BF	TDAHi	-	-	[Renner et al., 2008]
Gens lateralitat		CC	TDAHi/a	BAIAP2 rs8079626(A)/rs11657991(G)/rs7503597(G)/rs7210438 (C)	TDAHa-C: p=4,2e-4	[Ribases et al., 2009a]
LPHN3	taggSNPs	CC	TDAHa	rs6551665 rs1947274 rs1947274 rs2345039	p=3,46e-4 p=5,41e-4 p=5,41e-4 p=8,97e-4	[Arcos-Burgos et al.]
	43SNPs	CC	TDAHa-C	rs1868790(T)/rs6813183(C)/rs12503398(A)	p= 7,5e-05	[Ribases et al., 2010]

CC: estudi d'associació cas-control poblacional, BF: estudi d'associació basat en famílies, HRR: **Risc relatiu d' haplotip** TDAHi: TDAH infantil, TDAHa: TDAH adult, TDAH-C: TDAH combinat, TDAH-I: TDAH inatent, TDAH-H: TDAH hiperactiu.

2.3.1.2 Estudis d'associació a escala genòmica (GWAS)

A diferència dels estudis d'associació amb gens o regions candidates, en què es parteix d'una hipòtesi prèvia sobre llur implicació en el trastorn, els GWAS són una aproximació lliure d'hipòtesis que permeten la identificació de noves variants de risc que participen en la fisiopatologia del trastorn estudiat. Aquesta aproximació es basa en la selecció de l'ordre de centenars de milers o milions de variants polimòrfiques de tipus SNP distribuïdes al llarg de tot el genoma en funció dels patrons de LD subjacents establerts pel projecte HapMap [Frazer et al., 2007]. La majoria d'aquestes variants no estan situades dins de regions codificants, que només comprenen de l'ordre d'un 1% del genoma.

Quatre dels GWAS realitzats en TDAH en població infantil han analitzat una mostra de TDAH de 958 trios d'ètnia caucàsica formats pels progenitors i un fill menor d'edat amb TDAH del subtipus combinat, i va ser dut a terme pel consorci internacional "*International Multicenter ADHD Genetics* (IMAGE)" [Brookes et al., 2006a; Lasky-Su et al., 2008a; Neale et al., 2008]. Posteriorment aquestes dades genotípiques s'han reanalitzat tenint en compte diversos paràmetres fenotípics [Lasky-Su et al., 2008b; Neale et al., 2008; Neale et al., ; Sonuga-Barke et al., 2008]. Únicament s'ha realitzat un GWAS en població adulta amb TDAH [Lesch et al., 2008]. En tots ells s'han identificat diversos SNPs nominalment associats ($p<0.05$) al TDAH, però en cap cas se supera la correcció de Bonferroni per múltiples comparacions, de l'ordre de $p<10^{-8}$. A taula 2 es mostren els SNPs identificats en més d'un GWAS o bé nominalment associats al TDAH en un GWAS i en estudis de gens candidats o en anàlisis de lligament anteriors, o bé SNPs que s'han relacionat en estudis previs amb altres trasntorns psiquiàtrics [Franke et al., 2009].

Taula 2. SNPs nominalment associats al TDAH mitjançant GWAS

SNP	Posició	Gen	GWAS en TDAH	Estudis Associació	Lligament	Relació altres trastorns
rs9676447	Intró	*NUCB1*	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	-
rs964647	80 kb 3 '	*CNR1*	[Lasky-Su et al., 2008a; Neale et al., 2008]	[Lu et al., 2008; Ponce et al., 2003]	[Ogdie et al., 2004; Zhou et al., 2008]	-
rs9389835	Intergènic	-	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	-
rs876477	Intró	*KCNIP4*	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	Esquizofrènia [Sullivan et al., 2008] Trastorn bipolar [Sklar et al., 2008]
rs6570426	Intergènic	-	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	[Romanos et al., 2008; Zhou et al., 2008]	Esquizofrènia [Lerer et al., 2003]
rs9484448	Intergènic	-	[Neale et al., 2008]	-	[Romanos et al., 2008; Zhou et al., 2008]	Esquizofrènia [Lerer et al., 2003]
rs9608617	Intergènic	-	[Neale et al., 2008]	-	-	Esquizofrènia [Lewis et al., 2003]
rs6657749	Intró	*PTPN14*	[Neale et al., 2008]	-	-	Esquizofrènia [Sklar et al., 2008]

rs17722514	Intergènic	-	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	-
rs11221064	Intergènic	-	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	Esquizofrènia [Lewis et al., 2003]
rs6919857	5 kb 3'del gen *PEX7* 27 kb 5'del gen *MAP3K5*		[Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	-
rs17754282	Intergènic	-	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	-
rs7187223	Intergènic 203 kb 3'	*CDH13*	[Lasky-Su et al., 2008a; Lesch et al., 2008; Neale et al., 2008]	Adicció metamfetamina [Uhl et al., 2008]	[Zhou et al., 2008]	Esquizofrènia [Sullivan et al., 2008] Autisme [Christian et al., 2008]
rs3782309	Intró	*ITPR2*	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	Esclerosi lateral amiotròfica [van Es et al., 2007]
rs1541665	Intró	*KCNIP1*	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	Esquizofrènia [Lewis et al., 2003]; [Sullivan et al., 2008]
rs12505502	Intró	*MGC48628*	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	Trastorn bipolar [Sklar et al., 2008]
rs922781	Intró	*RORA*	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	[Faraone et al., 2008]	Esquizofrènia [Sullivan et al., 2008]
rs4241112	Intergènic	-	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	Esquizofrènia[Lewis et al., 2003]
rs1018040	Intergènic	-	[Lasky-Su et al., 2008a]	-	-	Anorèxia Nerviosa [Devlin et al., 2002]
rs7577925	Intró	*NAP5* (FLJ34870)	[Lasky-Su et al., 2008a]	-	-	Esquizofrènia[Lewis et al., 2003]
rs522958	Intergènic	-	[Lasky-Su et al., 2008a]	-	-	Diabetis tipus 2 [Zeggini et al., 2007]
rs8047014	5 kb *3'HAS3* i 20 kb *5'TMCO7*		[Lasky-Su et al., 2008a]	-	[Zhou et al., 2008]	-
rs130575	Intergènic	-	[Lasky-Su et al., 2008a]	-	-	Esquizofrènia[Lewis et al., 2003], Dependència a la Nicotina [Kelsoe et al., 2001; Saccone et al., 2007].
rs17641078	Exó	*DMRT2*	[Lasky-Su et al., 2008a]	-	-	Autisme [Allen-Brady et al., 2009] Dependència Nicotina [Li et al., 2008].
rs363512	Intró	*GRIK1*	[Lasky-Su et al., 2008a]	-	[Lasky-Su et al., 2008]	Trastorn Bipolar (Wellcome Trust Case Control Consortium 2007)
rs552655	Intró	*GFOD1*	[Lasky-Su et al., 2008a]	-	-	Esquizofrènia[Lewis et al., 2003]
rs11786458	Intergènic	-	[Lasky-Su et al., 2008a]	-	-	Esquizofrència [Stefansson et al., 2002]
rs11790994	Intergènic	-	[Lasky-Su et al., 2008a]	-	[Zhou et al., 2008]	-
rs10895959	Intergènic	-	[Lasky-Su et al., 2008a]			Esquizofrènia [Lewis et al., 2003]
rs478597	Intró	*NOS1*	[Lasky-Su et al., 2008a]	[Reif et al., 2009]	-	Alzheimer i Esquizofrenia [Galimberti et al., 2008]
rs7495052	Intró	*SLCO3A1*	[Lasky-Su et al., 2008a]	-	-	Autisme [Szatmari et al., 2007], Depressió [Holmans et al., 2004] Dependència a Nicotina [Berrettini et al., 2008]
rs17281813	Intró	*ZNF423*	[Lasky-Su et al., 2008a]	-	[Zhou et al., 2008]	-
rs13330107	Intergènic	-	[Lasky-Su et al., 2008a]	-	[Zhou et al., 2008]	-
rs272000	50kb 5'	*DPP10*	[Lasky-Su et al., 2008a]	-	-	Esquizofrènia, Trastorn Bipolar i Autisme [Lewis et al., 2003] [Sullivan et al., 2008]
rs17367118	Intergenic	-	[Lasky-Su et al., 2008a]	-	-	Esquizofrènia[Lewis et al., 2003]
rs11719664	Intró	*ZNF385D*	[Lasky-Su et al., 2008a]	-	-	Esquizofrènia[Lewis et al., 2003] Trastorn Bipolar [Sklar et al., 2008]
rs6791644	Intró	*FHIT*	[Lasky-Su et al., 2008a]	-	-	Esquizofrència [Sullivan et al., 2008] Autisme [Marshall et al., 2008]
rs17651978	Intró o 5' UTR	*FOXP1*	[Lasky-Su et al., 2008a]	-	-	Esquizofrència [Sullivan et al., 2008]
rs10767942	Intergènic		[Lasky-Su et al., 2008a]	-	-	Autisme [Duvall et al., 2007]; [Szatmari et al., 2007]
rs7992643	15 kb 5'	*CLYBL*	[Lasky-Su et al., 2008a]	-	-	Trastorn Bipolar [Detera-Wadleigh et al., 1999] Esquizofrènia [Lewis et al., 2003]
rs11590090	60 kb 5'	*FAM19A3*	[Lasky-Su et al., 2008a]	-	-	Esquizofrència [Sullivan et al., 2008]
rs1202199	Intró	*MBOAT1*	[Lasky-Su et al., 2008a]	-	-	Esquizofrènia[Lewis et al., 2003]
rs8041675	Intró	*MEIS2*	[Lasky-Su et al., 2008a]	-	[Bakker et al., 2003; Zhou et al., 2008]	-
rs10227331	40kb 5'	*PTPRN2*	[Lasky-Su et al., 2008a]	-	-	Autisme [Trikalinos et al., 2006] Esquizofrènia [Sklar et al., 2008; [Sullivan et al., 2008]

rs2769967	Intergènic	-	[Lasky-Su et al., 2008a]	-	[Zhou et al., 2008]	-
rs1471225	30 kb 5'	*KIAA0574*	-	-	[Zhou et al., 2008]	Autisme [Christian et al., 2008; Marshall et al., 2008]
rs7172689	Intró	*IL16*	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	-
rs2587695	Intró	*PCDP1 = MGC33657*	[Lesch et al., 2008]	-	-	Esquizofrènia[Lewis et al., 2003]
rs864643	3'UTR/Intró	*MOBP*	[Lesch et al., 2008]	-	-	Abús de drogues[Albertson et al., 2004; Mitkus et al., 2008] Esquizofrènia [Lewis et al., 2003]
rs2199161	Intró	*MAP1B*	[Lesch et al., 2008]	-	[Zhou et al., 2008]	-
rs2677744	Intró	*MAN2A2*	[Lesch et al., 2008]	-	-	Autisme [Szatmari et al., 2007]i Depressió [Holmans et al., 2004]
rs7175404	Intró	*AK094352*	[Lesch et al., 2008]	-	-	Depressió [Holmans et al., 2004]
rs10983238	Intró	*ASTN2*	[Lesch et al., 2008]	Adicció metamfetamina [Uhl et al., 2008]	-	Esquizofrènia[Sullivan et al., 2008] Autisme [Marshall et al., 2008]
rs2281597	Intró	*CSMD2*	[Lesch et al., 2008]	Adicció metamfetamina [Uhl et al., 2008]	-	-
rs2502731	Intró	*DNM1*	[Lesch et al., 2008]	-	-	Autisme [Szatmari et al., 2007]
rs412050	5'	*PPM1F*	[Lesch et al., 2008]	-	-	Esquizofrènia[Lewis et al., 2003] Adicció Nicotina [Saccone et al., 2007]
rs220470		*ITGAE*	[Lesch et al., 2008]	-	[Zhou et al., 2008]	-
rs4964805	Intró	*NT5DC3*	[Lesch et al., 2008]	[Rommelse, 2008]	-	Neurosi [Fullerton et al., 2003]
rs1555322	Intró	*MMP24*	[Lesch et al., 2008]	-	-	Autisme [Allen-Brady et al., 2009]
rs7164335		*ITGA11*	[Lesch et al., 2008]	-	-	Autisme [Szatmari et al., 2007]
rs11594082	Intró	*CDH23*	[Lesch et al., 2008]	-	-	Esquizofrènia[Sullivan et al., 2008]
rs7995215	Intró	*GPC6*	[Lesch et al., 2008]	-	-	Trastorn Bipolar [Sklar et al., 2008] Esquizofrènia [Marshall et al., 2008; Sullivan et al., 2008]
rs2241685	Intró	*MYT1L*	[Lesch et al., 2008]	-	-	Esquizofrènia [Vrijenhoek et al., 2008]
rs13395022	Intró	*CTNNA2*	[Lesch et al., 2008; Neale et al., 2008]	-	-	Esquizofrènia[Lewis et al., 2003; Sullivan et al., 2008]
rs10786284	Intró	*TLL2*	[Lesch et el., 2008; Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	-
rs2237349	-	*CREB5*	[Lesch et al., 2008]	-	-	Trastorn neurotic [Fullerton et al., 2003] Esquizofrènia [Sullivan et al., 2008]
rs10514604		*ATP2C2*	[Lesch et al., 2008]	-	[Zhou et al., 2008]	-
rs2842643	5'	*TFEB*			[Zhou et al., 2008]	Esquizofrènia [Lewis et al., 2003]
rs3893215	Intró	*KCNC1*	[Lesch et al., 2008]	-	-	Autisme [Duvall et al., 2007; Trikalinos et al., 2006]
rs11646411	Intró	*CDH13*	[Lesch et al., 2008] [Lasky-Su et al., 2008a; Neale et al., 2008]	-	[Zhou et al., 2008]	Adicció metamfetamina [Uhl et al., 2008]
rs3799977	Intró	*SUPT3H*	[Lasky-Su et al., 2008a]	-	[Zhou et al., 2008]	Esquizofrènia [Lewis et al., 2003]
rs9845475	20 kb 3'	*TRIM71*	[Lasky-Su et al., 2008a]	-	-	Esquizofrènia [Lewis et al., 2003]
rs9451437	15kb 5'	*MAP3K7*	[Lasky-Su et al., 2008a]	-	[Zhou et al., 2008]	-
rs6968385	Intergènic	-	[Lasky-Su et al., 2008a]	-	-	Autisme [Trikalinos et al., 2006]i Esquizofrènia [Walsh et al., 2008]
rs1325154	Intergènic	-	[Lasky-Su et al., 2008a]	-	-	Dependència Nicotina [Li et al., 2008].
rs874426	Intró	*NAV2*	[Lasky-Su et al., 2008a; Neale et al., 2008]	-	-	Autisme [Duvall et al., 2007; Szatmari et al., 2007; Trikalinos et al., 2006] Trastorn Bipolar [Sklar et al., 2008]
rs4810685	Intró	*SULF2*	[Lasky-Su et al., 2008a]	-	-	Autisme [Allen-Brady et al., 2009]
rs11752175	Intró	*KIF6*	[Sonuga-Barke et al., 2008]	-	[Zhou et al., 2008]	Esquizofrènia [Lewis et al., 2003; Sullivan et al., 2008]
rs2360997	25 Kb 3'	*ESRRB*	[Sonuga-Barke et al., 2008]	-	-	Autisme [Marshall et al., 2008]
rs10049246	Intró	*AK309325*	[Sonuga-Barke et al., 2008]	-	-	[Allen-Brady et al., 2009]
rs4875598	Intergènic	-	[Sonuga-Barke et al., 2008]	-	[Zhou et al., 2008]	-

Finalment, el grup de Neale i Col. ha publicat l'any 2010 una meta-anàlisi utilitzant les dades del GWAS realitzat previament en TDAH i ampliant el nombre de mostres, amb un total de 2,064 trios, 896 casos i 2,445 controls, però no han identificat cap SNP associat al TDAH [Neale et al., 2011].

2.3.2 Estudis de lligament genètic

En els darrers anys, i amb la finalitat d'identificar regions cromosòmiques que continguin gens implicats en el TDAH, s'han dut a terme estudis de lligament a gran escala (Taula 3) [Arcos-Burgos et al., 2004; Bakker et al., 2003; Fisher et al., 2002; Hebebrand et al., 2006; Ogdie et al., 2006; Smalley et al., 2002] . Diversos estudis meta-analítics realitzats a partir d'anàlisis de lligament amb famílies amb nens TDAH indiquen que la variabilitat genètica existent entre poblacions dificulta la identificació de *loci* comuns. Fins ara s'ha identificat un *locus* comú de risc pel TDAH al cromosoma 5p13 [Bakker et al., 2003; Smalley et al., 2002] i un altre en una regió de 64Mb-83Mb situada al cromosoma 16 [Zhou et al., 2008]. Finalment, el treball publicat l'any 2007 per Faraone i col·l no va permetre identificar regions de lligament implicades en el TDAH [Faraone et al., 2008f].

Taula 3. Estudis de lligament genètic en famílies amb TDAH

Regió cromosòmica	LOD-score	Referència
10q26	>1,5	[Fisher et al., 2002]
11q	0,41	[Hebebrand et al., 2006]
11q23	2,5	[Arcos-Burgos et al., 2004]
12q	2,10	[Hebebrand et al., 2006]
12q23	>1,5	[Fisher et al., 2002]
12p13	2,6	[Fisher et al., 2002]
	> 1,0	[Ogdie et al., 2006; Ogdie et al., 2004; Ogdie et al., 2003]
15q15	3,5	[Bakker et al., 2003]
15q21	1,81	[Faraone et al., 2008]
16p13	>1,5	[Fisher et al., 2002]
	4	[Smalley et al., 2002]
	3,7	[Ogdie et al., 2004]
16q23	3,1	[Asherson et al., 2008]
17p	1,39	[Hebebrand et al., 2006]
17p11	2,8	[Arcos-Burgos et al., 2004]
	3,63	[Ogdie et al., 2004; Ogdie et al., 2003]
	3,0	[Fisher et al., 2002]
5q13	4,16	[Romanos et al., 2008]
5p13	> 1,5	[Fisher et al., 2002]
	2,6	[Hebebrand et al., 2006]
	> 1,0	[Ogdie et al., 2004; Ogdie et al., 2003]
5p12	>1,5	[Fisher et al., 2002]
5p17	2,59	[Hebebrand et al., 2006]
5q33.3	2,4	[Arcos-Burgos et al., 2004]
6q	0,58	[Hebebrand et al., 2006]
6q12	3,3	[Ogdie et al., 2006; Ogdie et al., 2004; Ogdie et al., 2003]
	>1,5	[Fisher et al., 2002]
7p	0,92	[Hebebrand et al., 2006]
7p13	3,0	[Bakker et al., 2003]
8q11	1,85	[Faraone et al., 2008]
9q	0,68	[Hebebrand et al., 2006]
9q22	2,13	[Asherson et al., 2008]
9q33	2,1	[Bakker et al., 2003]

2.4 Interacció gen- ambient

En els darrers anys ha crescut l'interès per reduir l'heterogeneïtat de les mostres de TDAH en els estudis genètics tot considerant els diferents subgrups clínics, diferents endofenotips associats al TDAH o mitjançant l'estudi de possibles interaccions genètico-ambientals [Caspi et al., 2008; Nigg et al., 2005; Sonuga-Barke et al., 2008; Thapar et al., 2007]. Aixi, s'han realitzat diversos estudis que demostren la relació existent entre certs gens del sistema dopaminèrgic, el consum

de nicotina i alcohol durant l'embaràs i el risc de desenvolupar el trastorn [Becker et al., 2008; Brookes et al., 2006; Neuman et al., 2007].

2.5 Models animals del TDAH

Els models múrids del TDAH presenten certs avantatges respecte als estudis en humans o en altres primats, com ara (i) l'homogeneïtat genètica entre ratolins, (ii) el manteniment és menys costós i per tant permet estudiar un major nombre d'animals que l'estudi de primats no humans, (iii) la seva neurologia és molt coneguda i (iv) l'investigador pot tenir un control més eficient sobre les variables ambientals com ara la dieta, l'entorn o l'aprenentatge. El sistema nerviós dels rosegadors és molt més senzill que el sistema nerviós humà i, per tant, no permet estudiar certs comportaments cognitius com el llenguatge. Tot i així, els mecanismes bàsics de comportament són molt similars als de l'ésser humà i proporcionen informació sobre la neuroquímica subjacent a aspectes específics del comportament. Els diferents models animals del TDAH se centren en caràcters específics de la simptomatologia i ofereixen per tant una visió específica del trastorn tant a nivell de la simptomatologia com a nivell de del sistema de neurotransmissió afectat (noradrenèrgic, serotoninèrgic o dopaminèrgic). Així, a causa de la classificació per subtipus clínic del TDAH, és molt complicat trobar un model animal específic del trastorn, i la major part dels models animals estudiats són models d'hiperactivitat. A continuació es descriuen els diferents models múrids d'hiperactivitat estudiats fins ara:

a. *Ratolí Coloboma:* El ratolí Coloboma, que presenta una deleció de ~2cM d'una regió que inclou els gens *Snap*, *Pclb* i *Jag1,* és el model animal més acceptat d'hiperactivitat [Hess et al., 1992]. El fenotip característic del ratolí és una hiperactivitat espontània de l'ordre de 10 vegades superior als controls. El gen *Snap* codifica la proteïna de 25 KD associada al sinaptosoma (Snap-25), una proteïna específica de neurones que participa en la neurosecreció sinàptica. L'administració d'Snap-25 al model animal reverteix la hiperactivitat però no la resta de característiques fenotípiques que presenta el ratolí, tot suggerint que hi ha una relació causal entre el gen Snap-25 i la fisiopatologia del trastorn. Diversos estudis d'associació entre TDAH i *SNAP-25* recolzen aquesta idea.

b. *Rates amb hipertensió espontània (SHR):* Les rates SHR presenten les característiques conductuals del TDAH, que inclouen una disminució de l'atenció sostinguda (no causada per problemes sensorials), impulsivitat i hiperactivitat que va incrementant amb el temps.

c. Rata lesionada per l'administració de la neurotoxina *6-hidroxidopamina (6-OHDA)*, que afecta les neurones catecolaminèrgiques i produeix una lesió que té un efecte directe sobre els seus nivells d'activitat [Davids et al., 2002; Davids et al., 2003].

d. Model genoanul·lat (*knock-out*) *SLC6A3 (-/-)* que presenta un fenotip amb unes 6 vegades més activitat que els ratolins control. Aquests ratolins presenten una estimulació dopaminèrgica continuada per la no recaptació de dopamina de l'espai sinàptic com a conseqüència del dèficit del transportador d'aquest neurotransmisor [Gainetdinov et al., 1999].

Finalment, a diferència de tots els anteriors models basats en la hiperactivitat, el model de dèficit d'execució de tasques *5-CSRT,* presenta problemes de manteniment sostingut de l'atenció, és utilitzat com a model d'inatenció en l'estudi del TDAH.

3. GENS CANDIDATS A PARTICIPAR EN LA SUSCEPTIBILITAT AL TDAH ESTUDIATS EN AQUEST TREBALL

En el treball que es presenta s'ha estudiat la contribució al fenotip TDAH de gens implicats en els sistemes de neurotransmissió dopaminèrgica i serotoninèrgica, així com gens que codifiquen factors neurotròfics.

3.1 Factors neurotròfics i TDAH

3.1.1 Factors neurotròfics

Els factors neurotròfics es classifiquen en dos grups funcionals en funció dels mecanismes d'acció i de les vies de transducció de senyal sobre les quals realitzen la seva acció:

(j) Grup heterogeni de molècules que formen part de la superfamília de les citocines que inclouen al factor neurotròfic ciliar (CNTF), el factor inhibidor de leucèmia (LIF), l'oncostatina M (OSM) i la interleucina 6 (IL6) [Chao et al., 2006; HuangiReichardt, 2001; Thome et al., 1998].

(ii) Factors de creixement neuronal (NGF) o neurotrofines, que inclouen el factor de creixement neuronal (NGF), el factor neurotròfic derivat de cervell (BDNF), la neurotrofina 3 (NT3) i la neurotrofina-4/5 (NT4/5). Els seus efectes estan mediats a través de l'elevada especificitat pels receptors neurotròfics tirosina cinases (NTRKs: TrkA, TrkB i TrkC) i pel receptor de baixa afinitat no selectiu p75NGFR (Figura 7). Les neurotrofines són un grup de factors neurotròfics específics del sistema nerviós dels vertebrats que juguen un paper essencial en la supervivència, diferenciació i proliferació neuronal durant el desenvolupament del SNC i perifèric. Aquestes molècules estimulen el creixement axonal i tenen influència sobre les connexions axó-teixit diana per a l'establiment de connexions sinàptiques al sistema nerviós adult [Connor and Dragunow, 1998]. Controlen la plasticitat i eficiència sinàptica (Caixa 7) i són essencials per al manteniment de les funcions neuronals [Altar et al., 1997].

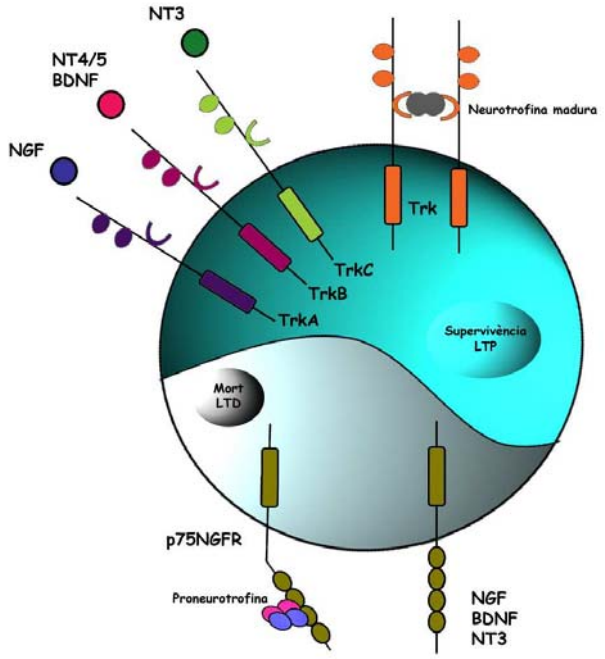


Figura 7. Les accions de les neurotrofines es produeixen a través de dos sistemes de senyalització dels receptors trasnmembrana.Cada receptor de neurotrofines - TrkA TrkB, TrkC i el receptor de neurotrofines p75 - es caracteritza per afinitats específiques pel factor de creixement nerviós (NGF), el factor neurotròfic derivat del cervell (BDNF), la neurotrofina 3 (NT3) i la neurotrofina 4/5 (NT4/5). Es considera que els dos tipus de receptors, Trk (a dalt) i p75NGFR (a baix), obtenen respostes biològiques oposades, a través de la seva unió diferencial a la forma madura de les neurotrofines (Trk), o bé, a als precusors de neurotrofines (p75NGFR). LTD: depressió a llarg termini; LTP: la potenciació a llarg termini (Figura adaptada de [Lu et al., 2005])

Les neurotrofines són secretades de forma constitutiva o regulada per part de neurones i cèl·lules neuroendocrines i de forma únicament constitutiva per cèl·lules no neuronals. Un cop s'uneixen al domini d'unió del seu receptor NTRK específic, aquest dimeritza, s'autofosforila i s'inicien cascades complexes de senyalització intracel·lular que finalment determinaran canvis transcripcionals com a resposta de la cèl·lula diana [Barbacid, 1995].

El patró d'expressió de les diferents neurotrofines és específic d'algunes regions del SNC i el seu radi d'acció inclou determinades subpoblacions neuronals. Així, l'expressió del NGF està restringida a l'hipocamp i al neocòrtex, tot actuant sobre les neurones colinèrgiques del prosencèfal basal que projecten cap a l'hipocamp i l'escorça [Maisonpierre et al., 1991]. NT3 s'expressa quasi de forma ubiqua al SNC, majoritàriament a l'escorça, hipocamp, tàlem i cerebel, i actua sobre el manteniment de la supervivència de les neurones noradrenèrgiques [ZhouiRush, 1994].D'altra banda, NT4/5 s'expressa en etapa postnatal a l'hipocamp, neocòrtex, cerebel i als nuclis del tàlem i la seva funció depèn de l'activitat de BDNF [Friedman et al., 1998]. Finalment, BDNF s'expressa de forma abundant a l'hipotàlem, neocòrtex, amígdala i cerebel i manté la supervivència i la diferenciació de les neurones colinèrgiques, noradrenèrgiques,

dopaminèrgiques, serotoninèrgiques, gabaèrgiques i neuropeptídiques de l'hipotàlem [Akbarian et al., 2002; Horger et al., 1999; Mamounas et al., 2000; Yamada et al., 2002a; Yamada et al., 2001; Yamada et al., 2002b].

Caixa 7. Mecanismes que participen en la neuroplasticitat i els mecanismes d'aprenentatge

Potenciació a llarg termini o LTP: Són aquells canvis anatòmics o funcionals que es produeixen durant els processos d'aprenentatge que consisteixen en gran part en la potenciació presinàptica associada a l'activació postsinàptica. Aquesta potenciació pot durar un temps prolongat (hores o dies).

Depressió a llarg termini o DLP: Són els canvis anatòmics o funcionals que es produeixen durant els processos d'aprenentatge pels quals es genera una disminució selectiva i perllongada de la força o activitat sinàptica.

3.1.2 Contribució dels factors neurotròfics al TDAH

La implicació de les neurotrofines en el desenvolupament neuronal, l'eficiència sinàptica i la plasticitat neuronal del SNC, el seu paper modulador dels sistemes de neurotransmissors i els resultats obtinguts en diversos estudis genètics, clínics, farmacològics i en models múrids, suggereixen que els gens que codifiquen aquests factors neurotròfics, principalment BDNF, podrien ser bons candidats per al desenvolupament i manteniment de diferents trastorns psiquiàtrics tals com el TDAH, l'esquizofrènia, l'abús de substàncies, trastorns afectius o trastorns de la conducta.

Els models múrids demostren que els ratolins genoanul.lats pel gen Bdnf en homozigosi, *Bdnf* (-/-), moren a la segona setmana de l'estadi embrionari. En canvi, els ratolins genoanul.lats heterozigots *Bdnf* (+/-) i genoanul.lats condicionals *Bdnf* (-/-), en què el gen s'elimina de manera condicional en el temps i/o espai, mostren agressivitat, ansietat, deficiències en l'aprenentatge i hiperactivitat en comparació amb els ratolins control. A més, reduccions dels nivells de Bdnf al cervell del ratolí a l'edat adulta tenen com a conseqüència una discapacitat de les funcions de l'hipocamp, mentre que si la pèrdua de la neurotrofina es produeix durant l'estadi embrionari les conseqüències fenotípiques són molt més greus a nivell d'aprenentatge i d'hiperactivitat [Kernie et al., 2000; Linnarsson et al., 1997; Lyons et al., 1999; Rios et al., 2001]

Hi ha també evidències farmacològiques de la implicació dels factors neurotròfics en l'etiologia del TDAH. S'ha observat que els fàrmacs psicoestimulants, com el metilfenidat i l'amfetamina, així com els antidepressius tricíclics utilitzats en el tractament del TDAH, modulen l'expressió de BDNF i del seu receptor NTRK2 [Chase et al., 2007; Meredith et al., 2002; Meredith and Steiner, 2006]. La modulació de BDNF i altres factors neurotròfics, com NT3 i

CNTF, deguda a l'administració de psicoestimulants, indueix neuroadaptació i canvis en l'activitat locomotora a través de vies de neurotransmissió implicades en el TDAH, com els sistemes dopaminèrgic, serotoninèrgic i noradrenèrgic [Altar et al., 1997; Hall et al., 2003; Horger et al., 1999; Martin-Iverson et al., 1994].

Per altra banda, hi ha diversos estudis d'associació que relacionen els factors neurotròfics amb el TDAH. Aquests estudis s'han centrat majoritàriament en dos SNPs del gen *BDNF*, l'rs6265 (p.Val66met) i el rs2030324 (-270C/T) i un SNP del gen *NGF,* l'rs6330 (p.Ala35Val) [Friedel et al., 2005; Kent et al., 2005; Kim et al., 2007; Schimmelmann et al., 2007].

Finalment, s'ha descrit que un individu portador d'una inversió cromosòmica que provoca haploinsuficiència del gen *BDNF* presenta símptomes d'hiperactivitat, deficiències en la memòria, llenguatge, atenció i càlcul mental. Alhora, s'ha relacionat una mutació *de novo* de pèrdua de sentit a *NTRK2* amb fenotips severs d'obesitat, retard en el desenvolupament i deteriorament de l'atenció, memòria i aprenentatge [Gray et al., 2006; Yeo et al., 2004].

3.1.3 *Brain-Derived Neurotrophic Factor* (BDNF) i TDAH

El gen *BDNF* està situat a la regió cromosòmica 11p13-14 i està organitzat en 13 exons que codifiquen per un precursor peptídic de 247 aminoàcids conegut com a pro-BDNF, que mitjançant proteòlisi passa a la forma madura de la neurotrofina, de 153 aminoàcids, coneguda com a mBDNF (Figura 8).

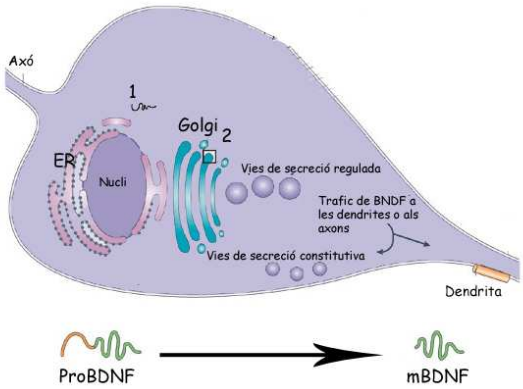


Figura 8. Síntesi de BDNF. El gen *BDNF* està format per 13 exons, que donen lloc a com a mínim 19 transcrits alternatius que difereixen en la seva regió 5'. (1) BDNF se sintetitza al reticle endoplasmàtic (ER) com a proBDNF (precursor de BDNF), i posteriorment es dirigeix al aparell de golgi on s'uneix a diverses proteïnes intracel.lulars que faciliten el plegament adequat del domini madur de BDNF (2). Un dels dominis de la proteina madura de BDNF s'uneix a la carboxipeptidasa E (CPE) i s'integra en vesícules per a la via de secreció regulada. En absència d'aquest motiu, BDNF és secretat per la via constitutiva. Un vegada s'ha decidit el tipus de secreció, BDNF és transportat al lloc apropiat d'alliberament, ja sigui a les dendrites o als axons (Figura adaptada de [Lu et al., 2005])

Fins ara s'han dut a terme un gran nombre d'estudis d'associació cas-control de tipus familiar o poblacional entre variants genètiques de BDNF i TDAH (Taula 4) [Brookes et al., 2006; Conner et al., 2008; Friedel et al., 2005; Kent et al., 2005; Lanktree et al., 2008; Lee et al., 2007; Oades et al., 2008; Schimmelmann et al., 2007; Xu et al., 2007]. També hi ha diversos GWAS que han detectat associacions nominals del TDAH amb aquet gen [Anney et al., 2008; Lasky-Su et al., 2008; Neale et al., 2008; Sonuga-Barke et al., 2008].El polimorfisme més estudiat ha estat l'SNP rs6265 (p.Val66Met), localitzat a la regió que codifica la forma inmadura de BDNF. Tot i estar localitzat a la regió de pro-BDNF i no alterar l'activitat biològica intrínseca de la proteïna madura, la variant rs6265A (p.Met66) dóna lloc a un processament intracel·lular anòmal de la proteïna que afecta la seva secreció regulada depenent de senyal com a neurotrofina madura mantenint constant la secreció constitutiva de BDNF [Egan et al., 2003]. A més, l'al·lel p.Met66 d'aquest polimorfisme està associat a concetracions elevades de BDNF en sèrum [Lang et al., 2009], fet que concorda amb els resultats obtinguts en estudis d'associació en què s'ha identificat

associació entre la variant Val66 i trasstorn bipolar i depressió [Sklar et al., 2002] així comTDAH [Lanktree et al., 2008; Xu et al., 2007b].

Taula 4. Estudis d'associació de tipus familiar i poblacional realitzats amb l'SNP rs6265 (p.Val66Met) en poblacions de TDAH infantil i TDAH adult.

TDAH	Al·lel associat	Estudi	Mida de la mostra	P-valor	Subtipus clínic	Referència
Infantil	p.Val66	BF	341 trios	P= 0,02**	81% C, 9% I, 10% H	[Kent et al., 2005]
	-270C/p.Val66***	BF	135 trios + 35 duos	Haplotip: P=0,032	78% C+ 22% I	[Xu et al., 2007b]
	-270C/p.Val66***	BF	Meta-anàlisi #	Haplotip: P=0,016	100% C	[Xu et al., 2007b]
	No associació	CC	88 casos + 96 controls	-	-	[Friedel et al., 2005]
	No associació	BF	468 casos de 294 famílies	-	72% C + 21.4% I + 6% H	[Schimmelmann et al., 2007]
	No associació	BF	315 casos de 266 famílies	-	62% C + 24% I + 14% H	[Lee et al., 2007]
	No associació	BF	674 casos amb 808 germans	-	100% C	[Brookes et al., 2006a]
Adult	p.Val66	BF	268 casos de 83 famílies	Al·lels: P=0,036 Haplotip&: P=0,0086	-	[Lanktree et al., 2008]
	p.Val66	CC+BF	121 casos + 83 famílies + controls inferits	Al·lels: P=0,0096 Haplotp&: P=0,025	-	[Lanktree et al., 2008]
	No associació	CC	121 casos + 80 controls	-	-	[Lanktree et al., 2008]
	No associació	Cohort&&	143 subjectes	-	-	[Conner et al., 2008]

* C: TDAH combinat; I: TDAH inatent; H: TDAH hiperactiu/impulsiu **Transmissió paterna (p=0,0005). ***Transmissió disminuïda de la combinació -270T/p.Val66. # Meta-anàlisi amb dues poblacions de Taiwan (135 trios i 35 duos) i UK (116 trios i 64 duos). & Haplotip en el gen *BDNF* que inclou els SNPs rs4923463, rs6265 (p.Val66Met), rs11030104, rs2049045 i rs7103411. && Estudi d'associació entre l'SNP rs6265 (p.Val66Met) i puntuacions del TDAH (inclou els qüestionaris ADHD-SB, WURS-k i l'entrevista Wender-Reimherr). BF: Estudi d'associació cas-control poblacional; CC: Estudi d'associació basat en famílies.

3.2 El sistema serotoninèrgic i el TDAH

3.2.1 Sistema serotoninèrgic

La serotonina (5-hidroxitriptamina, 5HT) és un neurotransmissor monoaminèrgic que juga un paper essencial en les funcions biològiques, fisiològiques i conductuals del SNC, tot incloent l'activitat motora, la conducta alimentària, la conducta sexual, l'humor, la son, la termoregulació, el control de la pressió sanguínia i les activitats respiratòries i cardiovasculars [FinkiGothert, 2007]. Els cossos cel·lulars de les neurones serotoninèrgiques s'agrupen en petits clústers als nuclis de la rafe localitzats al llarg del tronc de l'encèfal i innerven pràcticament la totalitat del SNC (Figura 9).

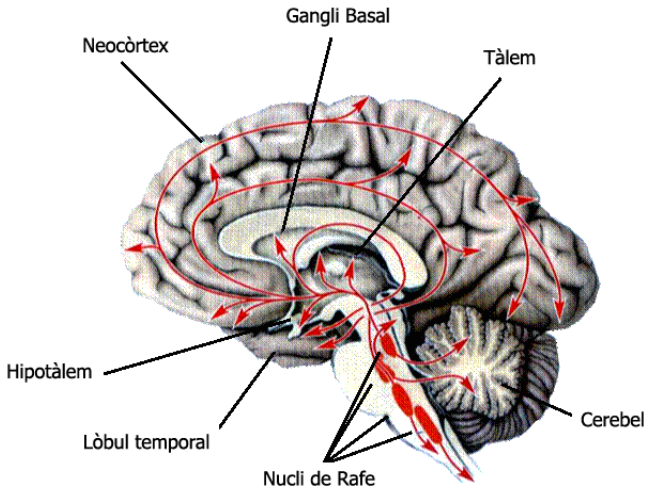


Figura 9. Vies de senyalització serotoninèrgica. Projeccions des del nucli de la rafe fins gairebé tot el SNC.

La 5HT se sintetitza a partir de l'aminoàcid essencial L-triptòfan a través de l'acció conjunta dels enzims triptòfan hidroxilasa (TPH) i descarboxilasa d'aminoàcids aromàtics (AADC o DDC). Un cop la 5HT ha realitzat la seva acció és degradada per l'enzim monoamina oxidasa (MAO), l'aldehid deshidrogenasa i l'alcohol deshidrogenasa per generar entre d'altres metabòlits, l'àcid 5-hidroxiindolacètic (5HIAA) (Figura 10). La concentració de 5HT en els teixits depèn del balanç metabòlic de síntesi i degradació [Tyce, 1990]. El 5HIAA s'utilitza com a mesura del metabolisme serotoninèrgic. L'exocitosi de la 5HT a l'espai sinàptic es realitza mitjançant vesícules de

secreció i està modulada per autoreceptors somatodendrítics i per altres neurotransmissors com l'acetilcolina, l'àcid gamma-aminobutíric (GABA), la histamina i la noradrenalina.

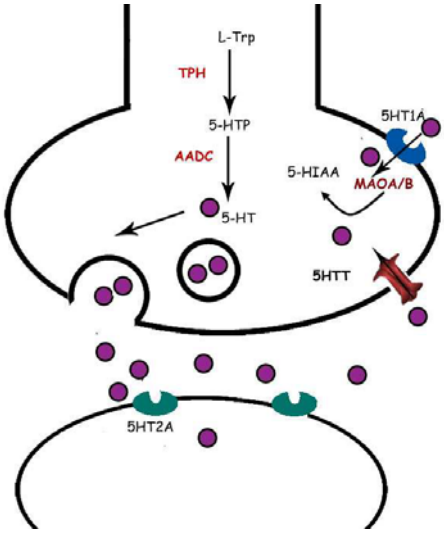


Figura 10. Esquema de la via de síntesi, secreció i degradació de la serotonina.
L-Trp: L-Triptòfan, TPH:Triptòfan hidroxilasa, 5-HTP:5-Hidroxi-L-triptòfan, AADC: 5-Hidroxitriptòfan decarboxilasa, 5-HT: Serotonina, 5-HIAA: àcid 5-hidroxiindolacètic, 5-HT1A, 2A

L'acció serotoninèrgica està mediada per la unió del neurotransmissor a diferents receptors serotoninèrgics que es classifiquen en 7 grups en funció del perfil farmacològic, el patró d'expressió, l'estructura proteica i els diferents mecanismes transductors de senyal (5HT1, 5HT2, 5HT3, 5HT4, 5HT5, 5HT6 i 5HT7) [Altshuler et al.] (Caixa 8). Els efectes dels neurotransmissors sobre els receptors són dependents de la seva localització pre- o postsinàptica. Així, els receptors 5HT1A, 5HT1B, 5HT1D i 5HT3 estan tant a la zona pre- com postsinàptica, mentre que els receptors 5HT1E, 5HT1F, 5HT2, 5HT4, 5HT5, 5HT6 i 5HT7 es localitzen únicament a la regió postsinàtpica. La variabilitat de la distribució dels receptors serotoninèrgics, així com els diferents mecanismes d'acció són factors reguladors de l'acció serotoninèrgica.

L'activitat serotoninèrgica finalitza amb la recaptació d'aquest neurotransmissor a les terminals de les neurones presinàptiques. Aquesta eliminació activa de 5HT a les terminacions nervioses està mediada per una única proteïna coneguda com a transportador presinàptic de serotonina (SLC6A4/5HTT) que s'expressa exclusivament a les neurones serotoninèrgiques dels nuclis de rafe [Chang et al., 1996]. L'activitat d'aquesta molècula transmembrana regula, juntament amb els autoreceptors presinàptics 5HT1A i 5HT1B, la concentració de serotonina a

l'espai sinàptic i per tant, té influència sobre la magnitud i durada de la transmissió, regulant així l'activitat de la serotonina [LeschiMossner, 1998].

Caixa 8. Receptors de serotonina, distribució i mecanismes d'acció

Receptor	Distribució al SNC	Mecanisme d'acció
5TH1A	Hipocamp, amígdala, septe, hipotàlem, nucli de rafe	Inhibició de l'activitat adenilat ciclasa obrint canals de K^+
5HT1B	Hipocamp, amígdala, septe, nucli de rafe, nucli estriat, escorça	Inhibició de l'activitat adenilat ciclasa
5HT1D	Hipocamp, amígdala, septe, nucli estriat, substància nigra, ganglis basals, col·licle superior	Inhibició de l'activitat adenilat ciclasa
5HT1E	Hipocamp, amígdala, septe, escorça	Inhibició de l'activitat adenilat ciclasa
5HT1F	Hipocamp, amígdala, septe, nucli estriat, escorça	Inhibició de l'activitat adenilat ciclasa
5HT2A	Hipocamp, amígdala, septe, nucli estriat, escorça	Estimulació de la fosfolipasa C tancant canals de K^+
5HT2B	Hipocamp, amígdala, septe, hipotàlem, nucli estriat, escorça	Estimulació de la fosfolipasa C
5HT2C	Septe, hipotàlem, nucli de rafe, escorça, substància nigra, plexe coroide, mèdul·la espinal	Estimulació de la fosfolipasa C
5HT3	Hipocamp, amígdala, septe, nucli de rafe, escorça, plexe coroide	Canal iònic regulat per lligand
5HT4	Hipocamp, amígdala, septe, nucli estriat, substància nigra	Estimulació de l'activitat adenilat ciclasa
5HT5A	Hipocamp, amígdala, septe, hipotàlem	Inhibició de l'activitat adenilat ciclasa
5HT5B	Hipocamp, amígdala, septe, nucli de rafe	-
5HT6	Hipocamp, amígdala, septe, nucli estriat	Estimulació de l'activitat adenilat ciclasa
5HT7	Amígdala, septe, hipotàlem, nucli de rafe, escorça	Estimulació de l'activitat adenilat ciclasa

3.2.2 Contribució del sistema serotoninèrgic al TDAH

El sistema serotoninèrgic també és considerat com a candidat a participar en l'etiologia del TDAH per la seva influència en el comportament agressiu i impulsiu [Halperin et al., 1994] i pel seu paper en el desenvolupament del cervell. A més, els primers estudis bioquímics realitzats en pacients amb TDAH demostren que aquests presenten nivells reduïts tant de 5HT com del seu transportador 5HTT [Rapoport et al., 1974; Stoff et al., 1987] i que el principal metabòlit de la serotonina, 5HIAA, està associat als símptomes d'hiperactivitat, agressivitat i impulsivitat [Castellanos et al., 1994; Halperin et al., 1997; Spivak et al., 1999]. D'altra banda, el defecte de triptòfan, aminoàcid essencial per a la síntesi de 5HT, produeix alteracions en l'aprenentatge i la memòria en individus de la població general [Park et al., 1994].

Diversos estudis farmacològics recolzen també la implicació de la 5HT en el TDAH. Tot i que en el tractament del TDAH s'utilitzen principalment fàrmacs psicoestimulants, hi ha evidències que agonistes serotoninèrgics, inclosos els inhibidors dels recaptadors de serotonina, els antidepressius tricíclics i els inhibidors de la monoamina oxidasa, redueixen els símptomes del TDAH [Popper et al., 1984; Rubinstein et al., 2006; Wilens et al., 2002]. A més, altres estudis realitzats tant en humans com en ratolins donen suport a la relació del sistema

serotoninèrgic amb la hiperactivitat i amb el sistema de recompensa [Fletcher, 1995; Kuczenski et al., 1987; Layer et al., 1992; McMahoniCunningham, 1999; Rocha et al., 2002] Així, els ratolins *Slc6a3* -/-, deficients en *Slc6a3*, experimenten una disminució dependent del sistema serotoninèrgic en la hiperactivitat en resposta al tractament amb psicoestimulants, i l'administració d'agonistes de 5HT atenua marcadament la hiperactivitat en aquest model animal [Gainetdinov et al., 1999]. Per altra banda, els ratolins *5ht1b* -/- són hiperactius, agressius i tenen comportaments desinhibits [Bouwknecht et al., 2001; Brunner et al., 1999; Zhuang et al., 1999], i els ratolins *5ht4* -/- tenen un comportament d'exploració de nous caràcters atenuat [Compan et al., 2004].

Finalment, diversos estudis d'associació impliquen el sistema serotoninèrgic en l'etiologia del TDAH (Taula 5). Els gens més estudiats han estat *5HT1B*, *5HT2A* i *SLC6A4* (*5HTT* o *SERT*) [Thapar et al., 2007]. La gran majoria d'aquests estudis s'han realitzat amb pacients infantils i tan sols cinc d'ells s'han dut a terme amb mostres adultes [Grevet et al., 2006; Muller et al., 2008; Retz et al., 2008; Ribases et al., 2009b].

La major part dels estudis d'associació publicats han relacionat el TDAH amb diferents polimorfismes del gen *SLC6A4* [Grevet et al., 2006; Heiser et al., 2007; Retz et al., 2008; Zhao et al., 2005], principalment el polimorfisme funcional 5HTTLPR que consisteix en una inserció/deleció de 44pb a la regió promotora del gen [Lesch et al., 1996]. En concret, l'al·lel llarg o inserció, que determina una major eficiència en la transcripció del gen, s'ha associat al TDAH [Curran et al., 2005; Kent et al., 2002; Manor et al., 2001; Seeger et al., 2001], resultat que no s'ha replicat en altres estudis [Brookes et al., 2006b; Grevet et al., 2006; Heiser et al., 2007; Langley et al., 2007; Wigg et al., 2006; Xu et al., 2005b]. Tot i que els resultats en la identificació de l'al·lel de risc han estat poc consistents, un segon polimorfisme al gen *SLC6A4*, un VNTR de 17pb a l'intró 2, s'ha associat també al TDAH [Banerjee et al., 2006; Heiser et al., 2007; Kent et al., 2002; Kim et al., 2005a; Xu et al., 2005b; Zoroglu et al., 2002].

Taula 5. Estudis d'associació cas-control poblacional i familiar realitzats entre TDAH i els gens *SLC6A4*, *5HT1B* i *5HT2A*.

Gen	Polimorfisme	Estudi	Tamany de la mostra	P-value	Subtipus clínic	Reference
SLC6A4	**5HTTLPR**	CC	687 casos vs 1013 controls	0,019	Aproximació QTL	[Curran et al., 2005]
		BF	98 famílies	0,008	C	[Manor et al., 2001]
		CC[a]	-	0,008	C + I + HI	[Kent et al., 2002]
		BF	126 famílies	0,036	28,6%C + 27,8%I + 7,9HI + 35,7%NOS	[Kim et al., 2005a]
		BF	293 famílies	0,016	41,0% C + 52,5% I + 6,5 % HI	[Li et al., 2007]
		CC	71 casos vs 128 controls	0,018	-	[Zoroglu et al., 2002]
		CC	101 casos vs 163 controls	0,004	C	[Seeger et al., 2001]
		BF	243 famílies	NS	65,6% C + 22,4% I + 6,2% HI	[Guimaraes et al., 2007]
		BF	102 famílies	NS	69% C + 27% I + 4 % HI	[Heiser et al., 2007]
		BF	46 trios + 9 duos	NS	-	[Banerjee et al., 2006]
		BF	113 famílies	NS	81% C + 8% I + 11 % HI	[Kent et al., 2002]
		BF	409 famílies	NS	78% C + 22% I	[Xu et al., 2005b]
		BF	209 famílies	NS	62% C + 24% I + 14 % HI	[Wigg et al., 2006]
		BF	150 trios	NS	-	[Langley et al., 2003]
		CC	150 casos vs 121 controls	NS	-	[Langley et al., 2003]
	5HTTVNTR	CC	46 trios + 5 duos	0,008[b]	-	[Banerjee et al., 2006]
		CC	71 casos vs 128 controls	0,001	-	[Zoroglu et al., 2002]
		CC	-	NS	C + I + HI	[Kent et al., 2002]
		BF	113 famílies	NS	81% C + 8% I + 11 % HI	[Kent et al., 2002]
		BF	293 famílies	NS	41,0% C + 52,5% I + 6,5 % HI	[Li et al., 2007]
		BF	150 famílies	NS	-	[Langley et al., 2003]
		CC	150 casos vs 121 controls	NS	-	[Langley et al., 2003]
		CC	687 casos vs 1013 controls	NS	Aproximació QTL	[Curran et al., 2005]
	rs3813034	BF	113 famílies	0,04	81% C + 8% I + 11 % HI	[Kent et al., 2002]
		BF	209 famílies	NS	62% C + 24% I + 14 % HI	[Wigg et al., 2006]
		BF	409 famílies	NS	78% C + 22% I	[Xu et al., 2005]
	Ile425Val	BF	209 famílies	NS	62% C + 24% I + 14 % HI	[Wigg et al., 2006]
	rs1050565	CC	687 casos vs 1013 controls	0,0045	Aproximació QTL	[Curran et al., 2005]
	rs1487871	CC	687 casos vs 1013 controls	0,033	Aproximació QTL	[Curran et al., 2005]
	rs2020930	CC	687 casos vs 1013 controls	0,035	Aproximació QTL	[Curran et al., 2005]
	rs140701	CC	687 casos vs 1013 controls	0,013	Aproximació QTL	[Curran et al., 2005]
		BF	674 famílies	NS	C	[Brookes et al., 2006]
	rs2020937	CC	687 casos vs 1013 controls	0,027	Aproximació QTL	[Curran et al., 2005]
	rs2020939	CC	687 casos vs 1013 controls	NS	Aproximació QTL	[Curran et al., 2005]
	rs6354	CC	687 casos vs 1013 controls	NS	Aproximació QTL	[Curran et al., 2005]
		BF	674 famílies	NS	C	[Brookes et al., 2006]
	rs2020942	CC	687 casos vs 1013 controls	NS	Aproximació QTL	[Curran et al., 2005]
		BF	674 famílies	NS	C	[Brookes et al., 2006]
	rs1872924	CC	687 casos vs 1013 controls	NS	Aproximació QTL	[Curran et al., 2005]
	rs140700	CC	687 casos vs 1013 controls	NS	Aproximació QTL	[Curran et al., 2005]
		BF	776 casos TDAH	NS	C	[Brookes et al., 2006]

	3'UTR SNP	CC	687 casos vs 1013 controls	NS	Aproximació QTL	[Curran et al., 2005]
	rs1042173	BF	674 famílies	NS	C	[Brookes et al., 2006]
	D17S1294	BF	179 famílies	0,002[d]	77% C + 15% I + 8% HI	[Hawi et al., 2005]
5HT1B	**rs6296**	BF	273 famílies	0,0065	-	[Hawi et al., 2002]
		BF	115 famílies	0,03[d]	57% C + 24% I + 19% HI	[Quist et al., 2003]
		BF	617 famílies	0,0009 [e]	-	[Smoller et al., 2006]
		BF	229 famílies	0,0056[f]	61,5% C + 32,4% I + 6,1% HI	[Smoller et al., 2006]
		BF	110 famílies	NS	94% C + 6% I	[Bobb et al., 2005]
		CC	163 casos vs 129 controls	NS	94% C + 6% I	[Bobb et al., 2005]
		BF	329 bessons dizigòtics	NS	Aproximació QTL	[Mill et al., 2005]
		BF	358 famílies	NS	41% C + 52.5% I + 6.5% HI	[Li et al., 2005]
		BF	102 famílies	NS	69% C + 27% I + 4 % HI	[Heiser et al., 2007]
		BF	203 famílies	NS	60% C + 26% I + 14% HI	[Ickowicz et al., 2007]
		BF	674 famílies	NS	C	[Brookes et al., 2006]
	rs6298	BF	110 famílies	NS	94% C + 6% I	[Bobb et al., 2005]
		CC	163 casos vs 129 controls	NS	94% C + 6% I	[Bobb et al., 2005]
		BF	358 famílies	NS	41% C + 52,5% I + 6,5% HI	[Li et al., 2005]
		BF	229 famílies	NS	61,5% C + 32,4% I + 6,1% HI	[Smoller et al., 2006]
		BF	203 famílies	NS	60% C + 26% I + 14% HI	[Ickowicz et al., 2007]
		BF	674 famílies	NS	C	[Brookes et al., 2006]
5HT2A	**rs6313**	BF	195 famílies	0,031	-	[Li et al., 2002]
		CC	323 casos vs 182 controls	0,003	-	[Li et al., 2002]
		BF	674 famílies	NS	C	[Brookes et al., 2006]
		BF	115 famílies	NS	-	[Quist et al., 2000]
		BF	102 TDAH famílies	NS	69% C + 27% I + 4 % HI	[Heiser et al., 2007]
		BF	110 famílies	NS	94% C + 6% I	[Bobb et al., 2005]
		CC	163 casos vs 129 controls	NS	94% C + 6% I	[Bobb et al., 2005]
		CC	70 casos vs 100 controls	NS	-	[Zoroglu et al., 2002]
	rs6314	BF	243 famílies	0,04	65.6% C + 22.4% I + 6.2% HI	[Guimaraes et al., 2007]
		BF	115 famílies	0,03	-	[Quist et al., 2000]
		BF	674 famílies	NS	C	[Brookes et al., 2006]
		BF	102 TDAH famílies	NS	69% C + 27% I + 4 % HI	[Heiser et al., 2007]
		BF	110 famílies	NS	94% C + 6% I	[Bobb et al., 2005]
		CC	163 casos vs 129 controls	NS	94% C + 6% I	[Bobb et al., 2005]
		BF	273 famílies	NS	-	[Hawi et al., 2002]
	rs6311	CC	41 pacients remetents vs 41 pacients no remetents	0,029[l]	-	[Li et al., 2006b]
		BF	674 famílies	NS	C	[Brookes et al., 2006]
		BF	243 famílies	NS	65.6% C + 22.4% I + 6.2% HI	[Guimaraes et al., 2007]
		BF	102 TDAH famílies	NS	69% C + 27% I + 4 % HI	[Heiser et al., 2007]
		BF	110 famílies	NS	94% C + 6% I	[Bobb et al., 2005]
		CC	163 casos vs 129 controls	NS	94% C + 6% I	[Bobb et al., 2005]
		CC	70 casos vs 100 controls	NS	-	[Zoroglu et al., 2002]
	rs3803189	BF	674 famílies	NS	C	[Brookes et al., 2006]

* C: TDAH combinat; I: TDAH inatent; HI: TDAH hiperactiu-impulsiu.

[a] Estudi combinat cas-control de tres estudis previs de [Kent et al., 2002; Manor et al., 2001; Seeger et al., 2001]

[b] Preferència transmissió materna (p=0,005).

[d] Quan es considera la transmissió paterna.

[e] Estudis familiars prèviament realitzats per [Quist et al., 2003] amb transmissió preferentment paterna (P=0,00005).

[f] Sobretransmissió de l'al·lel 861G al subtipus inatent (P=0,0056).

[l] Associació entre l'al·lel –1438A i remissió funcional.

CC: Estudi d'associació cas-control poblacional; BF: Estudi d'associació basat en famílies.

S'ha avaluat també la possible participació en el TDAH d'altres gens implicats en els mecanismes de síntesi i degradació de la serotonina, com el gen *TPH* en què no s'han identificat SNPs significativament associats amb el TDAH [Li et al., 2006; Sheehan et al., 2005; Walitza et al., 2005], o el gen *DDC*, també amb resultats negatius [Hawi et al., 2001].

3.3 El sistema dopaminèrgic i el TDAH

3.3.1 El sistema dopaminèrgic

La dopamina (DA) és un neurotransmissor catecolaminèrgic que actua com a neuromodulador tot alterant la resposta de les neurones diana a altres neurotransmissors i regulant l'activitat de canals iònics dependents de voltatge [Girault i Greengard, 2004]. El SNC conté dos grans grups de neurones dopaminèrgiques, (i) un grup localitzat a la substància nigra que innerva els nuclis caudat i putamen i (ii) un altre grup neuronal situat a l'àrea tegmental ventral que innerva el nucli estriat i l'escorça prefrontal (Figura 11)

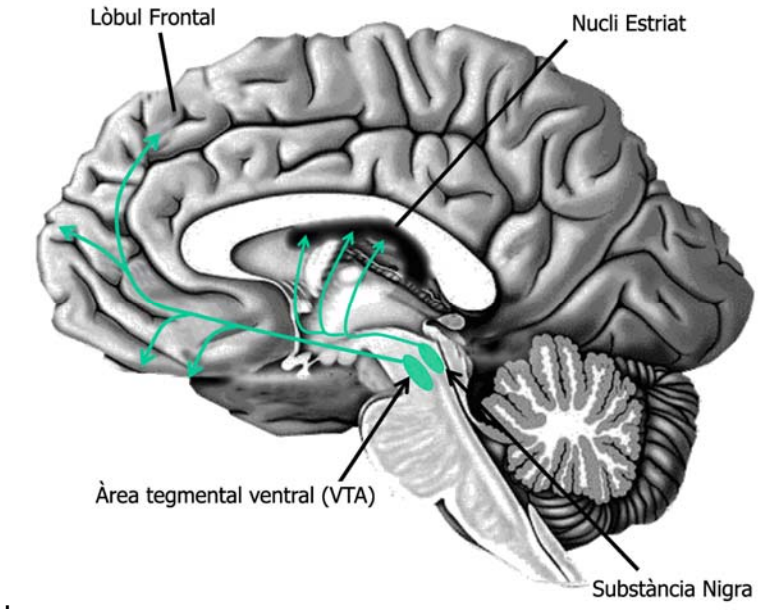


Figura 11. Vies de senyalització dopaminèrgica.

La DA és un dels neurotransmissors més importants del SNC i té un paper fonamental en la regulació de diverses funcions que inclouen les funcions motores neuroendocrines, motivacionals i afectives. A més, la DA s'ha relacionat amb el consum de drogues com la cocaïna, les amfetamines o altres psicoestimulants i amb els circuits de recompensa [Bjorklund i Dunnett, 2007].

El procés de síntesi de DA s'inicia amb la conversió de fenilalanina a tirosina gràcies a l'enzim feninalanina hidroxilasa (PAH). A continuació, la tirosina s'oxida a L-dihidrofenilalanina (L-DOPA) per l'acció de l'enzim tirosina hidroxilasa (TH), pas limitant de la síntesi de DA, noradrenalina i adrenalina al cervell. L'enzim DOPA-decarboxilasa (AADC o DDC) converteix L-DOPA en DA que a la vegada és convertida en noradrenalina per l'acció enzimàtica de la

dopamina hidroxilasa (DBH) (Figura 12). Un cop sintetitzada, la DA s'emmagatzema en vesícules sinàptiques i és alliberada a l'espai sinàptic.

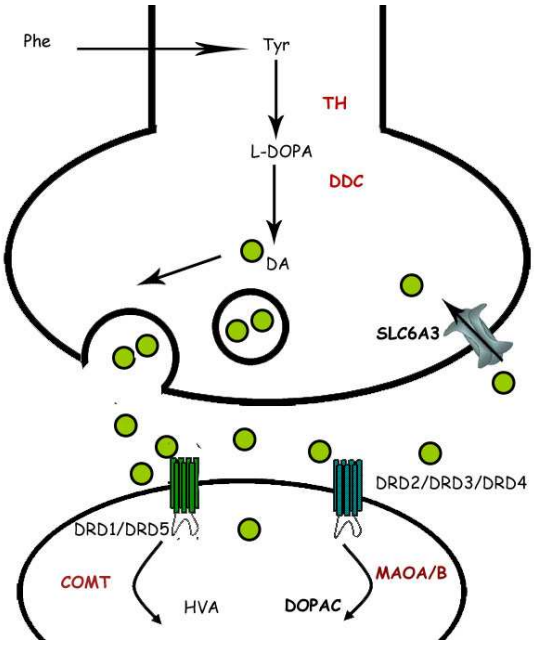


Figura 12. Via de síntesi i degradació de dopamina.

COMT: catecol-O-metiltransferasa, DA: Dopamina, DDC: DOPA-decarboxilasa, HVA: Àcido homovanílic, MAOA/B: Monoamina oxidasa, Phe: Fenilalanina, Tyr: Tirosina, TH:Tirosina Hidroxilasa.

Hi ha cinc tipus de receptors de DA que es diferencien en funció de les seves propietats estructurals i farmacològiques (Caixa 9). S'agrupen en dues categories: receptors similars al receptor D1 (DRD1), que activen l'adenilat ciclasa (receptors D1 (DRD1) i D5 (DRD5)); i els receptors similars al receptor D2, que inhibeixen la via d'adenilat ciclasa tot inhibint la formació d'AMPc (receptors D2 (DRD2), D3 (DRD3) i D4 (DRD4)) que s'expressen majoritàriament a l'escorça prefrontal [Girault et al., 2004].

Caixa 9. Receptors de la dopamina.

Receptors	Distribució al SNC	Mecanismes d'acció
DRD1	S'expressa per tot el SNC	Estimulació de l'activitat adenilat ciclasa
DRD2	Nucli caudat putamen i Nucli accumbens	Inhibició de l'activitat adenilat ciclasa
DRD3	Nucli accumbens i Nucli estriat.	Inhibició de l'activitat adenilat ciclasa
DRD4	Escorça frontal, mesencèfal i amígdala.	Inhibició de l'activitat adenilat ciclasa
DRD5	Sistema límbic	Estimulació de l'activitat adenilat ciclasa

Per altra banda, el transportador de dopamina (SLC6A3/DAT1) s'encarrega de la recaptació de la DA, per ser degradada alternativament pels enzims catecol-O-metiltransferasa (COMT) o monoamina oxidasa (MAOA i MAOB). Alguns trastorns neurològics o psiquiàtrics com la malaltia de Parkinson, l'addicció a drogues, el TDAH o l'esquizofrènia, entre d'altres, s'han associat a anomalies en la via de senyalització dopaminèrgica. La manifestació clínica és diferent en funció del tipus d'alteració (dèficit o guany de DA) i de la localització anatòmica de la disfunció.

3.3.2 Contribució del sistema dopaminèrgic al TDAH

Diferents línies d'investigació apunten al sistema de neurotransmissió dopaminèrgic com el principal candidat a contribuir a la patologia del TDAH per la seva funció al SNC com a regulador del control motor, de les funcions cognitives i dels circuits de recompensa. En aquest sentit, estudis de ressonància magnètica han identificat anomalies a les àrees cerebrals riques en innervacions dopaminèrgiques en individus infantils amb TDAH [Durston et al., 2005; Ernst et al., 1999] i els nivells de SLC6A3 en pacients amb TDAH estan alterats en regions implicades en el trastorn, com el nucli estriat i l'escorça frontal [Castellanos et al., 1996; Cheon et al., 2003; Jucaite et al., 2005]. Per altra banda, diversos models animals suggereixen també la participació del sistema dopaminèrgic en el TDAH centrant la seva atenció en els receptors de DA (*Drd1*, *Drd2*, *Drd3*, *Drd4* i *Drd5*), el transportador de dopamina (*Slc6a3*) i l'enzim dopamina β-hidroxilasa (*Dbh*). Així, el model de rata hiperactiva espontània (SHR) mostra alteracions en l'expressió de *Slc6a3*, mentre que el ratolí *Coloboma* redueix la seva hiperactivitat i l'efecte de l'administraació d'amfetamines quan el receptor *Drd2* és bloquejat de manera selectiva [Leo et al., 2003; Watanabe et al.]. A més, els ratolins deficients pels gens *Slc6a3* (Slc6a3 -/-) i Drd3 (Drd3 -/-) presenten hiperactivitat espontània [Accili et al., 1996; Giros et al., 1996], mentre que els ratolins Drd2-/- i Drd4-/- mostren reduccions en la seva activitat motora [Baik et al., 1995; Rubinstein et al., 1997]. A més, els ratolins genoanul.lats Dbh -/- i Drd4 -/- són hipersensibles a l'amfetamina o al consum d'etanol, cocaïna i a la hiperactivitat produïda pel consum de metaamfetamina [Rubinstein et al., 1997]. Finalment, els ratolins Drd1 -/- presenten reducció locomotora sota els efectes de la cocaïna, i els ratolins Drd5 -/- mostren nivells baixos d'immobilitat i reduccions en la hiperactivitat induïda per agonistes dopaminèrgics [Holmes et al., 2001]. Per acabar, l'efecte sobre el sistema dopaminèrgic dels fàrmacs psicoestimulants utilitzats en el tractament del TDAH, com el metilfenidat i les amfetamines, que incrementen els nivells sinàptics de dopamina

mitjançant el bloqueig del transportador, ha despertat l'interès dels investigadors envers la via dopaminèrgica.

S'han realitzat nombrosos estudis d'associació cas-control de tipus familiar i poblacional centrats en gens del sistema dopaminèrgic com a possibles factors de risc pel TDAH en mostres infantils i/o adultes (Taules 1 i 6). Alguns d'aquests gens, com *DRD4, DRD5, SCL6A3, DBH* i *COMT,* s'han estudiat en diferents meta-anàlisis i han mostrat resultats d'associació positius [Gizer et al., 2009; Thapar et al., 2007; Thapar et al., 2005]. Un dels gens més estudiats ha estat *DRD4,* situat a la regió cromosòmica 11p15.5 i organitzat en 4 exons. Dos dels polimorfismes d'aquest gen s'han analitzat en molts estudis a causa del seu possible efecte funcional: una variació de tipus VNTR amb una unitat de repetició de 48pb situada a l'exó 3 i una duplicació/deleció de 120pb a la regió promotora del gen. Els primers estudis d'associació suggerien que l'al·lel de set repeticions (7R) del polimorfisme VNTR és un factor de risc pel TDAH en diverses poblacions [Castellanos et al., 1998; Comings et al., 1999; Curran et al., 2001; Gabriela et al., 2009; Langley et al., 2004; Mill et al., 2001; Muglia et al., 2000]. Tot i que alguns grups han obtingut dades poc consistents, aquests resultats s'han replicat en posteriors estudis d'associació individuals i en meta-anàlisis [Faraone et al., 2001; Faraone et al., 2005; Gizer et al., 2009; Johansson et al., 2008; Li et al., 2006;[Nikolaidis and Gray]. Per altra banda, l'altre polimorfisme del gen *DRD4*, dup120pb, també ha estat objecte de diversos estudis d'associació que donen resultats molt controvertits [Barr et al., 2001; Bhaduri et al., 2006; Brookes et al., 2005; Kereszturi et al., 2007; Kustanovich et al., 2004; McCracken et al., 2000; Todd et al., 2001].

Juntament amb *DRD4*, el gen *SLC6A3/DAT1*, que codifica el transportador de dopamina, ha estat un dels gens dopaminèrgics més analitzats en relació al TDAH. Principalment s'hi han avaluat dos polimorfismes de tipus VNTR, un localitzat a l'intró 8 i l'altre localitzat a la regió 3'UTR del gen. Tot i les evidències farmacològiques que recolzen la implicació d'aquest gen en la patologia del TDAH, els estudis d'associació mostren resultats inconsistents entre ells. Finalment, s'han dut a terme diversos estudis per avaluar els diferents receptors de dopamina, així com els enzims que estan implicats en la seva síntesi o degradació, sense mostrar variables de risc inequívocament relacionades amb el TDAH.

Taula 6. Principals estudis d'associació de tipus familiar i poblacional realitzats entre el TDAH i gens del sistema dopaminèrgic

Gen i Polimorfisme	Estudi	Mida mostral	Fenotip associat	p-valor	Referència
SLC6A3					
VNTR 40 pb a 3'UTR	BF	152 casos, 102 famílies	TDAH	-	[Muglia et al., 2002]
VNTR 40 pb a 3'UTR (9/10)	CC	122 casos, 67controls	TDAH-H	p=0,01	[Barkley et al., 2006]
VNTR 40 pb a 3'UTR; VNTR 30 pb a intró 8	CC	122 casos, 174 controls	TDAH	-	[Bruggemann et al., 2007; Johansson et al., 2008]
VNTR 40 pb a 3'UTR	CC	358 casos, 340 controls	TDAH	-	Johansson et al., 2008]
VNTR 40 pb a 3'UTR; VNTR 30 pb a intró 8. (haplotip 9-6)	CC	216 casos, 528 controls	TDAH	p=0,0011	[Franke et al., 2008]
VNTR 40 pb a 3'UTR; VNTR 30 pb a intró 8.	CC	1440 casos, 1769 controls. Meta-anàlisi	TDAH	p=0,03,	[Franke et al.]
SLC6A3, DRD4					
VNTR 40 pb a 3'UTR de *SLC6A3*, VNTR 48 pb a exó 3 de *DRD4*	BF	563 casos	TDAH	-	[Biederman et al., 2009]
DRD4					
VNTR 48 pb a exó 3 (7R)	CC i BF	66 casos, 66 controls; 44 famílies	TDAH-C	p= 0,003	[Muglia et al., 2000]
VNTR 48 pb a exó 3; Indel 120 pb a promotor	Lligament	14 famílies	TDAH	p= 0,0467	[Arcos-Burgos et al., 2004]
VNTR 48 pb a exó 3	BF	96 famílies	TDAH	-	[McGough et al., 2005]
VNTR 48 pb a exó 3	CC	122 casos, 67 controls	TDAH-H	-	[Barkley et al., 2006]
VNTR 48 pb a exó 3	CC	358 casos, 340 controls	TDAH	-	[Johansson et al., 2008]
VNTR 48 pb a exó 3 (7R)	CC i BF	Meta-anàlisi	TDAH	CC: p<0,0001 BF: p=0,02	[Faraone et al., 2001]
VNTR 48 pb a exó 3 (7R)	CC i BF	Meta-anàlisi	TDAH	p=2e-12	[Li et al., 2006a]
VNTR 48 pb a exó 3, Indel 120 pb a promotor	CC i BF	Meta-anàlisi	TDAH	p=0,00007	[Gizer et al., 2009]
DRD3					
rs6280 (p.Ser9Gly)	BF	39 famílies	TDAH	-	[Muglia et al., 2002]
rs2399504, rs7611535, rs1394016, rs6280 (p.Ser9Gly), rs167770	CC	60 casos	TDAH-H	p=0,00017	[Glessner et al., 2009]
DRD2					
rs1800497 (TaqIA C>T)	CC	85 casos amb dependència a alcohol, dels quals 32,9% amb TDAH	-	-	[Kim et al., 2006a]
rs1800497 (TaqIA C>T)	CC	49 TDAH-Autisme	TDAH	-	[Sizoo et al.]
DRD5					
(CA)n al promotor	BF i CC	119 famílies 88 casos, 88 controls	TDAH	p=0,023	[Johansson et al., 2008; Squassina et al., 2008]
(CA)n al promotor	CC	358 casos, 340 controls	TDAH-C, I	p =0,02	[Johansson et al., 2008;]
DBH					
rs2519152	CC	112 casos,112 controls	TDAH	-	[Inkster et al., 2004]
rs2519152	CC	122 casos, 67 controls	TDAH-H	p=0,021	[Barkley et al., 2006]
rs1611115 (-1021C>T)	CC	407 casos, 387	TDAH	-	[Hess et al., 2009]
COMT					
rs4680 (p.Val158Met)	Regressió	203 controls	Fenotip inatent i hiperactiu-impulsiu	Inatenció p=0,008 Hiperactivitat/impulsivitat p=0,039	[Reuter et al., 2006]
rs4680 (p.Val158Met)	Regressió	85 alcohòlics 33% TDAH	-	-	[Reuter et al., 2006]
rs4680 (p.Val158Met)	Regressió	110 casos	TDAH	-	[Kim et al., 2006a]
rs4680 (p.Val158Met), rs4818	Regressió	184 homes TDAH	TDAH	-	[Brookes et al., 2006a]
rs6269, rs4633, rs4818, rs4680, (p.Val158Met)	CC	435 casos, 383 controls	TDAH-H	p=0,007 Hiperactiu/impulsiviu	[Retz et al., 2008]

CC: Estudi d'associació cas-control poblacional; BF: Estudi d'associació basat en famílies.
H: Hiperactiu; I: Inatent; C: Combinat

SLC6A3					
VNTR 40 pb a 3'UTR	BF	152 casos, 102 famílies	TDAH	-	[Muglia et al., 2002]
VNTR 40 pb a 3'UTR (9/10)	CC	122 casos, 67controls	TDAH-H	p=0,01	[Barkley et al., 2006]
VNTR 40 pb a 3'UTR; VNTR 30 pb a intró 8	CC	122 casos, 174 controls	TDAH	-	[Bruggemann et al., 2007; Johansson et al., 2008]
VNTR 40 pb a 3'UTR	CC	358 casos, 340 controls	TDAH	-	Johansson et al., 2008]
VNTR 40 pb a 3'UTR; VNTR 30 pb a intró 8. (haplotip 9-6)	CC	216 casos, 528 controls	TDAH	p=0,0011	[Franke et al., 2008]
VNTR 40 pb a 3'UTR; VNTR 30 pb a intró 8.	CC	1440 casos, 1769 controls. Meta-anàlisi	TDAH	p=0,03,	[Franke et al.]
SLC6A3, DRD4					
VNTR 40 pb a 3'UTR de *SLC6A3*, VNTR 48 pb a exó 3 de *DRD4*	BF	563 casos	TDAH	-	[Biederman et al., 2009]
DRD4					
VNTR 48 pb a exó 3 (7R)	CC i BF	66 casos, 66 controls; 44 famílies	TDAH-C	p= 0,003	[Muglia et al., 2000]
VNTR 48 pb a exó 3; Indel 120 pb a promotor	Lligament	14 famílies	TDAH	p= 0,0467	[Arcos-Burgos et al., 2004]
VNTR 48 pb a exó 3	BF	96 famílies	TDAH	-	[McGough et al., 2005]
VNTR 48 pb a exó 3	CC	122 casos, 67 controls	TDAH-H	-	[Barkley et al., 2006]
VNTR 48 pb a exó 3	CC	358 casos, 340 controls	TDAH	-	[Johansson et al., 2008]
VNTR 48 pb a exó 3 (7R)	CC i BF	Meta-anàlisi	TDAH	CC: p<0,0001 BF: p=0,02	[Faraone et al., 2001]
VNTR 48 pb a exó 3 (7R)	CC i BF	Meta-anàlisi	TDAH	p=2e-12	[Li et al., 2006a]
VNTR 48 pb a exó 3, Indel 120 pb a promotor	CC i BF	Meta-anàlisi	TDAH	p=0,00007	[Gizer et al., 2009]
DRD3					
rs6280 (p.Ser9Gly)	BF	39 famílies	TDAH	-	[Muglia et al., 2002]
rs2399504, rs7611535, rs1394016, rs6280 (p.Ser9Gly), rs167770	CC	60 casos	TDAH-H	p=0,00017	[Glessner et al., 2009]
DRD2					
rs1800497 (TaqIA C>T)	CC	85 casos amb dependència a alcohol, dels quals 32,9% amb TDAH	-	-	[Kim et al., 2006a]
rs1800497 (TaqIA C>T)	CC	49 TDAH-Autisme	TDAH	-	[Sizoo et al.]
DRD5					
(CA)n al promotor	BF i CC	119 famílies 88 casos, 88 controls	TDAH	p=0,023	[Johansson et al., 2008; Squassina et al., 2008]
(CA)n al promotor	CC	358 casos, 340 controls	TDAH-C, I	p =0,02	[Johansson et al., 2008;]
DBH					
rs2519152	CC	112 casos,112 controls	TDAH	-	[Inkster et al., 2004]
rs2519152	CC	122 casos, 67 controls	TDAH-H	p=0,021	[Barkley et al., 2006]
rs1611115 (-1021C>T)	CC	407 casos, 387	TDAH	-	[Hess et al., 2009]
COMT					
rs4680 (p.Val158Met)	Regressió	203 controls	Fenotip inatent i hiperactiu-impulsiu	Inatenció p=0,008 Hiperactivitat/impulsivitat p=0,039	[Reuter et al., 2006]
rs4680 (p.Val158Met)	Regressió	85 alcohòlics 33% TDAH	-	-	[Reuter et al., 2006]
rs4680 (p.Val158Met)	Regressió	110 casos	TDAH	-	[Kim et al., 2006a]
rs4680 (p.Val158Met), rs4818	Regressió	184 homes TDAH	TDAH	-	[Brookes et al., 2006a]
rs6269, rs4633, rs4818, rs4680, (p.Val158Met)	CC	435 casos, 383 controls	TDAH-H	p=0,007 Hiperactiu/impulsiviu	[Retz et al., 2008]

CC: Estudi d'associació cas-control poblacional; BF: Estudi d'associació basat en famílies.
H: Hiperactiu; I: Inatent; C: Combinat

Hipòtesis i Objectius

Aquest treball té per objectiu principal aprofundir en el coneixement dels factors genètics implicats en l'etiologia del TDAH. Els objectius concrets proposats han estat els següents:

Hipòtesi 1

Donades les següents evidències:

1. El factor neurotròfic derivat de cervell, BDNF, participa en el control de l'aprenentatge, l'activitat locomotora i l'agressivitat.
2. Els models animals deficients en *Bdnf* mostren un fenotip més agressiu i hiperactiu que els ratolins control.
3. El tractament farmacològic utilitzat en el TDAH, que inclou psicoestimulants o antidepressius tricíclics, modula l'expressió de BDNF i del seu receptor NTRK2.
4. BDNF modula diferents sistemes de neurotransmissió implicats en l'etiologia del TDAH.
5. Diferents estudis d'associació suggereixen que *BDNF* és un factor de risc pel TDAH.

Es planteja la següent hipòtesi:
Variants genètiques en el gen *BDNF* participen en l'etiologia del TDAH.

Objectiu 1

Analitzar la implicació del polimorfisme de tipus SNP rs6265 (p.Val66Met), localitzat a la regió que codifica la forma inmadura de BDNF, en l'etiologia del TDAH en una mostra d'individus adults mitjançant un estudi d'associació cas-control en quatre grups poblacionals i un estudi de tipus meta-anàlisi.

Hipòtesi 2

Donades les següents evidències:

1. Els pacients amb TDAH presenten baixes concentracions plasmàtiques de serotonina (5-HT) i del seu transportador 5-HTT.
2. Els nivells plasmàtics d'àcid 5-hidroxiindolacètic (5HIAA), el metabòlit principal de la 5-HT, s'han relacionat amb símptomes d'agressivitat, hiperactivitat i impulsivitat.
3. Agonistes serotoninègics i inhibidors de la recaptació o degradació de serotonina redueixen la simptomatologia del TDAH.
4. El model animal genoanul·lat (*knock-out*) pel receptor de serotonina 1b (*5-ht1b* -/-) presenta hiperactivitat, agressivitat i desinhibició.
5. Diferents estudis d'associació suggereixen que diversos gens del sistema serotoninèrgic, com *SLC6A4*, *5HT2A* i *5HT2B*, són factors de risc pel TDAH.

Es planteja la següent hipòtesi:

Variants genètiques en el gen *SLC6A4* (*5-HTT* o *SERT*) estan implicades en l'etiologia del TDAH.

Objectiu 2

Analitzar la contribució al TDAH de vuit polimorfismes (5-HTTLPR i set SNPs) del gen que codifica el transportador de serotonina *SLC6A4* en població adulta mitjançant un estudi d'associació cas-control en diferents grups poblacionals i un estudi de tipus meta-anàlisi.

Hipòtesi 3

Donades les següents evidències:

1. La dopamina (DA) és un neurotransmissor que s'expressa en diferents àrees cerebrals que s'han relacionat amb el TDAH com el nucli estriat i l'escorça frontal.
2. La DA juga un paper fonamental en la regulació de les funcions motores i cognitives així com en els circuits de recompensa.
3. S´'han identificat anomalies a les regions riques en innervacions dopaminèrgiques en pacients amb TDAH mitjançant ressonància magnètica.
4. Els pacients amb TDAH presenten nivells alterats del transportador dopaminèrgic SLC6A3/DAT1 a les regions cerebrals implicades en el trastorn.
5. Els models murins genoanul·lats per diferents receptors dopaminèrgics (*Drd1, Drd2, Drd3, Drd4* i *Drd5*) o el transportador de DA (*Slc6a3*) mostren alteracions en l'activitat locomotora.
6. Els fàrmacs psicoestimulants que s'utilitzen en el tractament del TDAH, com el metilfenidat o l'amfetamina, afecten principalment el sistema dopaminèrgic tot bloquejant el transportador de DA i incrementant els nivells d'aquest neurotransmissor a l'espai sinàptic.
7. Diversos estudis d'associació suggereixen que gens del sistema dopaminèrgic com *DRD4, SLC6A3/DAT1, DRD1* i *DRD5*, entre d'altres, són factors de risc pel TDAH.

Es planteja la següent hipòtesis:
Variants genètiques en els gens que codifiquen els receptors dopaminèrgics (*DRD1, DRD2, DRD3, DRD4* i *DRD5)*, el transportador de DA *(SLC6A3/DAT1)* i enzims implicats en la síntesi (*TH*) o degradació (*DBH* i *COMT*) de DA estan implicades en l'etiologia del TDAH.

Objectiu 3a

Avaluar la contribució al TDAH de 9 gens del sistema dopaminèrgic (*DRD1, DRD2, DRD3, DRD4, DRD5, SLC6A3, TH, DBH i COMT*) en població infantil i adulta mitjançant estudis d'associació de tipus cas-control seguint criteris de màxima cobertura genètica basada en els patrons de desequilibri de lligament.

Objectius 3b i 3c

Avaluar la contribució al TDAH en adults de dos polimorfismes de tipus no-SNP en el gen que codifica el receptor de dopamina D4 (*DRD4),* una repetició en tàndem (VNTR) a l'exó 3 i una duplicació/deleció (Dup/Del) a la regió promotora del gen, i de dos polimorfismes de tipus VNTR a l'intro 8 i a la regió 3' no traduïda (3'UTR) del gen que codifica el transportador dopaminèrgic *SLC6A3/DAT1* mitjançant estudis d'associació cas-control en quatre grups poblacionals i estudis de tipus metanàlisi. Analitzar també el possible paper de la interacció entre aquests dos gens en el TDAH.

Resultats

INFORME DELS DIRECTORS SOBRE LA CONTRIBUCIÓ DEL DOCTORAND A LES PUBLICACIONS D'AQUESTA TESI DOCTORAL

Títol de la Tesi: "**Factors genètics de susceptibilitat al Trastorn per Dèficit d'Atenció amb Hiperactivitat (TDAH)**"
Autor: Cristina Sánchez Mora
Directors: Dr. Bru Cormand i Rifà, Dra. Marta Ribasés Haro

ARTICLE 1 (CAPÍTOL 1)

Sánchez-Mora C, Ribasés M, Ramos-Quiroga JA, Casas M, Bosch R, Boreatti-Hümmer A, Heine M, Jacob CP, Lesch KP, Fasmer OB, Knappskog PM, Kooij JJ, Kan C, Buitelaar JK, Mick E, Asherson P, Faraone SV, Franke B, Johansson S, Haavik J, Reif A, Bayés M, Cormand B.

Meta-analysis of brain-derived neurotrophic factor p.Val66Met in adult ADHD in four European populations.

American Journal of Medical Genetics part B Neuropsychiatric Genet.ics. 2010;153B(2):512-23.

Índexs de qualitat: SCI2009=3,481, quartil 2 de la categoria GENETICS & HEREDITY

Aportació personal a l'article: Disseny de l'estudi. Cerca bibliogràfica. Extracció d'ADN de casos i controls de la mostra espanyola. Preparació de les mostres espanyoles i genotipació del polimorfisme rs6265 (p.Val66Met) del gen *BDNF*. Anàlisi estadística. Elaboració del primer esborrany del manuscrit i participació en l'edició final.

ARTICLE 2 (CAPÍTOL 2)

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ARTICLE 3 (CAPÍTOL 3a)

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Barcelona, 20 de març de 2011

Signat pels directors:

Dr. Bru Cormand Rifà

Dra. Marta Ribasés Haro

CAPÍTOL 1: TDAH i factor neurotròfic derivat de cervell (*BDNF*)

Article 1

Meta-anàlisi en quatre poblacions europees del polimorfisme p.Val66Met del factor neurotròfic derivat de cervell en TDAH adult

RESUM

Antecedents i propòsit: Els factors neurotròfics participen en el desenvolupament, supervivència i manteniment funcional de les neurones i en els processos de neuroplasticitat del sistema nerviós central. S'ha observat que els fàrmacs psicoestimulants utilitzats en el tractament del TDAH modulen l'expressió de BDNF. És per això que molts estudis han proposat que BDNF podria participar en l'etiologia del TDAH. Els estudis d'associació prèviament publicats sobre la contribució del polimorfisme de tipus SNP p.Val66Met (rs6265) del gen *BDNF* a l'etiologia del TDAH mostren resultats contradictoris. L'objectiu d'aquest treball ha estat avaluar el paper d'aquest polimorfisme en el risc de patir TDAH a l'edat adulta.

Mètodes: Metaanàlisi d'estudis d'associació cas-control en quatre poblacions europees en un total de 1445 pacients adults amb TDAH i 2247 controls aparellats per edat i sexe.

Resultats: No es van identificar diferències significatives en les distribucions genotípiques o al·lèliques del polimorfisme estudiat entre casos i controls en cap de les quatre poblacions estudiades individualment ni en unificar les poblacions mitjançant un estudi meta-analític.

Conclusions: L'estudi d'associació dut a terme no recolza la contribució del polimorfisme p.Val66Met (rs6265) del gen *BDNF* al TDAH en població adulta.

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Meta-analysis of brain-derived neurotrophic factor p.Val66Met in adult ADHD in four European populations.

American Journal of Medical Genetics part B Neuropsychiatric Genetics 2010;153B(2):512-23

RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART B
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Meta-Analysis of Brain-Derived Neurotrophic Factor p.Val66Met in Adult ADHD in Four European Populations

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Attention-deficit hyperactivity disorder (ADHD) is a multifactorial, neurodevelopmental disorder that often persists into adolescence and adulthood and is characterized by inattention, hyperactivity and impulsiveness. Before the advent of the first genome-wide association studies in ADHD, genetic research had mainly focused on candidate genes related to the dopaminergic and serotoninergic systems, although several other genes had also been assessed. Pharmacological data, analysis of animal models and association studies suggest that *Brain-Derived Neurotrophic Factor* (*BDNF*) is also a strong candidate gene for ADHD. Several polymorphisms in *BDNF* have been reported and studied in psychiatric disorders but the most frequent is the p.Val66Met (rs6265G > A) single nucleotide polymorphism (SNP), with functional effects on the intracellular trafficking and secretion of the protein. To deal with the inconsistency raised among different case–control and family-based association studies regarding the p.Val66Met contribution to ADHD, we performed a meta-analysis of published as well as unpublished data from four different centers that are part of the International Multicentre Persistent ADHD CollaboraTion (IMpACT). A total of 1,445 adulthood ADHD patients and 2,247 sex-matched controls were available for the study. No association between the p.Val66Met polymorphism and ADHD was found in any of the four populations or in the pooled sample. The meta-analysis also showed that the overall gene effect for ADHD was not statistically significant when gender or comorbidity with mood disorders were considered. Despite the potential role of *BDNF* in ADHD, our data do not support the involvement of p.Val66Met in the pathogenesis of this neuropsychiatric disorder. © 2009 Wiley-Liss, Inc.

Key words: attention-deficit hyperactivity disorder; adult ADHD; BDNF; case–control association study; meta-analysis

INTRODUCTION

Attention-deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder that often persists into adolescence and adulthood and is characterized by inattention, hyperactivity and impulsiveness and causes significant social, educational and psychological problems in childhood and adulthood. Heritability of ADHD has been estimated at 39–91% and it is considered a complex disorder with genetic and environmental risk factors [Faraone et al., 2005]. The genetic causes of ADHD are still unknown, but so far genetic research has mainly focused on candidate genes of the dopaminergic and serotoninergic systems [Comings et al., 2000; Ribasés et al., 2009]. In addition to these neurotransmission pathways, neurotrophic factors, which participate in development, survival and functional maintenance of neurons, may be involved in neuroplasticity changes that take place in the central nervous system and may also contribute to the genetic predisposition to ADHD. Among these, *Brain-Derived Neurotrophic Factor* (*BDNF*) is a strong candidate gene. Psychostimulant and antidepressant drugs commonly used for ADHD treatment modulate the expression of *BDNF* and its specific receptor *NTRK2* [Meredith et al., 2002; Chase et al., 2007]. In addition, heterozygous *Bdnf* knockout mice display hippocampal-dependent learning deficiencies, aggressiveness, anxiety, and hyperactive locomotor behavior when compared with wild-type littermates, which interestingly can be rescued in an enriched environment [Linnarsson et al., 1997; Kernie et al., 2000; Rios et al., 2001; Chourbaji et al., 2004]. Chronic infusion of Bdnf into the substantia nigra of animal models alters locomotion activity, whereas its intracerebroventricular administration decreases locomotion, motility and rearing [Martin-Iverson et al., 1994; Kobayashi et al., 1997]. Finally, several association studies have tested the possible involvement of *BDNF* in ADHD, although the results are controversial. Some of these studies focus solely on *BDNF* or a few candidate genes (Table SI; [Friedel et al., 2005; Kent et al., 2005; Brookes et al., 2006; Lee et al., 2007; Schimmelmann et al., 2007; Xu et al., 2007; Conner et al., 2008; Lanktree et al., 2008; Oades et al., 2008]), whereas others are GWAS [Neale et al., 2008; Sonuga-Barke et al., 2008; Lasky-Su et al., 2008a,b; Anney et al., 2008b].

The *BDNF* gene is located at 11p13-14, is organized in 13 exons, and encodes a 247 amino acid precursor peptide (pro-BDNF) that is proteolytically cleaved to form the mature protein of 153 amino acids. Several polymorphisms of *BDNF* have been reported and studied in a number of psychiatric disorders. The most frequent is the rs6265 (p.Val66Met) SNP located within the pro-BDNF region, which goes along with increased BDNF serum concentrations in Met allele carriers [Lang et al., 2009]. Egan et al. [2003] demonstrated that although it is located in the pro-BDNF polypeptide and does not alter the intrinsic biological activity of the mature protein, the rs6265G > A sequence variant impairs the intracellular processing and secretion of the mature neurotrophin. In addition, as it was recently shown that mice also secrete proBDNF, especially

while the brain is developing, these data point towards an impact of p.Val66Met on neuronal development, especially in the hippocampus [Yang et al., 2009].

To deal with the inconsistency raised among different case–control and family-based association studies regarding the contribution of *BDNF* p.Val66Met to ADHD, we aimed to perform a meta-analysis of published as well as unpublished case–control data from four different centers (Germany, The Netherlands, Norway and Spain) integrated in the International Multicentre persistent ADHD CollaboraTion (IMpACT), which focuses its interest on adulthood ADHD.

MATERIALS AND METHODS

Patients and Controls

In total, 1,445 adult ADHD patients and 2,447 controls of Caucasian origin from four European countries (Spain, Germany, Norway, and The Netherlands) were recruited by members of the International Multicentre Persistent ADHD CollaboraTion (IMpACT). Consensus eligibility criteria for the current study across all sites were a diagnosis of ADHD according to the diagnostic criteria of DSM-IV (Diagnostic and Statistical Manual for Mental Disorders-IV), onset before the age of 7 years via retrospective diagnosis (which was confirmed by a family member, wherever possible), lifelong persistence and current diagnosis. Patients were extensively examined by experienced psychiatrists in adult ADHD using open and semi-structured interviews. Other psychiatric disorders were evaluated with the Structured Clinical Interview of DSM-IV for axis-I and axis-II disorders (SCID-I, SCID-II) or semi-structured interviews. Severity of ADHD in adulthood was measured by means of standards rating scales. Most of the clinical sample has been described before [Bekker et al., 2005; Kooij et al., 2005; Jacob et al., 2007; Franke et al., 2008; Johansson et al., 2008; Ramos-Quiroga et al., 2008; Ribasés et al., 2008]. Diagnosis was blind to genotype (see Table SII for a detailed description of the instruments and procedures used by the different sites).

Most controls (except for the Norwegian samples and part of the German samples, see Table SII) were screened for the presence of ADHD and those scoring high on symptoms of the disorder were excluded.

The study was approved by the ethics committee of each participating institution and informed consent was obtained from all subjects in accordance with the Helsinki Declaration.

DNA Isolation and Genotyping

Genomic DNA was isolated either from saliva using the Oragene™ DNA Self-Collection kit from DNA Genotek (DNA Genotek Inc., Ottawa, Canada) or from peripheral blood lymphocytes by the salting-out procedure [Miller et al., 1988].

Germany and Norway: genotyping of rs6265 was accomplished using the Sequenom MassArray genotyping platform (Sequenom, San Diego, CA).

Netherlands: The rs6265 polymorphism was genotyped using TaqMan-based genotyping. Genotyping was carried out in a volume of 10 µl containing 10 ng of genomic DNA, 5 µl of ABgene Mastermix (2×; ABgene Ltd., Hamburg, Germany), 0.125 µl of the Taqman assay (assay ID: Taqman assay: C_11592758_10; reporter 1: VIC-C-allele; reporter 2: FAM-T-allele; Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands) and 3.875 µl of H_2O. Amplification was performed on a 7500 Fast Real-Time PCR System starting with 15 min at 95°C, followed by 50 cycles of 15 sec at 95°C and 1 min at 60°C. Genotypes were scored using the algorithm and software supplied by the manufacturer (Applied Biosystems).

Spain: rs6265 was genotyped with the SNPlex platform (Applied Biosystems, Foster City, CA) at the Barcelona node of the Centro Nacional de Genotipado (CeGen, www.cegen.org) as previously described [Tobler et al., 2005].

Statistical Analysis

We first assessed Hardy–Weinberg equilibrium (HWE) in the control groups from each IMpACT node using a χ^2 test. To determine the best genetic model to be used we estimated the three possible odds ratios (ORs) and their 95% confidence intervals (CI): OR1 (Val/Val vs. Met/Met), OR2 (Val/Val vs. Val/Met) and OR3 (Met/Met vs. Val/Met) in the pooled sample (Table SIII). Thus, if $OR1 = OR3 \neq 1$ and $OR2 = 1$, a recessive model is suggested; $OR1 = OR2 \neq 1$ and $OR3 = 1$ indicates a dominant model; and $OR1 > OR3 > 1$ (or $OR1 < OR2 < 1$ and $OR1 < OR3 < 1$) suggests a codominant model. Prior to the pooling of the four individual studies, we compared genotype and allele frequencies among cases and controls from each separate IMpACT node using a χ^2 test with the SNPassoc R package [Gonzalez et al., 2007]. To combine the individual study results, we conducted meta-analyses using the Meta R package (cran.r-project.org/web/packages/meta/index. html). We first tested for heterogeneity among studies using the Q-statistic, which is a weighted sum of the squares of the deviations of individual study OR estimates from the overall estimate. When the ORs are homogeneous, Q follows a χ^2 distribution with $r - 1$ (r is the number of studies) degrees of freedom (df). If $P_Q < 0.10$, the heterogeneity was considered to be statistically significant [Lau et al., 1997; Fleiss, 1981]. Inconsistency across studies was quantified with the I^2 metric ($I^2 = Q - df/Q$), which can be interpreted as the percentage of total variation across several studies due to heterogeneity. I^2 takes values between 0% and 100%, with higher values denoting a greater degree of heterogeneity (0–25%: no heterogeneity; 25–50% moderate heterogeneity; 50–75%: large heterogeneity; 75–100% extreme heterogeneity) [Zintzaras and Hadjigeorgiou, 2004]. When no heterogeneity was present, the pooled OR was estimated using fixed-effects model (Mantel and Haenszel, 1959). Otherwise, random effects models (Laird and Mosteller, 1990) were applied to obtain the pooled OR. Random effects modeling assumes a genuine diversity in the results of various studies and it incorporates a between-study variance into the calculations (Whitehead, 2002). The results of the association tests are given as pooled ORs (which estimate the genotype-induced risk of adult ADHD), with the corresponding 95% confidence intervals (CIs). $P < 0.05$ was considered statistically significant. Additionally, we conducted other meta-analytical studies in which we split the sample by gender or by clinical subtype (combined and inattentive). Because ADHD is highly comorbid with mood disorders and *BDNF* has been extensively tested for association with this psychiatric entity [Gratacòs et al., 2007;

Verhagen et al., 2008], we also considered ADHD patients with and without mood disorders.

RESULTS

A total of 1,445 adult ADHD patients and 2,247 controls from four IMpACT nodes were available for the meta-analysis. Table I shows the clinical description of the samples included in the study. Genotype distributions of the p.Val66Met SNP showed no significant departure from HWE when each control group as well as the pooled control sample were considered ($P > 0.05$). No significant differences were observed when genotype or allele frequencies of the p.Val66Met SNP were compared between ADHD patients from each country and their sex-matched unrelated controls (Table II). To determine the best genetic model we calculated the three ORs. The estimated ORs in the pooled sample were $OR_1 = 0.93$ (95% CI: 0.66–1.31), $OR_2 = 0.99$ (95% CI: 0.86–1.14), and $OR_3 = 1.07$ (95% CI: 0.75–1.52). No heterogeneity was detected for OR_1, OR_2, or OR_3 across samples ($P = 0.43$, $P = 0.35$, and $P = 0.51$, respectively) (Table SIII). The fact that none of the ORs significantly deviated from 1 did not allow to choose a particular genetic model based on the pattern of the odds ratios. Thus, comparisons of genotype frequencies between cases and controls were performed under different models (codominant, dominant, recessive, overdominant, and log-additive). The dominant one was eventually selected for the meta-analysis because (1) it was the one that produced lower *P*-values in most comparisons, (2) it produced the lowest Akaike Information Criterion (AIC) values, and (3) it was the only one to show nominal *P*-values in some particular comparisons (see below). Therefore, a Met66 dominant model with fixed effects was used to perform a meta-analysis, which showed a lack of a statistically significant effect of the p.Val66Met *BDNF* SNP on adulthood ADHD ($P = 0.86$; OR = 0.99 (95% CI: 0.86–1.13); Table III).

We then considered the combined and inattentive subtypes separately and we did not observe statistically significant effects of p.Val66Met BDNF on ADHD subtypes in either of the four populations (Table SIV). The pooled analysis under a fixed-effects model showed no significant effect of the polymorphism on ADHD subtypes either (Table IV). The hyperactive/impulsive subtype was not studied because of the limited sample size (6.43% from the total sample; Table I).

The separate analysis of males and females displayed a nominal association between p.Val66Met and ADHD in females from the Spanish cohort ($P = 0.0363$, OR = 0.50 (95% CI: 0.26–0.96); Table SV). Nevertheless, after having discarded heterogeneity, the pooled analysis under a fixed-effects model showed that the overall gene effect on ADHD was neither statistically significant in females ($P = 0.51$; OR = 0.93 (95% CI: 0.75–1.14)) nor in males ($P = 0.72$; OR = 1.03 (95% CI: 0.85–1.24); Table V).

We then separated ADHD patients according to comorbidity with mood disorders and observed nominal association between *BDNF* and ADHD with mood disorders in some of the populations (Germany, genotypes: $P = 0.035$, OR = 1.33 (95% CI: 1.02–1.75) and alleles: $P = 0.035$, OR = 1.28 (95% CI: 1.01–1.64); Table SVI) and without mood disorders in others (Netherlands: genotypes: $P = 0.047$, OR = 0.60 (95% CI: 0.36–0.99); Table SVI). However, after discarding heterogeneity, the meta-analysis under a fixed-effects model showed that the overall gene effect was not significant in any of the two clinical groups considered (ADHD with mood disorders: $P = 0.24$, OR = 1.11 (95% CI: 0.93–1.31); ADHD without mood disorders: $P = 0.20$, OR = 0.88 (95% CI: 0.73–1.07); Table VI).

TABLE I. Descriptive Characteristics of the IMpACT Samples: Adult ADHD Patients and Controls Without ADHD From Four European Countries

Study	Germany	Netherlands	Norway	Spain[a]	Pool
Cases					
Female	282 (46.45%)	95 (50%)	208 (47.7%)	58 (23.5%)	643 (44.49%)
Male	325 (53. 54%)	96 (50%)	228 (52.29%)	153 (76.5%)	802 (55.50%)
Total	607	191	436	211	1,445
ADHD subtype					
Combined type	408 (67.21%)	172 (90.07%)	230 (52.75%)	139 (65.87%)	949 (65.67%)
Hyperactive/impulsive type	48 (7.90%)	5 (2.61%)	30 (6.88%)	10 (4.73%)	93 (6.43%)
Inattentive type	151 (24.87%)	14 (7.32%)	73 (16.74%)	62 (29.4%)	300 (20.76%)
Sub-threshold	—	—	97 (22.24%)	—	97 (6.71%)
Unknown	—	—	6 (1.39%)	—	6 (0.43%)
Age (mean and SD)	31 (10.26)	42 (10.7)	34 (10.6)	35 (10.17)	35 (4,6)
Mood disorders	323 (53.21%)	119 (62.30%)	299 (68.57%)	81 (38.38%)	822 (56.8%)
Controls					
Female	390 (46, 42%)	243 (50%)	237 (47.78%)	116 (27.29%)	986 (43.8%)
Male	450 (53, 57%)	243 (50%)	259 (52.2%)	309 (72.7%)]	1261 (56.1%)
Total	840	486	496	425	2,247
Age (mean and SD)	31 (10.4)	62 (18.06)	27 (7.2)	43 (16.36)	40 (15.7)

[a]Published data [Ribasés et al., 2008].

TABLE II. Comparison of Genotype and Allele Frequencies of the BDNF p.Val66Met Polymorphism in 1,445 Adulthood ADHD Patients and 2,247 Sex-Matched Unrelated Controls From Four European Countries

	Genotypes[a]												Alleles[b]	
	Cases, n (%)				Controls, n (%)				Genotype AA vs. AG + GG		Genotype AG + AA vs. GG		Allele A vs. allele G	
	AA	AG	GG	Sum	AA	AG	GG	Sum	P	OR (95% CI)	P	OR (95% CI)	OR (95% CI)	P
Germany	25 (4.1)	189 (31.1)	393 (64.7)	607	39 (4.6)	284 (33.8)	517 (61.5)	840	0.45	0.88 (0.53–1.47)	0.63	1.14 (0.92–1.42)	1.12 (0.93–1.35)	0.22
Netherlands	5 (2.6)	67 (35.1)	119 (62.3)	191	12 (2.5)	152 (31.3)	322 (66.3)	486	0.62	1.06 (0.37–3.06)	0.91	0.84 (0.59–1.19)	0.88 (0.65–1.18)	0.38
Norway	22 (5.0)	132 (30.3)	282 (64.7)	436	17 (3.4)	155 (31.2)	324 (65.3)	496	0.46	1.50 (0.78–2.86)	0.21	0.97 (0.74–1.26)	0.93 (0.74–1.17)	0.53
Spain	10 (4.7)	79 (37.4)	122 (57.8)	211	18 (4.2)	141 (33.2)	266 (62.6)	425	0.51	1.12 (0.51–2.48)	0.24	0.81 (0.58–1.14)	0.86 (0.65–1.13)	0.28
Pool	62 (4.3)	467 (32.3)	916 (63.4)	1,445	86 (3.8)	732 (32.6)	1,429 (63.6)	2,247	0.77	1.13 (0.81–1.57)	0.48	0.99 (0.86–1.13)	0.98 (0.87–1.1)	0.72

[a]AA = MetMet; AG = MetVal; GG = ValVal.
[b]A = Met; G = Val.

DISCUSSION

To our knowledge, this is the first meta-analysis performed to evaluate the association between *BDNF* and adulthood ADHD. No evidence of association was found between the p.Val66Met variation and this neuropsychiatric disorder. Although most of the previous studies considered childhood ADHD samples (Table SI), our results are in agreement with those described by Friedel et al. [2005] in 88 childhood ADHD patients and 96 controls, Schimmelmann et al. [2007] in 468 children from 294 families comprising one or more affected sibs, Lee et al. [2007] in 315 ADHD children from 266 nuclear families, or Brookes et al. [2006] in 674 DSM-IV combined type probands with 808 siblings, in which no association between the p.Val66Met SNP and ADHD was detected. However, other association studies have reported a possible contribution of *BDNF* to ADHD (Table SI). In this regard, Kent et al. [2005] demonstrated a preferential paternal transmission of the p.Val66 allele in 341 childhood ADHD trios and Xu et al. [2007] found no association between p.Val66Met and ADHD but showed evidence for a decreased transmission of the −270T/p.Val66 haplotype in two independent samples of ADHD child probands. In addition, two studies considered the *BDNF* gene in adulthood ADHD and while Lanktree et al. [2008] found evidence for a p.Val66 contribution, Conner et al. [2008] showed no association between this polymorphism and adult ADHD scores (Table SI). Two genome-wide association studies (GWAS) have been reported in ADHD so far, one of them in 343 adult ADHD patients and 304 controls using a DNA pooling approach [Lesch et al., 2008] and the other one in 958 affected family trios with child ADHD sibs considering either ADHD as a categorical trait or related quantitative measures such as ADHD symptoms, age at onset of ADHD symptoms or conduct problems [Neale et al., 2008; Lasky-Su et al., 2008a,b; Anney et al., 2008b]. All of them included the p.Val66Met variation (rs6265) and other polymorphic sites within the *BDNF* gene, but they did not achieve genome-wide significance.

All these conflicting findings may either reflect that the variant is not associated with ADHD or, alternatively, that the results vary because of factors such as reduced statistical power due to limited sample sizes in some of the reported studies (Table SI), genetic heterogeneity introduced or increased by different diagnostic criteria, different frequencies of ADHD subtypes, comorbidity and/or distinct ethnic origin among the studied samples, as differences in *BDNF* allelic frequencies have been reported among ethnic groups [Shimizu et al., 2004]. The lack of association between *BDNF* and adulthood ADHD detected in our study, however, raises several considerations:

(1) Genetic effects for common variants may be reduced and difficult to detect in association studies of small sample sizes. The present meta-analysis, that considers relatively large well-characterized clinical samples that fulfilled DSM-IV criteria for ADHD and were evaluated using a common set of diagnostic instruments, improves the statistical power to detect association (>95%) with respect to the analyses of previous datasets. Our strategy also allowed examination of heterogeneity between the different series of patients and controls included in the study. In this regard, we did not detect heterogeneity of ORs

TABLE III. Genetic Effect of the p.Val66Met Polymorphism on Adulthood ADHD Using a Fixed-Effects Model

	Cases		Controls						
Study	**GG**	**Total**	**GG**	**Total**	**Weight (%)**	**OR M-H, fixed, 95% CI**	***z***	***P***	**OR M-H, fixed, 95% CI**
1. Germany	393	607	517	840	37.95	1.14 (0.92–1.42)			
2. Netherlands	119	191	322	486	17.00	0.84 (0.59–1.19)			
3. Norway	282	436	324	496	26.57	0.97 (0.74–1.27)			
4. Spain	122	211	266	425	18.48	0.81 (0.58–1.14)			
Fixed-effects model	916	1,445	1,429	2,247	100	0.98 (0.86–1.13)			

Test of heterogeneity: $Q = 3.84$, $df = 3$, $P = 0.28$; $I^2 = 21.9\%$ (0–88%).

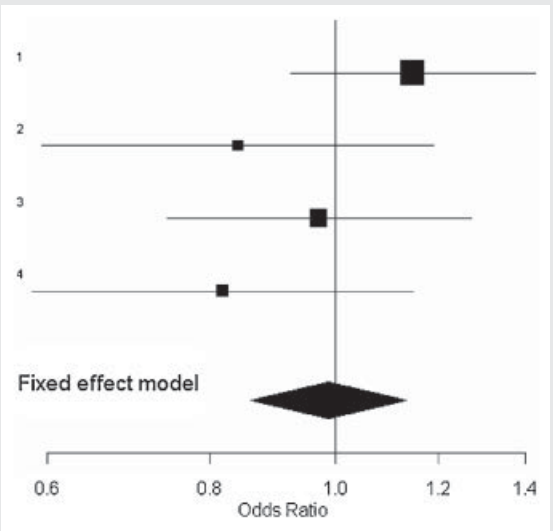
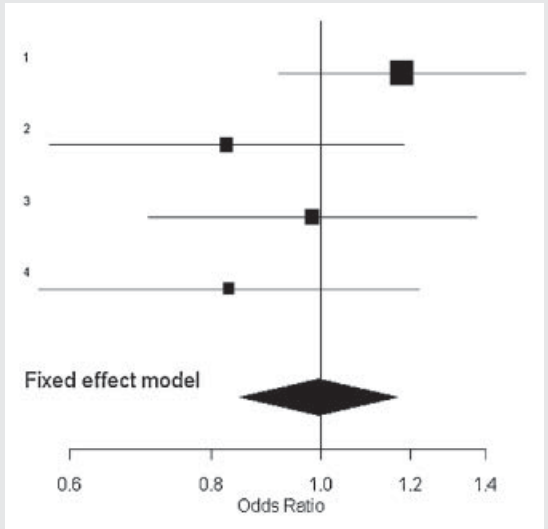


TABLE IV. Genetic Effect of the p.Val66Met Polymorphism on (a) Adulthood ADHD, Combined Subtype (949 Cases and 2,247 Controls) and Adulthood ADHD, Inattentive Subtype (299 Cases and 2,247 Controls), Using a Fixed-Effects Model

	Cases		Controls						
Study	**GG**	**Total**	**GG**	**Total**	**Weight (%)**	**OR M-H, fixed, 95% CI**	***z***	***P***	**OR M-H, fixed, 95% CI**
(a)[a]									
1. Germany	274	407	534	840	37.51	1.18 (0.91–1.51)			
2. Netherlands	106	172	321	486	21.21	0.82 (0.57–1.18)			
3. Norway	155	230	336	496	22.86	0.98 (0.70–1.37)			
4. Spain	79	140	259	425	18.42	0.83 (0.56–1.22)			
Fixed-effects model	614	949	1450	2,247	100	0.99 (0.84–1.16)	−0.05	0.95	
(b)[b]									
1. Germany	89	151	534	840	51.24	0.82 (0.57–1.17)			
2. Netherlands	12	14	321	486	1.97	3.08 (0.68–13.9)			
3. Norway	43	73	336	496	27.17	0.68 (0.41–1.12)			
4. Spain	37	61	259	425	19.62	0.98 (0.57–1.71)			
Fixed-effects model	181	299	1450	2,247	100	0.86 (0.67–1.10)	−1.16	0.24	

[a]Test of heterogeneity: $Q = 3.67$, df = 3, $P = 0.29$; $I^2 = 18.3\%$ (0–87.5%).
[b]Test of heterogeneity: $Q = 3.87$, df = 3, $P = 0.27$; $I^2 = 22.6\%$ (0–88.1%).



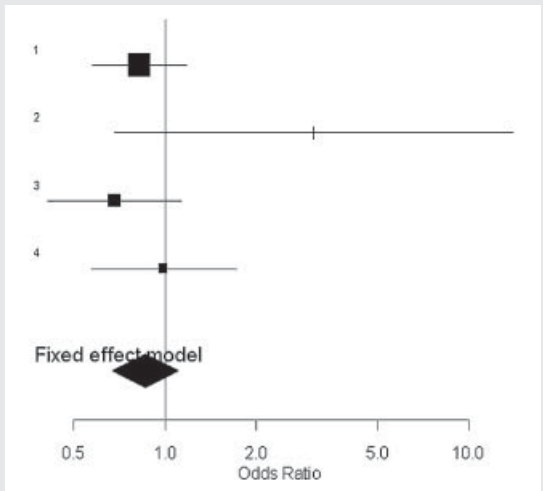
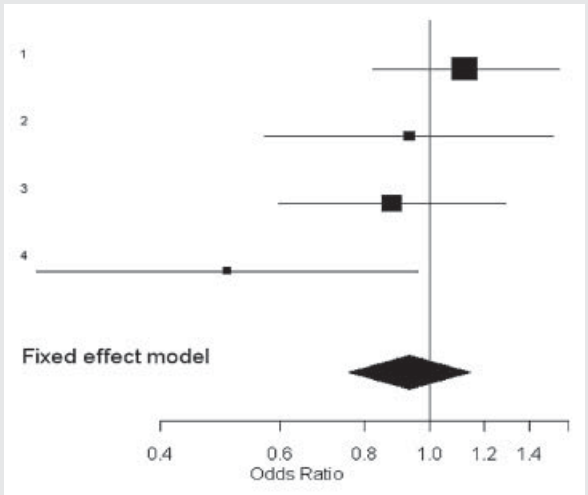


TABLE V. Genetic Effect of the p.Val66Met Polymorphism on (a) Females (643 Cases and 986 Controls) and (b) Males (802 Cases and 1261 Controls) with Adulthood ADHD Using a Fixed-Effects Model

	Cases		Controls						
Study	**GG**	**Total**	**GG**	**Total**	**Weight (%)**	**OR M-H, fixed, 95% CI**	***z***	***P***	**OR M-H, fixed, 95% CI**
(a)[a]									
1. Germany	180	282	238	390	38.91	1.13 (0.82–1.54)			
2. Netherlands	59	95	155	243	17.78	0.93 (0.56–1.51)			
3. Norway	128	208	153	237	29.62	0.87 (0.59–1.29)			
4. Spain	30	58	79	116	13.69	0.50 (0.26–0.95)			
Fixed-effects model	397	643	625	986	100	0.93 (0.75–1.14)	−0.66	0.51	
(b)[b]									
1. Germany	213	325	279	450	37.08	1.16 (0.86–1.56)			
2. Netherlands	60	96	167	243	16.31	0.75 (0.46–1.24)			
3. Norway	154	228	171	259	23.90	1.07 (0.73–1.56)			
4. Spain	92	153	187	309	22.71	0.98 (0.66–1.46)			
Fixed-effects model	519	802	804	1261	100	1.03 (0.85–1.24)	0.36	0.72	

[a]Test of heterogeneity: $Q = 2.23$, df $= 3$, $P = 0.52$; $I^2 = 40\%$ (0–79.6%).
[b]Test of heterogeneity: $Q = 2.23$, df $= 3$, $P = 0.52$; $I^2 = 40\%$ (0–79.6%).



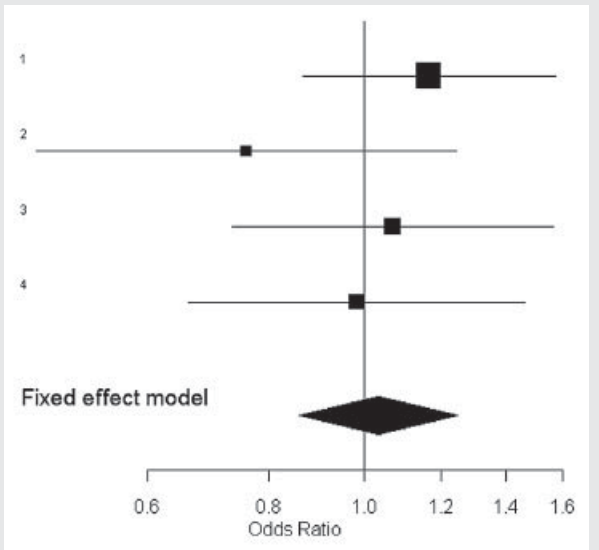
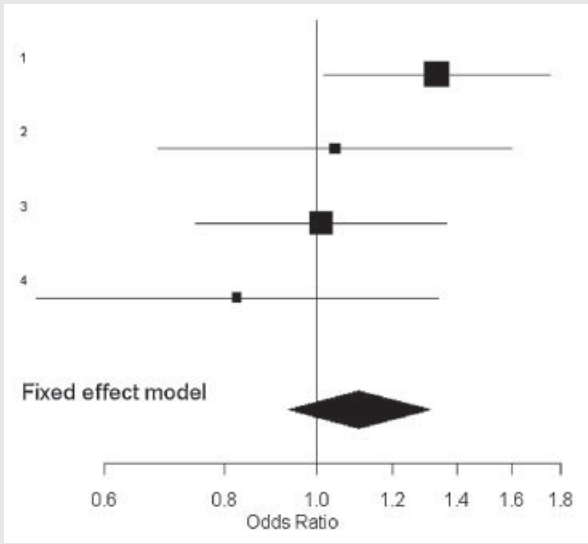
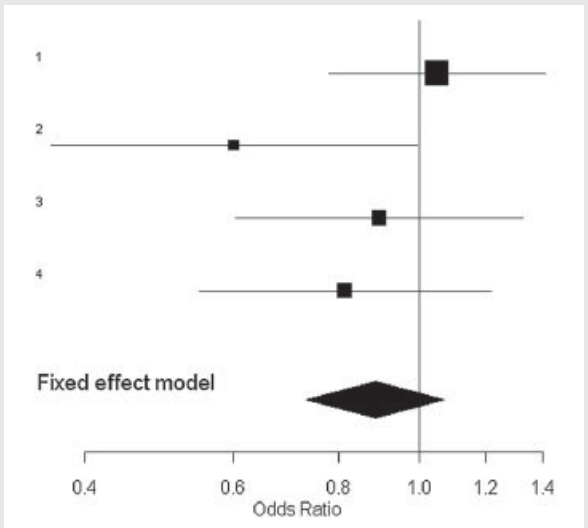


TABLE VI. Genetic Effect of the p.Val66Met Polymorphism on (a) Adulthood ADHD With Mood Disorders (822 Cases and 2,247 Controls) and (b) Adulthood ADHD Without Mood Disorders (580 Cases and 2,247 Controls) Using a Fixed-Effects Model

	Cases		Controls						
Study	GG	Total	GG	Total	Weight (%)	OR M-H, fixed, 95% CI	*z*	*P*	OR M-H, fixed, 95% CI
(a)[b]									
1. Germany	220	323	517	840	36.23	1.33 (1.01; 1.75)			
2. Netherlands	80	119	322	486	16.42	1.04 (0.68; 1.60)			
3. Norway	196	299	324	496	33.21	1.01 (0.74; 1.36)			
4. Spain	47	81	266	425	14.14	0.82 (0.50; 1.33)			
Fixed-effects model	543	822	1,429	2,247	100	1.10 (0.93; 1.31)	1.17	0.24	
(b)[b]									
1. Germany	151	241	517	840	37.58	1.04 (0.77; 1.40)			
2. Netherlands	39	72	322	486	16.62	0.60 (0.36; 0.99)			
3. Norway	86	137	324	496	22.79	0.89 (0.60; 1.32)			
4. Spain	75	130	266	425	23.01	0.81 (0.54; 1.21)			
Fixed-effects model	351	580	1,429	2,247	100	0.88 (0.73; 1.06)	−1.26	0.20	

[a]Test of heterogeneity: $Q = 3.64$, df = 3, $P = 0.30$; $I2 = 17.6\%$ (0–87.4%).
[b]Test of heterogeneity: $Q = 3.71$, df = 3, $P = 0.29$; $I2 = 19\%$ (0–89.6%).





or evidence that a single study accounted for the significance or magnitude of the pooled study.

(2) The proportions of the different ADHD subtypes differ among the reported studies. Thus, while most patients described by Kent et al. [2005] (81%) or Xu et al. [2007] (100% UK and 78% Taiwan) were combined ADHD, a lower proportion was seen in the other studies (<70%), including the present meta-analysis (66%). If the *BDNF* contribution to ADHD was subtype-specific, these differences could account for the distinct results between studies.

(3) Gender differences among studies may also explain controversial results, as a recent study suggests that hyperactivity may be gender-specific [Kim et al., 2007]. In this regard, male *BDNF* conditional knock-out mice exhibit hyperactivity, whereas females display normal locomotor activity with a prominent increase in depression-like behaviors [Monteggia et al., 2007]. Although we found nominal association between *BDNF* and ADHD in Spanish females, the pooled analysis stratified for gender showed that the overall gene effect on ADHD was not statistically significant.

(4) Other studies have reported a gene-specific parent of origin effect in ADHD. In this regard, Kent et al. [2005] demonstrated a significant preferential paternal transmission of the p.Val66 allele to children with ADHD. Nevertheless, other groups did not find evidence for a preferential paternal transmission of *BDNF* risk alleles in ADHD [Kim et al., 2007; Anney et al., 2008a]. This aspect, however, could not be assessed in the present case–control association study.

(5) Genetic variants in *BDNF* have generally been linked to a number of other psychiatric phenotypes, that may share genetic risk factors with ADHD, such as schizophrenia, eating disorders, bipolar disorder or depression [Neves-Pereira et al., 2002; Sklar et al., 2002; Green et al., 2006; Gratacòs et al., 2007; Verhagen et al., 2008; Duncan et al., 2009]. Since comorbidity with these disorders is frequently observed in ADHD (e.g., substantial proportion of our ADHD sample showed comorbidity with mood disorders (56.8%)), in contrast to most of the previous studies we tested directly whether this comorbidity might bias association results for ADHD [Kent et al., 2005; Xu et al., 2007; Lanktree et al., 2008]. Although we observed a nominal association between *BDNF* and ADHD with mood disorders (in the German sample) or without mood disorders (in the Dutch sample), the pooled analysis showed that the overall gene effect was not significant in any of these two groups.

(6) Most of the previous studies reporting a *BDNF* contribution to the susceptibility to ADHD include only children samples, which may suggest a childhood-specific association between this neurotrophic factor and ADHD. Only one study reports association between BDNF and adult ADHD, although the sample size is limited [Lanktree et al., 2008]. In this regard, our group identified a childhood-specific association between ADHD and the BDNF specific receptor, *NTRK2* [Ribasés et al., 2008] that might support a differential genetic component in childhood ADHD with or without remission of the phenotype across life span.

(7) Although it has been reported that the p.Val66Met polymorphism alters the intracellular trafficking and activity-dependent secretion of BDNF [Egan et al., 2003], this SNP may not be functionally involved in ADHD, although it is still possible that other *BDNF* sequence variants do contribute to the phenotype.

In conclusion, although *BDNF* remains as a candidate risk factor for ADHD, we found no evidence of association between the p.Val66Met SNP and adulthood ADHD using a meta-analysis strategy in large well-characterized clinical samples from four European populations. Further dissection of clinical phenotypes as well as full genetic coverage, in terms of LD, of the *BDNF* gene in larger cohorts of children as well as adult patients may improve our knowledge about the involvement of *BDNF* in the susceptibility to this complex neuropsychiatric disorder.

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Table SI. Overview of family-based (FB) and case-control (CC) association studies with the *BDNF* p.Val66Met polymorphism in child and adult ADHD samples.

ADHD sample	Associated allele	Study design	N	P-value	ADHD sample*	Reference
Childhood	Val66	FB	341trios of ADHD	P= 0.02**	81% C + 9% I + 10% H	Kent et al. 2005
	-270C/p.Val66***	FB	135 trios + 35 duos	Haplotype: P=0.032	78% C+ 22% I	Xu et al. 2007
	-270C/p.Val66***	FB	pooled sample****	Haplotype: P=0.016	100% C	Xu et al. 2007
	No association	CC	88 cases + 96 controls	-	-	Friedel et al. 2005
	No association	FB	468 probands from 294 families	-	72% C + 21.4% I + 6% H	Schimmelman et al. 2005
	No association	FB	315 probands from 266 families	-	62% C + 24% I + 14% H	Lee et al. 2007
	No association	FB	674 probands with 808 siblings	-	100% C	Brookes et al. 2006
Adulthood	p.Val66	FB	268 ADHD probands from 83 families	Alleles: P=0.036 Haplotype[&]: P=0.0086	-	Lanktree et al. 2008
	p.Val66	CC+FB	121 cases + 83 families + inferred controls	Alleles: P=0.0096 Haplotype[&]: P=0.025	-	Lanktree et al. 2008
	No association	CC	121 cases + 80 controls	-	-	Lanktree et al. 2008
	No association	Cohort[&&]	143 subjects	-	-	Conner et al. 2008

* C: Combined ADHD; I: Inattentive ADHD; H: Hyperactive-Impulsive ADHD
** Preferential paternal transmission (p=0.0005)
*** Decreased transmission of the -270T/p.Val66 (-270C>T/p.Val66Met) allelic combination.
**** Pooled sample: Taiwanese sample (135 trios and 35 duos) and UK sample (116 trios and 64 duos)
& *BDNF* SNPs within the haplotype : rs4923463, rs6265 (p.Val66Met), rs11030104, rs2049045 and rs7103411.
&& Association study between the p.Val66Met SNP and scores of adult ADHD, that include the ADHD-SB questionnaire, WURS-k and the Wender-Reimherr Interview.

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Table SII. Instruments used to evaluate the phenotype of patients by the different sites contributing to IMpACT.

	Germany*	Norway**	Spain***	Netherlands****
Clinical interview	X	X	X	X
CAADID-II			X	
WURS	X	X	X	
CAARS	X		X	
ASRS		X		
ADHD-RS			X	X
ADHD Screening checklist			X	
CGI			X	
SDI			X	
SCID-I	X		X	
SCID-II	X		X	
BDI	X		X	
MDQ		X		
TPQ	X			
NEO PI-R	X			
Fagerström scale	X			
STAI			X	
MCMI-II			X	

*IMPACT Germany: Patients have been extensively examined using an open interview by an experienced psychiatrist (M.H, A. B.-H., and C.P.J., who supervised the complete ascertainment procedure) as well as by the Structured Clinical Interview of DSM-IV for axis-I and axis-II disorders (SCID-I, SCID-II) also allowing the registration of co-morbid conditions [Fydrich et al., 1997; Wittchen et al., 1997]. Severity of A-ADHD was measured by means of the WURS-k interview [Retz-Junginger et al., 2002; Wender, 1995]. Where available, chart reviews have been performed and information obtained from relatives, school reports etc. were taken into consideration. Depressive symptoms were rated by the Beck Depression Inventory (BDI) [Beck et al., 1961]. Personality assessment was done using the NEO PI-R and TPQ questionnaires [Costa et al., 1995; Cloninger et al., 1991]; impulsivity was assessed by the I7 questionnaire [Eysenk, 1993] and nicotine abuse was quantified using the Fagerström scale [Fagerstrom, 1978]. Eligibility criteria for the study were A-ADHD according to the diagnostic criteria of DSM-IV, onset before the age of 7 years via retrospective diagnosis, life-long persistence, current diagnosis and age at recruitment between 18 and 65 years. Exclusion criteria were the restricted appearance of lack of concentration, hyperactivity, impulsivity to the duration of any other axis-I disorder, as well as current diagnosis of not withdrawn drug/alcohol abuse/dependence, lifetime diagnosis of bipolar-I disorder, schizophrenia, or any other psychotic disorder, and mental retardation (IQ level< 80; MWT-B < 13 points). Further details on the sample can be obtained from Jacob et al. [Jacob et al., 2007]. The control group was recruited in the Lower Franconia area in Germany and consisted of healthy volunteers of Caucasian origin. About 40% of them were extensively interviewed for absence of ADHD and did not fulfill DSM-IV ADHD criteria, while the remaining controls consisted of unscreened blood donors and University staff not explicitly screened for absence of psychiatric disorders. However, the scope of the study was explained to these individuals.

**IMPACT Norway: All patients were formally diagnosed with ADHD before inclusion into the project, but subtype data were not systematically available at the time of the primary diagnosis. Severity of past and current ADHD symptoms in patients was evaluated using the Wender Utah Rating Scale (WURS) [Ward et al., 1993] and the ASRS. Furthermore, the referring clinicians filled in additional data on diagnosis, treatment history and treatment response, filled in the Mood Disorder Questionnaire (MDQ) [Hirschfeld et al., 2000] and responded to 31 additional questions regarding socio-demographic variables and comorbid conditions in participants and their first degree relatives. The ASRS and WURS versions used in this study have also been used in earlier publications [Johansson et al., 2008; Halmøy et al., 2008]. The control sample included 496 sex-matched unrelated subjects, 269 of which had no previous diagnosis of ADHD and were recruited using a

random selection of persons born in Norway from 1967 to 1989, whereas the rest were healthy blood donors in whom no information about ADHD symptoms was available.

***IMPACT Spain: The diagnosis of ADHD in adulthood was evaluated with the Structured Clinical Interview for DSM-IV Axis I and II Disorders (SCID-I and SCID-II) [Fydrich et al., 1997; Wittchen et al., 1997] and the Conners Adult ADHD Diagnostic Interview for DSM-IV (CAADID Part II) [Epstein et al., 2001]. In addition to this, the following instruments were used to characterize the patients in this cohort: Severity of ADHD symptoms was evaluated with the long version of the Conners' ADHD Rating Scale (self-report form CAARS-S:L and observer form CAARS-O:L) [Conners et al., 1999], the ADHD Rating Scale (ADHD-RS) [DuPaul et al., 1998], the ADHD Screening Checklist and the WURS [Wender, 1995] for retrospective symptomatology. The level of impairment was measured with the Clinical Global Impression (CGI) [Conners et al., 1999] and the Sheehan Disability Inventory (SDI) [Sheehan, 1983]. For the evaluation of psychiatric symptoms patients filled in the BDI [Beck et al., 1961], the State Trait Anxiety Inventory (STAI) [Spielberger et al., 1986] and the Millon Clinical Multiaxial Inventory (MCMI-II) [Avila-Espada 1998]. Full-Scale IQ was estimated with the Vocabulary and Block Design subtests of the WAIS-III [Wechsler, 1997]. Patients also completed the Digit span, Arithmetic, Letter-Number Sequencing and Symbol Search subtests of the WAIS-III, the Conners Continuous Performance Test (CPT) [Conners, 1985] the California Verbal Learning Test (CVLT) [Delis et al., 1987], the Logical Memory I-II and Visual Memory I-II of the WMS-Rand the Trailmaking Test (Parts A and B) [Reitan, 1958]. The control sample consisted of unrelated Spanish Caucasian blood donors for whom ADHD was excluded based on DSM-IV criteria. They were recruited at the Blood and Tissue Bank of Hospital Universitari Vall d'Hebron and approximately matched for sex the ADHD group.

****IMPACT Netherlands: Subjects were included if a clinical diagnosis of adult ADHD with childhood onset was established. Prior to inclusion, all patients underwent a standard clinical assessment consisting of a psychiatric evaluation by experienced psychiatrists using a semi-structured diagnostic interview for ADHD and comorbid disorders, the Dutch version of structured diagnostic interviews for retrospective diagnosis of ADHD. For current ADHD symptoms during the last 6 months, a Dutch version of the DSM-IV ADHD Rating Scale, (ADHD-RS) based on the 18 DSM-IV items for ADHD, was used [Kooij et al., 2005; DuPaul, 1998]. The ADHD Rating Scale has been used in epidemiologic and clinical research in adults in the United States and in The Netherlands [Kooij et al., 2005; Murphy and Barkley, 1996]. To be given a full diagnosis of adult ADHD, subjects had to (A) meet at least 6 out of 9 DSM-IV criteria of inattention and/or hyperactivity/impulsivity for a diagnosis of ADHD in childhood and at least 5 out of 9 criteria in adulthood, (B) describe a chronic persisting course of ADHD symptoms from childhood to adulthood, and (C) endorse a moderate to severe level of impairment attributed to the ADHD symptoms. A cut-off point of 5 of 9 criteria was set for adult diagnosis of ADHD based on literature and epidemiological data using the same DSM-IV ADHD Rating Scale [Kooij et al., 2005]. In order to obtain information about lifetime ADHD symptoms and impairment, the patient, the partner and if available the parents were interviewed. Information on school reports was examined in order to sustain the diagnosis in childhood. Diagnostic criteria have been described in more detail elsewhere [Bekker et al., 2005; Kooij et al., 2005]. Controls were also evaluated using the ADHD-RS and a cut-off of 4 or more symptoms was used to exclude controls from the study.

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Table SIII. Genetic model of the p.Val66Met polymorphism in the pooled study.

Fixed effects model Mantel-Haenszel method					
	OR (95% CI)	**Q**	**d.f.**	**p**	**I^2**
OR 1 (ValVal vs MetMet)	0.93 (0.66-1.30)	2.77	3	0.428	0% (0%-83.4%)
OR 2 (ValVal vs ValMet)	0.98 (0.85-1.14)	3.31	3	0.346	9.3% (0%-86.1%)
OR 3 (MetMet vs ValMet)	1.07 (0.75-1.51)	2.30	3	0.512	0% (0%-80.1%)

Table SIV. Comparison of genotype and allele frequencies of the *BDNF* p.Val66Met polymorphism in (a) 949 adult combined subtype ADHD patients and 2247 sex-matched unrelated controls and (b) 299 adult inattentive subtype ADHD patients and 2247 sex-matched unrelated controls from four European countries.

(a)

	Genotypes*													Alleles**	
	Cases n (%)				Controls n (%)					Genotype AA vs AG+GG		Genotype AG+AA vs GG		Allele A vs Allele G	
	AA	AG	GG	Sum	AA	AG	GG	Sum	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
Germany	14 (3.4)	119 (29.2)	274 (67.3)	407	32 (3.8)	274 (32.6)	534 (63.6)	840	0.41	0.90 (0.47-1.70)	0.73	1.19 (0.92-1.51)	0.18	1.15 (0.92-1.42)	0.21
Netherlands	5 (2.9)	61 (35.5)	106 (61.6)	172	12 (2.5)	153 (31.5)	321 (66.0)	486	0.58	1.18 (0.41-3.41)	0.75	0.82 (0.57-1.17)	0.29	0.86 (0.63-1.16)	0.32
Norway	10 (4.3)	65 (28.3)	155 (67.4)	230	16 (3.2)	144 (29.0)	336 (67.7)	496	0.74	1.37 (0.61-3.08)	0.45	0-98 (0.70-1.36)	0.92	0.95 (0.71-1.27)	0.73
Spain	6 (4.3)	55 (39.3)	79 (56.4)	140	20 (4.7)	146 (34.4)	259 (60.9)	425	0.59	0.91 (0.36-2.31)	0.83	0.83 (0.56-1.23)	0.36	0.98 (0.65-1.23)	0.49
Pool	35 (3.7)	300 (31.6)	614 (64.7)	949	80 (3.6)	717 (31.9)	1450 (64.5)	2247	0.97	1.04 (0.69-1.55)	0.86	1.01 (0.86-1.17)	0.90	1 (0.88-1.15)	0.96

(b)

	Genotypes*													Alleles**	
	Cases n (%)				Controls n (%)					Genotype AA vs AG+GG		Genotype AG+AA vs GG		Allele A vs Allele G	
	AA	AG	GG	Sum	AA	AG	GG	Sum	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
Germany	8 (5.3)	54 (35.8)	89 (58.9)	151	32 (3.8)	274 (32.6)	534 (63.6)	840	0.48	1.41 (0.64-3.13)	0.40	0.81 (0.57-1.17)	0.28	0.84 (0.62-1.12)	0.23
Netherlands	0 (0)	2 (14.3)	12 (85.7)	14	12 (2.5)	153 (31.5)	321 (66.0)	486	0.21	0	0.40	1.38 (0.68-14.2)	0.09	2.9 (0.68-12.3)	0.09
Norway	5 (6.8)	25 (34.2)	43 (58.9)	73	16 (3.2)	144 (29.0)	336 (67.7)	496	0.21	2.13 (0.75-6.03)	0.17	0.68 (0.41-1.13)	0.14	0.69 (0.46-1.04)	0.08
Spain	4 (6.6)	20 (32.8)	37 (60.7)	61	20 (4.7)	146 (34.4)	259 (60.9)	425	0.83	1.42 (0.47-4.31)	0.55	0.96 (0.55-1.66)	0.89	0.92 (0.59-1.45)	0.73
Pool	17 (5.7)	101 (33.8)	181 (60.5)	299	80 (3.6)	717 (31.9)	1450 (64.5)	2447	0.15	1.63 (0.95-2.80)	0.08	0.84 (0.65-1.07)	0.17	0.83 (0.68-1.02)	0.08

*AA=MetMet; AG=MetVal; GG=ValVal
**A=Met; G=Val

Table SV. Comparison of genotype and allele frequencies of the *BDNF* p.Val66Met polymorphism in (a) adult ADHD females (643 cases and 986 controls) and (b) adult ADHD males (802 cases and 1261controls) from four European countries.

(a)

Female ADHD															
	Genotypes*													Alleles**	
	Cases n (%)				Controls n (%)					Genotype AA vs AG+GG		Genotype AG+AA vs GG		Allele A vs Allele G	
	AA	AG	GG	Sum	AA	AG	GG	Sum	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
Germany	14 (5)	88 (31.2)	180 (63.8)	282	14 (3.6)	138 (35.4)	238 (61)	390	0.40	1.40 (0.66-2.99)	0.38	1.12 (0.81-1.53)	0.45	1.04 (0.8-1.36)	0.75
Netherlands	3 (3.2)	33 (34.7)	59 (62.1)	95	6 (2.5)	82 (33.7)	155 (63.8)	243	0.91	1.29 (0.32-5.26)	0.72	0.93 (0.57-1.51)	0.77	0.93 (0.61-1.41)	0.72
Norway	8 (3.8)	72 (34.6)	128 (61.5)	208	7 (3.0)	77 (32.5)	153 (64.6)	237	0.75	1.31 (0.47-3.69)	0.60	0.87 (0.59-1.29)	0.51	0.89 (0.64-1.23)	0.46
Spain	2 (3.4)	26 (44.8)	30 (51.7)	58	6 (5.2)	31 (26.7)	79 (68.1)	116	0.06	0.65 (0.13-3.35)	0.60	0.50 (0.26-0.96)	0.03	0.65 (0.38-1.11)	0.12
Pool	27 (4.2)	219 (34.1)	397 (61.7)	643	33 (3.3)	328 (33.3)	625 (63.4)	986	0.60	1.27 (0.75-2.13)	0.37	0.93 (0.75-1.14)	0.50	0.93 (0.78-1.1)	0.38

(b)

Male ADHD															
	Genotypes*													Alleles**	
	Cases n (%)				Controls n (%)					Genotype AA vs AG+GG		Genotype AG+AA vs GG		Allele A vs Allele G	
	AA	AG	GG	Sum	AA	AG	GG	Sum	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
Germany	11 (3.4)	101 (31.1)	213 (65.5)	325	20 (4.4)	139 (30.9)	291 (64.7)	450	0.75	0.75 (0.36-1.59)	0.45	1.04 (0.76-1.40)	0.80	1.06 (0.82-1.37)	0.63
Netherlands	2 (2.1)	34 (35.4)	60 (62.5)	96	6 (2.5)	70 (28.8)	167 (68.7)	243	0.49	0.84 (0.17-4.24)	0.83	0.75 (0.46-1.25)	0.27	0.82 (0.54-1.26)	0.37
Norway	14 (6.1)	60 (26.3)	154 (67.5)	228	10 (3.9)	78 (30.1)	171 (66.0)	259	0.37	1.63 (0.71-3.74)	0.24	1.07 (0.73-1.56)	0.72	0.98 (0.71-1.34)	0.88
Spain	8 (5.2)	53 (34.6)	92 (60.1)	153	9 (2.9)	106 (34.3)	194 (62.8)	309	0.46	1.84 (0.70-4.86)	0.22	0.89 (0.60-1.33)	0.58	0.86 (0.62-1.2)	0.38
Pool	35 (4.4)	248 (30.9)	519 (64.7)	802	45 (3.6)	393 (31.2)	823 (65.3)	1261	0.66	1.23 (0.79-1.94)	0.36	0.98 (0.81-1.17)	0.79	0.96 (0.82-1.12)	0.59

*AA=MetMet, AG=MetVal and GG=ValVal
**A=Met, G=Val

Table SVI. Comparison of genotype and allele frequencies of the *BDNF* p.Val66Met polymorphism in 2247 unrelated controls and (a) 822 adult ADHD patients with mood disorders or (b) 580 adult ADHD patients without mood disorders from four European countries.

(a)

ADHD with mood disorders															
	Genotypes*													Alleles**	
	Cases n (%)				Controls n (%)					Genotype AA vs AG+GG		Genotype AG+AA vs GG		Allele A vs Allele G	
	AA	AG	GG	Sum	AA	AG	GG	Sum	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
Germany	11 (3.4)	92 (28.5)	220 (68.1)	323	39 (4.6)	284 (33.8)	517 (61.5)	840	0.10	0.74 (0.37-1.46)	0.37	1.33 (1.02-1.75)	0.03	1.28 (1.01-1.62)	0.03
Netherlands	2 (1.7)	37 (31.1)	80 (67.2)	119	12 (2.5)	152 (31.3)	322 (66.3)	486	0.86	0.67 (0.15-3.05)	0.59	1.04 (0.68-1.61)	0.83	1.06 (0.73-1.55)	0.74
Norway	17 (5.7)	86 (28.8)	196 (65.6)	299	17 (3.4)	155 (31.2)	324 (65.3)	496	0.27	1.70 (0.86-3.39)	0.13	1.01 (0.74-1.36)	0.93	0.94 (0.73-1.21)	0.62
Spain	5 (6.2)	29 (35.8)	47 (58.0)	81	18 (4.2)	141 (33.2)	266 (62.6)	425	0.63	1.46 (0.53-4.047)	0.47	0.81 (0.5-1.33)	0.41	0.82 (0.55-1.23)	0.34
Pool	35 (4.3)	244 (29.7)	543 (66.1)	822	86 (3.8)	732 (32.6)	1429 (63.6)	2247	0.27	1.13 (0.75-1.68)	0.56	1.11 (0.94-1.31)	0.19	1.07 (0.93-1.23)	0.37

(b)

ADHD without mood disorders															
	Genotypes*													Alleles**	
	Cases n (%)				Controls n (%)					Genotype AA vs AG+GG		Genotype AG+AA vs GG		Allele A vs Allele G	
	AA	AG	GG	Sum	AA	AG	GG	Sum	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
Germany	12 (5.0)	78 (32.4)	151 (62.7)	241	39 (4.6)	284 (33.8)	517 (61.5)	840	0.91	1.07 (0.55-2.07)	0.84	1.04 (0.77-1.40)	0.76	1.02 (0.8-1.31)	0.86
Netherlands	3 (4.2)	30 (41.7)	39 (54.2)	72	12 (2.5)	152 (31.3)	322 (66.3)	486	0.13	1.71 (0.47-6.23)	0.43	0.59 (0.36-0.99)	0.04	0.66 (0.44-1)	0.05
Norway	5 (3.6)	46 (33.6)	86 (62.8)	137	17 (3.4)	155 (31.2)	324 (65.3)	496	0.84	1.05 (0.38-2.89)	0.93	0.89 (0.60-1.31)	0.56	0.92 (0.66-1.28)	0.60
Spain	5 (3. 8)	50 (38.5)	75 (57.7)	130	18 (4.2)	141 (33.2)	266 (62.6)	425	0.55	0.90 (0.33-2.48)	0.84	0.81 (0.54-1.21)	0.32	0.88 (0.63-1.23)	0.45
Pool	25 (4.3)	204 (35.2)	351 (60.5)	580	86 (3.8)	732 (32.6)	1429 (63.6)	2247	0.37	1.12 (0.71-1.76)	0.63	0.87 (0.72-1.05)	0.16	0.9 (0.77-1.05)	0.18

*AA=MetMet, AG=MetVal and GG=ValVal
**A=Met, G=Val

CAPÍTOL 2: TDAH i transportador de serotonina (*SLC6A4/5HTT)*

Article 2

Estudi d'associació multicèntric internacional del gen del transportador de serotonina en TDAH persistent

RESUM

Antecedents i propòsit: Diversos estudis a nivell bioquímic, farmacològic o molecular i alguns models animals recolzen la participació del sistema serotoninèrgic en l'etioliogia del TDAH. El polimorfisme funcional 5-HTTLPR, localitzat al promotor del gen que codifica el recaptador o transportador de serotonina *SLC6A4*/*5HTT*, s'ha relacionat prèviament amb diversos trastorns psiquiàtrics. Estudis funcionals demostren que l'al·lel S d'aquest polimorfisme redueix l'eficiència trasncripcional del gen. Els estudis d'associació prèviament publicats en què s'avaluava la possible participació del transportador de serotonina en la patologia del TDAH presenten resultats controvertits. L'objectiu d'aquest treball ha estat avaluar el paper de vuit polimorfismes del gen *SLC6A4* en pacients TDAH adults.
Mètodes: Estudi d'associació cas-control considerant vuit polimorfismes del gen *SCL6A4* (el polimorfisme funcional 5-HTTLPR a la regió promotora del gen i 7 SNPs) en una cohort de 448 pacients adults amb TDAH i 580 controls de Noruega, posterior rèplica mitjançant un estudi multicèntric en 1454 pacients i 1302 controls d'Alemanya, Espanya, Holanda i USA, i seqüenciació de les regions exòniques del gen en una mostra de 93 pacients amb TDAH.
Resultats: Es va identificar associació entre TDAH a l'edat adulta i el polimorfisme rs140700 (OR = 0,67; P = 0,01), principalment en dones (P = 0,00084). Es va observar també una sobrerepresentació propera a la significació de l'al·lel S del polimorfisme 5-HTTLPR en el grup de casos (OR = 1,19; P = 0,06) a la població de Noruega. Aquests resultats no es van replicar a l'estudi de meta-anàlisi. La seqüenciació de les regions codificants del gen en 93 pacients TDAH noruecs va permetre identificar un polimorfisme no descrit anteriorment (c.924T>C), 2 SNPs rars ja descrits (rs6352-p.Gly56Ala i rs6355-p.Lys605Asn), presents sempre en heterozigosi a la població estudiada i, finalment, un SNP comú a la regió 3'UTR representat a la població en estudi amb una MAF del 48%.
Conclusions: Aquest estudi no recolza la participació dels polimorfismes del gen *SLC6A4* analitzats en el risc de patir TDAH a l'edat adulta a la població estudiada.

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An international multicenter association study of the serotonin transporter gene in persistent ADHD

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Attention deficit hyperactivity disorder (ADHD) is a common behavioral disorder affecting children and adults. It has been suggested that gene variants related to serotonin neurotransmission are associated with ADHD. We tested the functional promoter polymorphism 5-HTTLPR and seven single nucleotide polymorphisms in *SLC6A4* for association with ADHD in 448 adult ADHD patients and 580 controls from Norway. Replication attempts were performed in a sample of 1454 Caucasian adult ADHD patients and 1302 controls from Germany, Spain, the Netherlands and USA, and a meta-analysis was performed also including a previously published adult ADHD study. We found an association between ADHD and rs140700 [odds ratio(OR) = 0.67; *P* = 0.01] and the short (S) allele of the 5-HTTLPR (OR = 1.19; *P* = 0.06) in the Norwegian sample. Analysis of a possible gender effect suggested that the association might be restricted to females (rs140700: OR = 0.45; *P* = 0.00084). However, the meta-analysis of 1894 cases and 1878 controls could not confirm the association for rs140700 [OR = 0.85, 95% confidence interval (CI) = 0.67–1.09; *P* = 0.20]. For 5-HTTLPR, five of six samples showed a slight overrepresentation of the S allele in patients, but meta-analysis refuted a strong effect (OR = 1.10, 95% CI = 1.00–1.21; *P* = 0.06). Neither marker showed any evidence of differential effects for ADHD subtype, gender or symptoms of depression/anxiety. In conclusion, our results do not support a major role for *SLC6A4* common variants in persistent ADHD, although a modest effect of the 5-HTTLPR and a role for rare variants cannot be excluded.

Keywords: Adult attention deficit hyperactivity disorder, comorbidity, depression, gender; *5-HTT*, 5-HTTLPR, serotonin, *SERT*, *SLC6A4*

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Attention deficit hyperactivity disorder (ADHD) is a common neurodevelopmental disorder. ADHD was initially considered a childhood condition, but recent studies have shown that symptoms often persist into adulthood (Faraone *et al*. 2006). Many genetic studies have focused on genes related to the dopaminergic system, principally because stimulant drugs, including methylphenidate and amphetamine, inhibit the dopamine (and norepinephrine) transporters. However, in mice lacking the dopamine transporter gene (*Dat1*) and exhibiting extreme hyperlocomotion (Giros *et al*. 1996), a calming effect of psychostimulants was still observed, which was not accompanied by any change in the dopamine level. It was therefore concluded that this effect was dependent on serotonergic neurotransmission (Gainetdinov *et al*. 1999), which was underscored by the calming effect of serotonin reuptake inhibitors in these animals. The serotonergic neurotransmission system is also considered a candidate for ADHD by its known influence on behavioral traits,

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such as aggression and impulsivity (Halperin *et al*. 1994; Lucki 1998), and by its role in brain development (Azmitia 2001).

The main regulator of synaptic serotonin concentration is the serotonin transporter, encoded by the *SLC6A4* gene (also known as *5-HTT* and *SERT*), mapped to human chromosome 17q11.1–q12 (Ramamoorthy *et al*. 1993). The most widely studied polymorphism in this gene is an insertion/deletion in the promoter region, the 5-HTTLPR. Functional studies on this polymorphism have demonstrated that the deletion [short (S)] variant reduces transcriptional efficiency of the gene (Lesch *et al*. 1996). *SLC6A4* has been implicated in a wide range of disorders with the shared feature of emotional dysregulation, such as depression and anxiety disorder (Murphy & Lesch 2008). It has been suggested that the long (L) allele is a risk variant for developing ADHD (Beitchman *et al*. 2003; Curran *et al*. 2005; Kent *et al*. 2002; Manor *et al*. 2001; Seeger *et al*. 2001; Zoroglu *et al*. 2002), although subsequent studies have been inconsistent (Brookes *et al*. 2006; Grevet *et al*. 2007; Heiser *et al*. 2006; Langley *et al*. 2003; Oades *et al*. 2008; Wigg *et al*. 2006; Xu *et al*. 2005), as shown in recent meta-analyses (Forero *et al*. 2009; Gizer *et al*. 2009).

The aim of this study was to examine the putative association between adult ADHD and variants in the *SLC6A4* gene region. Because it has been suggested that the 5-HTTLPR is associated with regulations of emotions, we also wanted to test if the S allele implicated in susceptibility to depression was differently associated in the group of ADHD patients who reported that they had experienced significant depression and anxiety. We first genotyped the 5-HTTLPR polymorphism and seven single nucleotide polymorphisms (SNPs) that tagged all common variants in the *SLC6A4* gene region in 448 clinically diagnosed adult Norwegian ADHD patients and 580 ethnically matched controls. We followed up the results by genotyping the two markers showing strongest association in an additional 1454 adult ADHD patients and 1302 controls from four populations from IMpACT, the International Multicentre persistent ADHD CollaboraTion. This co-operation was initiated in 2007 with the goal of promoting research on the genetics of adult ADHD and currently consists of research groups from Germany, Spain, the Netherlands, UK, USA and Norway. Additionally, we sequenced all coding exons in a subgroup of 93 Norwegian patients to search for possible coding variants with stronger effect.

Materials and methods

Subjects

The Norwegian sample consists of 448 Caucasians of Norwegian ancestry (237 males and 211 females) of more than 18 years of age, diagnosed with ADHD or hyperkinetic disorder using either the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) or International Statistical Classification of Diseases and Related Health Problems, 10th Revision (ICD-10) protocols. The majority was recruited by responding to invitation sent to their addresses, based on a Norwegian national registry of adult ADHD patients. The remaining patients were directly recruited from psychiatrists or outpatient clinics (Johansson *et al*. 2008). All patients provided written informed consent and filled in a questionnaire including the 18-item World Health Organization's Adult ADHD Self Report Scale (ASRS) (Kessler *et al*. 2005). ADHD combined, inattentive or impulsive/hyperactive subtypes were assessed using the ASRS with a cutoff of 17 or more on each subscale. Ten percent of the patients were defined as subthreshold and were excluded from subtype-specific analyses (Halleland *et al*. 2009). Depression and anxiety was extracted from self-reporting questionnaire data according to the following question: 'Have you experienced episodes of significant depression or anxiety?'. Patients with mental retardation were excluded from analyses.

The control group of 580 Norwegian adults (257 males and 323 females) consisted of 195 healthy blood donors (for whom only gender was known) and a random sample of 385 healthy volunteers (aged 18–40 years) recruited from all over Norway for the purpose of this study, described in more detail in Halmøy *et al*. (2010). No exclusion criteria were applied for the random controls.

Replication sample

The replication samples obtained through the IMpACT consortium included a total of 1454 cases and 1302 controls from Spain, Germany, the Netherlands and USA (Table 1). ADHD was diagnosed in accordance with the DSM-IV criteria; onset before the age of 7, lifelong persistence and current diagnosis. The depression and anxiety status was assessed by a psychiatrist (DSM-IV criteria) in the samples from the Netherlands and by the Structured Clinical Interview for DSM Disorders-1 in the German, Spanish and American samples. For more detailed description of the procedures and instruments used, please refer to the following references: Bekker *et al*. (2005), Franke *et al*. (2008), Jacob *et al*. (2007), Johansson *et al*. (2008), Kooij *et al*. (2005), Ramos-Quiroga *et al*. (2008) and Sanchez-Mora *et al*. (2009).

Genotyping

Norwegian sample

DNA was extracted from whole blood or saliva using the Oragene™ DNA Self-Collection Kit (DNA Genotek Inc., Ontario, Canada) and aliquoted into 96-well plates. Each plate contained DNA from both cases and controls and a minimum of two internal controls and two blank samples. The *SLC6A4* gene was tagged with seven SNPs based on HapMap build 35 (HAPLOVIEW software; Barrett *et al*. 2005), with minor allele frequency (MAF) threshold set to 5% and $r^2>0.8$ (pairwise tagging only). The SNPs were genotyped using the MassARRAY iPLEX System

Table 1: Characteristics of the samples studied

		Norway	Germany	The Netherlands	Spain	USA	Sum
Controls	Total (% males)	580 (44)	393 (50)	490 (49)	312 (65)	107 (43)	1882
Cases	Total (% males)	448 (53)	589 (53)	246 (49)	299 (72)	320 (41)	1902
Depression and/or anxiety	Total (% patients)	300 (67)	309 (79)*	164 (67)	150 (50)	168 (53)	1091
ADHD subtypes	Combined (%)	329 (73)	389 (66)	199 (81)	194 (65)	221 (69)	1332
	Inattentive (%)	46 (10)	146 (25)	22 (8.9)	88 (29)	89 (28)	391
	Hyperactive (%)	15 (3.3)	47 (8.0)	8 (3.3)	12 (4.0)	10 (3.1)	92

*Depression only, anxiety not specified.

(Sequenom, San Diego, CA, USA). The promoter insertion/deletion polymorphism, 5-HTTLPR, was amplified by the polymerase chain reaction (PCR) and genotyped by fragment analysis on the ABI3100 (Applied Biosystems, Foster City, CA, USA) using fluorescently labeled reverse primers (forward: 5′-GGCGTTGCCGCTCTGAATGC-3′; reverse: 5′-GAGGGACTGAGCTGGACAACCAC-3′) (Heils *et al*. 1996). The genotypes were automatically called using the GeneMapper software (Applied Biosystems), and they were subsequently manually inspected. Protocols for amplifications and fragment analysis are available upon request.

Replication samples from Spain, Germany, the Netherlands and USA

Genomic DNA was extracted from whole blood using the salting out method or from saliva using the Oragene™ DNA Self-Collection Kit (DNA Genotek Inc.). The 5-HTTLPR polymorphism was genotyped from each DNA sample using PCR. Amplification was performed with the primers according to the protocol described above and DNA products were resolved in 2% agarose gels. The SNP rs140700 was either genotyped in Norway (samples from Norway, Spain and the Netherlands) or in Germany (German samples) using the MassARRAY iPLEX System as described above. Genotyping for the replication samples from USA was conducted at the Psychiatric and Neurodevelopmental Genetics Unit of the Massachusetts General Hospital using a single base extension reaction with allele discrimination by MassARRAY mass spectrometry system (Sequenom, San Diego, CA, USA).

Statistical analyses

The statistical analyses of dichotomous traits were performed with the plink software (Purcell *et al*. 2007). All analyses were based on an additive allelic model. Stratification based on gender was achieved by subdividing the data in either male-only or female-only data files. Genotype distributions for all markers were consistent with Hardy–Weinberg Equilibrium, $P \geq 0.01$ for all countries. For the MassARRAY iPLEX analysis, 11 individuals were excluded because of low genotyping efficiency (missingness >0.3). Genotyping concordance was 100% ($n = 206$ comparisons) for this analysis, and the final genotyping call rate was >0.994. Analyses and visualization of linkage disequilibrium (LD) was performed with the haploview software (http://www.broadinstitute.org/haploview/haploview). The meta-analyses presented were performed with a random effects model (stata 8.2). Similar results were also obtained using the PLINK meta-analysis option (data not shown). For rs140700, the meta-analyses included Norwegian, German, Dutch, Spanish and American adult ADHD patients and controls from IMpACT. In the case of 5-HTTLPR, data from a published study on 312 Brazilian adult ADHD patients and 236 controls were also included (Grevet *et al*. 2007). Power calculations in the total sample were performed using the genetic power calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/cc2.html): Assuming an additive allelic model and using a significance level of 0.00625 (correction for eight markers tested), we had ~83% power to detect an effect at odds ratio(OR) = 0.75 for a disease allele frequency of 11% and 88% power to detect OR = 1.20 at a disease allele frequency of 40%.

All ORs estimated are presented for the minor allele. We termed $P < 0.05$ as nominally significant. All P values are presented without correction for multiple testing.

Sequencing

Ninety-three individuals from the Norwegian patient group were sequenced for the 13 protein-coding exons of *SLC6A4* (exon 3–15, NM_001045), including exon–intron boundaries, as well as the 3′UTR, all following a standard Sanger sequencing method. Primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3), and the sequence analysis was performed on an ABI3730 DNA Analyzer (Applied Biosystems). All sequences were manually inspected using the SeqScape software (Applied Biosystems).

Results

Single-SNP analysis in the Norwegian sample

Table 1 shows the demographics for the 448 cases and 580 controls of the Norwegian sample, together with those of the replication samples from Germany, the Netherlands, Spain and USA.

Figure 1 shows the pairwise LD structure of the *SLC6A4* gene with the 5-HTTLPR and the seven tag SNPs in the Norwegian individuals. LD is low between the promoter region (rs16965628 and 5-HTTLPR) and SNPs in the core gene region. No SNP, or two-marker haplotype combination, could efficiently tag the 5-HTTLPR in the Norwegian data set.

The comparison of allele frequencies between Norwegian cases and controls showed an association between rs140700 and ADHD [OR = 0.67; 95% confidence interval (CI) = 0.50–0.91; $P = 0.01$] and a trend for overrepresentation of the 5-HTTLPR S allele in the ADHD patients (OR = 1.19; 95% CI = 0.99–1.42; $P = 0.06$) (Table 2). The stratification analysis by gender suggested that this association was mainly restricted to females (Table 3). Thus, four markers were associated with ADHD in females in the stratified analysis; rs4583306, rs140700, rs8076005 and 5-HTTLPR (strongest association for rs140700, $P = 0.00084$).

Replication attempts and meta-analyses

We next genotyped the 5-HTTLPR and rs140700 markers in four additional case–control samples of European descent from IMpACT. Random effect meta-analyses were performed as shown in Fig. 2. The analysis for the 5-HTTLPR also includes data from the only previously published study on this polymorphism in adult Brazilian ADHD patients (Grevet *et al*. 2007). There was a trend for an association between the 5-HTTLPR S allele and ADHD, which did not reach statistical significance: $P = 0.060$ and OR = 1.10 (95% CI = 1.00–1.21). All case samples (except for the German sample) had similar effect sizes and no heterogeneity was detected among them ($P = 0.79$). The meta-analysis of rs140700 did not support the results observed in the Norwegian sample (OR = 0.85; 95% CI = 0.67–1.09; $P = 0.20$). The results for the population-specific allelic association tests can be found in Tables S1 and S2. Allele frequencies were very similar in all populations apart from the slightly higher frequency of the 5-HTTLPR S allele found in the Spanish population. Stratification by gender or ADHD subtypes did not affect the results (Tables S3 and S4).

Coexisting symptoms of depression/anxiety

As shown in Table 1, 67% of the Norwegian patients reported having experienced 'significant depression and/or anxiety'. In the other populations, the fractions of patients who had suffered from one or both of these conditions varied between 50 and 79%. Because the 5-HTTLPR S allele has been implicated in susceptibility to these psychiatric disorders, we next restricted the analysis to the group of ADHD patients with symptoms of depression/anxiety. However, the strength of association did not increase for any of the eight markers tested in the Norwegian sample (data not shown),

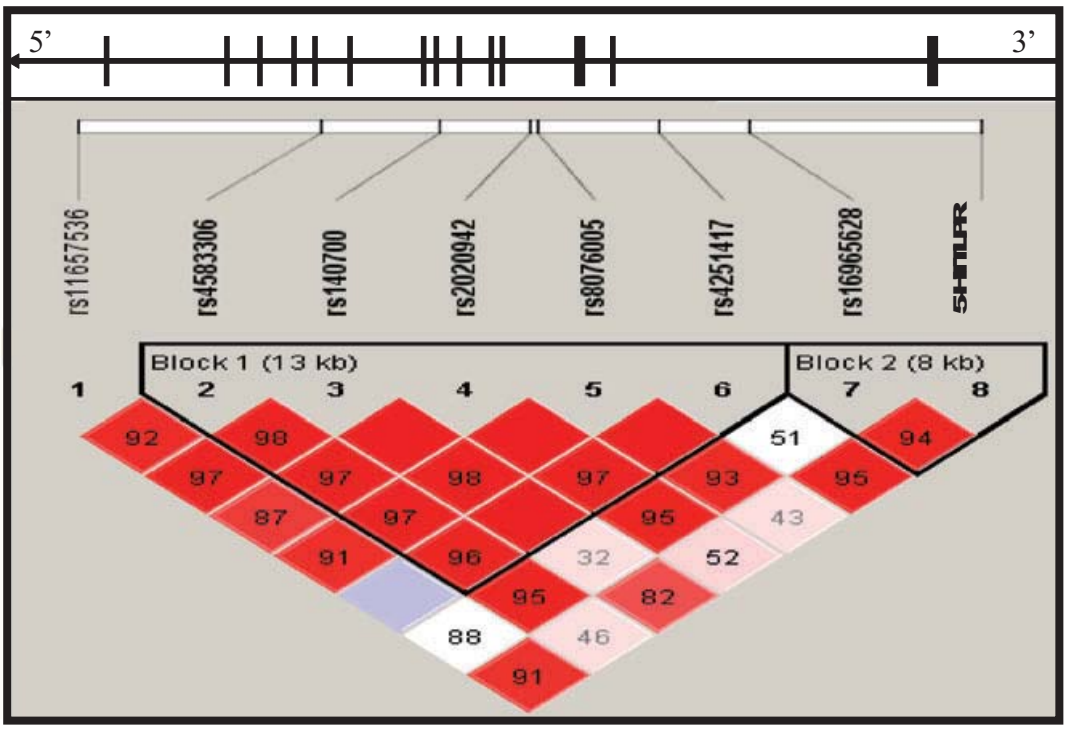

Figure 1: Upper part: SLC6A4, running 3′ to 5′, with exons (black boxes) and introns. Lower part: markers tested and pairwise LD (D′) between them. Haplotype blocks as defined by the method of Gabriel *et al*. (2002) are indicated.

Table 2: Individual markers, minor allele frequencies, *P* values and ORs for the comparison of allele frequencies between Norwegian ADHD cases and controls

Marker	Minor/major allele	MAF cases	MAF controls	*P*	OR (95% CI)
rs11657536	A/G	0.02	0.03	0.30	0.74 (0.42–1.31)
rs4583306	G/A	0.45	0.42	0.12	1.15 (0.97–1.37)
rs140700	A/G	0.08	0.11	0.011	0.67 (0.50–0.91)
rs2020942	A/G	0.37	0.38	0.43	0.93 (0.78–1.11)
rs8076005	G/A	0.18	0.20	0.27	0.88 (0.70–1.10)
rs4251417	A/G	0.10	0.11	0.30	0.86 (0.65–1.14)
rs16965628	G/C	0.08	0.08	0.86	0.97 (0.70–1.35)
5-HTTLPR	S/L	0.44	0.40	0.061	1.19 (0.99–1.42)

Table 3: Individual markers, minor allele frequencies, *P* values and ORs in females and males of the Norwegian sample

		Females				Males			
		MAF				MAF			
Marker	Minor/major allele	Cases	Controls	*P*	OR (95% CI)	Cases	Controls	P	OR (95% CI)
rs11657536	A/G	0.02	0.03	0.085	0.48 (0.20–1.13)	0.03	0.02	0.67	1.20 (0.52–2.74)
rs4583306	G/A	0.46	0.40	0.047	1.29 (1.00–1.65)	0.45	0.44	0.90	1.02 (0.79–1.31)
rs140700	A/G	0.05	0.11	0.00084	0.45 (0.27–0.72)	0.10	0.11	0.68	0.92 (0.60–1.39)
rs2020942	A/G	0.38	0.39	0.61	0.94 (0.73–1.21)	0.36	0.37	0.63	0.94 (0.72–1.22)
rs8076005	G/A	0.15	0.21	0.029	0.69 (0.50–0.96)	0.20	0.19	0.59	1.09 (0.80–1.50)
rs4251417	A/G	0.10	0.12	0.55	0.89 (0.60–1.31)	0.10	0.11	0.42	0.85 (0.56–1.27)
rs16965628	G/C	0.07	0.08	0.45	0.83 (0.52–1.34)	0.09	0.08	0.68	1.10 (0.70–1.74)
5-HTTLPR	S/L	0.44	0.37	0.016	1.36 (1.06–1.75)	0.44	0.44	0.98	1.00 (0.78–1.29)

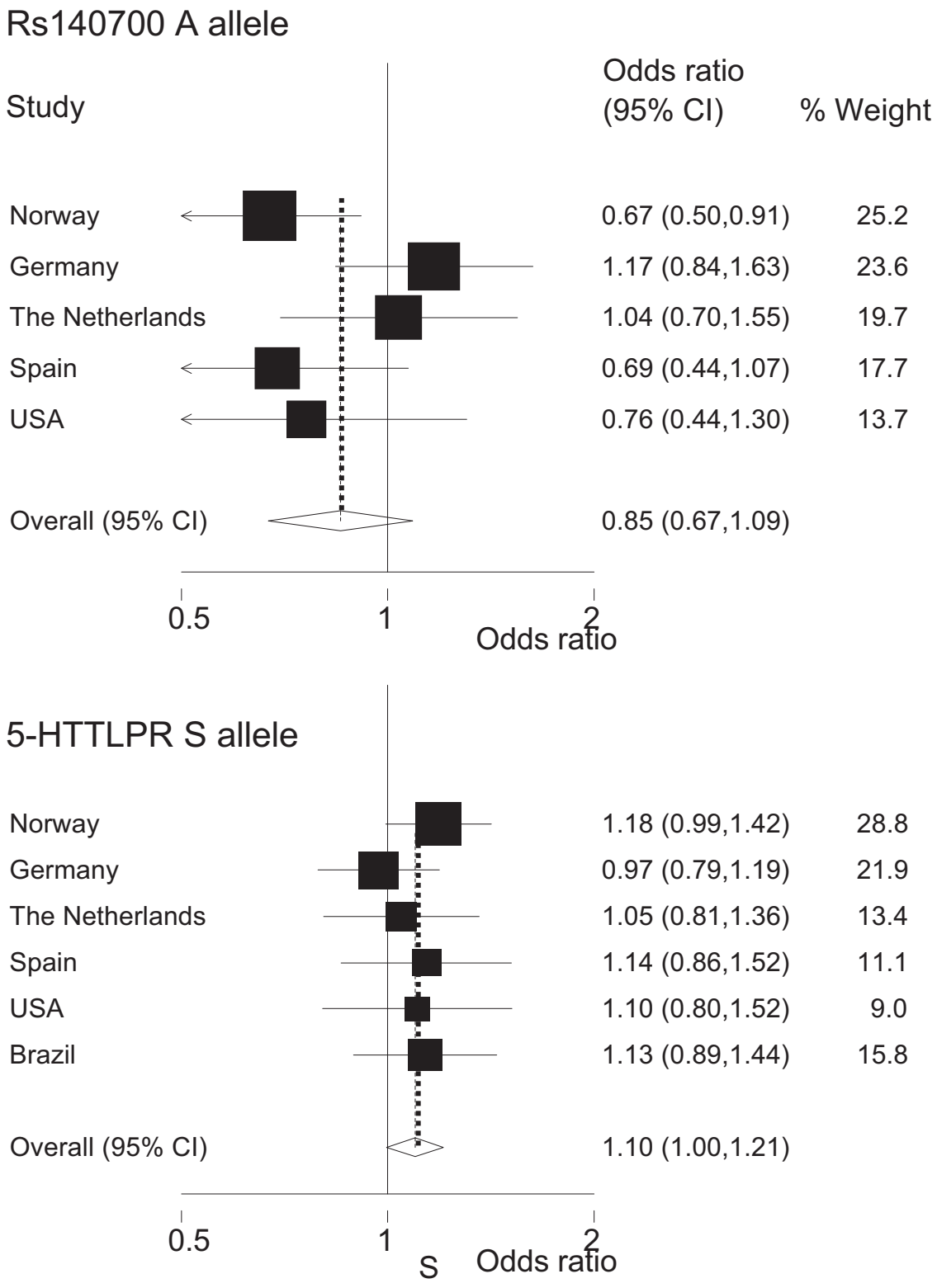

Figure 2: Meta-analysis of rs140700 in 1894 adult ADHD patients and 1878 controls (upper panel) and 5-HTTLPR in 1977 adult ADHD patients and 1650 controls (lower panel).

neither when all patients from the five IMpACT nodes were analyzed together (rs140700: $P = 0.08$; 5-HTTLPR: $P = 0.10$) (Fig. 3) nor after gender stratification (data not shown).

SLC6A4 sequencing for rare variants

Sequencing of all coding exons in 93 Norwegian patient samples revealed one silent mutation (c.924T>C) not previously described and two rare but already identified nonsynonymous variants (rs6352: p.Gly56Ala and rs6355: p.Lys605Asn), all present in the heterozygous state. Additionally, the common SNP rs1042173, located in the 3′UTR, was detected at a MAF of 48% in the sequenced samples (Table S5).

Discussion

The principal aim of this study was to investigate the possible association between adult ADHD and *SLC6A4* using a Norwegian sample for an exploratory analysis and replication in four other populations. We first genotyped seven tag SNPs and the promoter insertion/deletion 5-HTTLPR polymorphism in the Norwegian sample and found that the rare allele of rs140700 was associated with lower risk of ADHD (OR = 0.67; 95% CI = 0.50–0.91; $P = 0.01$). However, we were not able to replicate this finding in the other cohorts. We also found a trend toward an overrepresentation of the 5-HTTLPR S allele among the Norwegian cases (OR = 1.19; 95% CI = 0.99–1.42; $P = 0.06$). Replication attempts showed that the frequency of the S allele was slightly increased in cases as compared with controls in five of six populations (1977 patients and 1650 controls), including the previously reported Brazilian sample. Although results from the pooled analysis were not statistically significant (OR = 1.10; 95% CI = 1.00–1.21; $P = 0.06$), it is not possible to refute an association between the 5-HTTLPR S allele and persistent ADHD at the current time.

Contrary to our results, most of the initial studies on children with ADHD suggested that the long allele of 5-HTTLPR was associated with ADHD risk (Beitchman *et al*. 2003; Curran *et al*. 2005; Kent *et al*. 2002; Manor *et al*. 2001; Seeger *et al*. 2001; Zoroglu *et al*. 2002). These earliest studies looked at relatively small groups of patients (number of cases varied between 41 and 240). However, Faraone *et al*. (2005) used a meta-analysis approach to merge the results and found a pooled OR of 1.31 (95% CI = 1.09–1.59) for the L allele in childhood ADHD. More recently although, several studies have failed to find significant associations between ADHD and *SLC6A4*. Notably, the IMAGE multicenter study showed only a small and nonsignificant overtransmission of the L allele in 1020 families with 1166 ADHD cases (mainly combined subtype) (Xu *et al*. 2008). Furthermore, two very recent meta-analyses combining nearly fully overlapping sets of studies (Forero *et al*. 2009; Gizer *et al*. 2009) reached slightly different conclusions; only Gizer *et al*. were able to find a nominally significant effect ($P = 0.01$) for the L allele. Hence, considering the lack of consistent findings in childhood ADHD and taking into account the results from the present study of adult ADHD, it seems likely that the *SLC6A4* region does not harbor common susceptibility variants with a major effect on ADHD across the life span. However, we cannot rule out that the 5-HTTLPR or another variant in LD with this marker might be associated with ADHD, but with an effect size considerably lower than previously estimated. Based on our results, it can be estimated that a sample of more than 5600 cases and 5600 controls would be needed to achieve a power of 80% to detect an OR of 1.1 at the $P = 0.006$ level (study-wide significance after Bonferroni correction for eight tested markers).

Furthermore, it is also possible that *SLC6A4* polymorphisms might be important in subgroups of ADHD patients and/or influence other psychiatric symptoms among some ADHD patients. The 5-HTTLPR variant has been implicated in a wide range of psychiatric disorders, such as depression, anxiety, autism, bipolar disorder and obsessive compulsive disorder (OCD). Many of these diagnoses are overrepresented among adult ADHD patients (Caspi *et al*. 2003; Hariri *et al*. 2002; Sen *et al*. 2004). For example, as many as 67% of the Norwegian patients reported that they had experienced episodes of significant depression and/or anxiety. Similar numbers were also found using structured interviews in the other populations included in this study (range: 50–79%). We therefore tested the hypothesis that this subgroup of patients would be more likely to show an association with the 5-HTTLPR S allele than the patients who had not experienced such symptoms. However, restricting the analyses exclusively to the patients who reported depression/anxiety did not change the results (Fig. 3), neither did we find any difference when comparing patients with and without these symptoms. Still, if the 5-HTTLPR S allele is in fact related to these symptoms which are very frequent among adults with ADHD in particular, it could be part of the explanation of the somewhat different findings in adult and childhood samples.

It has been proposed that there are gender-specific variations in different aspects of serotonergic neurotransmission, such as the rates of central nervous system (CNS) serotonin synthesis (Nishizawa *et al*. 1997) and the density of certain serotonin receptors in the CNS (Biver *et al*. 1996; Costes *et al*. 2005). Likewise, other reports have suggested that the effects of tryptophan depletion and serotonin reuptake inhibitors, as well as the association between 5-hydroxyindoleacetic acid levels in cerebrospinal fluid and 5-HTTLPR genotypes, are different between females and males (Kornstein *et al*. 2000; Williams *et al*. 2003). Voyiaziakis *et al*. (2009) recently reported an association between a *SLC6A4* polymorphism and OCD in females, and for ADHD it has been suggested that variants in *SLC6A4* and several other ADHD candidate genes show dimorphic patterns of association between genders (Biederman *et al*. 2008). However, males have been highly overrepresented in most genetic studies performed on childhood ADHD, prohibiting investigation of gender effects. The IMAGE study consisted of almost 90% boys (Xu *et al*. 2008), which is very different from the almost 1:1 ratio found in the clinical adult samples included in this current study. It was therefore interesting to note that the Norwegian data suggested a

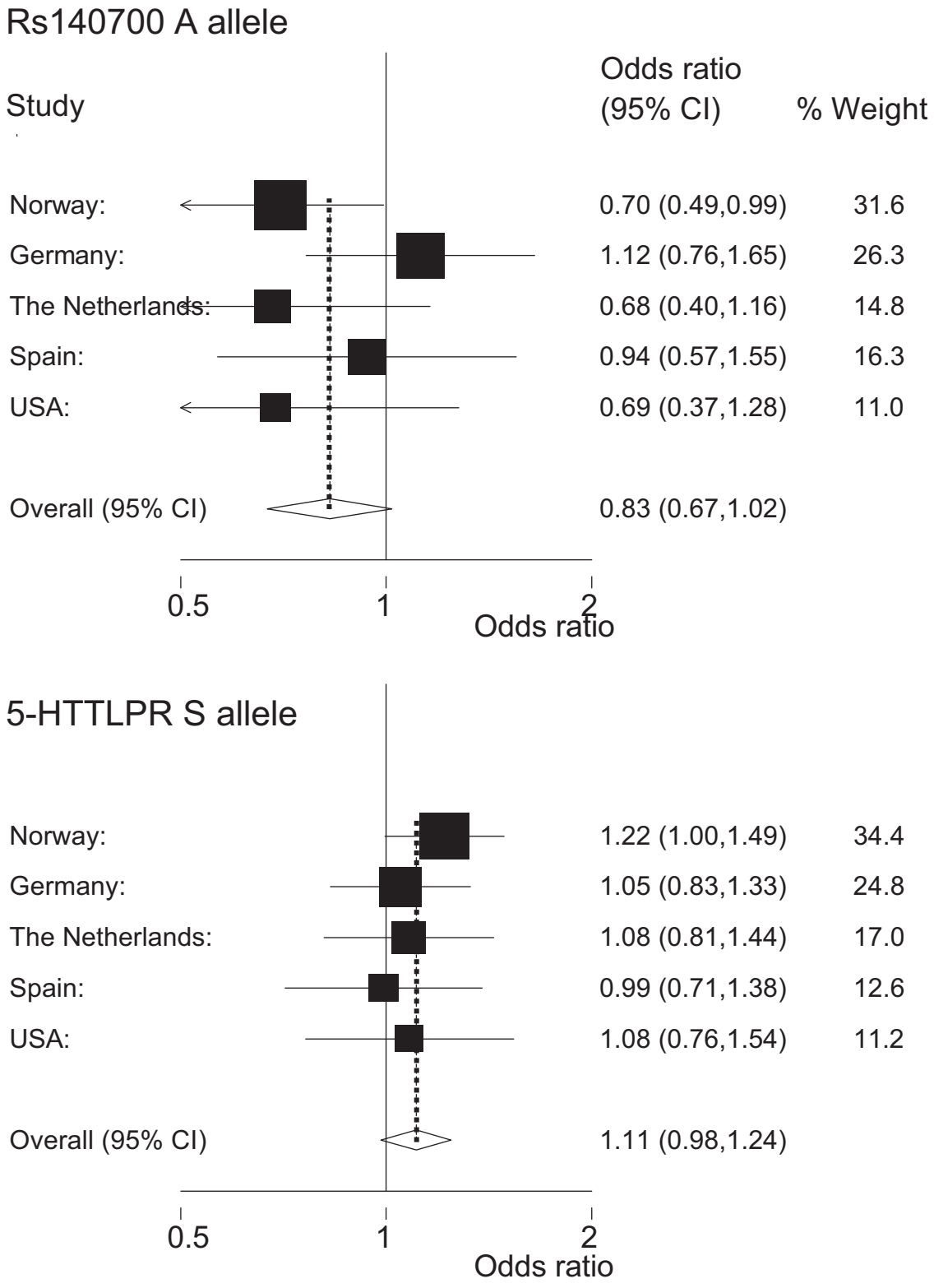

Figure 3: Meta-analysis of rs140700 and 5-HTTLPR restricted to ADHD patients with symptoms of depression/anxiety and controls.

serotonergic effect only in the women. This result was, however, not supported by the replication attempts. So while this illustrates that great care should be taken when analyzing multiple phenotypes, including possible gender effects (Ioannidis *et al*. 2009), it also emphasizes the importance of a more balanced gender recruitment into future studies of ADHD.

We cannot exclude that other factors, such as different recruitment strategies (childhood vs. adult samples), varying diagnostic traditions between countries or other sources of heterogeneity, might conceal a potential underlying genetic risk variant in the region and explain some of the inconsistent findings in the literature. However, the similar ADHD subtype distribution and occurrence of other psychiatric symptoms across IMpACT populations suggest that the total IMpACT sample used in the current study is rather homogeneous. Another limitation is that we have not excluded controls with ADHD symptoms in the Norwegian sample. However, the loss of power is relatively low if we assume that the prevalence of ADHD is no higher among our controls than the prevalence estimated in the general population.

The potential of gene–environment interactions has been much debated for the 5-HTTLPR. It has been suggested that carriers of the S allele exposed to traumatic life events exhibit more depressive symptoms (Caspi *et al*. 2003) and more commonly develop posttraumatic stress disorder (Xie *et al*. 2009) than individuals homozygous for the L allele. Considering ADHD, studies have found that the L allele has a protective effect on severity of the disorder for ADHD patients exposed to many adverse life events (Muller *et al*. 2008) and that the L allele reduce the patients' sensitivity to family environment (Sonuga-Barke *et al*. 2009). We can therefore not exclude the possibility of gene–environment interactions contributing to the etiology of ADHD in our populations. However, a very recent meta-analysis by Risch *et al*. (2009) pointed to the challenges associated with gene–interaction studies, and they were not able to detect any evidence of interaction between 5-HTTLPR genotype and stressful life events on depressive symptoms.

It has also been suggested that rare variants might contribute to common psychiatric traits (Dong *et al*. 2009; Elia *et al*. 2009; Walsh *et al*. 2008). We found nonsynonymous *SLC6A4* variants in 4 of 93 fully sequenced ADHD patients (4.3% carrier rate). These changes, although rare, have been previously described also in other populations and are probably unlikely to have strong impact on ADHD, and very large samples will be needed to test if any of these rare variants are involved in psychiatric disorders. Hence, until large-scale resequencing efforts have been performed (Manolio *et al*. 2009), it is not possible to exclude that rare *SLC6A4* coding variants might impact vulnerability toward psychiatric conditions, including ADHD.

In conclusion, our data show that there are no common variants within the *SLC6A4* gene region with a strong effect on adult ADHD across Caucasian populations. However, we cannot reject the possibility of the *SLC6A4* gene contributing to the disorder, for instance through a low effect size, through other psychiatric symptoms commonly associated with ADHD or by other rare variants.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1: The allelic distribution of 5-HTTLPR with *P* values and ORs in six adult ADHD populations.

Table S2: The allelic distribution of rs140700 with *P* values and ORs in five adult ADHD populations.

Table S3: 5-HTTLPR and rs140700 in all populations with gender stratification.

Table S4: 5-HTTLPR and rs140700 in cases with the respective subtypes vs. controls in four populations.

Table S5: SNPs found when sequencing the exons of SLC6A4 in DNA from 93 ADHD patients.

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Supplementary table 1 The allelic distribution of 5-HTTLPR with P-values and odds ratios in six adult ADHD populations

	N	Cases		Controls		S frequency			
	Cases/Controls	S	L	S	L	Cases	Controls	P value	OR (95% CI)
Norway	440/574	389	491	460	688	0.44	0.40	0.061	1.19 (0.99-1.42)
Germany	435/340	353	517	281	399	0.41	0.41	0.77	0.97 (0.79-1.19)
The Netherlands	217/248	186	248	207	289	0.43	0.42	0.73	1.05 (0.81-1.36)
Spain	255/149	263	247	144	154	0.52	0.48	0.37	1.14 (0.86-1.52)
USA	318/103	281	355	86	120	0.44	0.42	0.44	1.10 (0.81-1.61)
Brazil	312/236	297	327	210	262	0.45	0.44	0.31	1.13 (0.89-1.44)
Sum	1977/1650	1769	2185	1388	1912				

Supplementary table 2 The allelic distribution of rs140700 with P-values and odds ratios in five adult ADHD populations

	N	Cases		Controls		MAF			
	Cases/Controls	A	G	A	G	Cases	Controls	P value	OR (95% CI)
Norway	447/579	69	825	128	1030	0.08	0.11	0.011	0.67 (0.50-0.91)
Germany	589/392	104	1074	60	724	0.09	0.08	0.36	1.17 (0.84-1.63)
The Netherlands	245/488	40	450	77	899	0.08	0.08	0.86	1.04 (0.70-1.55)
Spain	299/312	36	562	53	571	0.06	0.08	0.10	0.69 (0.44-1.07)
USA	314/107	48	580	21	193	0.08	0.10	0.22	0.71 (0.40-1.23)
Sum	1894/1878	297	3491	339	3417				

Supplementary table 3 5-HTTLPR and rs140700 in all populations with gender stratification

	5-HTTLPR						rs140700					
	S allele frequency				N		A allele frequency				N	
	Cases	Controls	P	OR (95% CI)	Cases	Controls	Cases	Controls	P	OR (95% CI)	Cases	Controls
All individuals												
Norway	0.44	0.40	0.061	1.19 (0.99-1.42)	440	574	0.08	0.11	0.011	0.67 (0.50-0.91)	447	579
The Netherlands	0.43	0.42	0.73	1.05 (0.81-1.36)	217	248	0.08	0.08	0.86	1.04 (0.70-1.55)	245	488
Germany	0.41	0.41	0.77	0.97 (0.79-1.19)	435	340	0.09	0.08	0.36	1.17 (0.84-1.63)	589	392
Spain	0.52	0.48	0.37	1.14 (0.86-1.52)	255	149	0.06	0.08	0.10	0.69 (0.44-1.07)	299	312
USA	0.44	0.42	0.44	1.10 (0.81-1.61)	318	103	0.08	0.10	0.22	0.71 (0.4-1.23)	314	107
Females												
Norway	0.44	0.37	0.016	1.36 (1.06-1.75)	208	321	0.05	0.11	0.00084	0.45 (0.27-0.72)	211	323
The Netherlands	0.43	0.44	0.84	0.96 (0.67-1.39)	105	129	0.10	0.07	0.24	1.37 (0.81-2.34)	126	249
Germany	0.40	0.42	0.65	0.93 (0.70-1.25)	202	175	0.08	0.08	0.76	0.93 (0.58-1.49)	278	195
Spain	0.49	0.55	0.42	0.79 (0.44-1.41)	71	33	0.07	0.10	0.18	0.60 (0.28-1.27)	84	110
USA	0.40	0.43	0.82	0.94 (0.25-0.58)	131	58	0.08	0.11	0.16	0.58 (0.28-1.23)	129	61
Males												
Norway	0.44	0.44	0.98	1.00 (0.78-1.29)	232	253	0.10	0.11	0.68	0.92 (0.60-1.39)	236	256
The Netherlands	0.43	0.40	0.46	1.15 (0.79-1.67)	112	119	0.06	0.08	0.33	0.74 (0.40-1.36)	119	239
Germany	0.41	0.41	0.96	1.01 (0.76-1.34)	233	165	0.10	0.07	0.12	1.45 (0.90-2.33)	311	197
Spain	0.53	0.47	0.14	1.28 (0.92-1.78)	184	116	0.06	0.07	0.35	0.77 (0.44-1.33)	215	202
USA	0.47	0.40	0.28	1.31 (0.80-2.13)	187	45	0.07	0.09	0.95	0.97 (0.39-2.38)	185	46

Supplementary table 4 5-HTTLPR and rs140700 in cases with the respective subtypes versus controls in four populations

	5-HTTLPR						rs140700					
	S allele frequency						A allele frequency					
	Cases	Controls	P	OR	L95	U95	Cases	Controls	P	OR	L95	U95
Combined												
Norway	0.44	0.40	0.10	1.18	0.97	1.43	0.07	0.11	0.0035	0.59	0.42	0.84
Germany	0.41	0.41	0.82	0.97	0.78	1.22	0.08	0.08	0.80	1.05	0.73	1.50
The Netherlands	0.42	0.42	0.99	1.00	0.76	1.31	0.08	0.08	0.91	0.97	0.63	1.50
Spain	0.54	0.48	0.19	1.24	0.90	1.69	0.05	0.08	0.030	0.55	0.32	0.95
USA	0.44	0.42	0.57	1.10	0.78	1.58	0.09	0.10	0.53	0.83	0.47	1.48
Hyperactive												
Norway	0.47	0.40	0.47	1.31	0.63	2.71	0.07	0.11	0.45	0.57	0.14	2.44
Germany	0.44	0.41	0.71	1.09	0.68	1.75	0.11	0.08	0.38	1.37	0.68	2.76
The Netherlands	0.44	0.42	0.87	1.09	0.40	2.96	0.08	0.08	0.91	0.97	0.63	1.50
Spain	0.45	0.48	0.80	0.89	0.37	2.13	0.00	0.08	0.14	-	-	-
USA	0.25	0.42	0.24	0.50	0.16	1.59	0.00	0.10	0.99	-	-	-
Inattentive												
Norway	0.43	0.40	0.54	1.14	0.74	1.76	0.12	0.11	0.79	1.09	0.57	2.11
Germany	0.39	0.41	0.57	0.92	0.68	1.24	0.09	0.08	0.63	1.12	0.70	1.81
The Netherlands	0.53	0.42	0.20	1.56	0.79	3.08	0.12	0.08	0.36	1.56	0.60	4.08
Spain	0.48	0.48	0.96	0.99	0.67	1.46	0.09	0.08	0.80	1.08	0.60	1.94
USA	0.48	0.42	0.17	1.39	0.87	2.21	0.05	0.10	0.09	0.46	0.19	1.11

Supplementary table 5 SNPs found when sequencing the exons of SLC6A4 in DNA from 93 ADHD patients

Change	SNP Name	Position	MAF (n)	MAF in HapMap's CEU population	Amino Acid change
c.167G>C	rs6355	Exon 3	0.01 (2)	0.025[a]	p.Gly56Ala
c.924T>C	-	Exon 5	0.01 (2)	-	p.Gly308Gly
c.1815A>C	rs6352	Exon 14	0.01 (2)	0.00[b]	p.Lys605Asn
	rs1042173	3' UTR	0.48 (90)	0.43[c]	-

[a]HCB & JPT: 1.0%, [b]HCB & JPT: 7-8%, [c]HCB & JPT: ~80%

CAPÍTOL 3a: TDAH i sistema dopaminèrgic

Article 3

Anàlisi de sistemes candidats en TDAH: l'avaluació de 9 genes implicats en la neurotransmissió dopaminèrgica identifica associació amb *DRD1*

RESUM

Antecedents i propòsit: Diversos estudis farmacològics i genètics recolzen la participació del sistema de neurotransmissió dopaminèrgic en l'etiologia del TDAH. En base a aquesta informació es va avaluar la possible contribució al TDAH de nou gens implicats en la neurotransmissió dopaminèrgica (*DRD1, DRD2, DRD3, DRD4, DRD5, SLC6A3/DAT1, TH, DBH* i *COMT*).

Mètodes: Estudi d'associació cas-control poblacional i familiar considerant 61 SNPs seguint criteris de màxima cobertura genètica segons els patrons de desequilibri de lligament de cada gen en una mostra de 533 pacients amb TDAH (322 nens i 211 adults) i 533 controls aparellats per sexe i 196 famílies amb TDAH d'origen espanyol. Posterior rèplica en una mostra independent de 353 famílies d'origen alemany.

Resultats: Les anàlisis de marcadors individuals i d'haplotips a la mostra cas-control i a la mostra familiar proporcionen evidències de la contribució del gen *DRD1* que codifica el receptor dopaminèrgic D1 en el TDAH de tipus combinat a la població infantil (anàlisi d'haplotips cas-control: p = 8,8e-04, OR = 1,50 (1,18 -1,90); anàlisi d'haplotips familiar: p = 0,0061, OR = 1,73 (1,23-2,45)) però no a l'adulta. L' estudi de rèplica posterior realitzat en una mostra independent de 353 famílies alemanyes amb com a mínim un fill amb TDAH de tipus combinat recolza l'associació entre *DRD1* i TDAH combinat a la infància (p = 8,4e-05, OR = 3,67 (2,04-6,63)).

Conclusions: La rèplica de l'associació entre *DRD1* i TDAH combinat a les dues cohorts estudiades reforça la validesa dels resultats i dóna suport a la participació de *DRD1* en el TDAH infantil.

REFERÈNCIA

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Candidate system analysis in adhd: evaluation of 9 genes involved in dopaminergic neurotransmission identifies association with DRD1.

The World Journal of Biological Psychiatry (en premsa)

CANDIDATE SYSTEM ANALYSIS IN ADHD: EVALUATION OF 9 GENES INVOLVED IN DOPAMINERGIC NEUROTRANSMISSION IDENTIFIES ASSOCIATION WITH *DRD1*

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Running head: The Dopaminergic System and ADHD: Association Study

Keywords: Genetics, biological psychiatry, childhood ADHD, DRD1, association study

ABSTRACT

Objectives: Several pharmacological and genetic studies support the involvement of the dopamine neurotransmitter system in the etiology of attention-deficit hyperactivity disorder (ADHD). Based on this information we evaluated the contribution to ADHD of 9 genes involved in dopaminergic neurotransmission (*DRD1, DRD2, DRD3, DRD4, DRD5, DAT1, TH, DBH* and *COMT*). *Methods:* We genotyped a total of 61 tagging single nucleotide polymorphisms (SNPs) in in a sample of 533 ADHD patients (322 children and 211 adults), 533 sex-matched unrelated controls and additional 196 nuclear ADHD families from Spain. *Results:* The single- and multiple-marker analysis in both population and family-based approaches provided preliminary evidence for the contribution of *DRD1* to combined-type ADHD in children (P=8.8e-04; OR=1.50 (1.18-1.90) and P=0.0061; OR=1.73 (1.23-2.45)) but not in adults. Subsequently, we tested positive results for replication in an independent sample of 353 German families with combined-type ADHD children and replicated the initial association between *DRD1* and childhood ADHD (P=8.4e-05; OR=3.67 (2.04-6.63)). *Conclusions:* The replication of the association between *DRD1* and ADHD in two European cohorts highlights the validity of our finding and supports the involvement of *DRD1* in childhood ADHD.

INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by persistent and pervasive symptoms of hyperactivity, inattention and increased impulsivity that affects 5% to 6% of children (Polanczyk et al. 2007). Despite being one of the most prevalent childhood psychiatric disorders with persistence into adulthood in approximately 30-50% of patients, its etiology is poorly understood (Faraone et al. 2006; Faraone et al. 2000; Kessler et al. 2005; Kooij et al. 2005; Polanczyk et al. 2007).

Several lines of evidence imply aberrant dopaminergic neurotransmission as one underlying pathological mechanism of ADHD. Psychostimulant drugs that show successful therapeutic effects in ADHD, such as amphetamines or methylphenidate, block dopamine reuptake from the synaptic cleft through the blockage of the dopamine transporter (*SLC6A3*/*DAT1;* (Roman et al. 2002; Volkow and Swanson 2003)). In addition, alterations in striatal DAT1 have been identified in ADHD patients, and brain regions that are rich in dopamine activity, such as striatum, mid-brain and frontal cortex, are involved in the disorder (Castellanos et al. 1996; Cheon et al. 2003; Dougherty et al. 1999; Dresel et al. 2000; Ernst et al. 1998; Ernst et al. 1999; Faraone and Biederman 1998; Jucaite et al. 2005; Kim et al. 2002; Krause et al. 2000; Moll et al. 2000; Rubia et al. 1999; Spencer et al. 2005; Vaidya et al. 1998).
Animal studies also provided evidence for the implication of dopaminergic mechanisms in ADHD, pointing to genes encoding the dopamine receptors (*Drd1, Drd2, Drd3, Drd4* and *Drd5),* the dopamine transporter (*Dat1*) and dopamine beta-hydroxylase (*Dbh*). Spontaneously hyperactive rats (SHR) show altered *Dat1* expression, and the selective blockage of the D2 receptor in the hyperactive mutant mouse *Coloboma* eliminates hyperactivity and blocks the amphetamine-induced reduction in locomotor activity (Fan et al.; Leo et al. 2003; Watanabe et al. 1997). Interestingly, *Dat1* -/- and *Drd3* -/- knock-out mice show spontaneous hyperactivity (Accili et al. 1996; Giros et al. 1996), while reduced locomotor activity was observed in *Drd2* -/- and *Drd4*-/- mutant mice (Baik et al. 1995; Rubinstein et al. 1997). In addition, *Dbh* -/- and *Drd4* -/- mice display hypersensitivity to amphetamine (Weinshenker et al. 2002) or ethanol, cocaine and methamphetamine-induced hyperactivity, respectively (Rubinstein et al. 1997). Additionally, *Drd1* -/- mutant mice show reduced locomotor stimulant effects of cocaine and *Drd5* -/- knock-out mice exhibit lower levels of immobility and reduced response to the hyperactivity-inducing effects of dopaminergic agonists (Holmes et al. 2001; Xu et al. 1994a).

Variants in dopaminergic genes have also been identified as risk factors for ADHD through case-control and/or family-based association studies. Evidence for association has been reported by meta-analyses for variants in *DRD4*, *DRD5*, *DAT1* and *DBH* (Gizer et al. 2009; reviewed in Thapar et al. 2007 and Thapar et al. 2005).

Our group has recently focused on the association of adult and childhood ADHD with genetic variants in several candidate gene systems, covering entire functional networks such as the serotoninergic system (Ribases et al. 2009b), neurotrophins and their receptors (Ribases et al. 2008) and genes potentially involved in brain laterality (Ribases et al. 2009a). Along this line, and based on previously reported pharmacological, neuroimaging and genetic information, we have investigated 61 common sequence variants within 9 dopaminergic genes in childhood and adulthood ADHD. These genes encode the dopamine receptors (*DRD1, DRD2, DRD3, DRD4* and *DRD5*), the dopamine transporter (*DAT1*), the rate-limiting enzyme in dopamine synthesis (tyrosine hydroxylase, *TH*) and enzymes involved in dopamine degradation (dopamine beta-hydroxylase, *DBH*, and catechol-O-methyl transferase, *COMT)* (Table S1). To address this issue we performed case-control and family-based association studies in 533 ADHD patients (322 children and 211 adults), 533 sex-matched unrelated controls and 196 nuclear ADHD families from Spain. The results were then tested for replication in an independent sample of 353 nuclear ADHD families with combined-type ADHD from Germany.

METHODS

Subjects and Clinical Assessment

The clinical description of the sample of 2426 Caucasian subjects included in the present study is shown in Table S2. Diagnosis was blind to genotype. The study was approved by the ethics committee of each institution and informed consent was obtained from all subjects. A more detailed description of the different diagnostic instruments used was published previously (Ribases et al. 2009a; Ribases et al. 2008; Ribases et al. 2009b).

Original cohort:

Childhood ADHD sample:

Three hundred twenty-two children with ADHD (73.6% combined, 21.7% inattentive and 4.7% hyperactive-impulsive) and 322 sex-matched unrelated controls were recruited from two hospitals, Hospital Vall d'hebron and Hospital Mútua de Terrassa, located in the Barcelona area (Spain). Seventy-nine percent of patients and controls were male. The average age at assessment was 9.3 years (SD=2.6) for patients and 36.8 years (SD=17.0) for controls. Patients were evaluated with the Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (KSADS-PL) reported by parents. ADHD symptoms were assessed using the Conners' Parent Rating Scale and the Conners' Teacher Rating Scale. For the subsequent family-based study, one or both unscreened parents of a subset of 196 affected children suffering from combined-type ADHD, were available (both parents: n=137 and one parent: n=59). Eighty-three percent of these ADHD cases were males. The average age at assessment was 9.1 years (SD=2.8) for probands and 43 years (SD=7.9) for parents.

Adulthood ADHD sample:

The adulthood population consisted of 211 ADHD subjects (140 combined, 61 inattentive and 10 hyperactive-impulsive) and 211 sex-matched unrelated controls from Hospital Vall d'Hebron, Barcelona (Spain). Seventy-three percent of subjects were male. The average age at diagnosis was 29.8 years (SD=12.1) for patients and 44.2 years (SD=14.7) for controls. The ADHD diagnosis was based on the Structured Clinical Interview for DSM-IV Axis I and Axis II Disorders (SCID-I and SCID-II) and the Conners' Adult ADHD Diagnostic Interview for DSM-IV (CAADID). The level of impairment was measured by the Clinical Global Impression (CGI) included in the CAADID Part II and the Sheehan Disability Inventory.

Exclusion criteria for the adult and childhood Spanish patients cohorts were IQ<70; pervasive developmental disorders; schizophrenia or other psychotic disorders; the presence of mood, anxiety or personality disorders that might explain ADHD symptoms; adoption; sexual or physical abuse; birth weight <1.5 kg; and other neurological or systemic disorders that might explain ADHD symptoms. All controls consisted of Caucasian blood donors in which DSM-IV life-time ADHD symptomatology was excluded under the following criteria: 1) not having previously been diagnosed with ADHD and 2) answering negatively to the life-time presence of the following DSM-IV ADHD symptoms: a) often has trouble keeping attention on tasks, b) often loses things needed for tasks, c) often fidgets with hands or feet or squirms in seat and d) often gets up from seat when remaining in seat is expected. Due to ethics concerns, all subjects included as controls were adults.

Replication Cohort:

A total sample of 353 nuclear ADHD families from Germany (225 from the Department of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy, University Hospital, Würzburg, and 128 from the Departments of Child and Adolescent Psychiatry, Saarland University Hospital, Homburg, and Neurobehavioral Genetics, Institute of Psychobiology, Trier) with at least one child suffering from combined-type ADHD was assessed for replication. DNA was available from both parents of 304 probands, one parent of 49 probands and 17 siblings. Six siblings had combined, three inattentive and three hyperactive-impulsive ADHD. The other five siblings were healthy. Parents were not screened for ADHD or other mental disorder. Eighty-two percent of probands were males. The average age at assessment was 10.74 years (SD=2.47) for probands. The index child and siblings were included when at least 6 years old. All children were either assessed by the Kiddie-SADS-PL-German Version or the Kinder-DIPS, and parent and teacher ADHD DSM-IV based rating scales were obtained to ensure pervasiveness of symptoms. Exclusion criteria were IQ<80, co-morbid autistic disorders or somatic disorders (hyperthyroidism, epilepsy, neurological diseases, severe head trauma etc.), primary affective disorders, Tourette-syndrome, psychotic disorders or other severe primary psychiatric disorders, and birth weight below 2000 grams. Full phenotypic assessment methods of the sample were published previously (Palmason et al. 2010).

DNA isolation

Genomic DNA was isolated either from saliva using the Oragene DNA Self-Collection kit (DNA Genotek, kanata, Ontario, Canada) or from blood by the salting-out procedure or with magnetic bead technology with the Chemagic Magnetic Separation Mosule I anc Chemagic DNA kit (Chemagen, Baesweiler, Germany). DNA concentrations were determined using the PicoGreen dsDNA Quantitation Kit

(Molecular Probes, Eugene, Oregon). For the German samples, DNA was obtained by a routine desalting method.

SNP selection and genotyping

For the SNP selection, we used information from the Centre d'Étude du Polymorphisme Humain (CEPH) panel and from the HapMap database (www.hapmap.org; release 20) and considered the region spanning each candidate gene plus 3–5 kb flanking sequences. TagSNPs were selected at an r^2 threshold of 0.85 from all SNPs with minor allele frequency (MAF) > 0.15 for genes with fewer than 15 tagSNPs (*DRD1, DRD2, DRD3, DRD4, DRD5, TH* and *COMT*) and MAF > 0.25 for those genes with more than 15 tagSNPs (*DBH* and *DAT1*). A total of 68 tagSNPs (27 in multi-loci bins and 41 singletons) were chosen under these criteria with the LD-select software (Carlson et al. 2004). Additionally, rs2227850 located in exon 1 of *DRD5* was included in the analysis. The 69 selected SNPs were assessed with the automated assay design pipeline at ms.appliedbiosystems.com/snplex/snplexStart.jsp and a proper design could not be achieved for 5 SNPs, which translates into a design rate of 92.7%. All SNPs were genotyped using the SNPlex platform (Applied Biosystems, Foster City, CA, USA) as described (Tobler et al. 2005). Two HapMap samples were included in all genotyping assays and a concordance rate of 100% with HapMap data was obtained.

Statistical Analyses

A two-stage association study was performed: (i) We first tested association between ADHD and 61 variants in 9 dopaminergic genes by a case-control association study in two Spanish samples of adult and childhood ADHD patients and control subjects. Parents from 61% of children with ADHD were also available and those genes showing positive signals in the case-control study after applying the restrictive Bonferroni correction were analyzed using a family-based association approach (ii) Genes with SNPs showing significant association values were tested for replication in an independent sample of nuclear ADHD families from Germany. The analysis of minimal statistical power was performed *post hoc* using the Genetic Power Calculator software (Purcell et al. 2003), assuming an odds ratio (OR) of 1.3, prevalence of 0.05, significance level of 0.05, and the lowest MAF of 0.15. The presence of population substructures had been previously discarded in the Spanish case-control sample by means of genetic stratification testing using a panel of 45 unlinked non-genic SNPs (Ribases et al. 2009a; Ribases et al. 2008; Ribases et al. 2009b).

Case-control Association Study

Single-Marker Analysis:

The analysis of HWE (p>0.01) and the comparison of genotype and allele frequencies between cases and controls were performed using a Chi-square test with the SNPassoc R package (Gonzalez et al. 2007). Dominant and recessive models were considered for SNPs displaying nominal association when either genotypes under a codominant model or alleles were taken into account. Bonferroni correction for 244 tests in the initial association study, considering 61 SNPs, two age groups, and the comparison of genotype and allele frequencies, corresponds to a significance threshold of $p \leq 2.0e\text{-}04$.

Multiple-Marker Analysis:

The haplotype-based association study was restricted to genes including genetic variants associated with ADHD in the single-marker analysis. All the genotyped variants within these genes were considered. The best two-marker haplotype from all possible pairwise combinations was identified. Likewise, additional markers (up to four) were added in a stepwise manner to the initial two-SNP haplotype. Significance was estimated using 10,000 permutations with the UNPHASED software (Dudbridge 2003). Since the expectation-maximization algorithm does not accurately estimate low haplotype frequencies (Fallin and Schork 2000), haplotypes with frequencies <0.05 were excluded. We also tested the allelic combinations that showed positive association in the overall ADHD sample in the two diagnostic subgroups of combined and inattentive ADHD separately. The hyperactive/impulsive group was not considered due to its small sample size.

Family-based Association Study:

HWE (p>0.01) was confirmed for parental genotypes derived from the alleles not transmitted to the affected offspring. For the single and multiple-marker analyses, alleles or haplotypes transmitted and not transmitted from parents to the affected offspring were compared by the HRR strategy using the UNPHASED software (Dudbridge 2003). For the multiple-marker approach, we applied the same strategy described in the case-control study (see above). The best allelic combination was further considered in the Transmission Disequilibrium Test (TDT) using the PLINK software (Purcell et al. 2007).

RESULTS

Case-control association study

TagSNPs in 9 candidate genes of the dopaminergic system (*DRD1, DRD2, DRD3, DRD4, DRD5, DAT1*, *TH*, *DBH* and *COMT*) were analyzed in a Spanish sample of 322 children with ADHD and 322 sex-matched controls and 211 adults with ADHD and 211 sex-matched controls. Of the 64 SNPs selected for inclusion in the SNPlex assay, one was monomorphic and two had genotype calls < 90% and were excluded from the analysis (Table S1). The minimal statistical power calculated for the childhood sample was 36.4%, 28.3% and 6.7% for the dominant, codominant and recessive model of inheritance, respectively, and for the adult population it was 25.7%, 19.7% and 6.1% considering the same models.

In the single-marker analysis the comparison of genotype frequencies under a codominant model and allele frequencies showed nominal association between rs2070762 in the *TH* gene and both childhood and adulthood ADHD. In addition, four SNPs in *DRD1* displayed nominal associations with ADHD in the childhood dataset (rs835616, rs835541, rs863126 and rs265977; Table 1 and S3; Figure 1). After applying the Bonferroni correction, only rs265977 in *DRD1* remained associated with ADHD in children ($P_{codominant}$=2.0e-04; P_{allele}=2.1e-04, OR=1.77 (1.30-2.40)). To minimize the probability of type I errors, we further compared the 322 childhood cases with the 211 controls previously used in the adulthood comparison and confirmed the association between *DRD1* and ADHD in children (Table S4).

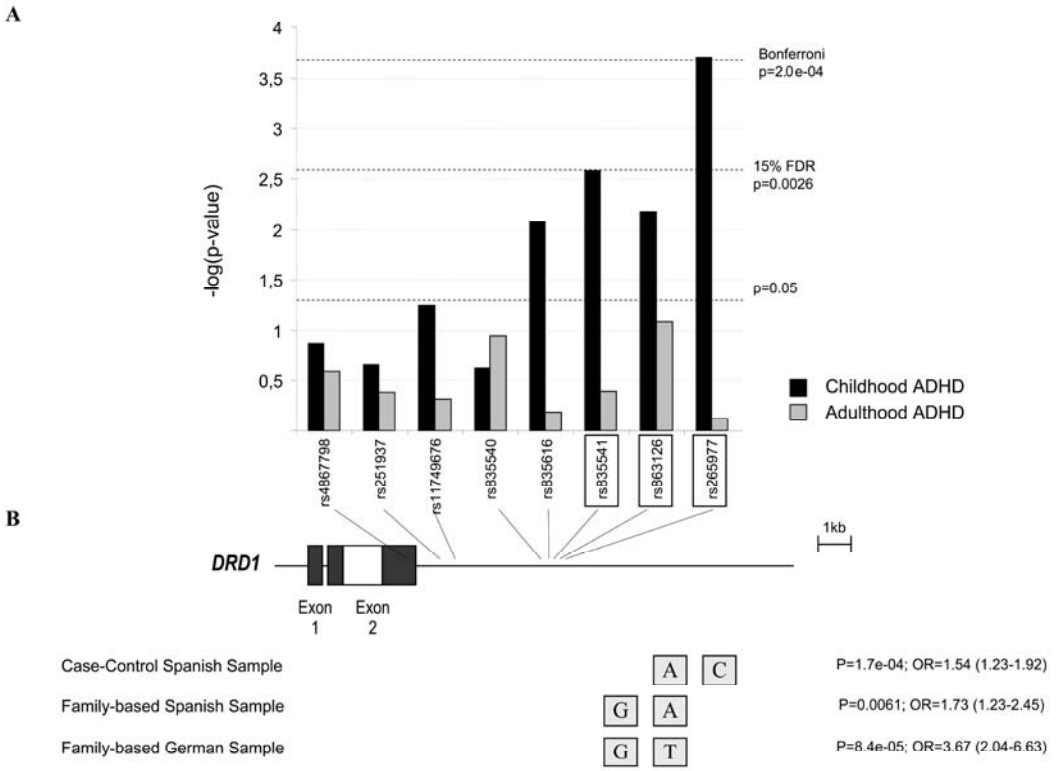

Figure 1. (a) Lowest level of significance, as –log(p-value) found in either the codominant genotypes or the alleles comparisons, of individual SNPs within the *DRD1* gene when 322 child ADHD patients (in black) and 211 adult ADHD patients (in gray) were compared to controls. SNPs in ADHD risk haplotypes associated with ADHD are boxed. (b) Diagram of the *DRD1* gene with the coding region in white and 5' and 3' untranslated regions in gray. Allelic combinations associated with combined ADHD in children in the Spanish and German samples through case-control or family-based association studies are shown. FDR = False Discovery Rate.

We then considered *DRD1* for the haplotype-based analysis only in the children dataset. The study of the eight *DRD1* SNPs revealed a two-marker haplotype (rs863126-rs265977) associated with childhood ADHD (Global P-value=4.36e-05; Table 2a) with an overrepresentation of the A-C allelic combination (P_{A-C}=1.7e-04; OR=1.54 (1.23-1.92)) and a reduced frequency of the A-T haplotype (P_{A-T}=1.4e-04; 1.77 (1.30-2.40); Figure 1) in the patients group. These differences were specific to the combined-type ADHD subgroup (Global P-value=4.4e-04; P_{A-C}=8.8e-04, OR=1.50 (1.18-1.90); P_{A-T}=0.0011, OR=1.74 (1.24-2.43); Table 2).

Table 1. Association study in 322 childhood ADHD patients (237 combined ADHD, 70 inattentive ADHD and 15 hyperactive-impulsive ADHD patients) and 322 sex-matched unrelated controls and 211 adult ADHD patients (140 combined ADHD, 61 inattentive ADHD and 10 hyperactive-impulsive ADHD patients) and 211 sex-matched unrelated controls.

		Genotypes													Alleles					
		Cases N (%)				Controls N (%)					Genotype 11 vs 12+22		Genotype 11+12 vs 22		Cases N (%)		Controls N (%)		Allele 2 vs allele 1	
Gene	SNP	11	12	22	Sum	11	12	22	Sum	P	OR (95% CI)	P	OR (95% CI)	P	1	2	1	2	OR (95% CI)	P
Children																				
DRD1	rs835616	142 (44.4)	141 (44.1)	37 (11.6)	320	177 (55.0)	117 (36.3)	28 (8.7)	322	0.026	1.53 (1.12-2.09)	0.0072	-	0.23	425 (66.4)	215 (33.6)	471 (73.1)	173 (26.9)	1.37 (1.09-1.75)[&]	0.0086
	rs835541	123 (38.6)	139 (43.6)	57 (17.9)	319	90 (28.0)	155 848.1)	77 (23.9)	322	0.011	1.61 (1.16-2.27)[&]	0.0043	-	0.060	385 (60.3)	253 (39.7)	335 (52.0)	309 (48.0)	1.40 (1.12-1.75)[&]	0.0026
	rs863126	149 (46.3)	129 (40.1)	44 (13.7)	322	119 (37.0)	169 (52.5)	34 (10.6)	322	0.0066	1.47 (1.07-2.0)[&]	0.016	-	0.23	427 (66.3)	217 (33.7)	407 (63.2)	237 (36.8)	-	0.24
	rs265977	244 (76.0)	75 (23.4)	2 (0.60)	321	209 (64.9)	98 (30.4)	15 (4.7)	322	2.0e-04*	1.72 (1.22-2.44)[&]	0.002	7.69 (1.75-33.3)[&]	7e-04	563 (87.7)	79 (12.3)	516 (80.1)	128 (19.9)	1.77 (1.30-2.40)	2.1e-04
TH	rs2070762	107 (33.5)	153 (48.0)	59 (18.5)	319	125 (38.8)	158 (49.1)	39 (12.1)	322	0.062	-	0.16	1.65 (1.06-2.55)	0.024	367 (57.5)	271 (42.5)	408 (63.4)	236 (36.6)	1.28 (1.02-1.59)[&]	0.033
Adults																				
TH	rs2070762	71 (34.1)	110 (52.9)	27 (13.0)	208	89 (42.2)	86 (40.8)	36 (17.1)	211	0.044	-	0.090	-	0.24	252 (60.6)	164 (39.4)	264 (62.6)	158 (37.4)	-	0.55

* Statistically significant P-values after applying Bonferroni correction (p≤2.0e-04); [&] When odds ratio < 1, the inverted score is shown.

Table 2. (a) Haplotype analysis of 8 *DRD1* SNPs in a clinical sample of 322 child ADHD patients and 322 controls using the UNPHASED software; (b) Haplotype distributions of the rs863126 and rs265977 *DRD1* SNPs.

(a)

	All ADHD (n=322)			Combined ADHD (n=237)			Inatentive ADHD (n=70)		
Marker * haplotype	**Global P-value**	**Best haplotype P-value (Adjusted P-value)**	**Risk Haplotype- OR**	**Global P-value**	**Best haplotype P-value (Adjusted P-value)**	**Risk Haplotype- OR**	**Global P-value**	**Best haplotype P-value (Adjusted P-value)**	**Risk Haplotype- OR**
7 8	**4.36e-05**	**1.4e-04 (8.9e-04)**	**1.54 (1.23-1.92)**	**4.4e-04**	**8.8e-04 (0.0044)**	**1.50 (1.18-1.90)**	**0.044**	**0.027 (0.065)**	**-**
4 7 8	9.19e-05	1.7e-04 (0.0012)	1.54 (1.24-1.93)	4.9e-04	9.0e-04 (0.0055)	1.50 (1.18-1.91)	0.096	-	-
1 4 7 8	1.3e-04	0.0019 (0.0142)	1.37 (1.08-1.74)	6.4e-04	0.011 (0.065)	-	0.11	-	-

In bold the best allelic combination (higher OR)

(b)

	All ADHD (n=322)			Combined ADHD (n=237)		
Marker * haplotype	**Cases**	**Controls**	Haplotype-specific P-value; OR (CI)	**Cases**	**Controls**	Haplotype-specific P-value; OR (CI)
7 8						
A T	79 (12.3)	128 (19.9)	1.4e-04; 1.77 (1.30-2.40)&	59 (12.5)	128 (19.9)	0.0011; 1.74 (1.24-2.43)&
A C	347 (54.1)	279 (43.3)	1.7e-04; 1.54 (1.23-1.92)	252 (53.4)	279 (43.3)	8.8e-04 ; 1.50 (1.18-1.90))
T C	216 (33.6)	237 (36.8)	-	161 (34.1)	237 (36.8)	-

*1-rs4867798; 4-rs835540; 7-rs863126; 8-rs265977. Numbering of markers correlates with their position on the gene in the 5' to 3' direction (see Figure 1 for the detailed gene location of the SNPs)
& Underepresented in ADHD cases

Family-based association study

The eight *DRD1* SNPs were further tested in two childhood combined-type ADHD family-based samples from Spain (n=196) and Germany (n=353). All patients from the Spanish trios were part of the previously studied case-control sample. The minimum statistical power calculated for the Spanish sample was 20.5% and 32.9%, respectively. Two SNPs, rs835616 and rs835540, had genotype call rates < 60% in both populations and were discarded form the family-based study.

Spanish sample:

The Haplotype Relative Risk (HRR) analysis showed no significant differences when individual *DRD1* markers were considered. However, the multiple-marker analysis showed evidence for association between combined ADHD and the two-marker haplotype rs835541-rs863126 (Global P-value=0.0029; Table 3a). We observed an overtransmission of the G-A allelic combination (P_{G-A}=0.0061; OR=1.73 (1.23-2.45)) and marginally significant evidence for nontransmission of the G-T haplotype to the affected offspring (P_{G-T}=0.021; OR=2.27 (1.17-4.39); Table 3b, Figure 1). Although only 24% of parents were informative, the TDT analysis confirmed the nominal association of *DRD1* with combined ADHD in children from Spain (P_{G-A}=0.042; OR=2.31 (1.20-4.43); Table 3c).

German sample:

No significant differences in transmission were observed in the single-marker analysis of *DRD1*. However, the multiallelic version of the HRR test confirmed the strong association between combined ADHD and the rs835541-rs863126 haplotype identified in the Spanish sample (Global P-value=1.2e-04; Table 3a). Interestingly, the analysis of individual haplotypes showed an excess of transmission of the G-T allelic combination to the ADHD probands (P_{G-T}=8.4e-05; OR=3.67 (2.04-6.63)) and a reduced transmission of the A-T haplotype (P_{A-T}=0.031; OR=1.46 (1.11-1.93); Table 3b, Figure 1). Consistently with the HRR results, the TDT analysis considering the 306 informative parents, also highlighted the *DRD1* association with combined-type ADHD in the German dataset (P_{G-T}=0.0024; OR=2.82 (1.46-5.45); data not shown).

Table 3. (a) Haplotype analysis of six *DRD1* SNPs in a clinical sample of 353 childhood combined ADHD trios from Germany and 196 childhood combined ADHD trios from Spain using the UNPHASED software; (b) Haplotype distributions of the rs265977 and rs863126 *DRD1* SNPs considering a Haplotype Relative Risk analysis.

(a)

	Spain (n=196)			Germany (n=353)			
Marker * haplotype	Global P-value	Best haplotype P-value (Adjusted P-value)	Risk Haplotype- OR	Marker * haplotype	Global P-value	Best haplotype P-value (Adjusted P-value)	Risk Haplotype- OR
6	0.11	0.11	-	6	0.056	0.057	-
6 7	0.0029	0.0061 (0.024)	1.73 (1.23-2.45)	6 7	1.2e-04	8.4e-05 (2.2e-04)	3.67 (2.04-6.63)
3 6 7	0.029	0.016 (0.076)	-	6 7 8	0.0044	0.0011 (0.0031)	2.47 (1.40-4.37)

(b)

	Spain (n=196)			Germany (n=353)		
Marker * haplotype	Cases	Controls	Haplotype-specific P-value; OR (CI)	Cases	Controls	Haplotype-specific P-value; OR (CI)
6 7						
G A	138 (52.4)	103 (38.8)	0.0061; 1.73 (1.23-2.45)	223 (45.0)	229 (45.0)	-
A T	73 (27.8)	72.3 (27.2)	-	121 (24.4)	159 (32.0)	0.031; 1.46 (1.11-1.93)&
G T	14 (5.2)	30 (22.2)	0.021; 2.27 (1.17-4.39)&	51 (10.3)	15 (3.0)	8.4e-05; 3.67 (2.04-6.63)
A A	39 (14.6)	61 (22.8)	-	101 (20.3)	93 (18.8)	-

*3-rs11749676; 6-rs835541; 7-rs863126; 8-rs265977. Numbering of markers correlates with their position on the gene in the 5' to 3' direction (see Figure 1 for the detailed gene location of the SNPs).
Markers rs835616 and rs835540 showed genotype call rates < 60% and were excluded from the family-based study
& Untransmitted to the affected ADHD offspring

DISCUSSION

We followed a hypothesis-driven approach based on presumed ADHD neurobiology and investigated the main components of the dopaminergic system for their involvement in the genetic susceptibility to ADHD through a two-step population and family-based association study design. The analysis of 9 dopaminergic candidate genes showed strong evidence for the contribution of *DRD1* to childhood combined ADHD in two independent datasets from Spain and Germany. Our study raises several methodological considerations that we discuss below:

(i) Standardized assessment of ADHD by structured interviews and rating scales were considered across the different population samples.

(ii) Rather than focus on single SNPs in dopaminergic genes previously associated with ADHD, the study design ensured a full coverage of the genes in terms of linkage disequilibrium (LD).

(iii) Since population-based association studies are particularly susceptible to stratification, cases and controls were previously tested for confounding population substructures by genotyping a set of 45 non-linked SNPs located in different chromosomes outside of any known gene (Ribases et al. 2009a; Ribases et al. 2008; Ribases et al. 2009b). Also, a follow-up family-based approach using case-parent triads was performed to confirm the initial association finding. The family-based study included a subset of the child patients used in the original case-control analysis and an independent cohort from Germany.

(iv) To minimize the probability of type I errors, we applied a robust and stringent approach for dealing with multiple comparisons across all statistical tests performed and focused only on the single association that remained statistically significant after the Bonferroni correction.

(v) The identification of the best allelic combination conferring susceptibility to ADHD through family-based association studies in two independent datasets from Spain and Germany converge in the same two-marker haplotype, rs835541-rs863126, of the *DRD1* gene. This risk haplotype was not identical but overlapped with the associated two-marker haplotype identified by the case-control approach in the Spanish cohort.

(vi) The relationship between *DRD1* and combined ADHD in childhood is less straightforward than expected since we detected a “flip-flop” phenomenon, with opposite allelic risk variants of the same haplotype associated with ADHD in the two populations under study (Spanish cohort: rs835541G-rs863126A; German cohort: rs835541G-rs863126T). Rather than statistical artifacts, they may be attributable to the presence of non-causal SNPs in LD with the genetic variant directly involved in the vulnerability to ADHD. The fact that SNPs displaying association in both the single and multiple-marker analyses are located in the 3’ region of the gene is in agreement with this hypothesis. Although the reason for such “flip-

flop" results are unknown, they may be due to differences in the genetic background of the two studied populations, either in terms of differential LD architectures and/or the presence of specific ADHD risk loci and environmental factors interacting with *DRD1* (Lin et al. 2007).

(vii) Despite the relatively limited sample size resulting in low power to detect genetic risk factors of small effect and the fact that parents included in the family-based approach were unscreened for ADHD, which may contribute to generate type II errors (false negatives), we identified *DRD1* as a risk factor for combined ADHD in two independent cohorts. However, since reduced sample sizes can provide imprecise estimates of the magnitude of the observed effects, further studies in larger samples would improve our knowledge about the role of *DRD1* in the disorder.

Previous studies support the connection of DRD1 with ADHD. Dopamine D1 receptor mutant mice exhibit locomotor hyperactivity and two animal models of ADHD, the Spontaneously Hypertensive rat (SHR) and the Naples High Excitability (NHE) rat, show alterations in the expression and/or function of DRD1 (Clifford et al. 1998; Russell 2002; Sagvolden et al. 1992; Viggiano et al. 2002; Xu et al. 1994a; Xu et al. 1994b). In addition, DRD1 antagonists reverse the methylphenidate effects on prefrontal cortex cognitive function in rats (Arnsten 2006; Arnsten and Dudley 2005) and several association studies and a genome-wide association scan (GWAS) in juvenile but not in adult ADHD (Lesch et al. 2008) also emphasized the potential impact of *DRD1* in ADHD as well as in inattentive and impulsive symptoms (Bobb et al. 2005; Brookes et al. 2006; Lasky-Su et al. 2008; Luca et al. 2007; Misener et al. 2004; Shaw et al. 2007; Taylor et al. 1997). Mutational screening of the gene, however, did not identify any potential functional variant directly involved in the disorder and suggests that the causal sequence variant may reside outside the *DRD1* coding region (Feng et al. 1998; Misener et al. 2004; Thompson et al. 1998). Finally, because *DRD1* was associated with childhood but not with adulthood ADHD, we reasoned that it may be implicated in those specific symptoms, such as hyperactivity or impulsivity, that decline with increasing age (Biederman et al. 2000; Hart et al. 1995; Rietveld et al. 2004). This putative *DRD1* influence on hyperactivity-impulsivity may explain the specific association detected only in the combined but not in the inattentive clinical subtype. These results suggest differential genetic influences contributing to stability versus remission of ADHD symptoms across the lifespan and support previous studies pointing to different genetic factors emerging at distinct developmental periods (Franke et al. 2010; Kuntsi et al. 2005). In this regard, we have previously identified several genetic risk factors in our Spanish cohort that are specifically associated with an age group or disease subtype (*BAIAP2* and *MAOB* in adults, *NT3* and *NTRK2* in children and *HTR2A* in combined ADHD) (Ribases et al. 2009a; Ribases et al. 2008; Ribases et al. 2009b). Alternatively, discrepancy between the combined and inattentive groups and between the age groups could also be attributed to limited statistical power,

distinct environmental influences, additional genetic risk factors, clinical heterogeneity and comorbid disorders co-occurring with ADHD.

In agreement with the involvement of the dopaminergic neurotransmission in ADHD, we have previously identified association between ADHD and two genes encoding enzymes involved in dopamine and serotonin synthesis (*Monoamine oxidase B; MAOB*) and degradation (*Dopa decarboxylase; DDC)* (Ribases et al. 2009b).

In summary, although the functional sequence variants in *DRD1* directly involved in the disorder remain to be uncovered, we have identified a significant childhood-specific association between the gene and combined ADHD in two independent samples. Follow-up studies may shed light on the possible involvement of *DRD1* in changes of ADHD symptoms across life span.

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STATEMENT OF INTEREST

None to declare.

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Table S1. Description of the SNPlex assay within 9 dopamine-related candidate genes for ADHD

Gene	Contig reference	Location	Length (kp)	N of exons	SNPs	Tag SNPs	SNP ID	Location	Exclusion criteria	Gene Coverage*	Other SNPs within the BIN**
DRD1	NM_00794	5q35.1	3.127	2	11	8	rs4867798	3'UTR		1	-
							rs251937	3'			rs703748
							rs11749676	3'			rs686, rs265978
							rs835540	3'	Genotype call < 60%[&]		-
							rs835616	3'	Genotype call < 60%[&]		-
							rs835541	3'			-
							rs863126	3'			-
							rs265977	3'			-
DRD2	NM_000795	11q23	65.56	8	35	10	rs4630328	Intron 1		1	rs17601612
							rs7131056	Intron 1			-
							rs4245146	Intron 1			rs4245147, rs4274224
							rs17529477	Intron 1			-
							rs2002453	Intron 1			rs1076562, rs2005313, rs2245805, rs4586205, rs4648318
							rs12363125	Intron 2			rs1076563, rs12364051, rs12800853, rs2587548, rs2734833, rs2734837, rs2734838, rs2734839, rs4587762, rs4938017, rs7122246, rs7131440
							rs2283265	Intron 5			-
							rs2242592	3'			rs2734841, rs2734842, rs6275, rs6279
							rs1554929	3'			rs10891549
							rs2234689	3'			-
DRD3	NM_000796	3q13.3	50.34	7	23	11	rs9825563	5'		0.91	-
							rs1800828	Intron 1			-
							rs6280	Exon 2			rs324026, rs7638876
							rs10934256	Intron 2			rs7633291
							rs167770	Intron 2			rs226082,rs324030,rs7625282,rs324029, rs324022, rs11721264

							rs167771	Intron 3			-
							rs324035	Intron 3			-
							rs9880168	Intron 3			-
							rs2134655	Intron 5			-
							rs3732790	3'			rs963468, rs3773679
							rs2399496	3'	Failed		rs9817063
DRD4	NM_000797	11p15.5	3.399	4	4	2	rs3758653	5'	SNPlex design	0.5	-
							rs936465	3'			rs4331145, rs11246226
DRD5	NM_000798	4p16.1	2.03	1	3	2	rs2227850	Exon 1	Monomorphic	0.5	
							rs10033951	3'			rs2867383
COMT	NM_000754	22q11.21	27.22	6	14	10	rs2020917	5'		0.9	rs737866
							rs933271	Intron 1			rs174675
							rs1544325	Intron 1			rs5993883
							rs740603	Intron 1			-
							rs740601	Intron 3			rs2239393
							rs4680	Exon 4			-
							rs4646316	Intron 5			-
							rs174696	Intron 5	SNPlex design		-
							rs165774	Intron 5			-
							rs9332377	Intron 5			-
DBH	NM_000787	9q34	22.98	12	22	14	rs2007153	Intron 1		0.86	-
							rs2797851	Intron 1			rs2519155, rs279785, rs2797849
							rs1548364	Intron 3			rs1611122, rs1611125, rs2873804
							rs2797855	Intron 5			rs1611123
							rs1541332	Intron 5			-
							rs2519154	Intron 5			-
							rs2797853	Intron 5			-
							rs6479643	Intron 6			-
							rs77905	Exon 9			-

							rs2097628	Intron 9	SNPlex design		rs2097629
							rs2073833	Intron 9			-
							rs1611131	Intron 10			-
							rs129883	3'			-
							rs129915	3'	SNPlex design		-
SLC6A3	NM_001044	5p15.3	52.63	15	19	9	rs2617605	Intron 2	Failed	0.78	-
							rs460700	Intron 4			rs464061, rs456082, rs409588, rs458860, rs464528, rs463379, rs456774, rs460000
							rs37020	Intron 6			rs464049, rs458334
							rs13161905	Intron 6			-
							rs27048	Intron 8			-
							rs6347	Exon 9			-
							rs11133767	Intron 13			-
							rs40184	Intron 14			-
							rs2975292	3'	SNPlex design		-
TH	NM_199292	11p15.5	7.876	14	4	3	rs10770140	5'		1	rs10770141
							rs6356	Exon 2			-
							rs2070762	Intron 13			-

* Analyzed tagSNPs/total tagSNPs within the gene

**SNPs within a haplotype block, as defined by the LD-select software (Carlson et al. Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. *Am J Hum Genet* 2004;74:106–120)

[&]SNP excluded from the family-based but not the case-control association study

Table S2. Description of the 2426 subjects included in the association study

Sample	Type of Study	Subjects				Clinical Subtype N (%)		
		Total	ADHD Subjects	Unaffected Subjects	Unscreened Subjects	Combined	Inattentive	Hyperactive-Impusive
Initial Sample								
Spain[1]	Case-control	644 subjects	322	322	-	237 (73.6)	70 (21.7)	15 (4.7)
Spain[2]	Case-control	422 subjects	211	211	-	140 (66.4)	61 (28.9)	10 (4.7)
Spain[1]	Family-based	196 families	196	-	333 [3]	196	-	-
Replication Sample								
Germany[1]	Family-based	353 families	365	-	662	359 (98.4)	3 (0.8)	3 (0.8)

[1]Samples including children ADHD patients
[2]Sample including adult ADHD patients
[3]Parents of the ADHD patients

Table S3. Nominal P-values observed when genotype and allele frequencies of 61 SNPs within 9 candidate genes were considered in 322 children with ADHD and 322 controls and 211 adults with ADHD and 211 controls.

		Children		Adults	
Gene	**SNP**	**Genotypes**	**Alleles**	**Genotypes**	**Alleles**
DRD1	**rs4867798**	0.14	0.23	0.45	0.26
	rs251937	0.22	0.56	0.41	0.73
	rs11749676	0.12	0.06	0.73	0.47
	rs835540	0.24	0.93	0.23	0.11
	rs835616	0.0256	0.0086	0.84	0.64
	rs835541	0.0112	0.0027	0.71	0.40
	rs863126	0.0066	0.24	0.18	0.08
	rs265977	0.0002	0.0002	0.74	0.86
DRD2	**rs4630328**	0.14	0.55	0.97	0.84
	rs7131056	0.78	0.53	0.69	0.47
	rs4245146	0.12	0.86	0.63	0.34
	rs17529477	0.25	0.10	0.19	0.30
	rs2002453	0.70	0.58	0.44	0.39
	rs12363125	0.56	0.43	0.05	0.81
	rs2283265	0.69	0.59	0.51	0.55
	rs2242592	0.81	0.71	0.83	0.61
	rs1554929	0.74	0.61	0.91	0.78
	rs2234689	0.96	0.81	0.68	0.40
DRD3	**rs9825563**	0.72	0.99	0.19	0.08
	rs1800828	1.00	0.93	0.25	0.32
	rs6280	0.76	0.77	0.06	0.09
	rs10934256	0.82	0.51	0.45	0.40
	rs167770	0.78	0.98	0.13	0.09
	rs167771	0.93	0.90	0.23	0.12
	rs324035	0.29	0.80	0.06	0.02
	rs9880168	0.07	0.79	0.10	0.38
	rs2134655	0.52	0.95	0.50	0.27
	rs3732790	0.51	0.81	0.35	0.40
DRD4	**rs936465**	0.98	0.85	0.60	0.32
DRD5	**rs10033951**	0.71	0.48	0.68	0.41
COMT	**rs2020917**	0.60	0.36	0.99	0.88
	rs933271	0.75	0.46	0.75	0.56
	rs1544325	0.27	0.10	0.60	0.57
	rs740603	0.21	0.18	0.97	0.94
	rs740601	0.27	0.12	0.71	0.88
	rs4680	0.30	0.15	0.76	0.62
	rs4646316	0.64	0.66	0.41	0.75
	rs165774	0.83	0.55	0.51	0.79
	rs9332377	0.25	0.80	0.52	0.37
DBH	**rs2007153**	0.17	0.05	0.96	0.80
	rs2797851	0.39	0.20	0.89	0.98

	rs1548364	0.42	0.43	0.29	0.95
	rs2797855	0.29	0.11	0.41	0.61
	rs1541332	0.08	0.31	0.28	0.49
	rs2519154	0.44	0.94	0.48	0.24
	rs2797853	0.14	0.47	0.33	0.63
	rs6479643	0.16	0.06	0.16	0.39
	rs77905	0.15	0.09	0.83	0.58
	rs2073833	0.92	0.76	0.41	0.82
	rs1611131	0.94	0.74	0.67	0.76
	rs129883	0.13	0.45	0.15	0.05
SLC6A3	**rs460700**	0.73	0.52	0.73	0.43
	rs37020	0.94	0.87	0.68	0.77
	rs13161905	0.71	0.46	0.83	0.55
	rs27048	0.75	0.48	0.33	0.24
	rs6347	0.73	0.52	0.84	0.76
	rs11133767	0.24	0.29	0.51	0.47
	rs40184	0.67	0.41	0.87	0.99
TH	**rs10770140**	0.82	0.74	0.45	0.26
	rs6356	0.33	0.69	0.41	0.73
	rs2070762	0.06	0.0328	0.73	0.47

Table S4. Association study in 322 childhood ADHD patients (237 combined ADHD. 70 inattentive ADHD and 15 hyperactive-impulsive ADHD patients) and 211 unrelated controls.

		Genotypes[&]													Alleles[&]	
		Cases N (%)				Controls N (%)					Genotype 11 vs 12+22		Genotype 22 vs 11+12		Allele 2 vs allele 1	
SNP		11	12	22	Sum	11	12	22	Sum	P	OR (95% CI)**	P	OR (95% CI)**	P	OR (95% CI)**	P
Children																
DRD1	rs835616	142 (44.4)	141 (44.1)	37 (11.6)	320	109 (51.7)	87 (41.2)	15 (7.1)	211	0.12	-	0.13	-	0.08	1.31 (1.00-1.72)[&]	0.05
	rs835541	123 (38.6)	139 (43.6)	57 (17.9)	319	61 (28.9)	99 (46.9)	51 (24.2)	211	0.058	1.51 (1.04-2.22)[&]	0.029	-	0.088	1.37 (1.07-1.76)	0.014
	rs863126	149 (46.3)	129 (40.1)	44 (13.7)	322	77 (36.5)	103 (48.8)	31 (14.7)	211	0.12	1.43 (1.00-2.04)[&]	0.046	-	0.79	-	0.11
	rs265977	244 (76.0)	75 (23.4)	2 (0.60)	321	138 (65.4)	65 (30.8)	8 (3.8)	211	0.0020	1.72 (1.16-2.5)[&]	0.0061	6.67 (1.43-33.33)[&]	0.0060	1.73 (1.23-2.44)	0.0015
TH	rs2070762	107 (33.5)	153 (48.0)	59 (18.5)	319	89 (42.2)	86 (40.8)	36 (17.1)	211	0.15	-	0.058	-	0.72	-	0.13

* When odds ratio < 1. the inverted score is shown

CAPÍTOL 3b: TDAH i sistema dopaminèrgic

Article 4

L'anàlisi multicèntrica de l'haplotip de VNTRs del gen *SLC6A3/DAT1* en TDAH persistent suggereix una implicació diferencial en TDAH infantil i adult

RESUM

Antecedents i propòsit: El transportador de dopamina ha estat objecte d'estudi en molts treballs sobre el TDAH pel fet que el tractament farmacològic dels pacients amb metilfenidat actua directament sobre aquesta proteïna. Dos polimorfismes de tipus VNTR en el gen corresponent (*SLC6A3/DAT1*), un localitzat a l'extrem 3'UTR i l'altre a l'intró 8, han estat objecte de diversos estudis d'associació sense resultats consistents. L'objectiu d'aquest treball és l'anàlisi de la participació d'aquests dos polimorfismes en el risc de patir TDAH

Mètodes Estudi d'associació cas-control en una població holandesa amb TDAH considerant dos polimorfismes de tipus VNTR a l'intró 8 i a la regió 3' no traduïda del gen *SLC6A3/DAT1* que codifica el transportador de dopamina. Metanaàlisi en una mostra de 1440 pacients adults amb TDAH i 1769 controls de quatre poblacions europees (Espanya, Holanda, Alemanya i Noruega).

Resultats: En l'estudi cas-control realitzat a la mostra holandesa es va identificar un haplotip de risc format pels al·lels 9R i 6R (3'UTR-Intró8) (p<0,001, OR: 1,5 (1,25-1,79)) que es replicà en un estudi de meta-anàlisi (p=0,03, OR:1,39 (1,03-1,88)).

Conclusions: L'estudi de rèplica realitzat en forma de meta-anàlisi recolza la contribució de l'haplotip 9R6R format pels dos polimorfismes del gen *SLC6A3* al risc de patir TDAH en població adulta. En estudis previs realitzats amb pacients TDAH infantils es detectava associació amb l'haplotip 10R6R.

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Multicenter Analysis of the *SLC6A3/DAT1* VNTR Haplotype in Persistent ADHD Suggests Differential Involvement of the Gene in Childhood and Persistent ADHD

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Attention deficit/hyperactivity disorder (ADHD) is one of the most common neuropsychiatric disorders with a worldwide prevalence around 4–5% in children and 1–4% in adults. Although ADHD is highly heritable and familial risk may contribute most strongly to the persistent form of the disorder, there are few studies on the genetics of ADHD in adults. In this paper, we present the first results of the International Multicentre Persistent ADHD Genetics CollaboraTion (IMpACT) that has been set up with the goal of performing research into the genetics of persistent ADHD. In this study, we carried out a combined analysis as well as a meta-analysis of the association of the *SLC6A3/DAT1* gene with persistent ADHD in 1440 patients and 1769 controls from IMpACT and an earlier report. *DAT1*, encoding the dopamine transporter, is one of the most frequently studied genes in ADHD, though results have been inconsistent. A variable number tandem repeat polymorphism (VNTR) in the 3′-untranslated region (UTR) of the gene and, more recently, a haplotype of this VNTR with another VNTR in intron 8 have been the target of most studies. Although the 10/10 genotype of the 3′-UTR VNTR and the 10-6 haplotype of the two VNTRs are thought to be risk factors for ADHD in children, we found the 9/9 genotype and the 9-6 haplotype associated with persistent ADHD. In conclusion, a differential association of *DAT1* with ADHD in children and in adults might help explain the inconsistencies observed in earlier association studies. However, the data might also imply that *DAT1* has a modulatory rather than causative role in ADHD. *Neuropsychopharmacology* advance online publication, 4 November 2009; doi:10.1038/npp.2009.170

Keywords: dopamine; transporters; psychiatry and behavioral sciences; neurogenetics

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INTRODUCTION

Attention deficit/hyperactivity disorder (ADHD) is one of the most common neuropsychiatric disorders in children worldwide. The prevalence of the disorder in children is estimated to be 4–5% (Polanczyk *et al*, 2007). Although ADHD is classically considered a disorder of children and adolescents, only a subset of affected individuals remit (Faraone *et al*, 2000; Faraone *et al*, 2006), and the prevalence in adults lies between 1 and 4% (Kessler *et al*, 2006; Polanczyk *et al*, 2007; Kooij *et al*, 2005). ADHD in adults causes a considerable burden to patients, their families, and society as a whole (Kessler *et al*, 2005a; Goodman, 2007; Bernfort *et al*, 2008). Adult patients have difficulties in social, educational, and professional fields, such as developing and maintaining stable social relationships, completing educational programs, and holding jobs.

Many twin and adoption studies have shown that ADHD symptoms in children are highly heritable, with about 76% contribution of heritable factors to phenotypic variance (Faraone *et al*, 2005). Although the heritability of the adult form of ADHD has not been formally established, the contribution of familial factors to this form of the disorder may be even larger than to childhood ADHD (Faraone, 2004). Numerous molecular genetic studies have been carried out to identify the genetic risk factors for ADHD. This has resulted in a limited number of significant and replicated findings, but all with modest effect sizes (for review see Faraone *et al*, 2005; Li *et al*, 2006) and explaining only a very small part of the genetic contribution to the disorder. Surprisingly, most molecular genetic studies report on children with ADHD, persistent ADHD has been largely neglected in genetics research so far.

In 2007, the International Multicentre Persistent ADHD CollaboraTion (IMpACT) was formed by researchers participating in the ADHD Molecular Genetics Network (Faraone, 2003; 2002). Currently, research groups from Germany, Norway, Spain, The Netherlands, the United Kingdom, and the United States participate in IMpACT. The collaboration was set up with the goal of performing and promoting research into the genetics of persistent ADHD. This publication presents its first results.

SLC6A3/DAT1, encoding the dopamine transporter, is probably the most studied gene in ADHD. The transporter is the direct target of stimulant-based medication effective in treating ADHD symptoms (Medori *et al*, 2008; Faraone *et al*, 2004). The polymorphism identified as a risk factor for ADHD is a variable number of tandem repeat (VNTR) in the 3′-untranslated region (UTR) of the gene. Since the original publication in 1995 (Cook *et al*, 1995), many studies have investigated the association of this VNTR with ADHD, with variable results. Meta-analyses of the data have also not been consistent, with the most comprehensive ones showing little or no significant effect (Maher *et al*, 2002; Faraone *et al*, 2005; Todd *et al*, 2005; Yang *et al*, 2007; Li *et al*, 2006). Because of the number of positive reports and because of significant evidence of heterogeneity between data sets, systematic differences between data sets might explain the apparent discrepancies (Li *et al*, 2006). Recently, two studies have suggested that a haplotype of two VNTRs in *DAT1*, including the 3′-UTR VNTR and a VNTR in intron 8, is more strongly associated with ADHD than the 3′-UTR VNTR alone (Asherson *et al*, 2007; Brookes *et al*, 2006). Recently, two studies also tested the involvement of the VNTR haplotype in persistent ADHD: whereas one of them did not find an association with the disorder (Bruggemann *et al*, 2007), the other concluded that the 9–6 haplotype, which differs from the 10–6 haplotype associated with childhood ADHD, has a role in the persistent disorder (Franke *et al*, 2008).

To resolve the apparent discrepancies in the literature, we performed a meta-analysis of published data and unpublished data from the IMpACT samples to further investigate the association between the *DAT1* VNTR haplotype and persistent ADHD.

MATERIALS AND METHODS

The study reported here has been carried out in accordance with the Declaration of Helsinki.

Patients and Controls, Assessment of Psychopathology at the IMpACT Nodes

All patients were evaluated by experienced psychiatrists and diagnosed with persistent ADHD according to DSM-IV (Diagnostic and Statistical Manual for Mental Disorders) criteria. Consensus eligibility criteria for this study across all study sites were a diagnosis of ADHD according to the diagnostic criteria of DSM-IV, onset before the age of 7 years by retrospective diagnosis (which was confirmed by a family member, wherever possible), life-long persistence and current diagnosis. Most controls were screened for the presence of ADHD too (see Supplementary File S1 for more detailed information). All subjects were of Caucasoid origin. Diagnosis was blind to genotype. A detailed description of the samples, instruments, and procedures used by the different sites is provided in Supplementary File S1 and Supplementary Table S1. In total, 1525 patients and 1711 controls are part of IMpACT. Studies were approved by the ethics committees of the participating institutions, and written informed consent was obtained from all patients and controls. Genotyping data for both *DAT1* VNTRs were available for 421 patients and 405 controls from IMpACT Germany, for 450 patients and 548 controls from IMpACT Norway, IMpACT Spain contributed 264 patients and 195 controls, and from IMpACT The Netherlands 269 patients and 532 controls with complete genotyping data were available. The total numbers of genotyped cases and controls were 1404 and 1680, respectively (see Table 1).

Genotyping of the Two *DAT1* VNTRs

Genotyping of the 40 bp VNTR located in the 3′-UTR of *DAT1* had been carried out earlier at the different IMpACT sites. Procedures and/or references can be found in Supplementary File S1. As part of quality control, all four sites each sent 24 DNA samples to Norway for genotyping of the *DAT1* 3′-UTR repeat, according to the high-resolution method used in Norway (see Supplementary File S1). Samples were dispensed to one common 96-well plate, and the operator was blinded to the sample ID and country of origin of the samples. Genotyping concordance between tests was 100% for all 96 samples. The intron 8 VNTR was

Table 1 Numbers of Cases and Controls Successfully Genotyped for both VNTRs in *DAT1*

	Cases	Cases included in analysis	Controls	Controls included in analysis
Samples from the International Multicentre Persistent ADHD CollaboraTion (IMpACT)				
Germany	421	406	405	393
Norway	450	432	548	530
Spain	264	249	195	184
Netherlands	269	238	532	491
Combined subtype	928 (70.0%)			
Inattentive subtype	256 (19.3%)			
Hyperactive/impulsive subtype	66 (5.0%)			
Unknown	75 (5.7%)			
Other samples				
Germany (26)	116	115	174	171
Total number of samples in this study				
	1520	1440	1854	1769

Only samples with common alleles were included in the analysis. The subtyping data refer to the samples from IMpACT included in the analysis only.

genotyped in The Netherlands (Norwegian, Spanish, and Dutch samples) or Germany according to the protocol used by the Dutch IMpACT partner (see Supplementary File S1).

Statistical Analysis

Hardy–Weinberg equilibrium (HWE) was assessed for all available samples using the Markov Chain Monte-Carlo approximation of the exact test implemented in the GENEPOP package V 3.3 (Raymond and Rousset, 1995), and genotype distributions were consistent with HWE for both polymorphisms in all four samples ($p > 0.01$). Before haplotype estimation, the VNTRs were recoded, lumping all rare alleles into one group, so that three alleles for the 3′-UTR VNTR (ie, the 9-repeat and 10-repeat alleles plus a rare alleles pool), and three alleles for the intron 8 VNTR (ie, the 5-repeat and 6-repeat alleles plus a rare alleles pool) were considered. Haplotypes were estimated using the haplo.em function implemented in the haplo.stats package (Schaid *et al*, 2002), which computes maximum likelihood estimates of haplotype probabilities, together with posterior probabilities of haplotype pairs for each subject. Haplotype frequencies are shown in Table 2. In the further analysis, we only considered the four most common haplotypes, ie, 10-6, 9-6, 9-5, and 10-5. All haplotypes included in the analysis had a posterior probability of 97% or higher.

A combined analysis was carried out including the samples of IMpACT only. A trend test was used to evaluate the ADHD risk conferred by carrying the 9-6 haplotype using basic χ^2 and logistic regression tests. We focused on the 9-6 haplotype because this had been implicated by one of our earlier studies (Franke *et al*, 2008). The effect of the 9/9 genotype *vs* all other genotypes was also tested using a χ^2-test. These tests used SPSS (version 16.0).

The meta-analysis examined the haplotype association of the 9-6 allelic combination with the risk of ADHD relative to all other alleles in the entire sample, and in a subsample of patients with combined or hyperactive/impulsive ADHD subtype. In addition, allelic and genotypic ORs were calculated for the two VNTRs, separately, with the most common allele or genotype as the reference.

To combine the individual study results, we conducted meta-analyses using RevMan (version 5.0.2) (The Cochrane Collaboration, 2008). The heterogeneity between studies was tested using the Q-statistic (Lau *et al*, 1997; Fleiss, 1981). Inconsistency across studies was quantified with the I^2 metric ($I^2 = Q - df/Q$) (Zintzaras and Hadjigeorgiou, 2004). When no heterogeneity was present, the pooled OR was estimated using fixed effects model (Mantel and Haenzsel, 1959). Otherwise, random effects model (DerSimonian and Laird, 1986) were applied to obtain the pooled OR (Whitehead, 2002). The results of the association tests are indicated as pooled ORs with the corresponding 95% confidence intervals (CIs) of the haplotype-/allele- or genotype-induced risk of persistent ADHD. $P < 0.05$ was considered statistically significant.

RESULTS

Within the IMpACT group, 1404 persistent ADHD patients and 1680 controls, in which both VNTRs had been genotyped, were considered for inclusion in the study (Table 1). Supplementary Table S2 shows the characteristics of the IMpACT patient samples.

The haplotype frequencies in the different samples are shown in Table 2. Of the total number of patients and controls, we included only those individuals that carried the four most common haplotypes for both VNTRs (ie, those containing the 9-repeat or 10-repeat allele of the 3′-UTR VNTR and the 5-repeat or 6-repeat allele of the intron 8 VNTR). This led to the exclusion of 80 patients and 85 controls (Table 1). Haplotype frequencies varied among countries, with the 9-6 allele being more frequent in the

Table 2 Frequencies of Haplotypes per Country (Haplotypes Coded as 'Other' Consist of a Mixture of Rare Haplotypes)

	IMpACT Germany	IMpACT Norway	IMpACT Spain	IMpACT The Netherlands	Germany (from the study by Bruggemann *et al*, 2007)
10-6 (%)					
Cases	68.7	65.5	59.6	63.9	68.1
Controls	68.7	67.9	60.5	69.2	71.1
Total	68.7	66.8	60.0	67.5	69.9
9-5 (%)					
Cases	17.5	19.0	17.7	15.5	17.2
Controls	17.1	21.0	20.9	16.1	15.4
Total	17.3	20.1	19.1	15.9	16.1
9-6 (%)					
Cases	9.2	9.0	15.5	11.1	10.5
Controls	9.8	4.9	13.2	6.4	9.1
Total	9.5	6.8	14.5	7.9	9.6
10-5 (%)					
Cases	2.8	4.0	4.4	3.8	3.7
Controls	3.0	4.6	2.6	4.5	3.6
Total	2.9	4.3	3.6	4.3	3.6
Other (%)					
Cases	1.8	2.4	2.8	5.8	0.5
Controls	1.5	1.7	2.8	3.9	0.9
Total	1.6	2.0	2.8	4.5	0.7

Table 3 Analysis of the Association of the 9-6 Haplotype Formed by the 3′-UTR and Intron 8 VNTRs of the *SLC6A3* Gene *vs* all Other (frequent) Haplotypes

Number of 9-6 alleles	Frequency (%)				
	Controls	Cases	Pearson's χ^2 *p*-value[a]	OR[b]	95% CI
0	1370 (85.7)	1062 (80.2)	20.363	1.5	1.25–1.79
1	221 (13.8)	244 (18.4)			
2	7 (0.4)	19 (1.4)			
Total	1598 (100)	1325 (100)			

[a]df = 2.
[b]Logistic regression analysis.

Spanish sample compared with the other Northern European samples (Table 2).

We first performed a combined analysis in the IMpACT sample only. In a sample of 1325 patients and 1598 controls, we evaluated if there was a difference in the distribution of the 9-6 haplotype between cases and controls. As shown in Table 3, this haplotype indeed was significantly more frequent in the cases ($\chi^2 = 20.36$; df = 2; $p < 0.001$). The allelic trend test showed a risk increase of 1.5 (95% CI 1.25–1.79) for carrying a 9-6 haplotype. Interestingly, an analysis of the 9/9 genotype *vs* all other genotypes showed essentially the same result (9/9 homozygotes = 99 (6.2%) in controls and 119 (9.0%) in cases; $\chi^2 = 8.15$; df = 1; $p = 0.005$; OR = 1.5, 95% CI 1.13–1.97).

Given the differences in haplotype frequencies between samples (Table 2), we considered a meta-analysis design more appropriate than a combined analysis for further analysis. In addition to the IMpACT samples, we also

included data from another published report on the *DAT1* VNTR haplotype in adults (Bruggemann *et al*, 2007) into this meta-analysis, which increased the total number of genotypes included further to 1440 patients with adult ADHD and 1769 controls. Meta-analysis of the data using a random effects model showed that the 9-6 haplotype was significantly associated with ADHD in adults, with an OR of 1.39 (95% CI 1.03–1.88), $p = 0.03$ (Figure 1). As *DAT1* has been suggested to be more relevant for those ADHD subtypes including hyperactivity (Diamond, 2007), we repeated the analysis excluding patients with inattentive subtype ADHD from the four IMpACT samples (see Table 1 for the numbers of samples included). As shown in Figure 2, the point estimate for the OR increased somewhat numerically, but nonsignificantly (OR 1.47, 95% CI 1.02–2.12).

We also analyzed the two VNTRs, separately, in the samples included in the haplotype analysis. For the VNTR in the *DAT1* 3′-UTR the homozygous 10/10 genotype, which is thought to be the risk factor for ADHD in children, did not show association with ADHD in adults (OR = 0.93, 95% CI 0.93–1.07) (Figure 3a). However, as in the combined analysis, we did observe an association of the homozygous 9/9 genotype with persistent ADHD (Figure 3b), with an effect size similar to the one observed for the 9-6 haplotype (OR 1.34, 95% CI 1.03–1.76), $p = 0.03$. The intron 8 VNTR by itself did not have any effect on ADHD risk in the adults (Supplementary Figure S1A–C).

DISCUSSION

In this study we investigated two VNTRs within the *SLC6A3/DAT1* gene and the haplotypes formed by them for association with persistent ADHD. The study included genotype information on 1440 patients, of whom 1100 were formerly unpublished ones from the IMpACT study group. Both the 9-6 haplotype (3′-UTR VNTR/Intron 8 VNTR) and the 9/9 genotype of the 3′-UTR VNTR showed association with the disorder in adults using two different analysis methods, ie, a combined analysis and a meta-analysis design. The intron 8 VNTR by itself did not seem to increase persistent ADHD risk.

The two VNTRs within *SLC6A3/DAT1* have both been suggested to influence the regulation of the gene (Brookes *et al*, 2007; Spencer *et al*, 2005; Guinda-Lini *et al*, 2006). However, *in vivo* and *in vitro* studies for the 3′-UTR VNTR have not been consistent, and the intron 8 VNTR has so far only been studied once. It might therefore also be possible that—instead of being directly involved in regulating gene expression—both VNTRs (incompletely) tag an unknown functional site, with the haplotype increasing the efficiency of the tagging (Asherson *et al*, 2007).

The finding of association with persistent ADHD for the 9-6 haplotype supports an earlier report in the Dutch IMpACT subsample (Franke *et al*, 2008). This finding, as well as the finding that the 9/9 3′-UTR VNTR genotype is associated with persistent ADHD, is contrary to findings in

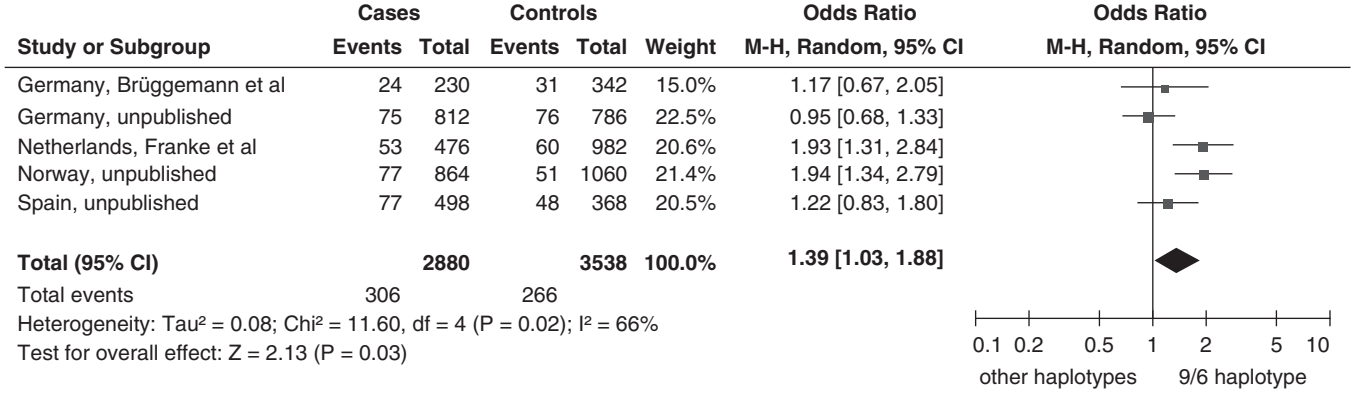

Figure 1 Forest plot showing the analysis of the 9-6 VNTR haplotype *vs* all other haplotypes.

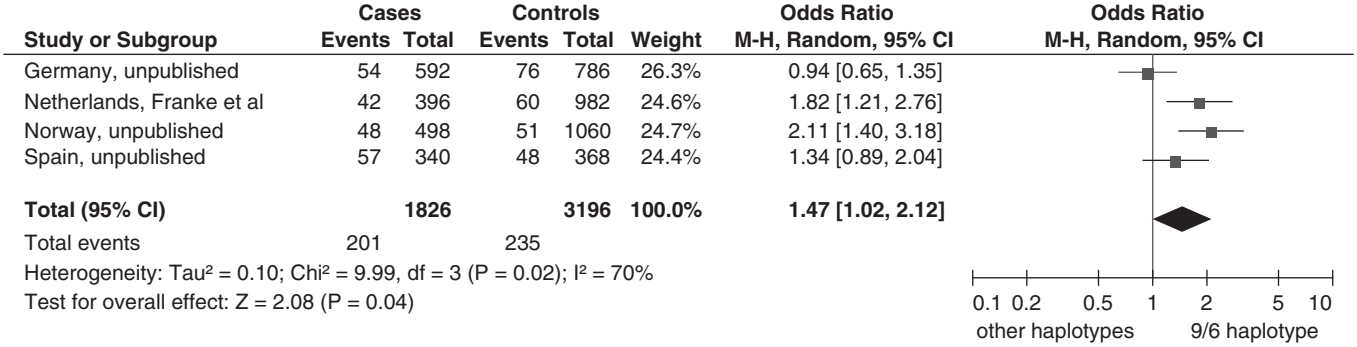

Figure 2 Forest plot showing the analysis of the 9-6 haplotype *vs* all others for patients with ADHD subtypes containing hyperactivity (combined subtype and hyperactive/impulsive subtype) only.

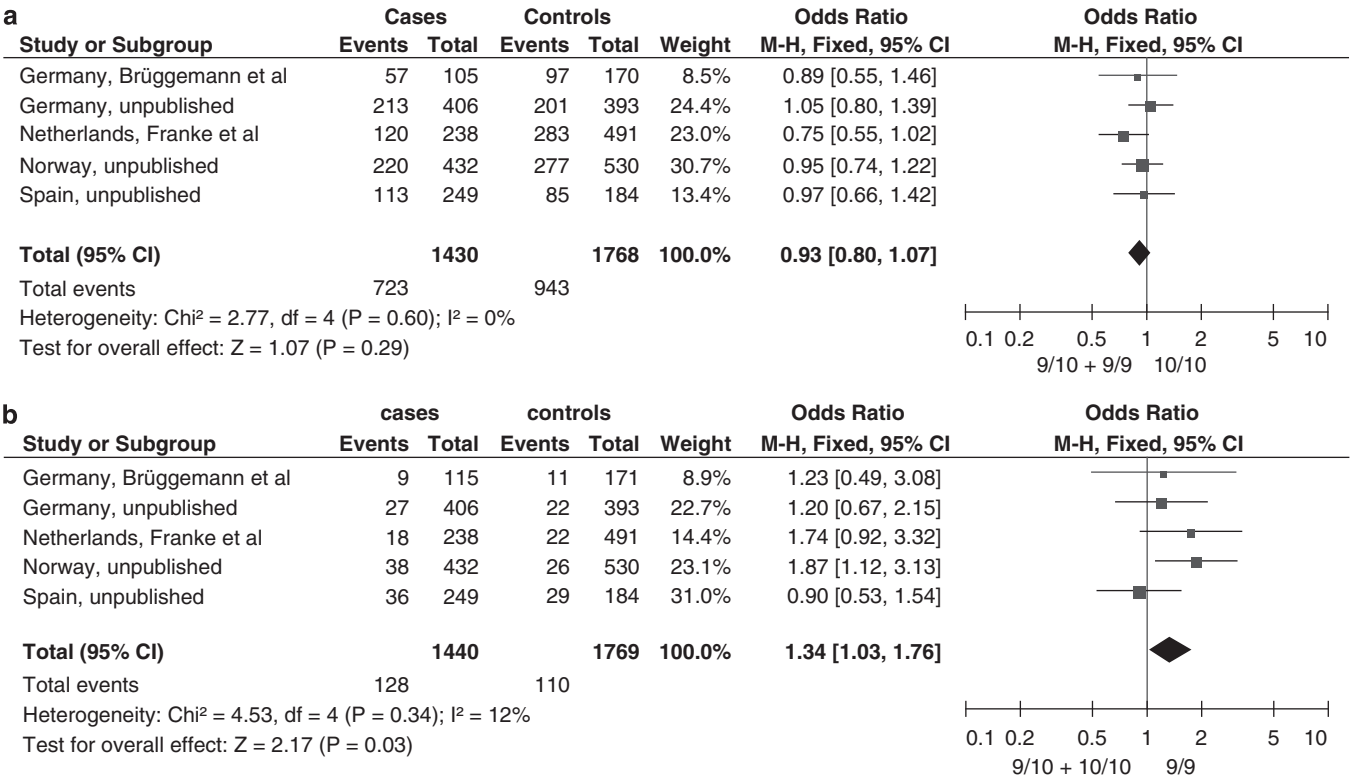

Figure 3 (a) Forest plot showing the analysis of the *DAT1* 3′-UTR VNTR 10/10 genotype *vs* all other genotypes. (b) Forest plot showing the analysis of the *DAT1* 3′-UTR VNTR 9/9 genotype *vs* all other genotypes.

children with ADHD, where the 10-6 haplotype and the 10/10 genotype have been suggested to be risk factors for the disorder (Brookes *et al*, 2006; Asherson *et al*, 2007; Faraone *et al*, 2005). This is not likely to reflect a mere population-specific effect, as the populations represented in this adult study are also represented (apart from the Norwegian sample) in the studies showing the effects of the 10-6 haplotype (eg, in the IMAGE study; Asherson *et al*, 2007). A major difference between most samples of childhood and adult ADHD is the gender distribution. Although in children the male to female ratio lies between 3 : 1 and 9 : 1 (Staller and Faraone, 2006), the gender distribution is much more equal in adults with ADHD (Kessler *et al*, 2006). However, it is unlikely that gender causes the differences in the findings for children and adults, as a male-specific analysis in this study did not change the results presented here (not shown). Another difference between samples of children and adults may be the comorbidity profile. Although comorbidity is the rule rather than the exception in both groups of patients, the prevalence of specific comorbid disorders may differ. This issue is further discussed below.

Despite an impressive number of studies already performed, the role of *DAT1* in ADHD, even in children, is still far from clear. The 3′-UTR VNTR has been investigated in multiple studies, but meta-analyses of the genotyping data are controversial (Maher *et al*, 2002; Faraone *et al*, 2005; Todd *et al*, 2005; Yang *et al*, 2007; Li *et al*, 2006). The same holds true for the current findings: although our meta-analysis included 1440 patients and 1854 controls, the *p*-values we report are only nominally significant (we did not carry out correction for multiple testing for the correlated tests). Hence, for both children and adults, the evidence for an association of the *DAT1* gene with ADHD is still far from reaching genome-wide significance. Apart from the possibility that the gene is simply not associated with ADHD after all, additional explanations for the limited significance of the findings are as follows: first, only a subgroup of patients might show the association with the gene. For example, data from studies by Diamond (2007) suggest that *DAT1* has a more important role in ADHD subtypes featuring hyperactivity symptoms than in the inattentive subtype. In this study, we did not find evidence to support this hypothesis. Also, *DAT1* might be linked to ADHD only in the absence or presence of a given comorbidity. This hypothesis recently found some support from the work within the IMAGE study, in which Zhou *et al* (2008) showed that *DAT1* was only associated with ADHD in children without comorbid conduct problems. This possibility needs to be tested in future studies. A second possible explanation, as mentioned above, is that the VNTRs and their haplotype incompletely tag the real ADHD risk variant in *DAT1*, or that additionally, other variants in or near the gene also exert an effect on ADHD risk. The latter view is again supported by the IMAGE study, which suggests that two different loci within *DAT1*, one 5′ and one 3′ site, influence ADHD risk in children (Brookes *et al*, 2008). As most studies up to now have studied children with the disorder, a third possible explanation is that age is an important factor to take into account when studying the role of *DAT1* in ADHD.

The putative differential association of *DAT1* with ADHD in children and adults might arise from different causes. Possibly, the 9/9 3′-UTR VNTR genotype and/or the 9-6 haplotype predispose(s) to a more severe ADHD phenotype characterized by persistence into adulthood. As only a subgroup of children with ADHD remit (Faraone *et al*, 2006; Barkley *et al*, 2006a; Kessler *et al*, 2005b), this subgroup might not be equally represented in all association studies of childhood ADHD. Some support for this hypothesis is provided by a prospective 13-year follow-up study indicating that more ADHD symptoms and externalizing behaviors were present in the 9/10 than in the 10/10 genotype for the group as a whole, and that the effects of the genotype became more pronounced with increasing age of the participants. Importantly, more individuals with a DSM diagnosis of ADHD in adulthood were found among those having the 9/10 genotype (53%) than among the 10/10 homozygous group (35%) (Barkley *et al*, 2006b). On the other hand, as dopamine transporter density decreases during life (Spencer *et al*, 2005) and ADHD symptoms are known to change during adolescence (Biederman *et al*, 2000), the differential association of *DAT1* with ADHD might reflect changing requirements on the dopaminergic system during life. Furthermore, adults more often than children consume cigarettes, alcohol or drugs, environmental factors that are known to influence the regulation of the dopamine transporter (Madras *et al*, 2005). An additional potential explanation might be that *DAT1* genotype effects on ADHD depend on the effect of another gene, which shows development-specific association with ADHD. A good candidate for this is *COMT*, encoding the catechol-O-methyltransferase, a major contributor to (prefrontal cortex) dopaminergic metabolism. Several recent studies suggest a double dissociation of dopamine effects, depending on *COMT* and *DAT1* genotype in dopamine-related brain activity (Bertolino *et al*, 2008; Yacubian *et al*, 2007). However, an involvement of *COMT* in ADHD has been suggested for both children and adults (eg, Lasky-Su *et al*, 2008; Halleland *et al*, 2008). Future studies of the *DAT1* VNTR haplotype might want to use brain imaging to investigate the neural substrates of the differences between children and adults. One would predict that these substrates are different for the different developmental stages. However, as this study is cross-sectional we cannot exclude the possibility that individuals seeking treatment as children are different from those seeking treatment as adults. This might be suggested by findings of other phenotypes associated with *DAT1*, such as decreased delinquency and promiscuous behavior in teenagers with the 9/9 genotype of the 3′-UTR VNTR (Guo *et al*, 2007).

In conclusion, our data bear the intriguing suggestion that the *DAT1* haplotype and genotype associated with ADHD in adults might be different from the one associated with the childhood disorder. A differential association of the *DAT1* gene with ADHD in children and in adults might help to explain the inconsistencies observed in association studies, where age is not commonly taken into account. However, the data might also imply that the gene has a role in modulating the ADHD phenotype, rather than causing it.

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DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

Supplementary File S1: information on samples and phenotyping

Supplementary Table S1: Instruments used to evaluate the phenotype of patients by the different sites contributing to IMpACT.

	Germany*	Norway**	Spain***	Netherlands****
Clinical interview	X	X	X	X
CAADID-II			X	
WURS	X	X	X	
CAARS			X	
ASRS		X		
ADHD-RS			X	X
ADHD Screening checklist			X	
CGI			X	
SDI			X	
SCID-I	X		X	
SCID-II	X		X	
BDI	X		X	
MDQ		X		
TPQ	X			
NEO PI-R	X			
Fagerström scale	X			
STAI			X	
MCMI-II			X	

*IMpACT Germany:

For this study, 437 patients were ascertained between 2004 and 2007 at the Department of Psychiatry, University of Würzburg. The sample consisted of in- and outpatients. The control group consisted of 418 healthy volunteers (of which 145 were screened for ADHD).

Patients have been extensively examined using an open interview by an experienced psychiatrist (J.R., M.H, A. B.-H., and C.P.J., who supervised the complete ascertainment

procedure) as well as by the Structured Clinical Interview of DSM-IV for axis-I and axis-II disorders (SCID-I, SCID-II) also allowing the registration of co-morbid conditions (60, 61). Severity of A-ADHD was measured by means of the WURS-k interview (62, 63). Where available, chart reviews have been performed and information obtained from relatives, school reports etc. were taken into consideration. Depressive symptoms were rated by the Beck Depression Inventory (BDI (64)). Personality assessment was done using the NEO PI-R and TPQ questionnaires (65, 66); impulsivity was assessed by the I7 questionnaire (67) and nicotine abuse was quantified using the Fagerström scale (68). Eligibility criteria for the study were A-ADHD according to the diagnostic criteria of DSM-IV, onset before the age of 7 years via retrospective diagnosis, life-long persistence, current diagnosis and age at recruitment between 18 and 65 years. Exclusion criteria were the restricted appearance of lack of concentration, hyperactivity, impulsivity to the duration of any other axis-I disorder, as well as current diagnosis of not withdrawn drug/alcohol abuse/dependence, lifetime diagnosis of bipolar-I disorder, schizophrenia, or any other psychotic disorder, and mental retardation (IQ level< 80; MWT-B < 13 points). Further details on the sample can be obtained from Jacob et al. (69). The control group was recruited in the Lower Franconia area in Germany and consisted of 418 healthy volunteers (50% male, mean age ± SD: 33 ± 11 years) of Caucasian origin. While the initial sample of 273 blood donors was not screened for a history of psychiatric disorders, psychiatric illness was ruled out by an extensive clinical interview in a further control sample of 145 subjects.

**IMpACT Norway:

The majority of the 453 patients (n = 338) was recruited from all parts of Norway, using a National Registry of adults diagnosed with ADHD in Norway during 1997-2005, as recently described {Halleland, 2008 940 /id;Johansson, 2007 860 /id;Halmøy, 2008 966 /id}. An additional 115 patients were recruited directly from clinicians during 2005-2007. The control group (n= 548) is comprised of 137 university students, 251 randomly selected people (in the age-range from 18 to 40 years) from the general population and 198 healthy blood donors (the latter not screened for ADHD).

All patients were formally diagnosed with ADHD before inclusion into the project, but subtype data were not systematically available at the time of the primary diagnosis. At the time of inclusion into the genetic study, 73% of the patients were classified as combined subtype, 11% as inattentive and 3% as hyperactive/impulsive subtype using the Adult ADHD Self Report Scale (ASRS, World Health Organization's (WHO) (70)) with a cut-off of 17 or more

on each subscale (10% were diagnosed as sub-threshold). In comparison, the corresponding proportions would have been 52% combined, 17% inattentive and 7% hyperactive/impulsive subtypes (22% sub-threshold) using a cut-off of 21 or more on each subscale. Severity of past and current ADHD symptoms in patients and controls was evaluated using the Wender Utah Rating Scale (WURS; (71)) and the ASRS. Furthermore, the referring clinicians filled in additional data on diagnosis, treatment history and treatment response in patients and both patients and controls filled in the Mood Disorder Questionnaire (MDQ, (72)) and responded to 31 additional questions regarding socio-demographic variables and comorbid conditions in participants and their first degree relatives. The ASRS and WURS versions used in this study have also been used in earlier publications (25, 28, 29).

***IMpACT Spain:

The clinical sample consisted of 360 out-patients with persistent ADHD recruited from the Adult ADHD Program of the Department of Psychiatry of the Hospital Universitari Vall d'Hebron (Barcelona) during 2004-2008 {Ribases, 2008 953 /id;Ribases, 2007 954 /id;Ramos-Quiroga, 2008 955 /id}. The control sample consisted of 195 unrelated blood donors with no DSM-IV ADHD symptoms.

The diagnosis of ADHD in adulthood was evaluated with the Structured Clinical Interview for DSM-IV Axis I and II Disorders (SCID-I and SCID-II (60, 61)) and the Conners Adult ADHD Diagnostic Interview for DSM-IV (CAADID Part II (73)). In addition to this, the following instruments were used to characterize the patients in this cohort: Severity of ADHD symptoms was evaluated with the long version of the Conners' ADHD Rating Scale (self-report form CAARS-S:L and observer form CAARS-O:L (74)), the ADHD Rating Scale (ADHD-RS (75)), the ADHD Screening Checklist (76) and the WURS (63) for retrospective symptomatology. The level of impairment was measured with the Clinical Global Impression (CGI (74)) and the Sheehan Disability Inventory (SDI (77)). For the evaluation of psychiatric symptoms patients filled in the BDI (64), the State Trait Anxiety Inventory (STAI (78)) and the Millon Clinical Multiaxial Inventory (MCMI-II (79)). Full-Scale IQ was estimated with the Vocabulary and Block Design subtests of the WAIS-III (80). Patients also completed the Digit span, Arithmetic, Letter-Number Sequencing and Symbol Search subtests of the WAIS-III, the Conners Continuous Performance Test (CPT (81)) the California Verbal Learning Test (CVLT (82)), the Logical Memory I-II and Visual Memory I-II of the WMS-Rand the Trail-making Test (Parts A and B (83)).

****IMpACT The Netherlands:

Patients (n=275) had been referred for assessment of ADHD to the outpatient clinic of GGZ Delfland in Delft, to Parnassia, psycho-medical centre in The Hague, or to the department of Psychiatry at the Radboud University Nijmegen Medical Centre in Nijmegen, the Netherlands. Most of the patients have been described before {Kooij, 2005 722 /id;Bekker, 2005 704 /id;Franke, 2008 946 /id}. Controls (n=550) were derived from the Nijmegen Biomedical Study (NBS, www.nijmegenbiomedischestudie.nl), a population-based survey conducted by the Departments of Epidemiology & Biostatistics and of Clinical Chemistry of the Radboud University Nijmegen Medical Centre {Hoogendoorn, 2006 725 /id}. The control group was frequency-matched for gender with the patient group.

Subjects were included if a clinical diagnosis of adult ADHD with childhood onset was established. Prior to inclusion, all patients underwent a standard clinical assessment consisting of a psychiatric evaluation by experienced psychiatrists using a semi-structured diagnostic interview for ADHD and comorbid disorders, the Dutch version of structured diagnostic interviews for retrospective diagnosis of ADHD. For current ADHD symptoms during the last 6 months, a Dutch version of the DSM-IV ADHD Rating Scale, (ADHD-RS) based on the 18 DSM-IV items for ADHD, was used (5, 75). The ADHD Rating Scale has been used in epidemiologic and clinical research in adults in the United States and in the Netherlands (5, 84). To be given a full diagnosis of adult ADHD, subjects had to (A) meet at least 6 out of 9 DSM-IV criteria of inattention and/or hyperactivity/impulsivity for a diagnosis of ADHD in childhood and at least 5 out of 9 criteria in adulthood, (B) describe a chronic persisting course of ADHD symptoms from childhood to adulthood, and (C) endorse a moderate to severe level of impairment attributed to the ADHD symptoms. A cut-off point of 5 of 9 criteria was set for adult diagnosis of ADHD based on literature and epidemiological data using the same DSM-IV ADHD Rating Scale (5). In order to obtain information about lifetime ADHD symptoms and impairment, the patient, the partner and if available the parents were interviewed. Information on school reports was examined in order to sustain the diagnosis in childhood. Diagnostic criteria have been described in more detail elsewhere (5, 33). Controls were also evaluated using the ADHD-RS and a cut-off of 4 or more symptoms was used to exclude controls from the study.

Genotyping of the two *DAT1* VNTRs

After DNA isolation by a standard desalting protocol, the German IMpACT sample was genotyped for the 40 base pair VNTR located in the 3' UTR of *DAT1* as previously described

{Hunnerkopf, 2007 903 /id}. The Intron 8 VNTR was genotyped according to a protocol used by the Dutch IMpACT partner, see below.

In Norway, the procedure was as follows: Genomic DNA was extracted either from whole blood, or from saliva using the Oragene™ DNA Self-Collection Kit from DNA Genotek (DNA Genotek Inc, Ontario, Canada) at the HUNT biobank (Levanger, Norway). Cases and controls were mixed on 96-well-plates with a minimum of two internal controls and two blank samples. Genotyping of the 3' UTR VNTR has been described before {Johansson, 2007 860 /id}. For genotyping of the Intron 8 VNTR we used the same primers as described below for the Dutch sample, except that the reverse primer was fluorescently labeled and PCR products were analyzed on an ABI 3100 sequencer using the GeneMapper software (Applied Biosystems). All genotype calls were also manually inspected. The results of genotyping of the 3' UTR VNTR in 358 patients and 340 controls have been reported earlier {Johansson, 2007 860 /id}.

In the Spanish IMpACT sample, the 3' UTR VNTR was genotyped from each DNA sample by PCR in a total volume of 10 µl, containing 75 ng of template DNA, 50 mM KCl, 10mM Tris-HCl, 1.5mM $MgCl_2$, 200 µM of dNTPs, 4pmol of each oligonucleotide and 1.5 U of Taq DNA polymerase. Amplification conditions consisted of an initial 2 min denaturation step at 94ºC, 38 cycles of 1 min at 94ºC, 1 min at 54ºC and 1 min at 72ºC, followed by a final extension of 10 min at 72ºC. Amplification products were resolved in 1.8% agarose gels. The VNTR in intron 8 was genotyped in the lab of the Dutch IMpACT partner, see below.

In The Netherlands, genotyping of the VNTRs in the 3' UTR of *DAT1* was carried out as described before {Brookes, 2006 782 /id;Boonstra, 2007 784 /id}. As for genotyping of the intron 8 VNTR we used a PCR-based method on 20 ng genomic DNA using 0.75 mM of forward (5'-GCTTGGGGAAGGAAGGG-3') and reverse primer (5'-TGTGTGCGTGCATGTGG-3'), respectively, 0.25 mM dNTPs and 6.25 U Taq DNA polymerase (Invitrogen, Breda, The Netherlands) in AmpliTaq PCR-buffer II (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) with 1.75 mM $MgCl_2$. The cycling conditions for amplification involved 10 min at 94 ℃, followed by 30 cycles of 1 min 94℃, 1 min at 64℃ and 1 min 72℃ and an extra 7 min at 72 ℃. Analysis on a 2 % agarose gel yielded distinct bands at 368 bp, 398 bp, 458 bp, 578 bp and 608 bp, with the smallest two bands being the most frequent ones (allele 5 and 6, formerly known as allele 2 and 3). The Intron 8 VNTR was coded as described earlier by Asherson et al. {Asherson, 2007 777 /id}, describing the number of repeats present in the repeat allele. Both genotyping assays have been validated earlier and 5% duplicates and blanks were taken along as quality controls during genotyping.

Supplementary Table S2: Demographics of the IMpACT patients

	IMpACT Germany	IMpACT Norway	IMpACT Spain	IMpACT The Netherlands
Number of subjects	406	432	249	238
Age (mean and range)	34 years (18-65)	34 years (18-71)	30 years (18-60)	35 years (18-62)
Males	53%	53%	73%	48%
Educational level				
Lower educational level	219 (63%)**	86 (20%)	85 (34%)	51 (21%)
High School	39 (11%)**	183 (42%)	101 (41%)	132 (56%)
University	71 (20%)**	99 (23%)	63 (25%)	15 (6%)
Unknown	20 (6%)**	63 (15%)	0 (0%)	40 (17%)
DSM IV axis I disorder				
ADHD combined type	262 (65%)	316 (73%)	160 (64%)	190 (80%)
ADHD hyperactive/impulsive type	34 (8%)	14 (3%)	10 (4%)	8 (3%)
ADHD inattentive type	107 (26%)	47 (11%)	79 (32%)	23 (10%)
unknown	3 (0.7%)	55 (13%)***	0 (0%)	17 (7%)
Any comorbid axis I disorder*	293 (84%)**	331 (77%)[1)]	163 (65%)	183 (82%)[4)]
Multiple (≥ 2) comorbid axis I disorders*	209 (60%)**	149 (34%)[1)]	56 (23%)	94 (42%)[4)]
Any mood disorder*	200 (57%)**	289 (67%)[1)2)]	83 (33%)	132 (59%)[4)]
Any anxiety disorder*	92 (27%)**	289 (67%)[1)2)]	63 (25%)	74 (33%)[4)]
Any substance use disorder*	157 (45%)**	44 (10%)	82 (33%)	55 (25%)[4)]
Bulimia Nervosa*	20 (6%)**	n.d.	3 (1%)	8 (3%)[4)]
Bipolar Disorder*	Excluded	46 (10%)[1)]	1 (0.4%)	n.d.
Co-morbid Borderline Personality Disorder	n.d.	n.d.	7 (3%)	34 (16%)[5)]

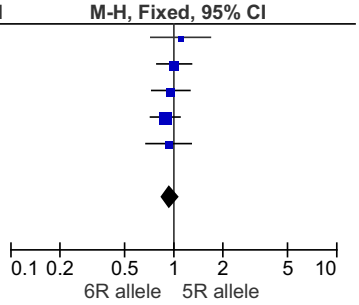
* past or present; ** data are available for 349 patients; *** of these, 43 subjects scored below threshold and 12 individuals had no information available.
[1)] self-report data
[2)] depression and/or anxiety
[3)] alcohol and/or other drugs
[4)] information not available for 14 patients
[5)] information not available for 26 patients

Supplementary Figure S1

A Forest plot of the analysis of the *DAT1* Intron 8 VNTR 6-repeat versus 5-repeat allele.

Study or Subgroup	cases Events	cases Total	controls Events	controls Total	Weight	Odds Ratio M-H, Fixed, 95% CI
Germany, Brüggemann et al	47	226	65	338	7.5%	1.10 [0.72, 1.68]
Germany, unpublished	165	812	159	786	23.5%	1.01 [0.79, 1.28]
Netherlands, Franke et al	100	476	213	982	20.1%	0.96 [0.73, 1.25]
Norway, unpublished	205	864	275	1060	34.4%	0.89 [0.72, 1.09]
Spain, unpublished	114	498	89	368	14.4%	0.93 [0.68, 1.28]
Total (95% CI)		**2876**		**3534**	**100.0%**	**0.95 [0.84, 1.07]**
Total events	631		801			

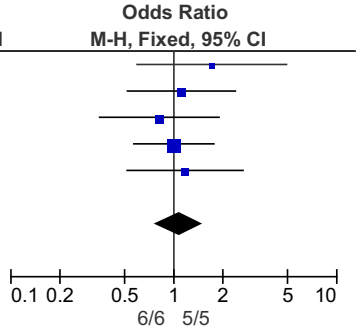
Heterogeneity: Chi² = 1.12, df = 4 (P = 0.89); I² = 0%
Test for overall effect: Z = 0.79 (P = 0.43)



B Forest plot of the analysis of the *DAT1* Intron 8 VNTR genotype 6/6 versus 5/5.

Study or Subgroup	cases Events	cases Total	controls Events	controls Total	Weight	Odds Ratio M-H, Fixed, 95% CI
Germany, Brüggemann et al	8	82	7	118	8.0%	1.71 [0.60, 4.93]
Germany, unpublished	15	271	13	260	19.3%	1.11 [0.52, 2.39]
Netherlands, Franke et al	8	154	20	318	19.1%	0.82 [0.35, 1.90]
Norway, unpublished	25	277	28	311	37.0%	1.00 [0.57, 1.76]
Spain, unpublished	17	169	10	115	16.5%	1.17 [0.52, 2.67]
Total (95% CI)		**953**		**1122**	**100.0%**	**1.07 [0.77, 1.51]**
Total events	73		78			

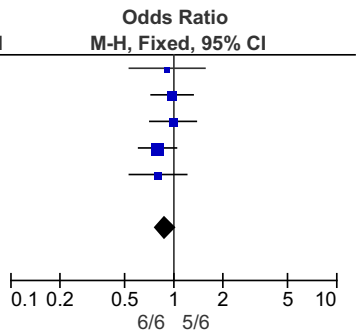
Heterogeneity: Chi² = 1.27, df = 4 (P = 0.87); I² = 0%
Test for overall effect: Z = 0.41 (P = 0.68)



C Forest plot of the analysis of the *DAT1* Intron 8 VNTR genotype 6/6 versus 5/6.

Study or Subgroup	cases Events	cases Total	controls Events	controls Total	Weight	Odds Ratio M-H, Fixed, 95% CI
Germany, Brüggemann et al	31	105	51	162	7.8%	0.91 [0.53, 1.56]
Germany, unpublished	135	391	133	380	24.4%	0.98 [0.73, 1.32]
Netherlands, Franke et al	84	230	173	471	19.9%	0.99 [0.71, 1.37]
Norway, unpublished	155	407	219	502	33.6%	0.79 [0.61, 1.04]
Spain, unpublished	80	232	69	174	14.3%	0.80 [0.53, 1.20]
Total (95% CI)		**1365**		**1689**	**100.0%**	**0.89 [0.76, 1.03]**
Total events	485		645			

Heterogeneity: Chi² = 1.77, df = 4 (P = 0.78); I² = 0%
Test for overall effect: Z = 1.54 (P = 0.12)



CAPÍTOL 3c: TDAH i sistema dopaminèrgic

Article 5

Explorant *DRD4* i la seva interacció amb *SLC6A3* com a possibles factors de risc pel TDAH: meta-anàlisi en quatre poblacions europees

RESUM

Antecedents i propòsit: S'han dut a terme un gran nombre d'estudis d'associació cas-control entre el gen *DRD4* que codifica el receptor dopaminèrgic D4 i el TDAH, tot i que no s'ha arribat a un consens quant a la seva participació, de forma individual o en interacció amb el gen *SLC6A3/DAT1*, en el risc de patir TDAH. Les variants polimòrfiques més estudiades han estat dos polimorfismes funcionals, una duplicació/delecció de 120pb situada a la regió promotora del gen i un VNTR de 48 pb situat a l'exó 3. L'objetiu d'aquest treball ha estat avaluar el paper d'aquests dos polimorfismes del gen *DRD4* en el TDAH en població adulta.

Mètodes: Estudi de meta-anàlisi de tipus cas-control en una mostra de 1608 pacients adults amb TDAH i 2352 controls de quatre poblacions europees (Espanya, Holanda, Alemanya i Noruega).

Resultats: Tot i que l'anàlisi de marcadors individuals no va mostrar associació entre TDAH i el gen *DRD4,* l'anàlisi d'haplotips va detectar associació entre TDAH de subtipus combinat i l'haplotip L-4R (dup120pb-VNTR48pb; p=0,02). A més, l'anàlisi de regressió logística no va mostrar evidències d'efectes epistàtics entre els gens *DRD4* i *SLC6A3* en l'etiologia del trastorn .

Conclusions: L'estudi de meta-anàlisi dut a terme recolza la contribució del gen *DRD4* al TDAH en població adulta.

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Exploring *DRD4* and its interaction with *SLC6A3* as possible risk factors for ADHD: A meta-analysis in four European populations.

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Exploring *DRD4* and its interaction with *SLC6A3* as possible risk factors for adult ADHD: A meta-analysis in four European populations

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Keywords: Attention-deficit hyperactivity disorder, case-control association study, neurotransmission, dopamine, psychiatric genetics

ABSTRACT

Attention-deficit hyperactivity disorder (ADHD) is a common behavioural disorder affecting about 4-8% of children. ADHD persists into adulthood in around 65% of cases, either as the full condition or in partial remission with persistence of symptoms. Pharmacological, animal and molecular genetic studies support a role for genes of the dopaminergic system in ADHD due to its essential role in motor control, cognition, emotion and reward. Based on these data, we analyzed two functional polymorphisms within the *DRD4* gene (120-bp duplication in the promoter and 48-bp VNTR in exon 3) in a clinical sample of 1,608 adult ADHD patients and 2,352 controls of Caucasian origin from four European countries that had been recruited in the context of the International Multicentre persistent ADHD CollaboraTion (IMpACT). Single-marker analysis of the two polymorphisms did not reveal association with ADHD. In contrast, multiple-marker meta-analysis showed a nominal association (P=0.02) of the L-4R haplotype (dup120bp-48bpVNTR) with adulthood ADHD, especially with the combined clinical subtype. Since we previously described association between adulthood ADHD and the dopamine transporter *SLC6A3* 9R-6R haplotype (3'UTR VNTR-intron 8 VNTR) in the same dataset, we further tested for gene x gene interaction between *DRD4* and *SLC6A3*. However, we detected no epistatic effects but our results rather suggest additive effects of the *DRD4* risk haplotype and the *SLC6A3* gene.

INTRODUCTION

Attention-deficit hyperactivity disorder (ADHD) is a common behavioural disorder affecting 4-8% of children [Polanczyk et al., 2007] and is characterized by inappropriate and impairing levels of hyperactivity, impulsivity and inattention. ADHD persists into adulthood in around 65% of cases either as the full condition or in partial remission with persistence of symptoms and with significant clinical impairment [Faraone et al., 2006; Lara et al., 2009]. A review of 20 twin studies in children estimated the heritability of ADHD to be around 76% [Faraone et al., 2005; Faraone and Mick., 2010].

Pharmacological studies, animal models and molecular genetic studies support a role for genes of the dopamine, serotonin and norepinephrine neurotransmitter systems in ADHD. Research, however, has mainly focused on the dopaminergic system due to its essential role in motor control, cognition and reward. In this regard, magnetic resonance imaging suggests abnormalities in ADHD-affected children in neuro-anatomical areas rich in dopaminergic innervations [Durston et al., 2005; Ernst et al., 1999]. In addition, methylphenidate, amphetamine and other psychostimulant drugs increase synaptic levels of dopamine and are effective in the control of ADHD symptoms through blockade of the dopamine transporter.

Among the different genes involved in dopaminergic neurotransmission, the dopamine receptor D4 (*DRD4*) has been widely considered in genetic studies of ADHD. The *DRD4* gene is located at chromosome 11p15.5, organized in 4 exons and encodes a G protein-coupled receptor belonging to the dopamine D2-like receptor family. This family of receptors is expressed predominantly in the prefrontal cortex. *DRD4* contains a number of polymorphisms, one of which, a variable number of 48-bp tandem repeats (48bpVNTR), is located in the third exon of the gene. This exon encodes the putative third cytoplasmic loop of the receptor and modulates the receptor's signal transduction properties by altering intracellular cyclic AMP levels [Van Tol et al., 1992]. This polymorphism, with a unit that is repeated from 2 (2R) to 11 (11R) times, shows considerable inter-ethnic heterogeneity [Chang et al., 1996; Van Tol et al., 1992]. Although its effect on the DRD4 function is still unknown, different pharmacological properties have been described for the distinct repeat lengths, with the 7R allele dampening the response of cells to dopamine [Asghari et al., 1995]. Other studies, however, showed no evidence of quantitative differences in G protein coupling among the *DRD4* 2R, 4R and 7R alleles [Kazmi et al., 2000].

Since the publication of the initial study by LaHoste et al. [1996] showing association between the 7R allele of the 48bpVNTR and ADHD in children, many case-control and

family-based association studies have been reported, although they show controversial results. After the first studies that identified the 7R as a risk allele for ADHD, most of the subsequent work solely tested this allele versus all the others [Castellanos et al., 1998; Comings et al., 1999; Curran et al., 2001; Gabriela et al., 2009; Langley et al., 2004; Mill et al., 2001; Muglia et al., 2000; Tahir et al., 2000]. Other studies differentiated between short (2R to 5R) and long alleles (6R to 11R) [Eisenberg et al., 2000; Hawi et al., 2000; Kotler et al., 2000; Manor et al., 2002]. However, only some of these studies focused on adult ADHD [Johansson et al., 2008; Muglia et al., 2000; Smith et al., 2003]. Johansson et al. [2008] found no association between the *DRD4* VNTR polymorphism and adulthood ADHD. On the other hand, Muglia et al. [2000] suggested a role of the 7R allele in adult ADHD and, finally, although performed in childhood ADHD samples, several meta-analyses have demonstrated a significant association between this *DRD4* allele and ADHD [Faraone et al., 2001; Faraone et al., 2005; Gizer et al., 2009; Li et al., 2006; Nikolaidis and Gray, 2009; Smith, 2010]. However, some of these meta-analyses detected significant heterogeneity in the effect size for *DRD4* in the different studies [Gizer et al., 2009; Li et al., 2006; Smith, 2010].

Seaman et al. [1999] subsequently identified a second common genetic variant in *DRD4*, the 120-bp duplication (dup120bp) located 1.2 kb upstream of the *DRD4* translation initiation codon. The duplication contains consensus sequences for several transcription factors and modulates transcription of the *DRD4* gene. In this regard, the 240-bp allele (long or L allele) showed enhanced binding capacity for the Sp1 transcription factor in a mobility shift assay and exhibited lower transcriptional activity than the 120-bp allele (short or S allele) in transfected cell lines [D'Souza et al., 2004; Kereszturi et al., 2007; Ronai et al., 2004]. Several studies have tested association between the *DRD4* dup120bp polymorphism and ADHD but results are also controversial, showing no association [Barr et al., 2001; Bhaduri et al., 2006; Brookes et al., 2005; Gizer et al., 2009; Todd et al., 2001] or identifying either the L or S alleles as risk factors for ADHD [Kereszturi et al., 2007; Kustanovich et al., 2004; McCracken et al., 2000]. Accordingly, a recent meta-analysis performed by Gizer et al. [2009] found no association between childhood ADHD and either allele. So far, Arcos-Burgos et al. [2004] were the only researchers to study the role of a haplotype of both polymorphisms in ADHD and indeed found significant evidence for association with the S-7R *DRD4* haplotype.

Since both the 48bpVNTR and the dup120bp polymorphisms in *DRD4* may affect the receptor function through changes in amino acid sequence or promoter activity and to challenge the inconsistencies raised among previous case-control and family-based association studies, we aimed to investigate the possible involvement of these

polymorphisms in ADHD in a large sample of adult patients and controls from Europe. We performed a meta-analysis of unpublished case-control data from four different countries (Germany, The Netherlands, Norway and Spain) integrated in the International Multicentre persistent ADHD CollaboraTion (IMpACT) in a sample of 1,608 adult ADHD patients and 2,352 controls. Also, as several studies have shown epistatic [Gabriela et al., 2009; Roman et al., 2001] or additive effects [Carrasco et al., 2006] between *DRD4* and *SLC6A3* in ADHD or hyperactive-impulsive symptoms [Auerbach et al., 2010], we used previously published *SLC6A3* genotype data from IMpACT [Franke et al., 2010] to assess their potential combined contribution to adulthood ADHD.

MATERIALS AND METHODS

Patients and Controls

In total, 1,608 adult ADHD patients and 2,352 controls of Caucasian origin from four European countries (Spain, Germany, Norway and The Netherlands) were recruited at four sites of IMpACT. Table I shows the clinical description of these patient cohorts. Diagnosis was blind to genotype. The study was approved by the ethics committee of each participating institution and informed consent was obtained from all subjects in accordance with the Helsinki Declaration.

Consensus eligibility criteria for the current study across all sites were a diagnosis of ADHD according to the diagnostic criteria of DSM-IV (Diagnostic and Statistical Manual for Mental Disorders-IV), onset before the age of 7 years via retrospective diagnosis (which was confirmed by a family member, wherever possible), lifelong persistence and current diagnosis. Patients were extensively examined by psychiatrists experienced in adult ADHD and were evaluated for other psychiatric disorders with the Structured Clinical Interview of DSM-IV for axis-I (and axis-II) disorders (SCID-I, SCID-II) or semi-structured interviews. Most controls (except for the Norwegian samples and part of the German samples) were screened for the presence of ADHD and those scoring high on symptoms of the disorder were excluded. For a more detailed description of the different diagnostic instruments see Sánchez-Mora et al. [Sanchez-Mora et al., 2009].

Table I. Descriptive characteristics of the IMpACT samples from four European countries.

	Germany	Netherlands	Norway	Spain	Pool	DRD4 Dup120bp	DRD4 48bp VNTR	DRD4 Dup120bp and 48bp VNTR
Controls								
Female	307 (53.6)	248 (50.7)	325 (55.6)	245 (34.8)	1125 (47.8)	872 (42.3)	813 (44.2)	845 (46.0)
Male	266 (46.4)	241 (49.3)	260 (44.4)	460 (65.2)	1227 (52.2)	1190 (57.7)	1027 (55.8)	990 (54.0)
Total	**573 (24.36)**	**489 (20.79)**	**585 (24.87)**	**705 (29.97)**	**2352**	**2062**	**1840**	**1835**
Age (mean and SD)	30.73 (9.8)	63.46 (18.07)	27.4 (7.16)*	45.26 (14.79)				
Cases								
Female	304 (48.8)	119 (50.0)	215 (47.4)	82 (28.0)	720 (44.8)	644 (44.5)	613 (43.6)	537 (43.2)
Male	319 (51.2)	119 (50.0)	239 (52.6)	211 (72.0)	888 (55.2)	803 (55.5)	794 (56.4)	709 (56.8)
Total	**623 (38.7)**	**238 (14.8)**	**454 (28.2)**	**293 (18.22)**	**1608**	**1447**	**1407**	**1246**
Age (mean and SD)	33.91 (10.12)	41.23 (11.35)	33.9 (11.6)	36.02 (16.83)				
ADHD subtype								
Combined type	422 (68.3)	202 (86.0)	327 (72,03)	194 (66.2)	1145 (71.2)	1037 (71.7)	991 (70.4)	883 (71.0)
Inattentive type	151 (24.4)	24 (10.2)	47 (10,35)	87 (29.7)	309 (19.2)	279 (19.3)	275 (19.5)	243 (19.5)
Hyperactivity/impulsive type	45 (7.3)	9 (3.8)	15 (3,30)	12 (4.1)	81 (5.04)	67 (4.6)	72 (5.1)	58 (4.7)
Sub-threshold	-	-	45 (9,91)	-	45 (2.8)	44 (3.0)	45 (3.2)	44 (3.5)
Unknown	-	-	20 (4,41)	-	20 (1.2)	20 (1.38)	20 (1.42)	16 (1.04)

* Calculated only for non-blood donors (n=365)

DNA Isolation and Genotyping

Genomic DNA was isolated either from saliva using the Oragene DNA Self-Collection Kit (DNA Genotek Inc., Canada) or from peripheral blood lymphocytes by the salting-out procedure [Miller et al., 1988].

Dup120bp DRD4 polymorphism:

Genotyping was carried out using standard PCR methods and amplification products were tested by electrophoresis on a 1.5% agarose gel and ethidium bromide staining (Germany, Spain and The Netherlands) or visualized on an ABI 3100 sequencer and automatically called using the GeneMapper software (Applied Biosystems) (Norway). Genomic DNA was amplified with primers 5'-GTTGTCTGTCTTTTCTCATTGTTTCCATTG-3' and 5'-GAAGGAGCAGGCACCGTGAGC-3' for the Spanish, German and Dutch samples and with a fluorescently labelled (FAM) reverse primer for the Norwegian samples. For the German, Spanish and Norwegian samples, PCR reactions were carried out in a final volume of 10 µl, containing 5 ng of genomic DNA, 0.5 pmol of each primer, 1µl PCRx Enhancer solution (10x; PCRx Enhancer System, Invitrogen), 1 µl PCRx Amplification Buffer (PCRx Enhancer System, Invitrogen), 0.2 µM of each dNTP, 0.5 mM $MgSO_4$ and 1 U of Taq polymerase. Amplification conditions consisted of an initial denaturation at 94º C for 1 min followed by 34 cycles of denaturation at 94º C for 1 min, annealing at 56.5º C for 1 min and extension at 72º C for 1 min, with a final extension step at 72ºc for 10 min. The amplification yielded distinct bands at 429 bp (short "S" allele) and 549 bp (long "L" allele). For Dutch samples, genotyping of the 120-bp tandem duplication

polymorphism was carried out using a PCR-based method as described by [Seaman et al., 1999]. PCR was performed on 62.5 ng genomic DNA using 0.4 μM of each of the primers described above, 0.25 mM dNTPs, 0.5 U Taq DNA polymerase (Invitrogen, Breda, The Netherlands) in a PCR buffer containing 10 mM Tris-HCl pH 8.0, 50 mM KCl, 0.1% Triton X-100 (v/v), 0.015% gelatin (w/v), 5% DMSO (v/v) and 1.5 mM $MgCl_2$. The cycling conditions were 10 min 92℃ followed by 35 cycles of 1 min 95℃, 1 min 58℃, 1 min 72℃. At the end of the protocol, 10 min at 72℃ were added.

48-bp VNTR DRD4 polymorphism:

Genotyping of the *DRD4* 48-bp VNTR polymorphism in exon 3 was performed according to the method used by Johansson et al. [2008] for samples from Norway and Spain. A protocol for PCR amplification and fragment analysis is available upon request. In short, DNA was amplified with the following primers: 5-CGTACTGTGCGGCCTCAACGA-3 and FAM-5-GACACAGCGCCTGCGTGATGT-3. The reverse primer was fluorescently labeled and PCR products were visualized on an ABI 3100 sequencer and automatically called using the GeneMapper software (Applied Biosystems). All genotype calls were also manually inspected. Analysis of the results using Genemapper showed fragment length at 598 bp (2 repeats), 646 bp (3 repeats), 694 bp (4 repeats), 742 bp (5 repeats), 790 bp (6 repeats), 836 bp (7 repeats), 884 bp (8 repeats), 930 bp (9 repeats) and 976 bp (10 repeats).

For the Dutch sample, the 48-bp repeat polymorphism was analyzed by simple sequence analysis on a genetic analyzer using primers as described earlier [Lichter et al., 1993]. For the PCR amplification, 50 ng of genomic DNA, 1.25 μM fluorescent labeled forward primer (5'-Vic-GCGACTACGTGGTCTACTCG-3') and 1.25 μM reverse primer with PIG tail (5'-AGGACCCTCATGGCCTTG-3'), 0.4 mM dNTPs, 1x GCI Buffer TaKaRa (Lucron Bioproducts BV, Gennep, The Netherlands), 0.5 U TaKaRa LA Taq™ (Lucron Bioproducts BV) and 1M betaine were used. The cycling conditions were 1 min 94℃ followed by 35 cycles of 30 sec 94℃, 30 sec 58℃, 1 min 72℃, with a final 5 min step at 72℃. The PCR product was diluted 10 times and 1 μl of the diluted PCR product together with 9.7 μl formamide and 0.3 μl GeneScan-600 Liz Size StandaardTM (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands) was analyzed on a 3730 Genetic Analyzer according to the protocol of the manufacturer. Analysis of the results using Genemapper showed fragment length at 378 bp (2 repeats), 426 bp (3 repeats), 474 bp (4 repeats), 522 bp (5 repeats), 570 bp (6 repeats), 618 bp (7 repeats) and 666 bp (8 repeats).

For the German sample, PCR amplification was performed using Vent polymerase (New England Biolabs: Ipswich Massachusetts, USA) and a high denaturing temperature (98 ℃ for 1 min) with a combined annealing and extension reaction for 5

min at 70 ℃. The primers were 5'-GCGACTACGTGGTCTAC TCG-3' and 5'-AGGACCCTCATGGCCTTG-3'. Thirty PCR cycles were performed and subsequently, the reaction mixture was electrophoresed on a 2% Metaphor gel (FMC) with ethidium bromide. The lengths of the resulting PCR products are the same as describe above for the Dutch sample.

Statistical analysis

Separate analysis of the four European samples:

We first performed a single- and multiple-marker analysis on the samples from the four IMpACT sites, separately, and then analyzed the pooled sample using a meta-analytical approach. Hardy-Weinberg Equilibrium (HWE) in the control groups from each IMpACT node as well as in the whole control sample was assessed using a χ^2 test with the HWE software v.1.05 (linkage.rockefeller.edu/soft/linkutil). Genotype and allele frequencies of the dup120bp and 48bpVNTR polymorphisms were compared between cases and controls from each separate IMpACT site using a χ^2 test with the SNPassoc R package and the statistical package SPSS 15.0, respectively [Gonzalez et al., 2007]. Since the 48bpVNTR polymorphism is multiallelic (from 2R to 11R alleles), rare (minor allele frequency <5%) genotypes and alleles were grouped in a single class as “others”. Haplotype frequencies were estimated using the PHASE software [Stephens et al., 2001] and values below 5% were grouped as “others” in the association analysis.

Meta-analysis:

To combine the individual results, we conducted a meta-analysis using the Meta R package (cran.r-project.org/web/packages/meta/index.html). The analysis of the minimal statistical power was performed post hoc using the Genetic Power Calculator software (pngu.mgh.harvard.edu/~purcell/gpc), assuming a dominant model, an odds ratio (OR) of 1.5, prevalence of 0.05, significance level of 0.05 and a minor allele frequency (MAF) of 0.158 [Purcell et al., 2003]. We first tested heterogeneity among studies using the Q-statistic [Lau et al., 1997; Zintzaras and Hadjigeorgiou, 2004]. When no heterogeneity was present, the pooled OR was estimated using a fixed-effects model [Mantel and Haenszel, 1959]. A random-effect model was considered in those cases where heterogeneity was detected [Laird and Mosteller, 1990]. Meta-analysis was performed considering the whole ADHD sample, but also split by gender or ADHD clinical subtypes. The hyperactive-impulsive clinical sample could not be evaluated due to insufficient sample size.

For the dup120bp polymorphism, we determined the best genetic model to be used by estimating the three possible ORs and their 95% confidence intervals (CI) in the meta-

analysis sample: OR1 (SS vs LL), OR2 (SS vs LS) and OR3 (LS vs LL). If OR1 = OR3 ≠ 1 and OR2 = 1, then a recessive model is suggested; OR1 = OR2 ≠ 1 and OR3 = 1 indicates a dominant model and OR1 > OR3 > 1 (or OR1 < OR2 < 1 and OR1 < OR3 < 1) suggests a codominant model. When none of the OR values significantly deviated from 1, then meta-analyses were performed for the three different genetic models.

For the 48bpVNTR we minimized multiple-testing by restricting the meta-analysis to alleles or genotypes showing frequency differences > 3% among cases and controls in at least one of the study populations. Thus, alleles 4R and 7R, and genotypes 4R4R, 4R7R and 7R7R were considered for meta-analysis. For the analysis of the dup120bp/48bpVNTR haplotype we only considered the carriers of the three major allelic combinations, S-4R, L-4R and L-7R. The EH software was used to test the presence of linkage disequilibrium between the two *DRD4* polymorphisms [Terwilliger and Ott, 1994].

*DRD4*SLC6A3 interaction analysis*

A logistic regression analysis was used to evaluate the independent and interactive effects of the *DRD4* and *SLC6A3* loci. For *DRD4* we considered the haplotype made up of the dup120bp L allele and the 4R allele of the 48bpVNTR polymorphism (L-4R), while for the *SLC6A3* gene we considered the previously described risk haplotype comprising the 9R allele of the VNTR in the 3'UTR and the 6R allele of the VNTR in intron 8 (9R-6R), using pre-existing genotype data on our multicenter cohort of patients and controls [Franke et al., 2010]. A stepwise logistic regression procedure was implemented to compare two different regression models by Likelihood-Ratio Test using the statistical package SPSS 15.0. In the first model we considered the affection status as a dependent variable and the *DRD4* and *SLC6A3* haplotypes as predictive variables. In the second model we included the interaction *DRD4*SLC6A3* as an independent variable.

RESULTS

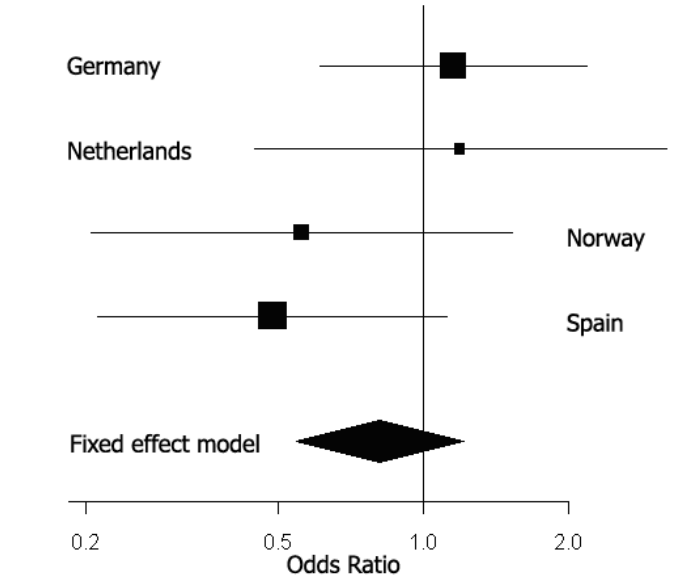
A total of 1,608 adult ADHD patients and 2,352 controls from four IMpACT sites were genotyped for the *DRD4* dup120bp and/or the 48bpVNTR polymorphisms. The clinical description of the samples included in the study is shown in Table I.

Single-marker analysis

For the *DRD4* dup120bp polymorphism, genotypes from 1,447 patients (90%) and 2,062 controls (88%) were available for the single-marker analysis. No significant departure from HWE was observed, neither in the control group from each population nor in the pooled sample ($p>0.05$). No significant association was detected when we compared genotype and allele frequencies between cases and controls from each separate site (Supplementary Table SI). Stratification of the ADHD samples according to gender or clinical subtype in the four separate populations resulted in a nominal association between the L allele of the dup120bp polymorphism and ADHD in males from Norway ($p=0.009$, OR=1.69 (1.14-2.56); Supplementary Table SII). Nominal association was also observed in two samples when we considered the combined clinical subtype (Supplementary Table SIII): Norway ($p=0.04$, OR=1.40 (1.01-1.96)) and Spain ($p=0.04$, OR=2.63 (0.91-7.69)). The evaluation of the best genetic model for the meta-analysis showed that none of the three ORs in the pooled sample significantly deviated from 1 ($OR1_{(SS\ VS\ LL)}$ = 1.23 (95%CI: 0.82-1.84), $OR2_{(SS\ VS\ LS)}$ =1.23 (0.81-1.86) and $OR3_{(LS\ VS\ LL)}$ =1.03 (95% CI: 0.88-1.20)) and, thus, we performed meta-analysis considering the dominant, recessive and codominant models. However, no significant association was found for dup120bp in the full ADHD sample (Table II), or when patients were subdivided by gender or clinical subtypes, in either of the three models (data not shown).

Table II. Genetic effect of the *DRD4* dup120bp polymorphism on adulthood ADHD: (a) using a dominant model (LS+LL vs SS), (b) recessive model (LL vs LS+SS) and (c) overdominant model (LS vs LL+SS).

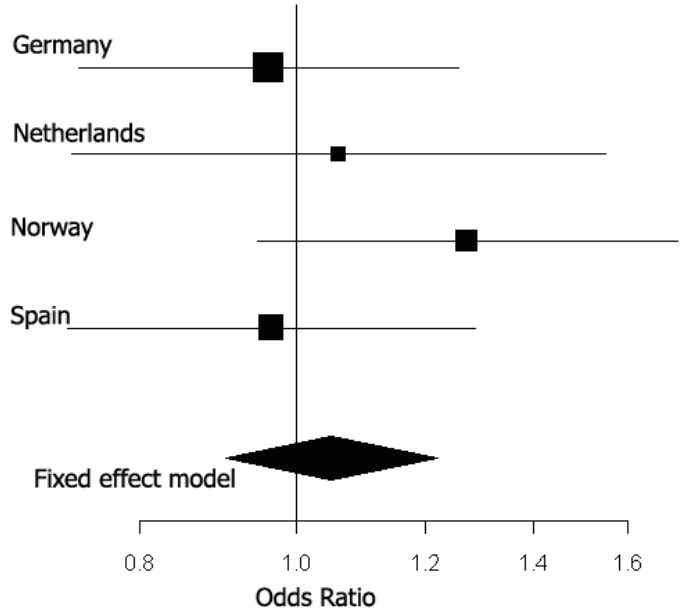
(a)



	Cases		Controls					
Study	**SS**	**Total**	**SS**	**Total**	**Weight (%)**	**OR M-H, Fixed,95% CI**	z	p
1. Germany	22	515	18	483	32.82	1.15 (0.61-2.17)		
2. Netherlands	6	189	13	485	13.03	1.19 (0.44-3.17)		
3. Norway	6	448	11	464	19.68	0.55 (0.20-1.52)		
4. Spain	7	295	30	630	34.48	0.48 (0.20-1.12)		
Fixed -effects model	**41**	**1447**	**72**	**2062**	**100**	**0.81 (0.54-1.21)**	**-1.02**	**0.30**

Test of Heterogeneity: Q= 3.74, df= 3, p= 0.29; I^2= 19.7% (0%-87.7%)

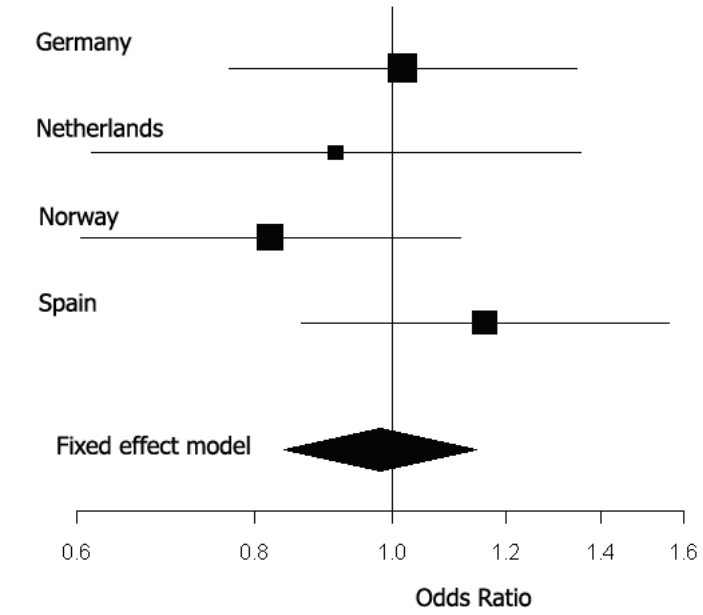
(b)



Study	Cases LL	Cases Total	Controls LL	Controls Total	Weight (%)	OR M-H, fixed,95% CI	z	p
1. Germany	356	515	338	483	32.69	0.96 (0.73-1.25)		
2. Netherlands	139	189	351	485	15.81	1.06 (0.72-1.55)		
3. Norway	344	448	355	464	23.19	1.27 (0.94-1.71)		
4. Spain	190	295	411	630	28.32	0.96 (0.72-1.28)		
Fixed -effects model	**1029**	**1447**	**1455**	**2062**	**100**	**1.05 (0.90-1.22)**	**0.63**	**0.52**

Test of Heterogeneity: Q=2.36, df= 3, p=0.50; I^2= 0% (0%-80.5%)

c)



Study	Cases LS	Cases Total	Controls LS	Controls Total	Weight (%)	OR M-H, fixed,95% CI	z	p
1. Germany	137	515	127	483	30.13	1.01 (0.76-1.34)		
2. Netherlands	44	189	121	485	16.30	0.91 (0.61-1.35)		
3. Norway	98	448	118	464	28.36	0.82 (0.60-1.11)		
4. Spain	98	295	189	630	25.21	1.16 (0.86-1.56)		
Fixed -effects model	**377**	**1447**	**555**	**2062**	**100**	**0.98 (0.83-1.14)**	**-0.24**	**0.80**

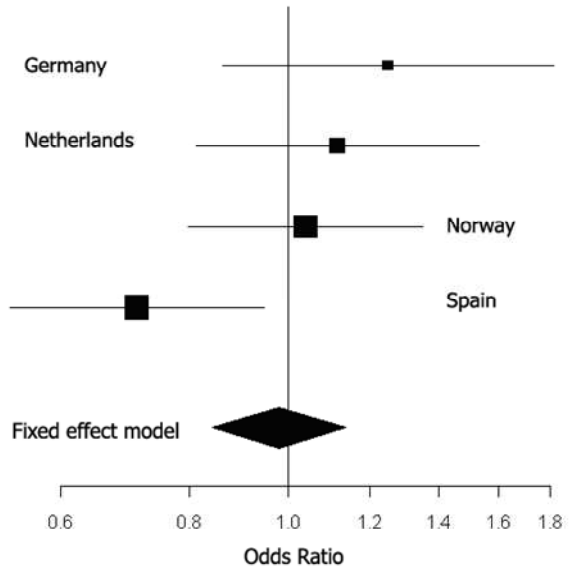
Test of Heterogeneity: Q=2.72 , df= 3, p= 0.43; I^2= 0% (0%-83.1%)

S: Short allele of the *DRD4* dup120bp polymorphism (120bp); L: Long allele of the *DRD4* dup120bp polymorphism (240bp)

A total of 1,407 patients (87.5%) and 1,840 controls (78.2%) had genotypes available for the *DRD4* 48bpVNTR polymorphism. Genotype frequencies did not deviate significantly from HWE in any of the cohorts ($p>0.05$). No significant association between the 48bpVNTR and ADHD was identified in any of the European samples, when studied separately (Supplementary Table SIV). As described above (see Materials and Methods), only 4R/4R, 4R/7R and 7R/7R genotypes were available for the meta-analysis. No association between the VNTR polymorphism and adult ADHD was seen in the full ADHD sample (Table III), even if gender or clinical subtypes were taken into account (data not shown).

Table III. Genetic effect of the *DRD4* 48bpVNTR polymorphism on adulthood ADHD: (a) Comparison of the 4R4R genotype vs others (b) Comparison of 7R7R genotypes vs others and (c) Comparison of the 4R7R genotypes vs others.

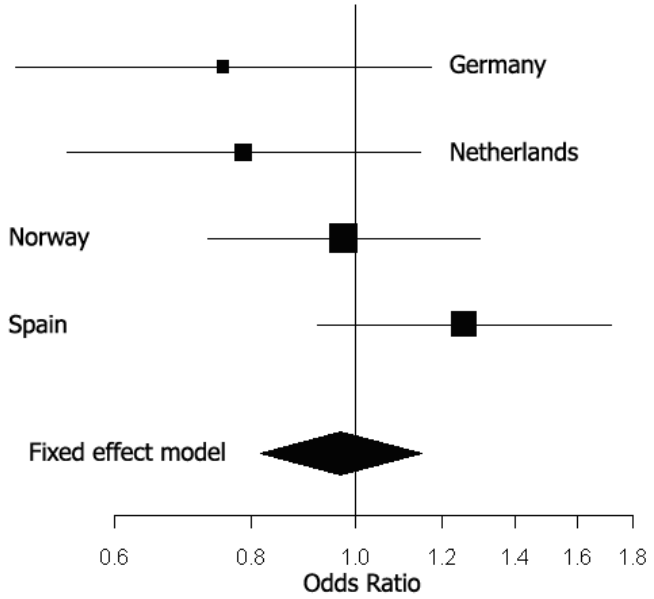
(a)



	Cases		Controls					
Study	**4R4R**	**Total**	**4R4R**	**Total**	**Weight (%)**	**OR M-H, Random,95% CI**	**z**	**p**
1. Germany	200	436	63	156	20.98	1.25 (0.86-1.81)		
2. Netherlands	102	236	193	476	24.39	1.11 (0.81-1.53)		
3. Norway	179	449	190	488	28.12	1.03 (0.79-1.35)		
4. Spain	109	289	290	631	26.51	0.71 (0.53-0.94)		
Fixed-effects model	**590**	**1410**	**736**	**1751**	**100**	**0.97 (0.84-1.13)**	**-0.27**	**0.78**

Test of Heterogeneity: Q= 7.34, df= 3, p=0.06; I^2= 59.1% (0%-86.4%)

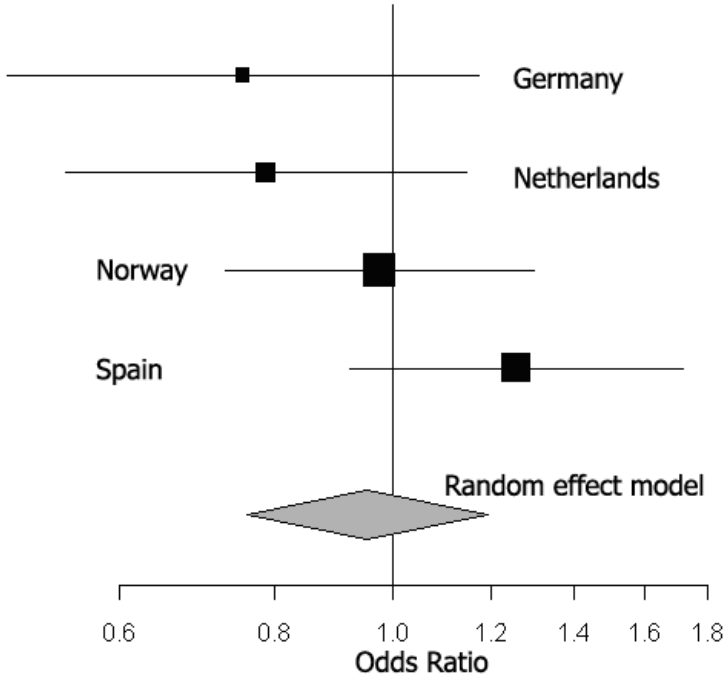
(b)



Study	Cases 7R7R	Cases Total	Controls 7R7R	Controls Total	Weight (%)	OR M-H, fixed,95% CI	z	p
1. Germany	29	436	7	156	16.90	1.41 (0.60-3.30)		
2. Netherlands	8	236	24	476	26.53	0.66 (0.29-1.19)		
3. Norway	20	449	26	488	41.20	0.82 (0.45-1.49)		
4. Spain	16	289	15	631	15.37	2.40 (1.17-4.93)		
Fixed-effects model	**73**	**1410**	**72**	**1751**	**100**	**1.12 (0.78-1.59)**	**0.63**	**0.52**

Test of Heterogeneity: Q=7.29, df=3, p=0.06; I^2= 58.9% (0%-86.3%)

(c)



Study	Cases 4R7R	Cases Total	Controls 4R7R	Controls Total	Weight (%)	OR M-H, Random,95% CI	z	p
1. Germany	89	436	37	146	23.0	0.75 (0.48-1.17)		
2. Netherlands	50	236	121	335	24.34	0.47 (0.32-0.69))		
3. Norway	124	429	136	488	21.64	1.19 (0.90-1.58))		
4. Spain	85	289	157	631	24.72	1.25 (0.92-1.71)		
Fixed-effects model	**348**	**1390**	**451**	**1600**	**100**	**0.87 (0.56-1.35)**	**-0.61**	**0.53**

Test of Heterogeneity: Q=19.3, df=3, p=0.0002; I^2= 84.5% (61.1%% - 93.8%)

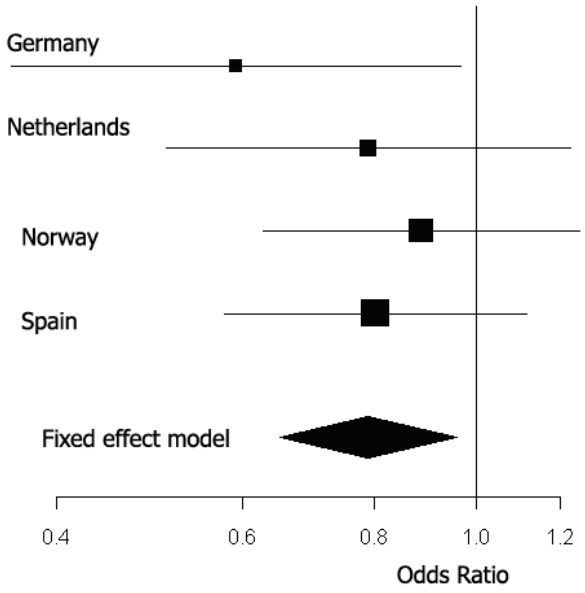
4R: 4 repeats of the *DRD4* 48bpVNTR polymorphism; 7R: 7 repeats of the *DRD4* 48bpVNTR polymorphism

Multiple-marker analysis

Linkage disequilibrium between the two studied polymorphisms in *DRD4* was assessed and shown to be negligible (p = 0.99). Genotypes from 1,246 patients (77%) and 1,835 controls (78%) were available for the two *DRD4* polymorphisms. Table IV summarizes the estimated haplotype frequencies for the four populations considered in the study.

The comparison of haplotype frequencies between cases and controls showed no association in any of the four separate cohorts. We subsequently performed a meta-analysis considering carriers of the three common *DRD4* haplotypes, S-4R, L-4R and L-7R, and, after discarding the presence of heterogeneity between the four samples using the Q-statistic, we observed an under-representation of the S-4R allelic combination in the ADHD sample (p=0.01; OR=0.78 (0.65-0.95); Table IV). We also performed multiple comparisons stratifying by gender and by clinical subtype and confirmed these differences in females (p= 0.04, OR= 0.73 (0.54-0.99); Supplementary Table SV). Interestingly, when we considered patients with the ADHD combined subtype only, we observed an increased frequency of carriers of the L-4R risk haplotype (p=0.02; OR=1.29 (1.04-1.61)) in addition to an under-representation of the S-4R allelic combination (p=0.01 OR= 0.75 (0.60-0.94)) in this clinical dataset (Table V).

Table IV. Pooled analysis considering S-4R carriers of the *DRD4* dup120bp-48bp VNTR haplotype in 1246 adulthood ADHD patients and 1835 controls.



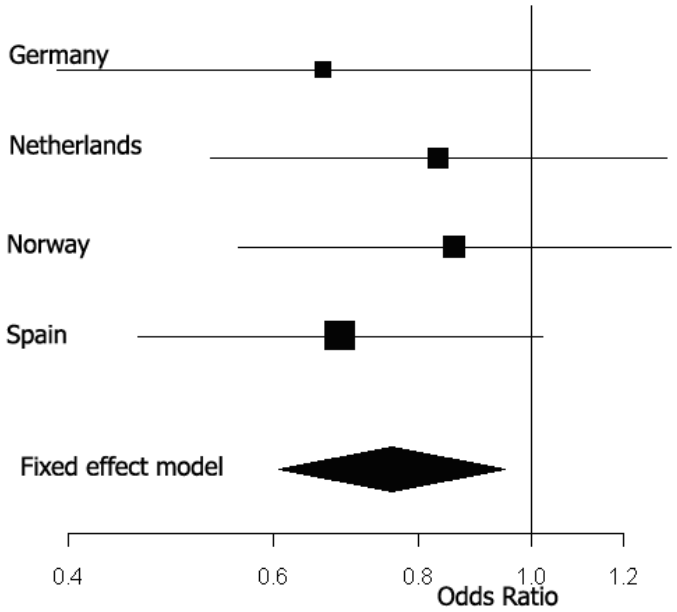
Study	Cases S-4R	Cases Total	Controls S-4R	Controls Total	Weight (%)	OR M-H, Fixed,95% CI	z	p
1. Germany	60	327	33	120	16.69	0.59 (0.65-0.96)		
2. Netherlands	32	187	99	478	19.53	0.79 (0.50-1.22)		
3. Norway	67	443	91	544	29.34	0.88 (0.62-1.25)		
4. Spain	62	289	176	693	34.44	0.80 (0.57-1.11)		
Fixed -effects model	**221**	**1246**	**399**	**1835**	**100**	**0.78 (0.65-0.95)**	**-2.39**	**0.01**

Test of Heterogeneity : Q= 1.78, df=3, p= 0.62; I^2= 0% (0%-74.1%)

S: Short allele of the dup120bp polymorphism (120bp); 4R: 4 repeats of the 48bpVNTR polymorphism

Table V. Pooled analysis considering the (a) S-4R and (b) L-4R carriers of the *DRD4* dup120bp-48bpVNTR haplotype in 788 combined ADHD patients and 1835 controls.

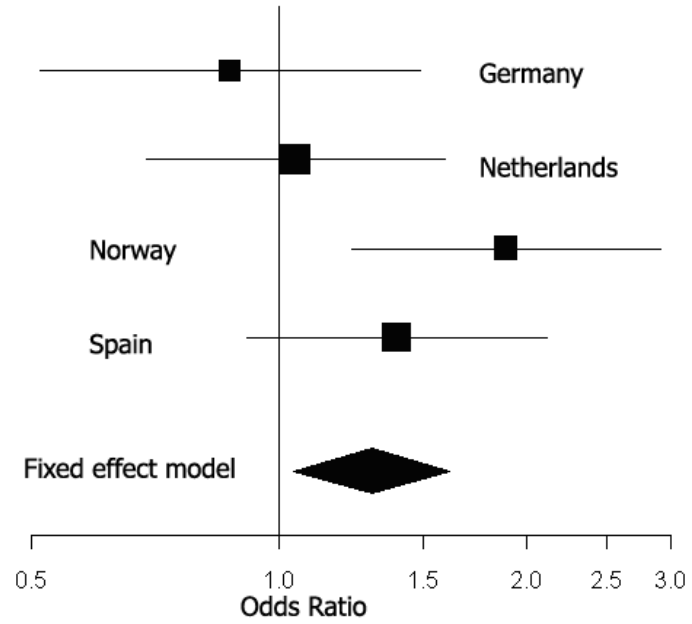
(a)



Study	Cases S-4R	Cases Total	Controls S-4R	Controls Total	Weight (%)	OR M-H, Fixed,95% CI	z	p
1. Germany	41	204	33	120	18.12	0.66 (0.39 - 1.12)		
2. Netherlands	30	168	99	478	23.08	0.83 (0.52 - 1.30)		
3. Norway	34	231	91	544	25.25	0.85 (0.56 - 1.31)		
4. Spain	36	190	176	693	33.54	0.68 (0.45 - 1.02)		
Fixed -effects model	**140**	**788**	**399**	**1835**	**100**	**0.75 (0.60 - 0.94)**	**-2.42**	**0.01**

Test of Heterogeneity : Q= 0.98 , df= 3 , p= 0.80; I^2= 0% (0%-53.2%)

(b)



Study	Cases L-4R	Cases Total	Controls L-4R	Controls Total	Weight (%)	OR M-H, Fixed,95% CI	z	p
1. Germany	153	204	93	120	20.27	0.87 (0.51-1.48)		
2. Netherlands	130	168	366	478	29.82	1.04 (0.68-1.59)		
3. Norway	196	226	422	544	22.77	1.88 (1.22-2.91)		
4. Spain	158	190	540	693	27.13	1.38 (0.91-2.11)		
Fixed -effects model	**637**	**788**	**1421**	**1835**	**100**	**1.29 (1.04-1.61)**	**2.31**	**0.02**

Test of Heterogeneity : Q= 6.14 , df= 3 , p= 0.10; I^2= 51.1% (0%-83.8%)

S: Short allele of the dup120bp polymorphism (120bp); L: Long allele of the dup120bp polymorphism (240bp); 4R: 4 repeats of the 48bpVNTR polymorphism

Interaction between *DRD4* and *SLC6A3*

Since we previously described an association of adulthood ADHD with the *SLC6A3* 9R-6R haplotype (3'UTR VNTR – Intron 8 VNTR) in the same dataset [Franke et al., 2010], we further tested for gene*gene interactions between the ADHD-associated haplotypes of *DRD4* and *SLC6A3*. Genotypes from 1,208 patients (75%) and 1,290 controls (55%) were available for the four polymorphisms in the two genes. Although no evidence for epistatic effects was detected, the simultaneous presence of the two risk haplotypes, *DRD4* L-4R and *SLC6A3* 9R-6R, increased the risk for ADHD in both the ADHD sample as a whole (p=3.04e-05; OR 1.66 (1.31-2.11)) and in the combined type clinical subtype (p=2.66e-05; OR=1.74 (1.35-2.26)). Thus, the OR for ADHD subjects carrying the *DRD4* L-4R haplotype rose from 1.09 (0.90-1.32) to 1.66 (1.31-2.11)) in those patients also carrying the *SLC6A3* 9R-6R allelic combination. Similar results were obtained in the combined type ADHD clinical subtype (Figure 1) and indicate additive effects between risk haplotypes at these two loci.

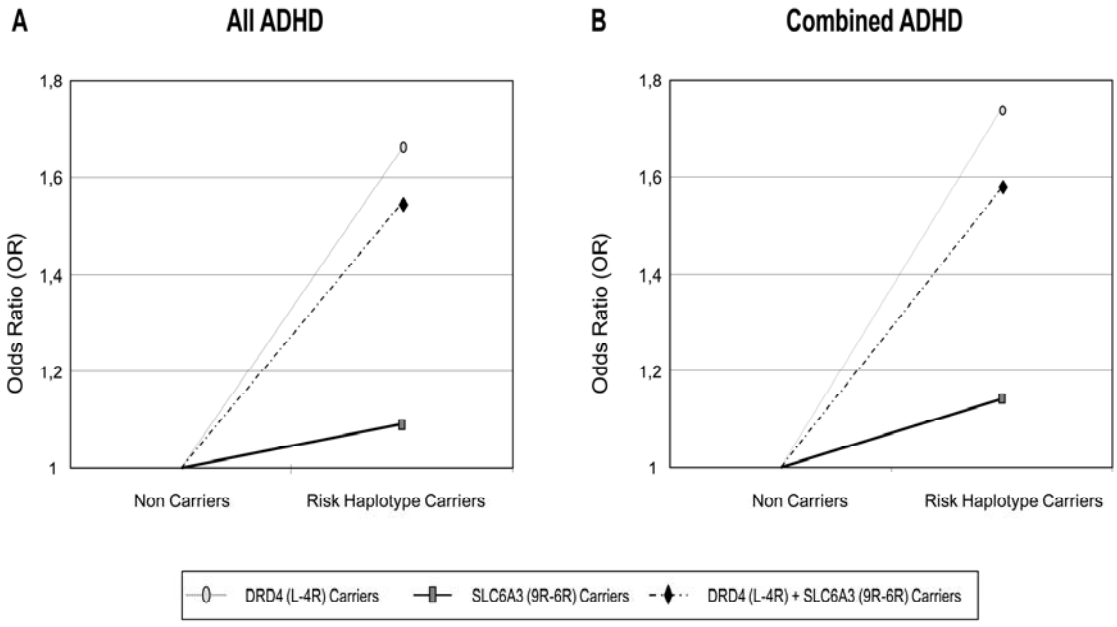


Figure 1. Graphical representation showing the analysis of gene*gene interactions between the *DRD4* and *SLC6A3* genes. The simultaneous presence of the two risk haplotypes, *DRD4* L-4R and *SLC6A3* 9R-6R, increased the risk for ADHD in both the pooled ADHD sample (A) and the combined clinical subtype sample (B). These results suggest additive effects of the risk haplotypes at these two loci on ADHD.

DISCUSSION

In the present study we performed a meta-analysis in a large sample of 1,608 adulthood ADHD patients and 2,352 unrelated controls from four European countries to evaluate the role of the *DRD4* dup120bp and 48bpVNTR polymorphisms in the persistent form of the disorder. Although nominal association with dup120bp was observed in some of the populations considered in this study, no evidence for a role of either polymorphism in ADHD was detected when they were considered separately in the entire sample. The multiple-marker analysis, however, supports a contribution of the L-4R (dup120bp-48bpVNTR) haplotype to adulthood ADHD, mainly to the combined clinical subtype, although these findings should be viewed with caution given the many (albeit highly correlated) statistical tests performed. These results support a connection between *DRD4* and the persistence of the ADHD symptoms across the life span, in line with previous follow-up studies [Biederman et al., 2009].

Many association studies between *DRD4* and ADHD have been performed, although results are often controversial. In this regard, although our data in the single-marker analysis are in agreement with previous reports showing no association between ADHD and the 48bpVNTR polymorphism [Bakker et al., 2005; Brookes et al., 2005; Carrasco et al., 2006; Johansson et al., 2008; Sonuga-Barke et al., 2008; Roman et al., 2001; Todd et al., 2001], they are contrary to others having reported on association between the 7R allele and the disorder [Biederman et al., 2009; Brookes et al., 2006; Faraone et al., 2001; Gizer et al., 2008; Gizer et al., 2009; Langley et al., 2009; Li et al., 2006; Maher et al., 2002; Nikolaidis and Gray, 2009; Rowe et al., 1998; Wohl et al., 2005]. Likewise, our results for the *DRD4* dup120bp polymorphism are in line with most of the literature, including a recent meta-analysis [Barr et al., 2001; Bhaduri et al., 2006; Brookes et al., 2005; Gizer et al., 2009; Kirley et al., 2004; Mill et al., 2003; Todd et al., 2001], but not with findings from the first two association analyses of this variation [McCracken et al., 2000].

Due to the heterogeneity of populations, methodologies and statistical tests used, it is difficult to establish direct comparisons between all these reports. In the case of the 48bpVNTR, allele frequencies of this multiallelic polymorphism vary considerably across ethnic groups. The 4R allele is the most prevalent one and appears in all populations. However, 7R is frequent among Americans but rare among Asians and 2R is frequent in Asia but uncommon among Americans [Chang et al., 1996; Ding et al., 2000; Van Tol et al., 1992; Wang et al., 2004]. In addition, some studies collapse different alleles into long and short categories (6R-8R versus 2R-5R) [Eisenberg et al., 2000; Manor et al., 2002] and others only consider the most frequent alleles among

Caucasians (4R and 7R) [Bellgrove et al., 2005; Roman et al., 2001]. Because grouping several frequent alleles in a single category or considering only variants described in previous analyses may result in loss of crucial genetic information, we included all frequent alleles separately and grouped the less frequent variants (<5%) into a single group.

Several other explanations could also account for the inconsistent findings observed among previously reported *DRD4* studies. Differences in sample size, comorbidities, and proportions of clinical subtypes or genders may result in discordant results. Limited sample sizes may provide imprecise or incorrect estimates of the magnitude of the observed effects. The present meta-analysis provides an adequate statistical power (>99%) to detect an association of small effect. Additionally, in the case of gender-specific associations, differences in the male:female proportion among studies may also contribute to variability. In this regard, although nominal and site-specific, we found male-specific association signals in the Norwegian sample when samples were stratified according to gender. Whether the different ADHD clinical subtypes share genetic risk factors has been still poorly explored. The association between ADHD and the *DRD4* risk haplotype detected in the present study was observed in the combined but not in the inattentive clinical subtype. Our results are in agreement with previous studies supporting the validity of the different DSM-IV subtypes, mainly the combined subtype, and suggesting the participation of differential genetic factors in distinct ADHD clinical groups [Larsson et al. 2006; Rasmussen et al. 2004; Ribasés et al. 2009; Sobanski et al. 2008]. Interestingly, Smith [2010] performed a meta-analysis of 28 association studies between ADHD and the 48bpVNTR in *DRD4* and observed that increases in the proportion of combined ADHD patients within an ADHD sample were associated with an increase in the magnitude of the effect. These results are consistent with the hypothesis that *DRD4* is more strongly associated with combined ADHD than with inattentive ADHD and are in line with the hypothesis that hypofunctioning in mesocortical and mesolimbic dopaminergic pathways better characterize the etiology of combined ADHD than inattentive ADHD (Sagvolden et al. 2005; Smith 2010). This view is supported by the fact that variation in *DAT1*, another dopaminergic gene, is also more strongly associated with combined ADHD than with inattentive ADHD (e.g. Waldman et al. 1998). Moroeover, it is possible that consideration of neuropsychological traits, comorbidities or personality data, not included in the present study, may help in the future to obtain better association signals with *DRD4* than the analysis of the sole ADHD condition. Finally, since most of previous research on the *DRD4* gene has focused on childhood patients (only four studies having considered the 48bpVNTR polymorphism in adulthood ADHD [Boonstra et al., 2008; Johansson et al.,

2008; Muglia et al., 2000; Smith et al., 2003], differential proportion of remitting and persisting ADHD within the children samples may also explain discordant results among studies [Brookes et al., 2006; Gornick et al., 2007; Langley et al., 2009]. In these regard, Shaw et al. [2007] showed in a longitudinal study that the 7R allele was associated with a better clinical outcome and with differences in cortical thickness in regions that are important in attention control. These neuroanatomical changes were most apparent early in development and resolved by late adolescence. In addition, Johansson et al. (2008) showed a trend towards protective effects of the 7R allele in adults with ADHD, results that suggest a different effect of the 7R allele in child and adulthood ADHD and could explain previous controversial results. However, another recent study by Biederman and co-workers [2009] showed increased risk of ADHD persistence due to the 7R allele.

The identification of different alleles at the same markers as susceptibility factors for ADHD in several studies and the failure to consistently replicate positive associations suggest that the dup120bp and the 48bpVNTR polymorphisms may not be themselves the causative variants that increase disease risk but are in linkage disequilibrium with a true causative variant within the same gene. Further deep-sequencing of this genomic region may allow identification of the functional *DRD4* variants directly involved in the genetic background of ADHD. In that regard, it is interesting to note that an increased burden of rare variants has been observed in the 7R allele of *DRD4* in children with ADHD [Grady et al., 2003].

In addition, we detected preliminary evidence of additive, but not epistatic, effects for *DRD4* and *SLC6A3* in ADHD. Since *SLC6A3* is expressed in subcortical regions whereas *DRD4* is expressed in frontal cortex, the two genes may indeed be expected to increase ADHD risk by acting in independent pathways [Durston et al., 2005]. However, it is difficult, to draw a final conclusion about the combined participation of these two genes in ADHD symptomatology, as several studies have described discordant results [Carrasco et al., 2006; Gabriela et al., 2009; Kim et al., 2005; Qian et al., 2007; Roman et al., 2001].

In summary, the results of the present study showed nominal association between the L-4R haplotype (dup120bp-48bpVNTR) and adulthood combined ADHD through a meta-analysis of four European populations. Our results also suggest additive effects between this *DRD4* risk haplotype and *SLC6A3*. Replication in other datasets is warranted to confirm these results and to better understand the involvement of the *DRD4* and *SLC6A3* genes in the predisposition to the persistent form of ADHD.

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CONFLICTS OF INTEREST

Netherlands: In the past 3 years, Jan K. Buitelaar has been a consultant to/member of advisory board of and/or speaker for Janssen Cilag BV, Eli Lilly, Bristol-Myer Squibb, Organon/Shering Plough, UCB, Shire, Medice and Servier. He is not an employee of any of these companies. He is not a stock shareholder of any of these companies. He has no other financial or material support, including expert testimony, patents or royalties. Sandra Kooij has received research grants from Janssen BV en Shire, and is on the speaker's bureau of Janssen and Eli Lilly. Martine Hoogman, Lambertus Kiemeney and Barbara Franke declare no conflicts of interest.
Norway: Jan Haavik has been a consultant for Janssen Cilag and Novartis.
Authors from Germany and Spain declare no conflicts of interest.

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Supplementary Table SI. Comparison of genotype and allele frequencies of the *DRD4* dup120bp polymorphism in 1447 adulthood ADHD patients and 2062 sex-matched unrelated controls from four European countries.

	Genotypes													Alleles	
	Cases n (%)				Control n (%)					Genotype LL VS (LS + SS)		Genotype (LL+LS) VS SS		Allele L VS S	
	LL	LS	SS	Sum	LL	LS	SS	Sum	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
Germany	356 (69.1)	137 (26.6)	22 (4.3)	515	338 (70)	127 (26.3)	18 (3.7)	483	0.89	1.04 (0.79-1.36)	0.76	1.15 (0.61-2.18)	0.66	1.05 (0.83-1.33)	0.67
Netherlands	139 (73.5)	44 (23.3)	6 (3.2)	189	351 (72.4)	121 (24.9)	13 (2.7)	485	0.86	1.06 (0.72-1.56)*	0.75	1.19 (0.45-3.18)	0.73	1.03 (0.74-1.43)	0.87
Norway	344 (76.8)	98 (21.9)	6 (1.3)	448	335 (72.2)	118 (25.4)	11 (2.4)	464	0.20	1.28 (0.94-1.72)	0.11	1.78 (0.65-5)	0.24	1.27 (0.97-1.66)	0.08
Spain	190 (64.4)	98 (33.2)	7 (2.4)	295	411 (65.2)	189 (30)	30 (4.8)	630	0.14	1.04 (0.78-1.39)	0.80	2.04 (0.89-4.76)	0.28	1.05 (0.82-1.35)	0.69
Pool	1029 (71.1)	377 (26.1)	41 (2.8)	1447	1435 (69.6)	555 (26.9)	72 (3.5)	2062	0.45	1.07 (0.92-1.23)	0.36	1.23 (0.84-1.81)	0.28	1.08 (0.95-1.23)	0.24

* When OR < 1, inverted score is shown

Supplementary Table SII. Comparison of genotype and allele frequencies of the *DRD4* dup120bp polymorphism in (a) adult ADHD males (803 cases and 1190 controls) and (b) adult ADHD females (644 cases and 872 controls) from four European countries.

(a)

	Genotypes													Alleles	
	Cases n (%)				Control n (%)					Genotype LL VS (LS + SS)		Genotype (LL+LS) VS SS		Allele L VS S	
	LL	LS	SS	Sum	LL	LS	SS	Sum	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
Germany	170 (65.6)	77 (29.7)	12 (4.6)	259	172 (70.8)	64 (26.3)	7 (2.9)	243	0.36	1.27 (0.87-1.85)	0.21	1.64 (0.63-4.23)	0.30	1.27 (0.92-1.75)*	0.15
Netherlands	64 (68.1)	26 (27.7)	4 (4.3)	94	171 (71.0)	62 (25.7)	8 (3.3)	241	0.84	1.15 (0.68-1.92)	0.60	1.29 (0.38-4.40)	0.68	1.15 (0.74-1.79)*	0.55
Norway	184 (77.6)	49 (20.7)	4 (1.7)	237	165 (67.1)	76 (30.9)	5 (2.0)	246	0.03	1.69 (1.14-2.56)*	0.009	1.20 (0.32-4.55)	0.77	1.55 (1.08-2.22)	0.01
Spain	138 (64.8)	70 (32.9)	5 (2.3)	213	299 (65.0)	137 (29.8)	24 (5.2)	460	0.16	1.01 (0.72-1.42)	0.95	2.27 (0.86-6.25)*	2.27	1.09 (0.81-1.46)	0.56
Pool	556 (69.2)	222 (27.6)	25 (3.1)	803	807 (67.8)	339 (28.5)	44 (3.7)	1190	0.69	1.06 (0.88-1.30)*	0.50	1.19 (0.72-1.96)*	0.48	1.07 (0.91-1.27)	0.41

(b)

	Genotypes													Alleles	
	Cases n (%)				Control n (%)					Genotype LL VS (LS + SS)		Genotype (LL+LS) VS SS		Allele L VS S	
	LL	LS	SS	Sum	LL	LS	SS	Sum	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
Germany	186 (72.7)	60 (23.4)	10 (3.9)	256	166 (69.2)	63 (26.2)	11 (4.6)	240	0.69	1.19 (0.80-1.75)*	0.39	1.18 (0.49-2.86)*	0.70	1.16 (0.83-1.62)	0.37
Netherlands	75 (78.9)	18 (18.9)	2 (2.1)	95	180 (73.8)	59 (24.2)	5 (2.0)	244	0.57	1.33 (0.75-2.38)*	0.31	1.03 (0.20-5.39)	0.97	1.26 (0.75-2.1)	0.37
Norway	160 (75.8)	49 (23.2)	2 (0.9)	211	170 (78)	42 (19.3)	6 (2.8)	218	0.24	1.13 (0.72-1.77)	0.59	2.94 (0.59-4.29)*	0.15	1.02 (0.68-1.52)*	0.93
Spain	52 (63.4)	28 (34.1)	2 (2.4)	82	112 (65.9)	52 (30.6)	6 (3.5)	170	0.78	1.11 (0.64-1.93)	0.70	1.47 (0.28-7.69)*	0.63	1.09 (0.81-1.46)	0.56
Pool	473 (73.4)	155 (24.1)	16 (2.5)	644	628 (72.0)	216 (24.8)	28 (3.2)	872	0.64	1.08 (0.85-1.35)*	0.53	1.30 (0.70-2.44)*	0.40	1.09 (0.89-1.33)	0.41

* When OR < 1, inverted score is shown

Supplementary Table SIII. Comparison of genotype and allele frequencies of the *DRD4* dup120bp polymorphism in (a) 941 combined ADHD patients and 2062 sex-matched unrelated controls and (b) 305 inattentive ADHD patients and 2062 sex-matched unrelated controls from four European countries.

(a)

	Genotypes													Alleles	
	Cases n (%)				Control n (%)					Genotype LL VS (LS + SS)		Genotype (LL+LS) VS SS		Allele L VS S	
	LL	LS	SS	Sum	LL	LS	SS	Sum	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
Germany	240 (69.0)	91 (26.1)	17 (4.9)	348	338 (70.0)	127 (26.3)	18 (3.7)	483	0.71	1.05 (0.78-1.41)	0.75	1.33 (0.67-2.61)	0.41	1.07 (0.83-1.38)*	0.56
Netherlands	122 (71.8)	42 (24.7)	6 (3.5)	170	351 (72.4)	121 (24.9)	13 (2.7)	485	0.85	1.03 (0.70-1.52)	0.87	1.33 (0.50-3.56)	0.57	1.05 (0.75-1.49)*	0.74
Norway	255 (78.5)	66 (20.3)	4 (1.2)	325	335 (72.2)	118 (25.4)	11 (2.4)	464	0.08	1.40 (1.01-1.96)*	0.04	2.12 (0.68-6.66)*	0.16	1.4 (1.04-1.89)	0.02
Spain	127 (65.5)	63 (32.5)	4 (2.1)	194	411 (65.2)	189 (30.0)	30 (4.8)	630	0.11	1.03 (0.73-1.44)*	0.85	2.63 (0.91-7.69)*	0.04	1.13 (0.85-1.52)	0.39
Pool	744 (71.1)	262 (25.3)	31 (3.0)	1037	1430 (69.4)	556 (27.0)	76 (3.7)	2062	0.35	1.11 (0.94-1.31)*	0.19	1.23 (0.80-1.88)*	0.32	1.11 (0.96-1.28)	0.14

(b)

	Genotypes													Alleles	
	Cases n (%)				Control n (%)					Genotype LL VS (LS + SS)		Genotype (LL+LS) VS SS		Allele L VS S	
	LL	LS	SS	Sum	LL	LS	SS	Sum	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
Germany	89 (69.0)	35 (27.1)	5 (3.9)	129	338 (70.0)	127 (26.3)	18 (3.7)	483	0.97	1.04 (0.38-2.85)	0.94	1.05 (0.67-1.62)	0.84	1.04 (0.72-1.49)*	0.82
Netherlands	12 (85.7)	2 (14.3)	-	14	351 (72.4)	121 (24.9)	13 (2.7)	485	0.40	2.27 (0.51-10)*	0.23	-	-	2.32 (0.55-9.9)	0.19
Norway	31 (66.0)	16 (34.0)	-	47	333 (71.8)	119 (25.6)	12 (2.6)	464	0.22	1.23 (0.65-2.33)	0.53	-	-	1.07 (0.60-1.88)*	0.80
Spain	54 (60.7)	32 (36.0)	3 (3.4)	89	411 (65.2)	189 (30.0)	30 (4.8)	630	0.49	1.19 (0.75-1.87)	0.46	1.58 (0.47-5.26)*	0.42	1.06 (0.72-1.56)*	0.74
Pool	186 (66.7)	85 (30.5)	8 (2.9)	279	1430 (69.4)	556 (27.0)	76 (3.7)	2062	0.40	1.13 (0.87-1.48)	0.36	1.29 (0.61-2.70)*	0.47	1.06 (0.84-1.33)*	0.58

* When OR < 1, inverted score is shown

Supplementary Table SIV. Comparison of (a) genotype and (b) allele frequencies of the *DRD4* 48bpVNTR polymorphism in 1410 adulthood ADHD patients and 1741 sex-matched unrelated controls from four European countries.

(a)

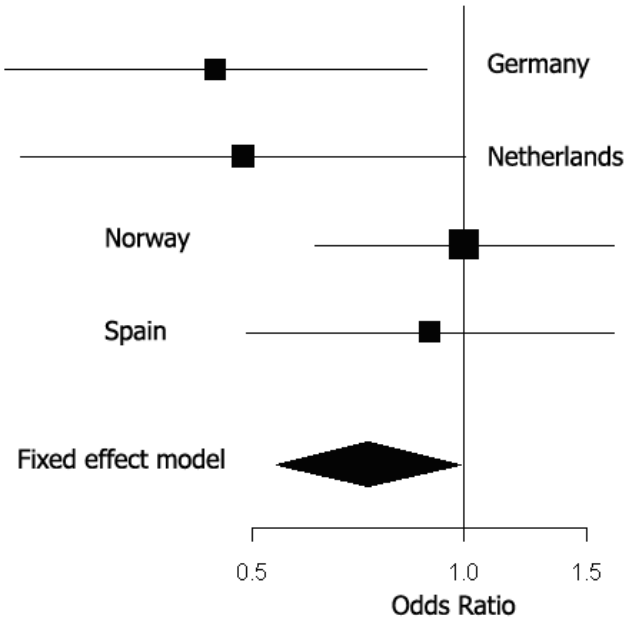
	Genotypes													
	Cases n (%)							Controls n(%)							
	2 4	3 4	4 4	4 7	7 7	Others*	Sum	2 4	3 4	4 4	4 7	7 7	Others*	Sum	p
Germany	52 (12.0)	24 (5.5)	200 (46.0)	89 (20.5)	29 (6.7)	41 (9.4)	435	19 (12.9)	7 (4.8)	64 (43.5)	36 (24.5)	7 (4.8)	14 (9.5)	147	0.887
Netherlands	30 (12.7)	11 (4.7)	102 (43.2)	50 (21.2)	8 (3.4)	35 (14.8)	236	52 (10.8)	23 (4.8)	197 (40.9)	123 (25.5)	24 (5.0)	63 (13.1)	482	0.672
Norway	40 (8.9)	31 (6.9)	179 (39.9)	124 (27.6)	20 (4.5)	55 (12.2)	449	62 (10.7)	29 (5.0)	226 (39.0)	160 (27.6)	30 (5.2)	73 (12.6)	580	0.747
Spain	35 (12.2)	11 (3.8)	108 (37.6)	84 (29.3)	16 (5.6)	33 (11.5)	287	81 (12.8)	24 (3.8)	280 (44.4)	156 (24.7)	15 (2.4)	75 (11.9)	631	0.091
Pool	157 (11.2)	77 (5.5)	589 (41.9)	347 (24.7)	73 (5.2)	164 (11.7)	1407	214 (11.6)	83 (4.5)	767 (41.7)	475 (25.8)	76 (4.1)	225 (12.2)	1840	0.516

(b)

	Alleles										
	Cases n (%)					Controls n (%)					
	2	4	7	Others*	Sum	2	4	7	Others*	Sum	p
Germany	52 (6.0)	565 (64.9)	147 (16.9)	106 (1.2)	870	19 (6.5)	190 (64.6)	50 (17.0)	35 (11.9)	294	0.988
Netherlands	30 (6.4)	295 (62.5)	66 (14.0)	81 (17.2)	472	52 (5.4)	592 (61.4)	171 (17.7)	149 (15.5)	964	0.269
Norway	40 (4.5)	553 (61.6)	164 (18.3)	141 (15.7)	898	62 (5.3)	703 (60.6)	220 (19.0)	175 (15.1)	1160	0.770
Spain	35 (6.1)	346 (60.3)	116 (20.2)	77 (13.4)	574	81 (6.4)	821 (65.1)	186 (14.7)	174 (13.8)	1262	0.036
Pool	157 (5.6)	10759 (62.5)	493 (17.5)	405 (14.4)	2814	214 (5.8)	2306 (62.7)	627 (17.0)	533 (14.5)	3680	0.944

*Genotypes freq.<0.05

Supplementary Table SV. Pooled analysis considering S-4R carriers of the *DRD4* dup120bp-48bp VNTR haplotype in 537 female adulthood ADHD and 845 controls from four European countries.



		Cases		Controls					
OR M-H, Fixed, 95% CI	Study	S-4R	Total	S-4R	Total	Weight (%)	OR M-H, Fixed,95% CI	z	p
	1. Germany	23	154	19	67	22.33	0.44 (0.22-0.88)		
	2. Netherlands	10	95	47	241	23.58	0.48 (0.23-1.00)		
	3. Norway	32	208	46	300	31.60	1.00 (0.61-1.63)		
	4. Spain	18	80	58	237	22.49	0.89 (0.49-1.63)		
	Fixed -effects model	**83**	**537**	**170**	**845**	**100**	**0.73 (0.54-0.99)**	**-2.02**	**0.04**

Test of Heterogeneity : Q= 5.26 , df= 3 , p= 0.15; I^2= 43% (0%-80.9%)

S: Short allele of the dup120bp polymorphism (120bp); 4R: 4 repeats of the 48bpVNTR polymorphism

Discussió

En els darrers anys s'ha produït un increment important en el número d'estudis genètics d'associació realitzats en TDAH. Des del reconeixement del trastorn com a malaltia complexa, a l'etiologia de la qual contribueixen factors genètics i ambientals, s'han identificat diversos gens de susceptibilitat, alguns dels quals han estat objecte d'estudi en aquest treball.

Els estudis d'associació de tipus cas-control, principal estratègia utilitzada en aquesta Tesi Doctoral, han patit una revolució en els darrers anys gràcies al desenvolupament de noves tecnologies de genotipació massiva i de metodologies d'anàlisi estadística i als avanços en el coneixement dels patrons de desequilibri de lligament del genoma humà en diferents poblacions. Així, les dades públiques del projecte HapMap (www.hapmap.org) i, més recentment, del projecte dels 1000 genomes (www.1000genomes.org) han determinat canvis fonamentals quant a les estratègies de selecció de marcadors polimòrfics, fent possible la utilització de criteris de màxima cobertura genètica. D'altra banda, l'increment de la magnitud dels estudis de genotipació massiva ha fet necessari augmentar la mida de la mostra i, alhora, ajustar els resultats obtinguts en cada estudi mitjançant tests estadístics de correccions per múltiples comparacions. Finalment, el gran nombre de resultats inconsistents obtinguts ha fet necessari la realització d'estudis conjunts amb poblacions independents mitjançant estratègies de meta-anàlisi.

Donat que inicialment es creia que el TDAH era un trastorn únicament infantil, gran part dels estudis realitzats s'han centrat en pacients en aquesta franja d'edat. No obstant, des de l'acceptació de la persistència del trastorn a l'edat adulta fa relativament pocs anys, ha augmentat el nombre d'estudis centrats en pacients adults. Fruit d'aquesta nova tendència, l'any 2007 es va constituir el consorci internacional *International Multicenter Persistent ADHD CollaboraTion* (IMpACT), inicialment integrat pels nodes holandès, espanyol, noruec i alemany i recentment ampliat a equips de recerca dels Estats Units, Gran Bretanya i Brasil, amb l'objectiu principal d'estudiar els factors genètics implicats en el TDAH a l'edat adulta.

La majoria d'estudis d'associació genètica del TDAH, tant aquells que han considerat gens o regions candidates com GWAS, no han mostrat dades concloents. La inconsistència entre els resultats podria ser conseqüència de les limitacions associades als estudis de les malalties complexes, que inclouen, entre d'altres: (I) fenòmens de penetració incompleta, (II) herència poligènica, en què la combinació de múltiples al·lels en múltiples gens amb un efecte individual menor sobre el fenotip i comuns a la població general participen en la predisposició genètica a la malaltia, (III) existència de fenocòpies, (IV) heterogeneïtat genètica, amb diferents subgrups genètics entre els individus de la mostra estudiada, cadascun dels quals es caracteritza

per variants en diferents gens de susceptibilitat (heterogeneïtat de *locus*) o diferents al·lels en un mateix locus (heterogeneïtat al·lèlica) i (V) heterogeneïtat clínica de la mostra. Aquesta falta de resultats amb rèpliques consistents en estudis independents posa de manifest les limitacions dels estudis d'associació, algunes de les quals es descriuen a continuació tant a nivell de la recollida de la mostra com de les anàlisis genètiques posteriors.

En aquest treball s'han estudiat diferents tipus de variants polimòrfiques candidates a contribuir al risc de patir TDAH, que inclouen: (i) polimorfismes no SNPs, com VNTRs o duplicacions/delecions, prèviament relacionats amb el TDAH en altres poblacions i potencialment rellevants a nivell funcional, mitjançant genotipació manual i (ii) SNPs en gens que codifiquen proteïnes implicades en la neurotransmissió o factors neurotròfics utilitzant majoritàriament criteris de cobertura genètica i tècniques de genotipació massiva.

1. CARACTERÍSTIQUES DE LA MOSTRA

Un dels aspectes més importants dels estudis d'associació en malalties complexes és la correcta caracterització fenotípica de la mostra en estudi. Les característiques clíniques dels pacients i dels controls que participen en aquest treball es resumeixen a les diferents publicacions.

1.1 Recollida de la mostra de TDAH

En el cas dels trastorns psiquiàtrics, pels quals en general no es disposa de marcadors biològics específics, la identificació de la patologia i el seu diagnòstic per part dels equips clínics especialistes es basa en dades subjectives recollides a través de tests com l'escala ASRS v1.1, instrument de cribratge de diagnòstic ràpid acceptat per l'Organització Mundial de la Salut (OMS) o avaluacions més exhaustives com els criteris DSM-IV. En l'estudi del TDAH és fonamental considerar els tres subtipus clínics de TDAH: combinat, inatent i hiperactiu-impulsiu, encara que la mida de la mostra sigui menor, i disminueixi el poder estadístic de l'estudi. En aquest sentit, en els diferents estudis que es presenten en aquest treball hem identificat tant factors de risc comuns en els diferents subtipus clínics (p.ex. l'haplotip de risc 9R6R del gen *SLC6A3*) com variants de risc implicades únicament en el subtipus combinat, com ara els haplotips de risc identificats en el gen *DRD*1 (rs863126A-rs265977C o rs835541G-rs863126T) o en el gen *DRD4* (dup120bp L - 48bpVNTR 4R). En els treballs en què hem fet anàlisis tot estratificant la mostra segons el subtipus clínic s'ha tingut en compte el subtipus combinat i el subtipus inatent, però no el subtipus hiperactiu/impulsiu, a causa de la seba baixa freqüència (6-21%).

1.2 Recollida de la població control: aparellament cas-control

Els estudis d'associació cas-control consisteixen a comparar la freqüència de determinats factors de risc genètic presents a la població general entre individus afectats i controls sans. Per tant, la selecció dels controls apropiats és un principi epidemiològic crucial per a la validesa d'aquest tipus d'estudi. Idealment, la mostra control ha de reflectir tant la component ètnica com genètica dels casos i, per assolir-ho, els controls s'han d'aparellar acuradament amb els casos [Cardon i Bell, 2001; Chanock et al., 2007]. Sinó fos així, qualsevol diferència en les freqüències al·lèliques o genotípiques identificada entre ambdós grups podria donar lloc a errors de tipus I (o falsos positius) i ser conseqüència de les migracions històriques, de diferències de gènere o d'altres processos independents de l'associació entre els marcadors analitzats i el fenotip estudiat [Cardon i Bell, 2001]. Tot i que no s'han identificat variants polimòrfiques específiques de sexe,

l'aparellament a nivell de gènere entre casos i controls és especialment crític en malalties com el TDAH en què la relació de sexes està molt esbiaixada (1:1 a 3:1 homes:dones en població adulta, i 3:1 a 9:1 nens:nenes en població infantil). En aquest treball la mostra control es va aparellar per gènere amb el grup de pacients, es va reclutar a la mateixa área geogràfica que els casos (Barcelona i voltants) i tots els individus eren de nacionalitat espanyola i d'ètnia caucàsica. A més, els individus control van ser entrevistats pel personal investigador mitjançant un qüestionari detallat sobre els antecedents personals i familiars de trets d''inatenció i hiperactivitat, i aquells que presentaven història personal o familiar en primer grau de TDAH van ser exclosos de l'estudi.

2. CONSIDERACIONS GENERALS SOBRE ELS ESTUDIS D'ASSOCIACIÓ CAS-CONTROL

En el context de l'anàlisi de les malalties complexes, en què diferents factors de risc intervenen en l'etiologia del trastorn, s'han d'optimitzar al màxim les variables condicionants de l'estudi per tal de reduir els errors de tipus I (falsos positius) i II (falsos negatius). A continuació es discuteixen diversos factors que poden influir en els resultats obtinguts en els estudis d'associació de tipus cas-control realitzats en aquest treball.

2.1 Determinació del fenotip

Tal i com s'ha esmentat a l'apartat 1, per tal de poder identificar variants genètiques de predisposició a un determinat fenotip complex és necessari homogeneïtzar al màxim les poblacions de casos i controls pel que fa al gènere, origen geogràfic, ètnia i, si és possible, edat. A més, sota la hipòtesi que hi ha variants genètiques de risc comunes i/o específiques de subtipus clínic, en l'estudi del TDAH és fonamental considerar els grups de TDAH combinat, inatent i hiperactiu-impulsiu.

2.2 Errors de genotipació

A diferència dels estudis basats en famílies, els estudis d'associació cas-control poblacionals són especialment vulnerables a errors de genotipació ja que no és factible detectar errors tècnics mitjançant anàlisi de segregació [Abou-Sleiman et al., 2006]. No obstant, generalment en els estudis en què es genotipa un nombre elevat de SNPs en una gran quantitat de mostres, petits errors de genotipació amb efecte aleatori comportarien només pèrdua del poder estadístic sense generar errors de tipus I [Abou-Sleiman et al., 2006; Gordon i Finch, 2005; Pompanon et al.,

2005]. Com en qualsevol mètode analític, la minimització de les variables que poden conduir a la generació d'errors afavoreix la qualitat del l'estudi.

A nivell tècnic, un dels mètodes més utilitzats per a la detecció de possibles errors de genotipació és la inclusió de mostres duplicades i de controls negatius [Abou-Sleiman et al., 2006], així com la utilització d'una segona tècnica de genotipació per validar l'exactitud dels resultats obtinguts [Chanock et al., 2007]. En aquest sentit, per cada polimorfisme estudiat en aquest treball s'han analitzat alguns individus per duplicat, mostres de referència de la col.lecció del CEPH (*Centre d'Étude du Polymorphisme Humain*) amb genotip conegut, i controls negatius que han proporcionat valors d'identitat propers al 100%.

Caixa 10. Equilibri de Hardy-Weinberg (HWE)

Tenint en compte que les freqüències al•lèliques es mantenen constants de generació en generació, una desviació respecte al HWE suggeriria l'existència d'un o més factors que afecten aquestes freqüències al•lèliques. L'efecte de la selecció natural, l'aparició d'una mutació de novo o la deriva gènica podrien donar lloc a una desviació de HWE, però errors en la genotipació també.
Segons el principi de Hardy-Weinberg, les freqüències genotípiques d'un determinat polimorfisme es poden calcular a partir de les freqüències al•lèliques. Si considerem els al•lels A1 i A2 d' un locus dicotòmic,

$p=f(A1), 0 \leq p \geq 1; q=f(A2), 0 \leq q \geq 1; p + q = 1$

i a la següent generació,

$(p + q)2 = p2 + 2pq + q2 = 1$
$p2=f(A1A1), 2pq=f(A1A2), q2=f(A2A2)$

A nivell analític, i sota la hipòtesi que una taxa elevada d'errors en la genotipació desviaria la distribució de les freqüències genotípiques de l'equilibri de Hardy-Weinberg (HWE) [Pompanon et al., 2005], hem contrastat el HWE a la mostra control (Caixa 10). En aquest treball el llindar de significació pel test de HWE s'ha establert a P=0,05 o P=0,01 en funció del nombre de polimorfismes considerats a cada publicació.

2.3 Estratificació poblacional

Una de les limitacions més importants dels estudis d'associació de tipus cas-control poblacional és la possible presència d'estratificació poblacional, és a dir, l'existència de diferències en les freqüències genotípiques, al·lèliques o haplotípiques entre les poblacions que es comparen per causes independents al fenotip estudiat, donant lloc a errors de tipus I o falsos positius.

Per aquest motiu, quan s'han dut a terme estudis en una única població en aquest treball, els casos i controls s'han aparellat per gènere i origen ètnic, essent tots ells de

nacionalitat espanyola i d'ètnia caucàsica. A més, abans de dur a terme els estudis d'associació, s'ha confirmat l'absència de subestructures poblacionals mitjançant la genotipació d'un grup de 48 SNPs intergènics independents localitzats a una distància mínima de 100 kb de qualsevol gen conegut [Sanchez et al., 2006]. L'anàlisi de les dades s'ha dut a terme utilitzant tres aproximacions diferents:

(i) El programa **STRUCTURE**, que utilitza genotips de múltiples *loci* per investigar l'estructura de la població i identificar la presència de subgrups genèticament diferenciats [Falush et al., 2003; Pritchard et al., 2000]. En els nostres estudis, la probabilitat que casos i controls pertanyin a un únic grup homogeni són superiors al 99%.

(ii) El **coeficient Fst,** utilitzat per valorar el grau de variabilitat genètica entre poblacions i dins d'una població [J, 1995]. Els valors del coeficient Fst calculats en els nostres estudis per a casos i controls conjuntament són propers a zero, tot indicant que no hi ha heterogeneïtat a la mostra

(iii) El **mètode de Pritchard i Rosenberg** per avaluar si les distribucions genotípiques per a aquests marcadors (sota models de codominància, dominància i recessivitat) són les mateixes en casos i controls [Pritchard et al., 2000]. En aquest cas, la comparació de les freqüències genotípiques als nostres estudis no ha revelat en cap cas diferències significatives, amb valors de P>0,05.

Per altra banda, en els estudis de tipus meta-anàlisi que s'han realitzat en aquest treball, que inclouen pacients i controls de quatre poblacions europees, s'ha avaluat la presència d'heterogeneïtat interpoblacional utilitzant **l'estadístic Q.** En aquest cas, quan s'ha detectat que no hi ha heterogeneïtat, s'han estimat les raons de probabilitats o riscs relatius (*odds ratios*, OR) de la suma de les diferents poblacions utilitzant el model d'efectes fixes [Mantel i Haenszel, 1959]. Alternativament, quan s'ha observat heterogeneïtat entre les poblacions, s'ha utilitzat el model d'efectes aleatoris [Laird i Mosteller, 1990], que assumeix l'existència d'heterogeneïtat i la té en consideració a l'hora de calcular l'efecte real del polimorfisme.

2.4 Selecció de polimorfismes i arquitectura gènica

Els estudis d'associació es basen en la premissa que els polimorfismes analitzats estan directament implicats en la malaltia (associació directa) o bé en desequilibri de lligament (LD) amb la variant rellevant a nivell funcional (associació indirecta). Així, els estudis d'associació directa es basen en l'anàlisi de polimorfismes potencialment causals amb rellevància funcional

prèviament establerta, que inclouen, entre d'altres, substitucions aminoacídiques situades en regions gèniques codificants, polimorfismes en zones reguladores de la transcripció o en zones implicades en processos de modificació post-transcripcionals, com el tall i empalmament d'exons (*splicing*) [Cordell i Clayton, 2005]. La principal limitació d'aquest tipus d'aproximació és el desconeixement de les possibles implicacions funcionals de la major part dels polimorfismes del genoma humà.

Alternativament, la selecció de polimorfismes en els estudis d'associació indirectes es fa en base als coneixements actuals sobre els patrons de LD del genoma humà (Figura 13) gràcies a la informació procedent del projecte internacional HapMap, l'objectiu del qual és determinar les freqüències de les variants polimòrfiques de tipus SNP i caracteritzar els patrons de LD al llarg del genoma humà a través de l'estudi de quatre poblacions d'orígens ètnics diferents: Estats Units amb origen centre-europeu (CEU), Nigèria (YRI), Xina (CHB) i Japó (JPT) [Frazer et al., 2007; Zondervan i Cardon, 2004]. Segons la informació disponible, els polimorfismes es troben espaiats homogèniament en el genoma, excepte en el cas de l'ADN mitocondrial i del cromosoma Y, aproximadament un 46% tenen al.lels de baixa freqüència (frquència de l'al.lel menor o MAF<0.05) i un 9% s'han identificat en un únic individu.

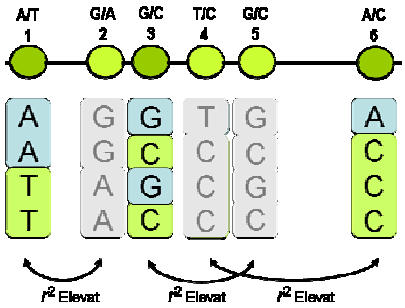


Figura 13. Selecció d'SNPs mitjançant criteris de LD en els estudis d'associació. Els SNPs 1, 3 i 6 proporcionen la màxima informació gènica assegurant la mínima redundància. Els SNPs 2, 4 i 5 estan en LD amb algun dels altres SNPs i per tant pràcticament no aporten nova informació. Adaptació de [Carlson et al., 2004]

L'objectiu dels mètodes indirectes és minimitzar la redundància genètica (tot limitant el nombre de variants a estudiar) però alhora evitar la pèrdua d'informació. Donada la seva elevada freqüència al genoma en comparació amb d'altres variants, com les VNTR o les duplicacions/delecions, actualment la majoria de polimorfismes utilitzats en els estudis

d'associació són de tipus SNP. Els criteris de selecció d'aquests es basen en la detecció de blocs o bins haplotípics o simplement en el grau de LD entre parelles de SNPs [Carlson et al., 2004; Gabriel et al., 2002; Gu et al., 2008]. En els estudis d'associació indirectes s'estima que la selecció de SNPs no correlacionats (amb valors de r^2 sempre inferiors a 0.80 en relació a qualsevol altre SNP de l'estudi) permet reduir la densitat de variants a analitzar entre un 75 i un 90% sense pèrdua essencial d'informació. Cal tenir en compte, però, que hi ha un cert número de SNPs que no han estat genotipats en el marc del projecte HapMap i que, en conseqüència, no s'estan considerant en els estudis basats en criteris de màxima cobertura genètica. Aquest fet que podria comportar pèrdues d'informació i provocar reduccions de l'ordre del 20% en el poder estadístic quan es consideren poblacions d'origen caucàsic [Gu et al., 2008].

En el nostre estudi presentem els resultats obtinguts a partir de l'anàlisi de variants genètiques seleccionades segons les dues estratègies: (i) màxima cobertura genètica en un sistema de gens candidats, com és el cas de la via de neurotransmissió dopaminèrgica, en què s'ha identificat el gen *DRD1* com a factor de susceptibilitat al TDAH, o bé (ii) selecció de variants potencialment funcionals com ara una VNTR o una duplicació/deleció al gen *DRD4* o el polimorfisme de tipus SNP p.Val66Met (rs6265) al gen *BDNF*.

En la selecció d'SNPs realitzada en aquest treball s'han emprat els programes LD-select [Carlson et al., 2004] i Haploview [Barrett et al., 2005], que utilitzen un algoritme per a identificar grups d'SNPs (anomenats bins haplotípics) representats per tagSNPs considerant valors mínims de MAF i r^2 establerts per l'usuari. L'estudi d'aquest grup reduït de tagSNPs és representatiu de la resta d'SNPs de la regió genòmica sota els criteris establerts. En el disseny de l'estudi del sistema dopaminèrgic s'han considerat els resultats de la segona fase del projecte HapMap i valors d'r^2<0,85 i MAFs superiors a 0,15 (en gens petits) o 0,25 (en gens grans). Per tant, marcadors amb MAF<0,15 o <0,25, en funció dels gens estudiats, no s'han seleccionat i, tot i que descartar informació és sempre poc desitjable, aquesta pèrdua és limitada en comparació amb els beneficis que s'obtenen en la simplificació de les anàlisis i l'increment del poder estadístic de l'estudi [Balding, 2006].

Tot i que la majoria dels estudis d'associació (inclosos els nostres) s'han basat fins ara en la hipòtesi "malalties comunes-variants comunes" (*common disease-common variant*), segons la qual les variants polimòrfiques causants de malalties complexes són variants comunes presents a la població general, hi ha noves línies d'investigació que recolzen la participació de variants rares (*common disease-rare variant*) en l'etiologia de les malalties complexes. En termes

generals, les estratègies metodològiques utilitzades en la detecció de les variants comunes i de variants rares són diferents (Caixa 11). En el cas de la identificació de les variants comunes, els estudis de genotipació a gran escala són els que més s'aproximen a les necessitats metodològiques per a la seva detecció.

Caixa 11. Variants comunes i variants rares en l'estudi de malalties complexes

Variants Comunes	Variants Rares
Estudis d'associació cas-control poblacionals o familiars, GWAS	Estudis de lligament i seqüenciació de gens candidats en nuclis familiars amb un o més individus afectats.
MAF >5%	0,1%<MAF<2-3%
1,2< OR <1,5	OR ≥ 2
No familiar	No familiar
Es troben en LD amb les variants causals	No es detecten per GWAS
Cal una mida mostral molt gran per reduir al màxim els errors de tipus I	Avaluació de la significació a través d'anàlisis funcionals
El sumatori de l'efecte de diferents variants té una contribució significativa al risc global de la malaltia	Tenen una contribució substancial al risc global de patir la malaltia
Penetrància massa baixa com per poder efectuar un tractament profilàctic	La penetrància és suficientment elevada com per justificar tractaments profilàctics
Difícil trobar una variant funcional	Les variants identificades són funcionals
És qüestionable la contribució a l'etiologia del trastorn	Contribueixen a la comprensió de l'etiologia del trastorn
Poden ser d'ajuda per a la recerca en variants rares candidates	El seu efecte pot estar modificat per variants comunes

Per altra banda, la seqüenciació en gens candidats seleccionats rigorosament o la seqüenciació d'exomes o genomes sencers en pacients amb fenotips molt específics, seguits d'un estudi funcional exhaustiu, és de moment l'estratègia més utilitzada per a la detecció de variants rares [Bodmer i Bonilla, 2008].

En els diferents estudis que es presenten en aquest treball únicament s'han seleccionat variants comunes com a possibles candidates al trastorn. Aquestes variants podrien estar directament relacionades amb el trastorn o, pel contrari, estar en LD amb altres variants, rares o comunes, que sí que tenen una vinculació directa amb el fenotip.

2.5 Estimació de les freqüències haplotípiques

Donat que en els estudis d'associació cas-control poblacionals no es disposa dels genotips dels progenitors, s'ha de recórrer a mètodes estadístics per tal d'estimar les freqüències haplotípiques a partir de les freqüències genotípiques. En els treballs presentats s'han utilitzat diferents aproximacions per tal d'estimar i identificar els haplotips de risc amb diferents programes que inclouen UNPHASED [Dudbridge, 2008], PLINK i PHASE [Stephens et al., 2001]

L'estimació de les freqüències haplotípiques es pot realitzar agrupant les poblacions de casos i controls o, alternativament, considerant els dos grups poblacionals de forma independent. La primera aproximació permet una millor estimació dels haplotips sota la hipòtesi de no associació, però podria introduir un biaix cap a aquesta hipòtesi i, per tant, generar errors de tipus II o falsos negatius. Contràriament, l'estimació de les freqüències haplotípiques en les poblacions de casos i controls de manera independent pot afavorir l'aparició d'errors de tipus I o falsos positius [Balding, 2006]. En aquest treball s'ha optat per estimar els haplotips en casos i en controls de forma separada, ja que l'estudi d'haplotips s'ha limitat a aquells gens que mostraven associació amb el fenotip estudiat a nivell genotípic o al·lèlic.

Fins ara no s'ha descrit cap algoritme estandarditzat per a la identificació dels haplotips de risc i la majoria d'estudis s'han limitat a l'estimació haplotips que consideren marcadors físicament consecutius. En aquest treball es va estimar les freqüències haplotípiques considerant aquella combinació haplotípica formada per dos SNPs que mostrava un major risc per al trastorn amb independència de si els marcadors eren físicament consecutius o no. A continuació, un cop fixat l'haplotip de risc de dos SNPs, es van anar afegint marcadors un a un fins a identificar la millor combinació de tres o quatre SNPs. Tot i que aquest mètode podria estar impedint la detecció d'altres combinacions haplotípiques més significatives, s'ha considerat l'estratègia més adient per tal de reduir el nombre de comparacions. A més, en alguns casos les limitacions computacionals impedeixen seguir una aproximació més oberta. No obstant, en ocasions aquesta aproximació ha permès identificar haplotips que no contenen alguns dels SNPs identificats en l'estudi de marcadors individuals, fet que suggereix que les variants identificades no són causals sinó que estan en LD amb les directament implicades en el trastorn.

2.6 Interaccions gen-gen i gen-ambient

Els estudis d'associació realitzats en malalties complexes com el TDAH, en què no hi ha un únic gen causal sinó que hi intervenen variants en diferents gens de susceptibilitat i també factors ambientals, tendeixen a ser simplistes i reduccionistes [Colhoun et al., 2003]. Així, les diferents vies implicades en el TDAH estan relacionades i intervenen conjuntament i de forma epistàtica en l'etiologia del trastorn. Aquesta epistasi, o efecte d'una variant al·lèlica en un *locus* sobre l'efecte en un fenotip determinat d'una altra variant al·lèlica en un segon *locus,* pot tenir efecte multiplicatiu o atenuant sobre el TDAH. Actualment hi ha molts mètodes estadístics que permeten detectar epistasi o interaccions gen-gen [Musani et al., 2007], entre els quals la

regressió logística és el més utilitzat. En aquest treball s'ha utilitzat aquesta aproximació amb l'objectiu d'avaluar possibles efectes additius o multiplicatius entre diferents gens, com *DRD4* i *SCL6A3/DAT1,* en el risc de patir TDAH. Tot i que no s'han detectat efectes epistàtics, aquest tipus d'estudi podria permetre detectar variants genètiques i ambientals de risc que a nivell individual no es trobarien relacionades amb el trastorn.

2.7 Mecanismes epigenètics

L'epigenètica ens explica aquella variabilitat heretable no dependent de la seqüència d'ADN. Els mecanismes epigenètics són els causants del silenciament o activació de gens mitjançant la seva acció sobre la seqüència d'ADN o sobre proteïnes reguladores. Fins ara s'han descrit quatre processos epigenètics principals: la metilació d'ADN, la remodelació de cromatina, ARNs no codificants i l'edició d'ADN o ARN ([Mehler, 2008]. En el marc dels estudis d'associació actuals és pràcticament impossible detectar els efectes epigenètics subjacents a les malalties complexes i és necessari el desenvolupament de noves estratègies per avaluar la seva participació en el TDAH. No obstant, de forma similar a altres patologies complexes, s'ha proposat que mecanismes com la metilació podrien tenir un paper rellevant en la susceptibilitat al TDAH [Lesch,] , hipòtesi que podria explicar la dificultat d'identificar de manera consistent gens implicats en el trastorn en els diferents estudis realitzats. En aquet sentit, hi ha estudis que demostren que la metilació de la regió promotora del gen de *BDNF* està relacionada amb situacions d'estrès o de por, així com en processos de memòria [Fuchikami et al., ; Munoz et al.]

2.8 Correccions per múltiples comparacions

La tendència actual en els estudis d'associació és augmentar el número de gens i variants estudiats, així com dur a terme anàlisi d'interaccions gen-gen i gen-ambient. Això implica un increment important en el número de comparacions que es realitzen, augmentant així la probabilitat de generar errors de tipus I o falsos positius. Per aquest motiu és necessari establir llindars de significació més restrictius que tinguin en compte el número de tests realitzats. No obstant, una correcció inadequada i massa restrictiva del llindar de significació podria incrementar els errors de tipus II o falsos negatius. En l'actualitat hi ha diferents aproximacions que s'utilitzen per resoldre aquest problema:

Correcció de Bonferrroni per múltiples comparacions: És el mètode més utilitzat en epidemiologia genètica, versàtil i simple però molt conservador, perquè requereix la independència dels tests

realitzats [Abou-Sleiman et al., 2006; Chanock et al., 2007; Gordon i Finch, 2005]. Aquesta correcció estableix un nou llindar de significació (α') segons el nombre de tests independents realitzats (n) seguint la relació α'= α/n, amb α=0,05. Això assegura una probabilitat d'error de tipus I no superior al 5% en cadascuna de les comparacions individuals. Un dels problemes principals de l'aplicació de la correcció de Bonferroni als estudis d'associació és que sovint no està clar el nombre d'hipòtesis independents que es contrasten, de manera que l'aplicació estricta d'aquest mètode és extremadament conservadora i tendeix a incrementar l'aparició d'errors de tipus II. En els estudis d'associació realitzats en aquest treball considerant SNPs seleccionats en base a criteris de LD (r^2>0.85), no podem assegurar la independència completa dels SNPs seleccionats. A més, els diferents grups d'edat (adults i infantils), els subtipus clínics (combinats, inatents i hiperactius-impulsius) i la comparació de les distribucions al·lèliques, genotípiques i haplotípiques tampoc són totalment independents i, per tant, l'aplicació de la correcció de Bonferroni pot resultar excessivament restrictiva.

Taxa de falsos descobriments (*False Discovery Rate*, FDR): Aquesta aproximació permet calcular la proporció de resultats positius falsos entre tots els resultats positius obtinguts, o dit d'una altra manera, la fracció d'hipòtesis nul·les rebutjades per error. El FDR considera la distribució dels nivells de significació assolits (valors de P) com un tot i discrimina entre la distribució uniforme (hipòtesi nul·la) i aquells valors que se'n desvien (hipòtesi alternativa), ajustant el nivell de significació.

Correccions basades en procediments computacionals: correccions per permutacions: Mitjançant aquest mètode es fixen les dades genotípiques i s'assigna el fenotip a cada individu de forma aleatòria amb l'objectiu de generar grups de dades que mantenen l'estructura de desequilibri de lligament però que compleixen la hipòtesi nul·la de no associació amb el fenotip. Aquest procés de permutació es realitza múltiples vegades (per exemple 10.000 permutacions en els nostres estudis de múltiples marcadors) i permet obtenir nous valors de significació ajustats. L'avantatge d'aquests mètodes és que es poden aplicar tant si les variables són independents com si estan relacionades.

En les anàlisis de marcadors individuals realitzades en aquest treball s'han utilitzat els mètodes de correcció de Bonferroni i FDR establint en el darrer cas un llindar del 15 % per *DRD1* en l'estudi del sistema dopaminèrgic (article 3), mentre que a l'anàlisi de múltiples marcadors o haplotips s'han corregit els valors de significació mitjançant permutacions. No obstant, cal tenir en compte que en els diferents estudis presentats s'ha utilitzat la mateixa població per identificar

variants de risc i, per tant, en teoria s'haurien de considerar les diferents comparacions realitzades a cada estudi a l'hora de corregir els valors de significació. Si consideréssim cadascun dels estudis realitzats a la mostra de TDAH que es presenta en aquest treball les correccions serien tant restrictives que cap marcador analitzat en l'estudi de marcadors individuals es mantindria associat al fenotip estudiat a l'estudi conjunt.

2.9. Estudis de rèplica

Els estudis de rèplica permeten avaluar en altres grups poblacionals l'associació positiva identificada en l'estudi original i determinar l'efecte genètic sobre un fenotip de forma més acurada que a l'estudi inicial. Els estudis de rèplica són necessaris per contrastar resultats i per establir la credibilitat de l'associació genotip-fenotip identificada [Abou-Sleiman et al., 2006; Chanock et al., 2007; Gordon i Finch, 2005]. Sovint aquest tipus d'estudis mostren resultats contradictoris possiblement com a conseqüència de l'heterogeneïtat intrínseca entre els diferents dissenys experimentals. Així, en relació als dos gens més estudiats en el TDAH, *DRD4* i *DAT1*, hi ha nombrosos estudis amb resultats inconsistents, que presenten diferències quant a les proporcions dels diferents subtipus clínics, les comorbiditats, les proporcions de gènere, així com diferències metodològiques. També és veritat, però, que a part de les diferències en el disseny dels estudis, els mecanismes de susceptibilitat i la contribució relativa d'un polimorfisme a una malaltia complexa poden diferir entre poblacions i per tant, resultats negatius en l'estudi de rèplica no necessàriament invaliden les troballes inicials. En aquest treball s'han realitzat diversos estudis per replicar l'associació entre TDAH i els gens *DRD1*, *BDNF*, *DRD4*, *SLC6A3/DAT1* i *SLC6A4*, i en el cas dels gens *DRD1* i *SLC6A3* l'associació s'ha confirmat.

Per altra banda, cal tenir present que els factors de risc identificats en malalties complexes com el TDAH tenen un efecte reduït sobre el conjunt del fenotip i, per tant, la mostra seleccionada per als estudis de rèplica ha de complir alguns requisits, que inclouen (i) tenir una mida prou gran com per a permetre la identificació de variants de risc d'efecte menor, (ii) utilitzar una mostra independent de l'estudi inicial, (iii) seleccionar una mostra amb un fenotip el més semblant possible al de la cohort original per minimitzar l'heterogeneïtat (en el cas del TDAH treballar amb el mateix subtipus clínic), (iv) treballar amb un poder estadístic igual o superior al de la mostra original i (v) analitzar els mateixos SNPs o amb SNPs que presentin un elevat grau de LD respecte als originals, amb r^2 properes a 1 [Bodmer i Bonilla, 2008].

2.10 Diferències genètiques entre poblacions

Atès que hi ha diferències genètiques entre poblacions, l'associació entre les variants polimòrfiques i la malaltia estudiada pot no mantenir-se en diferents contextos poblacionals. Si bé les freqüències al·lèliques de molts polimorfismes genètics varien significativament entre poblacions, com el polimorfisme VNTR48bp del gen *DRD4*, l'impacte biològic de les variants de risc per a una determinada patologia és generalment consistent entre els grups ètnics principals. No obstant això, s'han identificat diferències moderades en els efectes biològics de diverses variants polimòrfiques que podrien explicar la falta de rèplica en un percentatge significatiu dels estudis d'associació [Salas i Carracedo, 2007]. A més, tot i que diversos estudis indiquen que els tagSNPs sovint es transfereixen bé entre poblacions d'origen comú [Balding, 2006], un grup de tagSNPs seleccionats per assegurar la cobertura genètica d'una determinada població podria ser poc representatiu de la variabilitat del gen en una segona població. Recentment s'ha publicat un estudi de 21 gens relacionats amb els sistemes serotoninèrgic i dopaminèrgic en 39 poblacions que representen la diversitat genètica global de l'espècie humana [Gardner et al. 2008] i, sorprenentment, s'ha identificat homogeneïtat en les freqüències al·lèliques i similitud en els patrons de LD entre les diferents poblacions. Aquests resultats suggereixen que les diferències ètniques en si mateixes no poden explicar les discrepàncies identificades entre estudis de trastorns neurològics o psiquiàtrics.

En el cas dels estudis meta-analítics inclosos en aquesta Tesi Doctoral, el test Q que s'ha utilitzat per a detectar heterogeneïtat entre les diferents poblacions analitzades en cadascuna de les comparacions (Espanya, Holanda, Noruega i Alemanya en el cas dels articles 1 [Sánchez-Mora et al., 2010], 4 [Franke et al.,2009] i 5 [Sánchez-Mora et al], i Espanya, Holanda, Noruega, Alemanya i una població nord-americana a l' article 2 [Landaas et al., 2010]) indica que hi ha en general homogeneïtat interpoblacional, amb alguna excepció com és el cas de l'anàlisi del gen *SLC6A4,* en què es va haver d'utilitzar un model d'efectes aleatoris (*random-effect model*) en la meta-anàlisi [Landaas et al., 2010].

2.11 Poder estadístic

Els estudis d'associació han de tenir poder estadístic suficient per identificar les variants d'efecte menor implicades en l'etiologia del trastorn estudiat. El poder estadístic (o la probabilitat d'obtenir un resultat significatiu quan aquest resultat és cert) [Zondervan i Cardon, 2004] depèn de diversos factors: la magnitud de l'efecte de la variant que participa en el fenotip, la freqüència de

la variant funcionalment rellevant per a la malaltia i la de la variant analitzada, el grau de LD entre aquestes dues variants, la mida de la mostra i la prevalença de la malaltia [Abou-Sleiman et al., 2006]. No obstant, en general a les malalties complexes es desconeix molta d'aquesta informació. Així, generalment no se sap quin és l'efecte real de les variants genètiques sobre el fenotip global i es considera que les variants comunes tenen una contribució individual molt limitada. D'aquesta forma, s'estima que el risc relatiu dels polimorfismes amb freqüències al·lèliques superiors a 0,2 està dins del rang d'1,1 a 1,5, mentre que per a freqüències al·lèliques d'entre 0,05 i 0,2 el risc relatiu podria arribar fins a 3. Sembla lògic pensar que variants comunes amb freqüències al·lèliques elevades i un risc relatiu elevat explicarien una proporció gran de la causalitat de la malaltia, fet que s'ajustaria més aviat a allò que esperem a les malalties mendelianes que no a les complexes [Zondervan i Cardon, 2004].

Per altra banda, en els estudis d'associació indirectes, en què la selecció dels polimorfismes no causals ha d'assegurar la màxima cobertura genètica, el poder estadístic és màxim quan la freqüència al·lèlica del marcador estudiat i la del marcador de risc és la mateixa i estan en LD total (r^2=1 i D'=1) (Abou-Sleiman et al. 2006). A més, s'estima que per identificar ORs de l'ordre de 2 o 3, una mostra amb suficient poder estadístic hauria d'incloure de 500 a 1000 casos i controls. En canvi, per detectar marcadors amb efectes menors, amb ORs de 1,2 o 1,3, caldrien mostres del voltant de 5000 casos i controls [Zondervan i Cardon, 2004]. En condicions generals, un estudi que inclogui una mostra de 1000 casos i 1000 controls tindria un poder estadístic del 80% per detectar marcadors amb MAF>10% i OR de 1,5.

Hi ha un ampli ventall de programes que permeten estimar el poder estadístic tant per a estudis d'associació com per a estudis de lligament. Un dels més utilitzats és el *Genetic Power Calculator*, que té una interfase web i genera un arxiu de sortida (*output*) concís i útil [Purcell et al., 2003]. En l'estudi sobre el sistema dopamièrgic realitzat en aquesta Tesi Doctoral, vam assumir inicialment un risc relatiu de 1,5, una prevalença per a la malaltia de 0,05 i una MAF de 0,15, tot estimant un poder estadístic del 71,3% a la mostra de TDAH infantil i del 53,3% a la mostra de TDAH adult amb el model d'herència dominant. En resposta als requeriments dels revisors de la revista on es va enviar a publicar l'article, vam rebaixar el risc relatiu a un valor de 1,3, tot obtenint llavors valors de 36,4%, 28,3% i 6,7% per els models d'herència dominant, codominant i recessiu, respectivament, a la població infantil, i d'un 25,7%, 19,7% i 6,1% a la població adulta considerant els mateixos models d'herència. A l'estudi del gen *SLC6A4*, el poder estadístic estimat és d'un 83% assumint un risc relatiu de 1,2 amb una MAF de 0,00625. En

canvi, en els estudis de metanàlisi realitzats, el poder estadístic està al voltant del 90%, en presentar una mida mostral molt més gran.

2.12 Meta-anàlisi i biaix de publicacions

Les meta-anàlisis són anàlisis estadístiques conjuntes dels resultats obtinguts en diversos estudis. Consisteixen en mètodes quantitatius per combinar resultats de diferents treballs que avaluen l'associació dels mateixos marcadors amb un fenotip concret, amb l'objectiu de mesurar i intentar explicar les inconsistències entre els diferents estudis. Aquest tipus d'aproximació ofereix l'oportunitat de combinar dades acumulades en estudis retrospectius o generades en estudis prospectius [Kavvoura i Ioannidis, 2008]. No obstant, la selecció dels treballs inclosos ha de ser rigorosa, ja que la fiabilitat de l'estudi depèn de la qualitat i de la comparabilitat d'aquestes dades. En general, un dels principals problemes de les meta-anàlisis retrospectives és el biaix de publicacions, ja que la literatura tendeix a reflectir tan sols estudis que mostren associacions positives, mentre que una proporció elevada dels estudis negatius no s'arriben a publicar mai. Per tant, en els estudis de meta-anàlisi retrospectius s'observa una tendència sistemàtica cap a valors de P significatius, per sobre d'allò que s'esperaria per atzar [Salas i Carracedo, 2007] Aquesta preferència envers la publicació de resultats positius, encara que provinguin de dissenys no òptims, suposa una barrera per a la difusió dels estudis ben dissenyats però amb resultats negatius que, tot i difícils de publicar, són crucials per distingir les associacions positives reals de les falsament positives [Chanock et al., 2007]. En el treball que es presenta s'han realitzat meta-anàlisis que inclouen estudis no publicats prèviament de quatre poblacions TDAH independents per tal d'avaluar el paper dels gens *BDNF*, *DRD4*, *DAT1* i *SLC6A4* en el transtorn. En tots els casos, tant els mètodes de diagnòstic del trastorn com el disseny experimental de l'estudi, la metodologia i l'anàlisi han estat altament homogenis, fet que recolza la fiabilitat dels resultats obtinguts.

3. CONSIDERACIONS SOBRE ELS RESULTATS OBTINGUTS

3.1 *BNDF* i trastorn per dèficit d'atenció amb hiperactivitat: contribució del polimorfisme p.Val66Met

Hi ha molts estudis d'associació que impliquen el polimorfisme p.Val66Met (rs6265) del gen *BDNF*, que podria afectar el processament intracel·lular i la secreció de la neurotrofina, en l'etiologia de molts trastorns psiquiàtrics, com l'esquizofrènia, l'anorèxia o la depressió [Agartz et al., 2006; Chao et al., 2006; Connor i Dragunow, 1998; Egan et al., 2003; Ribases et al., 2005; Zhang et al., 2007]. Pel que fa al TDAH, els diferents estudis realitzats mostren resultats poc consistents (Taula 6). La meta-anàlisi que es presenta en aquesta Tesi no recolza la participació d'aquest polimorfisme en l'etiologia del TDAH. Aquests resultats negatius podrien ser deguts al fet que el polimorfisme p.Val66Met no estigui implicat en la susceptibilitat al trastorn o, alternativament, a l'existència d'heterogeneïtat metodològica entre els diferents estudis. En aquest sentit, cal tenir en compte algunes consideracions:

(i) La gran majoria dels estudis realitzats s'han centrat en població infantil amb TDAH, i únicament han considerat població adulta els estudis realitzats per Lanktree i col·l, que van identificar associació entre rs6265 i TDAH adult i Conner i col·l, que. no van identificar cap al·lel de risc implicat en TDAH adult [Conner et al., 2008; Lanktree et al., 2008]. La falta d'associació entre *BDNF* i TDAH que s'ha observat en aquest estudi podria explicar-se per un possible paper biològic d'aquest polimorfisme en l'evolució de la malaltia al llarg de la vida; és a dir, el SNP p.Val66Met participaria de forma específica en el TDAH en població infantil i no en població adulta. La implicació del receptor específic de BDNF, NTRK2, en TDAH infantil però no en adults identificada pel nostre grup recolza aquesta hipòtesi [Ribases et al., 2008]. Per altra banda, l'associació específica de subtipus clínic, concretament amb el subgrup de pacients amb TDAH de tipus hiperactiu-impulsiu, també podría explicar els resultats contradictoris obtinguts entre estudis. Així, donat que els subtipus clínics evolucionen al llarg de la vida i els símptomes d'hiperactivitat i, per tant, la proporció de pacients amb TDAH de subtipus hiperactiu-impulsiu, són més prevalents en població infantil, l'anàlisi del TDAH de forma global podria donar lloc a errors de tipus II o falsos negatius. Malauradament, donada la baixa proporció del subtipus hiperactiu-impulsiu a la nostra població adulta amb TDAH, l'efecte del polimorfisme p.Val66Met en aquest subtipus clínic no s'ha pogut avaluar.

(ii) Aquest estudi té un poder estadístic superior al 95%, fet que permetria la detecció de variants comunes d'efecte moderat associades al TDAH, com el polimorfisme p.Val66Met. No obstant, tot i utilitzar els mateixos criteris diagnòstics i descartar la presència d'heterogeneïtat, les quatre poblacions europees d'origen caucàsic analitzades mostren diferències en les freqüències al·lèliques d'aquest polimorfisme. Així, la població alemanya, que presenta una major representació de l'al·lel Val66 al grup de pacients, es comporta de manera diferent a la resta de poblacions, que presenten un excés de la variant Met66 en el grup de TDAH. Tot i que aquestes diferències podrien estar relacionades a fenòmens d'atzar, podrien afectar la tendència observada als altres tres grups poblacionals.

(iii) Les associacions identificades per altres autors entre el polimorfisme p.Val66Met i diversos trastorns comòrbids amb el TDAH, com la depressió, suggereixen que diferències en les proporcions d'aquestes comorbiditats a les poblacions TDAH estudiades podrien contribuir a la confusió dels estudis. En el nostre estudi, aproximadament el 57% dels pacients amb TDAH presenten depressió comòrbida. En subdividir-los en funció d'aquesta comorbiditat, es va identificar associació entre l'al·lel Val66 i TDAH+depressió a la població alemanya i de l'al·lel Met66 i TDAH sense depressió a la població holandesa, fet que recolza la necessitat de tenir en compte la comorbiditat en futurs estudis d'associació.

(iv) Finalment, tal i com suggereixen altres estudis d'associació [Kim et al., 2007] i estudis en models animals en què s'ha observat un efecte de BDNF dependent de gènere [Monteggia et al., 2007], les proporcions home:dona podrien tenir també alguna influència.

3.2 *SLC6A4* i trastorn per dèficit d'atenció amb hiperactivitat

Diversos estudis impliquen al sistema serotoninèrgic en l'etiologia del TDAH. Una de les variants polimòrfiques més estudiada ha estat el polimorfisme 5-HTTLPR del gen que codifica el transportador de serotonina, *SLC6A4 o 5HTT,* una inserció/deleció situada a la regió promotora del gen. Estudis funcionals realitzats amb aquest polimorfisme mostren que l'al·lel curt (S) redueix l'eficiència transcripcional del gen [Lesch et al., 1996] i està implicat en l'etiologia dels trastorns d'ansietat. Per altra banda, tot i que no hi ha dades genètiques concloents sobre la seva contribució al TDAH, l'al·lel llarg (L) s'ha identificat com a al·lel de risc en el TDAH en diversos estudis [Beitchman et al., 2003; Curran et al., 2005; Kent et al., 2002; Manor et al., 2001]. Els primers estudis realitzats tenien un escàs poder estadístic i utilitzaven un nombre limitat de pacients, mentre que en els últims anys s'han dut a terme diverses meta-anàlisis

d'estudis sobre població infantil. No obstant, en cap cas es van observar resultats sòlids favorables a l'associació [Faraone et al., 2005; Forero et al., 2009; Gizer et al., 2009; Xu et al., 2008]. Donats aquets antecedents que suggerien que la variant de risc del polimorfisme *5HTT-LPR* podria estar en LD amb la veritable variant de risc, vam voler avaluar la possible associació del gen *5-HTT* amb el TDAH en població adulta tot considerant aquest polimorfisme i set tagSNPs. A més, es van seqüenciar les regions exòniques del gen en un total de 93 pacients per tal d'identificar la variant directament implicada en el trastorn. Inicialment, es va identificar associació nominal entre el TDAH en adults i l'a·lel A del polimorfisme rs140700 al gen *SLC6A4* situat al intró 6, en una mostra de Noruega que no es va poder replicar en una meta-anàlisi de cinc poblacions diferents (Noruega, Alemanya, Holanda, Espanya i Estats Units). També es va descartar la participació dels diferents marcadors en el TDAH quan es va tenir en compte el gènere o la presència/absència de trastorns comòrbids com la depressió o l'ansietat. Per altra banda, la seqüenciació de les regions exòniques va permetre identificar una mutació sinònima (c.924T>C) no descrita anteriorment, dues variacions rares no sinònimes de tipus SNP (rs6352 p.Gly56Ala i rs6355 p.Lys605Asn) ja descrites i en heterozigosi i un SNP comú a la regió 3' UTR que presenta una MAF del 48% en la població d'estudi, totes elles interessants per ser estudiades en futurs estudis d'associació.

La gran majoria dels estudis realitzats en població infantil han identificat l'al·lel L del polimorfisme 5-HTTLPR com a factor genètic de risc [Curran et al., 2005; Kent et al., 2002; Manor et al., 2001; Seeger et al., 2001]. Aquests resultats es contraposen amb el nostre estudi realitzat en població adulta, en què observem una sobrerepresentació de l'al·lel S del polimorfisme a la població de casos, tot i que les diferències observades no són significatives. Aquestes diferències entre la població infantil i la població adulta suggereixen que el polimorfisme no conté variants comunes de susceptibilitat amb un efecte general sobre el trastorn al llarg de la vida. No podem descartar, però, que el polimorfisme 5-HTTLPR o una altra variació que estigui en LD amb aquest marcador estigui associada amb el TDAH. En base als resultats obtinguts, seria necessari una mostra de més de 5.600 casos i 5.600 controls per detectar, amb un poder estadístic del 80%, un OR de 1,1 a un nivell de significació de p = 0,006 corregit per Bonferroni.

3.3 Sistema dopaminèrgic i trastorn per dèficit d'atenció amb hiperactivitat

S'ha avaluat la possible implicació del sistema dopaminèrgic en el TDAH mitjançant dues aproximacions diferents: (i) un estudi d'associació de tipus cas-control poblacional i familiar en una població d'origen espanyol en què s'han analitzat 61 SNPs de 9 gens del sistema dopaminèrgic (*DRD1*, *DRD2*, *DRD3*, *DRD4*, *DRD5*, *SLC6A3*, *TH*, *COMT* i *DBH*) i el posterior estudi de rèplica en una mostra independent d'origen alemany i (ii) l'anàlisi de quatre polimorfismes no-SNP als gens *DRD4* i *SLC6A3* que s'han analitzat en dos estudis de meta-anàlisi independents en quatre poblacions europees.

3.3.1. Estudi cas-control de 9 gens del sistema dopaminèrgic

En aquest estudi, realitzat seguint criteris de màxima cobertura genètica, es va identificar associació entre TDAH infantil i el gen que codifica el receptor de dopamina, *DRD1,* a la cohort inicial d'origen espanyol. L'haplotip de risc identificat a la mostra espanyola (rs863126A-rs265977C) se solapa parcialment amb l'haplotip de risc identificat en una segona mostra de famílies d'origen alemany (rs835541G-rs863126T), amb descendència també en edat infantil. Tots dos haplotips contenen el SNP rs863126 però els al·lels de risc difereixen a les dues poblacions. Aquest fenomen, anomenat "flip-flop" [Lin et al., 2007], pel qual al·lels oposats d'un mateix marcador poden identificar-se com a al·lels de risc a un mateix fenotip, pot ser degut al fet que el marcador estudiat, en aquest cas el SNP rs863126, no sigui la variant de risc, sinó que estigui en LD amb la variant realment implicada en el trastorn. Diversos estudis previs recolzen la contribució de *DRD1* en el TDAH. Entre ells, tres models animals d'hiperactivitat que presenten alteracions en l'expressió o funció de *DRD1* (el model expontani SHR, el ratolí mutant genoanul.lat *DRD1* (-/-) i les rates NHE (Naples High-Excitability)) [Clifford et al., 1998; Faraone et al., 1998; Leo et al., 2003]. Per altra banda, agonistes de DRD1 reverteixen els efectes del metilfenidat a l'escorça prefrontal en rates [Arnsten i Dudley, 2005; Arnsten i Vijayraghavan, 2006] i diversos estudis d'associació [Bobb et al., 2005; Brookes et al., 2006; Lasky-Su et al., 2008a; Luca et al., 2007] i GWAS apunten al gen *DRD1* com a possible factor de risc de TDAH infantil però no a l'edat adulta [Lesch et al., 2008]. Aquests resultats recolzen la contribució del gen al TDAH, tot i que no s'ha identificat cap polimorfisme funcional o variant consistentment relacionada amb el trastorn en els diferents estudis, suggerint que la variant directament implicada està situada fora de la regió codificant de *DRD1* [Feng et al., 1998; Misener et al., 2004].

3.3.2. Estudi de meta-anàlisi dels gens *SLC6A3* i *DRD4*

Els gens del sistema dopaminèrgic que codifiquen el transportador de dopamina, *SLC6A3,* i el receptor dopaminèrgic D4, *DRD4*, són probablement els més estudiats en TDAH. Un gran nombre d'estudis d'associació han analitzat la possible contribució d'aquest dos gens al trastorn però, una vegada més, els resultats no són concloents. Per aquest motiu, s'han realitzat diverses meta-anàlisis amb l'objectiu d'unificar criteris i, tot i així, el resultat d'aquests estudis tampoc són consistents entre ells.

Diversos estudis en població infantil amb TDAH han identificat un haplotip de risc (10R6R) format pels dos polimorfismes tipus VNTR del gen *SLC6A3/DAT1* analitzats en aquest treball, un a la regió 3'UTR del gen i l'altre a l'intró8. No obstant, en el nostre treball hem identificat una combinació al·lèlica de risc diferent en considerar el grup de TDAH adult (9R6R). Aquests resultats suggereixen que diferents al·lels d'un mateix locus podrien estar implicats en el TDAH infantil o en la persistència del trastorn al llarg de la vida; és a dir, hi hauria una càrrega genètica diferencial entre el TDAH infantil i el TDAH adult. És probable que hi hagi factors de risc implicats únicament en el TDAH a la població infantil i en la remissió de la simptomatologia a l'edat adulta, mentre que d'altres variants estarien implicades en la persistència del trastorn al llarg de la vida. És necessari fer estudis longitudinals (seguiment d'una població de TDAH infantil fins a l'edat adulta) per a la confirmació d'aquestes hipòtesis. Estudis recents assenyalen que la susceptibilitat al TDAH és de fet, un procés dinàmic en què nous gens i factors ambientals van intervenint en diferents períodes del desenvolupament [Kuntsi et al., 2005; Mill et al., 2005; Rietveld et al., 2004].

Per altra banda, una de les variants del gen *DRD4* més estudiades en el TDAH ha estat el polimorfisme de tipus VNTR de 48 parells de bases situat a l'exó 3 del gen, que codifica el tercer domini transmembrana del receptor, regió d'unió a proteïna G mediadora en la transducció de senyal (Figura 14).

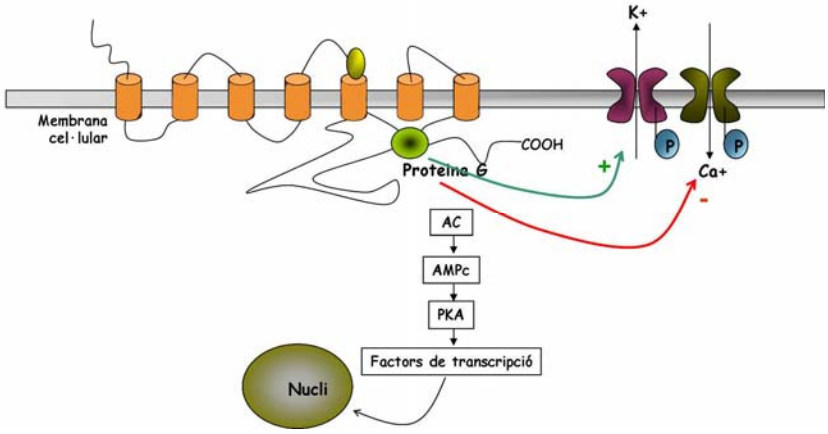


Figura 14. Receptor de dopamina *DRD4* i senyalització intracel.lular

AC:Adenilat ciclasa; AMPc: adenosina monofosfat cíclic; PKA: proteïna cinasa A.

Donat el seu possible efecte funcional [Asghari et al., 1995], aquest polimorfisme ha estat objecte d'estudi en múltiples poblacions amb TDAH i, mentre la gran majoria de treballs han identificat com a variant de risc l'al·lel 7R [Barkley et al., 2006; Bhaduri et al., 2006; Faraone et al., 2001; Kim et al., 2005b; Li et al., 2006; Lowe et al., 2004; Swanson et al., 2000], d'altres no recolzen la contribució d'aquest polimorfisme en el risc de patir el trastorn [Bakker et al., 2005; Brookes et al., 2005; Todd et al., 2001]. Cal destacar l'existència de diferències interètniques en les freqüències al·lèliques d'aquest polimorfisme, que podrien explicar les dades incongruents observades entre estudis. Per exemple, l'al·lel 4R està representat en totes les poblacions amb una freqüència elevada (mitjana global del 64,3%, rang del 16 al 96%), mentre que l'al.lel 7R, el segon més freqüent a nivell global (20,6%) és gairebé inexistent en població asiàtica (0-2%) però molt freqüent a Amèrica (48,3%). El tercer al.lel més freqüent, 2R (8,2% a nivell global), és comú a les poblacions asiàtiques (18,1%) però poc comú a Amèrica (2,9%) i Àfrica (1,7%). [Altshuler et al.]. Aquestes diferències en les freqüències al·lèliques posen de manifest una de les principals limitacions dels estudis de rèplica en poblacions independents.

Un segon polimorfisme del gen *DRD4* molt estudiat en TDAH perquè té possibles conseqüències funcionals, és una duplicació/deleció (Dup/Del) de 120pb situada a la regió promotora,. Així, diversos estudis recolzen que l'al·lel llarg de 240pb (L) disminueix l'activitat transcripcional del gen [D'Souza et al., 2004; Ronai et al., 2004]. L'anàlisi d'aquests dos polimorfismes de *DRD4* en funció del gènere i el subtipus clínic ens va permetre identificar una associació nominal entre l'al·lel L del polimorfisme Dup/Del i TDAH combinat o TDAH en el grup de dones. Tot i que aquests resultats no es van replicar a l'estudi de meta-anàlisi, l'anàlisi de

múltiples marcadors va mostrar associació entre la combinació al·lèlica L-4R (Dup/Del-48bpVNTR) i TDAH de subtipus clínic combinat. La identificació d'un haplotip de risc específic del subtipus clínic combinat posa de manifest que possiblement els diferents subtipus clínics presenten un patró genètic específic i per tant, en els diferents estudis de rèplica en què els resultats són poc consistents caldria tenir present les freqüències dels diferents subtipus clínics a les poblacions estudiades.

El fet que els dos gens, *SLC6A3* i *DRD4*, participen en la neurotransmissió dopaminèrgica i s'expressen en regions diferents del SNC (*SLC6A3* a les regions subcorticals i *DRD4* a l'escorça frontal) suggereix que les seves accions podrien tenir un efecte epistàtic en la contribució al risc de TDAH [Durston et al., 2005]. Diversos estudis que han avaluat l'existència d'epistasi entre els dos gens mostren resultats discordants sobre la participació combinada d'aquets dos gens en la simptomatologia del TDAH [Carrasco et al., 2006; Gabriela et al., 2009; Kim et al., 2005a; Qian et al., 2007; Roman et al., 2001]. En aquest sentit, el nostre treball mostra evidències d'efectes additius, però no epistàtics, entre *SLC6A3* i *DRD4* en l'etiologia del trastorn.

4. PERSPECTIVES DE FUTUR EN L'ESTUDI DE LA BASE GENÈTICA DEL TDAH

La major part de la recerca que s'ha fet fins ara sobre la base genètica del TDAH està basada en estudis de lligament genètic i, sobretot, en estudis d'associació de tipus cas-control. Els primers estudis d'associació se centraven en l'anàlisi de variants funcionals en gens candidats concrets i posteriorment han derivat cap a la utilització de criteris de cobertura genètica per seleccionar polimorfismes en gens candidats en funció dels patrons de LD, estratègia que implica l'estudi de variants no necessariament funcionals. El ràpid avenç de les noves tecnologies de genotipació massiva ha permès estudiar sistemes gènics que cobreixen vies funcionals senceres i des de fa pocs anys s'ha fet el salt a l'estudi de variants polimòrfiques que cobreixen tot el genoma (GWAS), i que representen la major part de la variabilitat existent. A partir de les reunions anuals de la Xarxa sobre Genètica Molecular del TDAH (*ADHD Molecular Genetics Network*), de la qual forma part el nostre grup de recerca, podem conèixer algunes de les perspectives immediates en la investigació sobre aquest trastorn, que es discuteixen a continuació.

4.1 Tècniques de seqüenciació massiva

La naturalesa intrínseca de la variabilitat genètica que hi ha darrere les malalties mendelianes o monogèniques (un únic canvi rar, en general en zona codificant, determina el fenotip) respecte a les malalties complexes o multifactorials (diversos canvis, comuns o rars, en diferents gens, donen susceptibilitat al trastorn en combinació amb factors ambientals) condiciona els enfocs experimentals que s'usen en un cas i en l'altre. Les tècniques de seqüenciació massiva s'han aplicat de moment a la identificació de variants rares en trastorns mendelians, i hi ha encara molt poques dades en malalties complexes, en què s'està explorant sobretot la hipòtesi "malaltia comuna/variant comuna" a través dels estudis d'associació. No obstant, poc a poc van apareixent treballs que contrasten la hipòtesi "malaltia comuna/variant rara", tant a partir de la identificació de CNVs rares com de canvis d'un únic nucleòtid [Elia et al.; Williams et al.; Berkel et al.]

Aquests darrers anys, l'adveniment de les noves tecnologies de seqüenciació massiva ha permès identificar en el genoma humà un gran número de variants rares (que per la seva baixa freqüència no es poden analitzar en els estudis d'associació cas-control clàssics) i de variants comunes no descrites anteriorment. Tot i que l'estudi de variants rares com a possibles candidates a contribuir als fenotips complexes és una via interessant, de moment les tecnologies

de seqüenciació massiva són encara relativament cares i per tant aquest tipus d'estudis se centren en general en casos familiars o en fenotips molt específics i ben delimitats.

4.1.1 Estudi d'exomes

Les regions codificants representen aproximadament un 1% del total del genoma humà. Els estudis de seqüenciació massiva de les regions exòniques, o exomes, són de gran utilitat per a la identificació de variants rares causants de malalties. L'estudi d'exomes permet identificar gens o al·lels implicats en malalties mendelianes i complexes o mutacions somàtiques esporàdiques en malalties com el càncer [Hoischen et al., ; Robison et al.]. Tot i que les variants relacionades amb les malalties multifactorials poden estar també fora dels exons, és probable que (de forma similar a les malalties mendelianes) la majoria d'aquests canvis afectin a la seqüència aminoacídica, i és per això que de moment, per minimitzar els elevats costos de la seqüenciació massiva, s'hagi optat per l'anàlisi d'exomes i no de genomes. No obstant, està clar que les regions reguladores també poden tenir un paper rellevant, i caldrà tenir-les en compte.

A l'última reunió de la Xarxa sobre Genètica Molecular del TDAH (*11th Annual Meeting of the ADHD Molecular Genetics Network*) celebrada a Florida (Estats Units) el mes de desembre de 2010, es van presentar els primers projectes de seqüenciació d'exomes en pacients amb TDAH, amb l'objectiu d'identificar variants rares en casos familiars o amb fenotips molt particulars. El consorci IMpACT (*International Multicenter Persistent ADHD Collaboration*), centrat en l'estudi del TDAH adult, va presentar una proposta per seqüenciar exomes en pacients noruecs, amb replicació dels resultats a la resta de poblacions del consorci, inclosa l'espanyola. Actualment, la limitació principal de la generalització dels estudis de seqüenciació massiva al TDAH i d'altres malalties complexes té relació amb la necessitat d'estudiar un número molt elevat de mostres per arribar a conclusions sòlides.

4.1.2 Estudis d'associació a escala genòmica (GWAS)

Sota la hipòtesi de malaltia comuna/variant comuna, els estudis de GWAS permeten analitzar variants comunes amb efecte moderat que incrementen el risc de desenvolupar un determinat fenotip complex. Aquesta aproximació és especialment interessant per aquells trastorns en què hi ha poques pistes sobre les bases fisiopatològiques subjacents, com és el cas del TDAH, fet que dificulta la tria de gens candidats. Fins a l'actualitat l'únic GWAS realitzat en TDAH en adults [Lesch et al., 2008] en un total de 343 casos i 304 controls amb una cobertura genètica de 500k

(500.000 SNPs) no ha permès identificar cap variant de risc clarament associada al fenotip, tot i que hi ha diverses variants que presenten associacions nominals. Per altra banda, en població infantil s'ha realitzat un estudi GWAS en uns 950 trios formats per dos progenitors i un fill o filla amb TDAH de subtipus combinat amb una cobertura genètica de 600K i no s'han identificat variants de risc després d'aplicar correccions per comparacions múltiples. No obstant, s'ha observat associació nominal entre TDAH infantil i alguns dels gens estudiats en aquest treball, com *DRD1* i *SLC6A3/DAT1,* entre d'altres [Lasky-Su et al., 2008a].

L'anàlisi de marcadors individuals en els GWAS és equivalent als estudis d'associació clàssics i, per tant, les dues aproximacions presenten limitacions similars: (i) Mida de la mostra. Es considera que per detectar un risc relatiu de l'ordre de 1,28 és necessària una la mostra de 3000 casos i 3000 controls [Hindorff et al., 2009]; (ii) Homogeneïtat clínica de la mostra. Per aquest motiu el GWAS realitzat en mostra infantil amb TDAH ha inclòs únicament pacients amb subtipuc clínic combinat [Lasky-Su et al., 2008a; Lasky-Su et al., 2008b]; (iii) A causa de l'elevat nombre de comparacions que es realitzen, el valor de significació corregit és de l'ordre de p=5x10e-8 [Pe'er et al., 2008], un valor molt astringent per reduir al màxim la probabilitat d'error de tipus I, però que pot afavorir l'aparició d'errors de tipus II o falsos negatius, (iv) Comorbiditat amb d'altres trastorns psiquiàtrics. Fins ara s'han publicat un total de 5 estudis GWAS en malalties psiquiàtriques que inclouen TDAH, esquizofrènia, trastorn bipolar, autisme i depressió i que presenten cert solapament en els resultats observats (Taula 4). Donat que hi ha certa comorbiditat entre ells, caldria considerar els trastorns comòbids en futurs estudis així com aprofundir en l'anàlisi d'endofenotips.

L'any 2009 el nostre grup de recerca va rebre finançament per a la realització d'un estudi GWAS d'1M en 700 casos i 700 controls amb l'objectiu de millorar els dissenys dels GWAS realitzats fins l'actualitat en TDAH a diferents nivells: (i) Selecció de pacients amb TDAH combinat sota la presumpció que els diferents subtipus clínics, tot i compartir factors genètics, presenten també diferències; (ii) Recollida dels casos i dels controls en una àrea geogràfica restringida al voltant de Barcelona per part de només dos grups clínics diferents que segueixen protocols de diagnòstic consensuats; (iii) Selecció dels controls segons criteris d'exclusió del TDAH; (iv) Realització d'estudis d'heretabilitat de símptomes de TDAH segons els criteris DSM-IV que permetran seleccionar els trets endofenotípics més heretables per incloure'ls a l'anàlisi GWAS; (v) Incorporació a l'anàlisi d'informació ambiental i fenotípica addicional, com dades de comorbiditat, valors neuropsicològics o factors ambientals de risc, per reflectir el màxim possible

la complexitat del trastorn; (vi) Rèplica dels resultats positius identificats en tres mostres independents d'Espanya, Alemanya i Colòmbia (n=3000); i (vi) Inclusió dels resultats del nostre GWAS en una meta-anàlisi de diferents GWAS en TDAH actualment en marxa en el marc de la *ADHD Molecular Genetics Network.*

4.3 Estudis de variacions del número de còpies (CNVs)

En aquest treball ens hem centrat en l'estudi de tres tipus de polimorfismes: canvis d'un únic nucleòtid o SNPs, delecions o duplicacions i VNTRs. Un estudi pioner publicat a finals de l'any 2010 ha avaluat variacions del número de còpies (CNVs) en una població amb TDAH [Williams et al.]. Els CNVs són una font important de variació en el genoma que en estudis previs s'han relacionat amb altres trastorns del neurodesenvolupament com l'autisme, l'esquizofrènia o un baix coeficient intel·lectual (IQ < 70) [Elia et al., ; Glessner et al., 2009; Kirov et al., 2009]. Encara que s'havia postulat que aquest tipus de polimorfisme podria estar implicat en l'etiologia del TDAH [Elia et al.], no hi ha cap estudi previ al publicat per Williams i col·l. en què s'identifiqui alguna variant de risc del tipus CNV. Aquest estudi mostra que la regió cromosòmica 16p13.11, prèviament associada a esquizofrènia [Ingason et al.], presenta un elevat nombre de CNVs que podrien estar implicats en l'etiologia del TDAH tant en l'estudi original de 410 pacients infantils i 1156 controls de Regne Unit (p corregida = 0,008) com en l'estudi de rèplica realitzat en 825 pacients TDAH i 35.243 controls d'una població independent (p corregida = 0,031). La regió identificada conté únicament set gens, entre els quals hi ha el gen *NDE1,* que participa en el neurodesenvolupament i interactua amb *DISC1*, que s'ha relacionat amb l'esquizofrènia. Aquests resultats obren un nou camí en la recerca de noves variants de risc implicades en l'etiologia del TDAH. Així, en el marc de l'estudi GWAS descrit a l'apartat anterior, el nostre grup pretén realitzar l'anàlisi de 4.878 CNVs distribuïts per tot el genoma.

4.4 Anàlisi de microRNAs

En malalties psiquiàtriques com el TDAH, alteracions en els elements reguladors que modulen la transcripció de gens implicats en processos de neurodesenvolupament o neurotranmissió poden ser de gran utilitat per entendre les funcions i processos cerebrals involucrats. Els microRNA (miRNA) són petits transcrits no codificants d'una longitud de 19-25 nucleòtids que regulen la traducció i estabilitat del RNA missatger (RNAm) i, per tant, l'expressió gènica, a través de la seva unió a seqüències diana complementàries a les regions no traduïdes dels RNAm [Filipowicz

et al., 2008]. S'estima que els miRNA regulen l'expressió d'aproximadament un 30% dels gens en el genoma humà, tot constituint un dels elements reguladors més importants. Canvis en miRNA s'han relacionat amb malalties com la Síndrome del Cromosoma X fràgil o alguns tipus de càncer [Gong et al., 2005; Melo et al., 2009]. En aquest context, polimorfismes situats a les regions complementàries dels miRNA o dels seus RNAm diana, o SNPs que regulen la producció de miRNA són candidats a contribuir a la susceptibilitat a malalties complexes. Així doncs, estudis d'associació que inspeccionin SNPs situats en aquestes regions podrien ser un recurs útil per a l'avaluació de l'efecte d'aquests miRNA en trastorns complexes com el TDAH. No obstant, cal considerar que les regions d'unió als miRNA estan molt conservades i presenten una baixa freqüència de SNPs.

De moment s'ha fet poca recerca en aquest camp en relació al TDAH. Així, s'ha publicat un estudi que mostra una major concentració del miRNA rno-let-7d a l'escorça prefrontal de rates hiperactives SHR respecte a rates control [Wu et al.,]. Aquest miRNA s'encarrega de la regulació, entre d'altres, de l'expressió de la galectina-3 (*LGALS3*), un factor de transcripció regulador de l'expressió de l'enzim Tirosina Hidroxilasa (TH) implicat en la síntesi de dopamina i candidat a contribuir al fenotip TDAH. Per altra banda, un altre estudi ha avaluat el possible paper de dos SNPs (rs3813034 i rs1042173), localitzats a 3'UTR del gen *SLC6A4* i prèviament associats al TDAH, en la regulació i l'estabilitat del RNAm a través de la unió de diversos miRNA. Els resultats d'aquest estudi demostren que aquests polimorfismes no estan situats a les seqüències d'unió del miRNA [Banerjee et al., 2009].

4.5 Estudis de fenotips intermedis

Els fenotips intermedis o endofenotips són caràcters heretables responsables de l'associació entre gens i fenotips clínics específics [Gottesman i Gould, 2003; Szatmari et al., 2007] i compleixen els següents criteris: cosegreguen amb la malaltia en els estudis familiars, estan associats a la malaltia en estudis cas-control i presenten valors de puntuació clínica més elevats en els familiars no afectats que en controls independents. Es creu que els endofenotips presenten menys complexitat genètica que els trastorns psiquiàtrics i, per tant, són més propers als processos biològics subjacents [Franke et al., 2009]. Diversos caràcters neurocognitius com la memòria de treball, el processament temporal o el control de la inhibició compleixen els criteris bàsics per ser considerats fenotips intermedis o endofenotips del TDAH [Bidwell et al., 2007; Doyle et al., 2005; Rommelse et al., 2008a; Rommelse et al., 2008b; Slaats-Willemse et al.,

2003]. Fins al moment s'ha evaluat l'associació entre diferents endofenotips i gens candidats al TDAH, que inclouen *DRD4, COMT, SLC6A3* o *DBH*, mitjançant estudis d'associació [Barkley et al., 2006; Kebir et al., 2009] i de neuroimatge funcional [Ehlis et al., 2008; Mier et al., ; Valera et al., 2005]. La consideració d'endofenotips en la re-anàlisi de resultats de GWAS poden ajudar a visualitzar associacions críptiques que no s'observaven quan es considerava el "fenotip global" de la malaltia. Seguint aquesta línia de treball, els estudis de GWAS realitzats en població infantil de TDAH han correlacionat variants genètiques amb diferents endofenotips [Neale et al., 2008]. Diverses característiques neurocognitives són prometedors fenotips intermedis, ja que les característiques principals del TDAH com són la manca d'atenció i la hiperactivitat estan conceptualment relacionades amb els dominis cognitius com ara la funció executiva, atenció, memòria i intel.ligència [Castellanos et al., 2002; Nigg et al., 2005]. La majoria dels estudis realitzats en endofenotips intermedis s'han centrat en TDAH infantil i han analitzat trets com el processament temporal (variabilitat de resposta), la memòria de treball (visual-espacial i verbal) i la inhibició (control d'interferències) [Bidwell et al., 2007; Doyle et al., 2005; Rommelse, 2008; Slaats-Willemse et al., 2003]. En TDAH adult s'han realitzat pocs estudis d'endofenotips intermedis [Boonstra et al., 2008]. Són per tant necessaris estudis d'aquest tipus en TDAH a l'edat adulta.

4.6 Estudis farmacogenètics

Durant els últims anys s'han publicat els resultats de diversos estudis per identificar els gens implicats en la resposta terapèutica al tractament del TDAH amb metilfenidat o en l'aparició d'efectes secundaris. La major part d'aquests estudis s'ha centrat en els gens *SCL6A3/DAT1* i *DRD4,* perquè estan implicats en el mecanisme d'acció del metilfenidat, però no han mostrat resultats consistents [Froehlich et al.; Gruber et al., 2009; Joober et al., 2007; Purper-Ouakil et al., 2005]. Recentment, el nostre grup ha publicat conjuntament amb investigadors de diversos centres internacionals el primer estudi que ha avaluat i identificat la participació del gen de la latrofinilina-3 (*LPHN3*) en la resposta a metilfenidat de pacients infantils amb TDAH [Arcos-Burgos et al., ; Ribases et al.]. No obstant, no hi ha dades sobre el possible paper dels gens implicats en la via metabòlica del metilfenidat, com la carboxilesterasa-1 (*CES1*), o gens rellevants en el neurodesenvolupament, com els factors neurotròfics, en la resposta diferencial dels pacients al tractament amb metilfenidat. Aquest tipus d'estudis poden ser de gran utilitat clínica, ja que poden ajudar a predir la resposta al metilfenidat (no tots els pacients responen a

aquest medicament) i a prevenir els efectes secundaris, com ara l'insomni, la irritabilitat i fins i tot la dependència als fàrmacs psicoestimulants. Els estudis realitzats fins ara presenten limitacions en relació a l'heterogeneïtat metodològica, estudis retrospectius versus prospectius o a nivell de la selecció de polimorfismes individuals, i en cap cas s'ha assegurat la cobertura genètica dels gens estudiats. Per això calen estudis amb mostres àmplies i que controlin les limitacions dels treballs previs, tot incorporant les recomanacions internacionals per a la realització d'estudis de farmacogenètica [Froehlich et al.,; Polanczyk i Jensen, 2008].

Conclusions

S'ha avaluat la participació en la susceptibilitat al trastorn per dèficit d'atenció amb hiperactivitat (TDAH) de variants polimòrfiques de tipus SNP, VNTR i duplicació/deleció localitzades en gens candidats que codifiquen proteïnes dels sistemes de neurotransmissió dopaminèrgica i serotoninèrgica i factors neurotròfics mitjançant estudis d'associació cas-control i meta-anàlisi.

1. L'estudi del polimorfisme p.Val66Met (rs6265) del gen *BDNF* en 4 poblacions europees mitjançant meta-anàlisi no recolza la participació d'aquesta variant en l'etiologia del TDAH adult.
2. L'anàlisi de la participació del gen que codifica el transportador de serotonina o *SLC6A4* mitjançant un estudi cas-control centrat en set SNPs i en el polimorfisme 5-HTTLPR, així com la seqüenciació completa del gen en mostres de quatre poblacions europees i un població d'origen americà, totes adultes, no recolza els resultats publicats prèviament que suggereixen la seva participació en l'etiologia del TDAH.
3. L'avaluació de la contribució de 9 gens implicats en el sistema dopaminèrgic al risc de patir TDAH en la infància i en l'edat adulta a través de l'estudi d'SNPs seguint criteris de cobertura genètica posa de manifest que el gen *DRD1* que codifica el receptor de dopamina D1 està associat a TDAH infantil de subtipus combinat. Aquests resultats reforcen l'existència de factors genètics de susceptibilitat en el TDAH que són específics de subtipus clínic i de grups d'edat.
4. L'estudi de dos polimorfismes de tipus VNTR localitzats a la regió 3'UTR i a l'Intró 8 del gen que codifica el transportador de dopamina o *SLC6A3/DAT1* mitjançant meta-anàlisi en quatre poblacions europees suggereix la participació d'aquest gen en el TDAH i evidencia l'existència de diferències genètiques entre les poblacions de nens i adults amb TDAH en identificar un haplotip de risc específic de la població adulta amb TDAH i diferent al descrit prèviament en població infantil.
5. L'estudi de meta-anàlisi de quatre poblacions europees adultes per avaluar la participació de dos polimorfismes funcionals de tipus VNTR i duplicació/deleció al gen *DRD4* que codifica el receptor de dopamina D4 posa de manifest la seva associació al TDAH. A més, s'ha identificat un efecte additiu entre els gens *DRD4* i *SLC6A3/DAT1* en el risc global de patir TDAH.

6. L'anàlisi dels sistemes de neurotransmissió dopaminèrgica i serotoninèrgica realitzat en aquest treball recolzen la hipòtesi que hi ha diferències genètiques entre la població de TDAH infantil i TDAH adult. Els resultats obtinguts posen de manifest que hi ha factors genètics implicats de forma lineal en el fenotip al llarg de la vida, d'altres que participarien en el desenvolupament de la persistència i, finalment, factors que estarien relacionats amb la remissió del trastorn.
7. Aquest treball exemplifica la necessitat d'analitzar sèries molt àmplies de pacients i de realitzar estudis de rèplica en diferents poblacions quan s'avaluen variants comunes d'efecte moderat sobre el risc global de patir una malaltia complexa.

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Annex

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Contribution of *LPHN3* to the genetic susceptibility to ADHD in adulthood: a replication study

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Attention-deficit/hyperactivity disorder (ADHD) is a common and highly heritable developmental disorder characterized by a persistent impairing pattern of inattention and/or hyperactivity-impulsivity. Using families from a genetic isolate, the Paisa population from Colombia, and five independent datasets from four different populations (United States, Germany, Norway and Spain), a highly consistent association was recently reported between ADHD and the *latrophilin 3* (*LPHN3*) gene, a brain-specific member of the LPHN subfamily of G-protein-coupled receptors that is expressed in ADHD-related regions, such as amygdala, caudate nucleus, cerebellum and cerebral cortex. To replicate the association between *LPHN3* and ADHD in adults, we undertook a case–control association study in 334 adult patients with ADHD and 334 controls with 43 single nucleotide polymorphisms (SNPs) covering the *LPNH3* gene. Single- and multiple-marker analyses showed additional evidence of association between *LPHN3* and combined type ADHD in adulthood [$P = 0.0019$; df = 1; odds ratio (OR) = 1.82 (1.25–2.70) and $P = 5.1e\text{-}05$; df = 1; OR = 2.25 (1.52–3.34), respectively]. These results further support the *LPHN3* contribution to combined type ADHD, and specifically to the persistent form of the disorder, and point at this new neuronal pathway as a common susceptibility factor for ADHD throughout the lifespan.

Keywords: Adult ADHD, attention-deficit/hyperactivity disorder, case–control association study, *LPHN3*

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Attention-deficit/hyperactivity disorder (ADHD) is a common developmental disorder characterized by a persistent pattern of inattention and/or hyperactivity-impulsivity (Biederman & Faraone 2005; Swanson *et al*. 1998). Data from twin, family and adoption studies show that genetic factors play an essential role in the etiology of the disorder (Albayrak *et al*. 2008; Faraone *et al*. 2005; Maher *et al*. 1999). The worldwide pooled prevalence of ADHD is around 5.3% in school-aged children (Polanczyk *et al*. 2007). Attention-deficit/hyperactivity disorder can persist into adolescence and adulthood with an estimated prevalence rate of 4.4% (Kessler *et al*. 2006) and higher familial aggregation, suggesting that genes may play a stronger role in the etiology of persistent than in remitting ADHD (Faraone *et al*. 2000a).

Although dopaminergic and serotonergic candidate genes have been extensively examined in ADHD (Faraone *et al*. 2005; Mick & Faraone 2008; Oades 2008; Ribases *et al*. 2009b; Swanson *et al*. 2007; Thapar *et al*. 2005), Arcos-Burgos *et al*. recently reported a consistent association between ADHD and common genetic variants within the *latrophilin 3* (*LPHN3*) gene, a brain-specific member of the LPHN subfamily of G-protein-coupled receptors that is expressed in ADHD-related regions, such as amygdala, caudate nucleus, cerebellum and cerebral cortex (Arcos-Burgos *et al*. in press; Krain & Castellanos 2006; Sugita *et al*. 1998). This association was first observed in a genetic isolate (Paisa population in Colombia) and was confirmed in a total sample comprising 2627 cases, 2531 controls and 1202 relatives from five different populations (Paisas, United States, Germany, Norway and Spain), supporting the implication of a new neuronal pathway in the predisposition to ADHD as well as in the response to treatment with stimulant medication (Arcos-Burgos *et al*. in press). Interestingly, although the specific role of *LPHN3* in ADHD remains unknown, different lines of investigation suggest the involvement of this family of G-protein-linked receptors in synaptic neurotransmitter release as well as in neurodegeneration in response to ischemia and hypoxia (Bin Sun *et al*. 2002; Ichtchenko *et al*. 1998; Krasnoperov *et al*. 1997; Sugita *et al*. 1998). *LPNH1* and *LPHN2* mediate the Ca^{2+}-independent neurotransmitter release from presynaptic nerve terminals induced by α-latrotoxin, a potent excitatory neurotoxin that stimulates synaptic vesicle exocytosis (Ichtchenko *et al*. 1998; Krasnoperov *et al*. 1997; Sugita *et al*. 1998). In addition,

the expression of LPHNs is highly regulated during postnatal brain development, with *LPNH3* exhibiting its highest expression levels immediately after birth (Kreienkamp *et al*. 2000). Although follow-up studies have shown that ADHD symptoms persist into adulthood in most children and adolescents with ADHD (Biederman *et al*. 1996; Faraone *et al*. 2002; Kuntsi *et al*. 2005; Polanczyk & Rohde 2007), genetic studies have mainly focused on pediatric samples. Thus, little is known about common susceptibility factors involved in the etiology of ADHD addressing the diagnostic continuity of the disorder throughout the lifespan (Franke *et al*. 2010; Ribases *et al*. 2008, 2009b). In this regard, six of the seven independent ADHD samples studied by Arcos-Burgos *et al*. consisted primarily of children and adolescents, and only the Norwegian clinical sample consisted exclusively of adult patients with ADHD (Arcos-Burgos *et al*. in press). To replicate the strong association between *LPHN3* and ADHD in an additional sample of adults with ADHD, we performed a case–control association study in 334 adults with ADHD and 334 controls with 43 single nucleotide polymorphisms (SNPs) covering, in terms of linkage disequilibrium (LD), the *LPNH3* gene.

Materials and methods

Subjects

We recruited 334 adult patients with ADHD at the Department of Psychiatry of the Hospital Universitari Vall d'Hebron of Barcelona (Spain) between 2004 and 2008 (65.3% combined type, 31.1% inattentive type and 3.6% hyperactive-impulsive type). All subjects met Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) criteria for ADHD and 71% were males ($n = 237$). Diagnosis was blind to genotype and was based on the Structured Clinical Interview for DSM-IV Axis I and II Disorders (SCID-I and SCID-II) and the Conners' Adult ADHD Diagnostic Interview for DSM-IV (CAADID Part I and II). A more detailed description of the different diagnostic instruments used was published previously (Ribases *et al*. 2009b). Exclusion criteria were IQ < 70, pervasive developmental disorders, schizophrenia or other psychotic disorders, ADHD symptoms due to mood, anxiety, dissociative or personality disorders, adoption, sexual or physical abuse, birth weight <1.5 kg and other neurological or systemic disorders that might explain ADHD symptoms. The control sample, consisting of 334 unrelated adult subjects from the same geographic area in whom DSM-IV ADHD symptoms were excluded, was matched for sex with the ADHD group. The average age at assessment was 30.2 years (SD = 12.4) for adult patients and 42.3 years (SD = 14.2) for controls. The study was approved by the ethics committee of Hospital Universitari Vall d'Hebron and informed consent was obtained from all subjects.

DNA isolation and quantification

Genomic DNA was isolated either from peripheral blood lymphocytes by the salting-out procedure or using magnetic bead technology with the Chemagic DNA kit (Chemagen AG, Baesweiler, Germany) or from saliva using the Oragene™ DNA Self-Collection kit (DNA Genotek Inc, Ottawa, Ontario, Canada).

SNP selection, plex design, genotyping and quality control

Information on the *Centre d'Etude du Polymorphisme Humain* (CEPH) panel from the HapMap database (release 22, March 2007) was used for SNP selection. To minimize redundancy and to ensure full genetic coverage, we evaluated the LD pattern of the *LPHN3* gene with LD-SELECT software version 1.0 (bioinfo.bsd.uchicago.edu/HapMap-LDSelect-Processor.html; Carlson *et al*. 2004). TagSNPs were selected at an r^2 threshold of 0.80 from all SNPs with minor allele frequency (MAF) > 0.2. Forty-six tagSNPs were chosen with these criteria (31 in multiloci bins and 15 singletons). Two additional SNPs with MAF < 0.2, rs1947275 and rs1397547, were also included in the analysis because they showed positive association with ADHD in a previous study by Arcos-Burgos *et al*. (in press). We evaluated the 48 selected SNPs with the automated assay design pipeline at ms.appliedbiosystems.com/snplex/snplexStart.jsp. A proper design could not be achieved for one SNP, which translates into a design rate of 97.9% (Table S1). All SNPs were genotyped using the SNPLEX™ platform (Applied Biosystems, Foster City, CA, USA). Two CEPH samples (NA10860 and NA10861) were included in all genotyping assays and a concordance rate of 100% with HapMap data was obtained ($n = 90$ genotypes).

Statistical analyses

The analysis of minimal statistical power was performed *post hoc* using the GENETIC POWER CALCULATOR software version 1.0 (Purcell *et al*. 2003), assuming codominant, dominant and recessive models, an odds ratio (OR) of 1.25, prevalence of 0.05, significance level of 0.05 and the lowest MAF of all the selected SNPs, 0.1 (Purcell *et al*. 2003). We carried out a case–control association study with single *LPNH3* markers followed by a haplotype-based analysis. The overall ADHD sample as well as two diagnostic subgroups of combined type and inattentive type ADHD were considered. The hyperactive-impulsive subgroup was too small to be studied separately.

Single-marker analysis

Analyses of Hardy–Weinberg equilibrium (HWE) ($P < 0.01$) in cases and controls separately and comparison of genotype and allele frequencies were performed using the SNPASSOC R package (Gonzalez *et al*. 2007). Dominant (11 vs. 12 + 22) and recessive (11 + 12 vs. 22) models were considered only for those SNPs displaying nominal association when either genotypes under a codominant model or alleles were taken into account. Correction for multiple testing was performed on the basis of the spectral decomposition (SpD) of matrices of pairwise LD between SNPs of the *LPHN3* gene and significance was set at $P < 0.002$ (Nyholt 2004). This method takes into account the level of LD between SNPs to establish the significance threshold.

Multiple-marker analysis

Rather than simplifying the study to physically contiguous SNPs, the best two-marker haplotype from all possible combinations was identified and additional markers (up to four, due to calculation constraints) were added in a stepwise manner to the initial two-SNP haplotype. Significance was estimated using 10 000 permutations with the UNPHASED software version 3.0.13 (Dudbridge 2003). As the expectation-maximization algorithm does not accurately estimate low haplotype frequencies (Fallin & Schork 2000), haplotypes with frequencies <0.05 were excluded. Subsequently, specific estimated haplotypes were assigned to individuals with the PHASE 2.0 software (Stephens *et al*. 2001). Population attributable risk (PAR) was calculated according to the STATSDIRECT STATISTICAL software (http://www.statsdirect.co.uk/help/statsdirect.htm#basics/attributable_risk.htm).

Results

We genotyped 47 tagSNPs spanning the *LPHN3* gene in a Spanish sample of 334 adult ADHD cases and 334 sex-matched unrelated controls. From the SNPs initially selected for inclusion in the SNPlex assay, five were discarded (one did not pass through the SNPlex design pipeline, three had genotype calls <90% and one had a significant departure from HWE; Table S1). The LD plot of

the 43 SNPs that were finally considered in the study is shown in Fig. 1. The minimal statistical power of the χ^2 test for the combined, inattentive and all ADHD patient subgroups was 15.2%, 10.9% and 18.1% considering a codominant model, 19.7%, 13.7% and 23.4% under a dominant model and 5.6%, 5.4% and 5.7% for the recessive model. Population stratification in this dataset was previously excluded by analyzing 48 unlinked anonymous SNPs located at least 100 kb distant from known genes (Ribases *et al*. 2008, 2009a,b).

The single-marker analysis identified one SNP in *LPHN3* displaying nominal association with ADHD: rs2122643 [SNP20; $P = 0.018$; df = 1; OR = 1.45 (1.07–1.97); Table 1; Fig. 1]. Once we subdivided patients according to clinical subtype, three SNPs, rs2122643 (SNP20), rs1868790 (SNP21) and rs6858066 (SNP25), were associated with combined type ADHD and two SNPs, rs4860106 (SNP35) and rs13115125 (SNP37), with inattentive type ADHD. However, after correcting for multiple testing, only rs6858066 (SNP25) remained associated with combined ADHD [$P = 0.0019$; df = 1; OR = 1.82 (1.25–2.70); Table 1 and Fig. 1].

We then performed a haplotype-based analysis within the combined type ADHD subgroup and all the associations described below remained significant when adjusted for multiple comparisons by a permutation test. The study of the 43 *LPHN3* SNPs showed a three-marker haplotype (rs1868790/rs6813183/rs12503398; SNP21/SNP28/SNP29) strongly associated with combined type ADHD (global *P*-value = 8.3e-04; df = 3; Table 2; Fig. 2). The analysis of the contribution of individual allelic combinations to this clinical subtype showed over-representation of the T-C-A haplotype [$P = 7.5e\text{-}05$; df = 1; OR = 2.06 (1.46–2.90)] and a trend toward under-representation of the A-G-G combination in this group of patients [$P = 0.018$; df = 1; OR = 1.38 (0.96–2.00); Table 2]. When we considered the frequency of individuals carrying the T-C-A risk haplotype, the association between *LPHN3* and combined type ADHD in adults was confirmed [$P = 5.1e\text{-}05$, df = 1; OR = 2.25 (1.52–3.34); data not shown]. Although these differences were not detected in the inattentive type subgroup, significant association was also observed when all ADHD patients were considered (global *P*-value = 0.0028; df = 3; Table 2), with over-representation of the same T-C-A risk haplotype [$P = 1.9e\text{-}04$; df = 1; OR = 1.80 (1.31–2.48); Table 2] and an increased frequency of carriers of this allelic combination in this clinical dataset [$P = 1.65e\text{-}04$; df = 1; OR = 2.01 (1.40–2.88)]. The PAR, estimating the proportion of combined type ADHD in the present study that is attributable to the *LPNH3* risk haplotype, was calculated as 12.35%.

Discussion

In this study, we replicated the recently described association between *LPHN3* and ADHD, supporting the involvement of this gene in the susceptibility to this psychiatric disorder in adults. As the majority of ADHD samples investigated by Arcos-Burgos *et al*. were pediatric (82.9%) (Arcos-Burgos *et al*. in press), our data provide further evidence of common susceptibility factors for ADHD extending from childhood to adulthood (Faraone *et al*. 2000a,b; Kuntsi *et al*. 2005; Polanczyk & Rohde 2007; Ribases *et al*. 2008, 2009b; Spencer *et al*. 2007).

The original study describing the association between *LPHN3* and ADHD identified a significantly associated area delimited by SNPs rs1901223 and rs1355368, located in the central part of the gene between introns 6 and 9. In this regard, the combined single- and multiple-marker family-based and case–control association studies performed with patients of the Paisa isolate showed association between ADHD and the *LPHN3* SNPs rs1901223, rs1376307, rs6813183 and rs1355368. A meta-analysis of seven independent samples from five populations confirmed the initial association. Although the associated SNPs were not the same (rs6551665, rs1947274 and rs2345039), they are located within the same region of the gene and have varying degrees of LD with the original SNPs (Fig. 2). Within the Arcos-Burgos *et al*. replication samples, only the Norwegian population included adult ADHD patients and its separate analysis showed evidence for significant associations between ADHD and rs1868790 and rs10446786, both in LD with SNPs also showing association in the Paisa population (Fig. 2) (Arcos-Burgos *et al*. in press).

The three SNPs of the risk haplotype identified in our Spanish adulthood ADHD sample, rs1868790 (SNP21), rs6813183 (SNP28) and rs12503398 (SNP29), are in common or in LD with SNPs identified in the Paisas, the Norwegians and/or the worldwide sample meta-analysis (Fig. 2) (Arcos-Burgos *et al*. in press). Thus, two of them, rs1868790 (SNP21) and rs6813183 (SNP28), were also associated with ADHD in the Norwegian or the Paisa samples, respectively, whereas the third, rs12503398 (SNP29), was in strong LD with two SNPs identified in the Colombian isolate ($D' > 0.87$; $r^2 > 0.68$; Fig. 2); also, SNP rs6813183 (SNP28) was in moderate LD with rs2345039 ($D' = 1$; $r^2 = 0.25$), one of the SNPs pinpointed in the previous meta-analysis by Arcos-Burgos *et al*. Although we have genotyped the other two markers highlighted in this meta-analysis, rs6551665 and rs1947274, they were not included in our final analysis because of abnormal HWE distribution of rs6551665 that was tagging rs1947274. However, these two SNPs are in strong LD with rs6858066 (SNP25), which is found associated in the single-marker analysis performed in our Spanish adult ADHD dataset ($D' > 0.95$ and $r^2 > 0.74$).

Whether the different ADHD clinical subtypes share genetic risk factors remains unclear. The strong association between ADHD and *LPHN3* detected in the present study was seen in the combined type (and to a lesser extent in the total ADHD sample) but not in the inattentive subgroup. Although the previous study by Arcos-Burgos *et al*. did not take into account diagnostic subgroups in the association of *LPHN3* with ADHD, our results are in agreement with previous studies supporting the validity of the DSM-IV distinction between the combined type and predominantly inattentive type, suggesting that differential genetic factors may participate in distinct ADHD clinical subgroups (Larsson *et al*. 2006; Lowe *et al*. 2004; Rasmussen *et al*. 2004; Ribases *et al*. 2008, 2009a,b; Smoller *et al*. 2006; Sobanski *et al*. 2008; Todd *et al*. 2001, 2005; Woo & Rey 2005). Furthermore, the

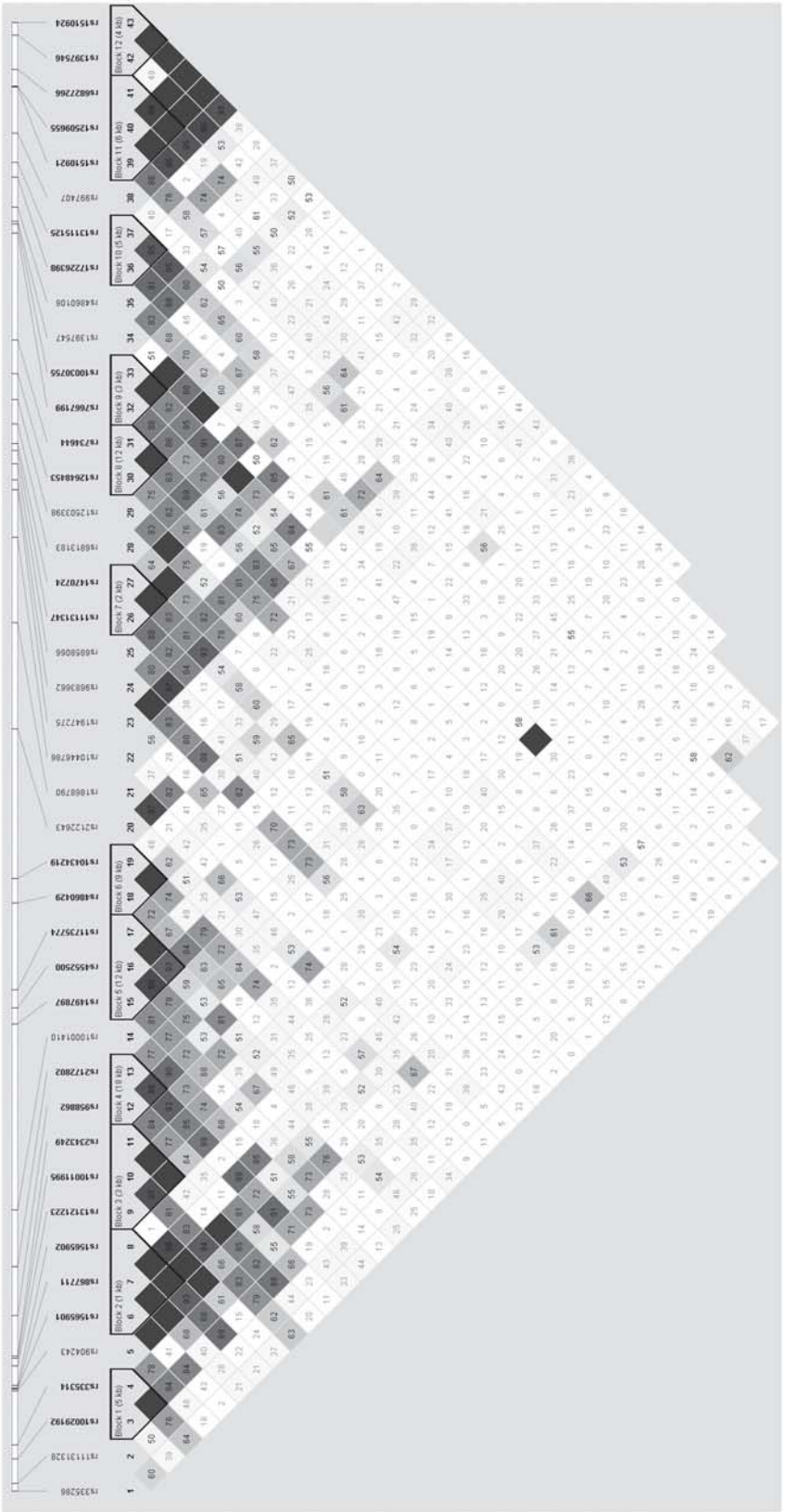

Figure 1: LD plot showing D′ values of the 43 *LPHN3* SNPs (from 5′ to 3′) in the control sample considered in the present study. Haploview v4.2 (http://www.broadinstitute.org/haploview) was implemented to determine the LD blocks within the gene using the confidence interval method. Asterisks and boxes indicate SNPs nominally associated with ADHD in the single- and multiple-marker analyses, respectively. Underlined, the SNP associated with ADHD in the single-marker analysis after correcting for multiple testing.

Table 1: Association study of 43 SNPs covering the *LPHN3* gene in 334 adult patients with ADHD (218 with combined type ADHD, 104 with inattentive type ADHD and 12 with the hyperactive-impulsive type) and 334 sex-matched unrelated controls

	Genotypes													Alleles	
	Cases *n* (%)				Controls *n* (%)					Genotype 11 vs. 12 + 22		Genotype 11 + 12 vs. 22		Allele 2 vs. allele 1	
SNP*	11	12	22	Sum	11	12	22	Sum	*P* (df = 2)	OR (95% CI)†	*P* (df = 1)	OR (95% CI)†	*P* (df = 1)	OR (95% CI)†	*P* (df = 1)
All ADHD															
rs2122643 (20)	165 (49.5)	143 (42.9)	25 (7.5)	333	196 (58.7)	117 (35.0)	21 (6.3)	334	0.06	1.45 (1.07–1.97)	0.018	—	0.53	1.31 (1.02–1.67)	0.032
Combined ADHD															
rs2122643 (20)	70 (32.6)	101 (47.0)	44 (20.5)	215	129 (39.3)	152 (46.3)	47 (14.3)	328	0.10	—	0.11	—	0.061	1.32 (1.02–1.67)	0.034
rs1868790 (21)	106 (48.6)	92 (42.2)	20 (9.2)	218	196 (58.7)	117 (35.0)	21 (6.3)	334	0.06	1.50 (1.06–2.12)	0.020	—	0.21	1.39 (1.06–1.82)	0.017
rs6858066 (25)	75 (34.6)	96 (44.2)	46 (21.2)	217	75 (22.5)	183 (54.8)	76 (22.8)	334	0.0066	1.82 (1.25–2.70)*	0.0019‡	—	0.069	1.32 (1.03–1.69)*	0.025
Inattentive ADHD															
rs4860106 (35)	26 (25.2)	60 (58.3)	17 (16.5)	103	123 (36.9)	144 (43.2)	66 (19.8)	333	0.019	1.77 (1.07–2.02)	0.021	—	0.45	—	0.27
rs13115125 (37)	20 (19.4)	60 (58.3)	23 (22.3)	103	105 (31.4)	147 (44.0)	82 (24.6)	334	0.018	1.93 (1.12–3.32)	0.013	—	0.63	—	0.21

*SNP number according to Fig. 1 is shown in brackets.

†When odds ratio <1, the inverted score is shown.

‡Correction for multiple testing on the basis of the SpD of matrices of pairwise LD between SNPs of the *LPHN3* gene ($P < 0.002$).

Table 2: Haplotype analysis of 43 *LPHN3* SNPs in a clinical sample of 334 adult patients with ADHD and 334 controls using the UNPHASED software version 3.0.13; haplotype distributions in the two-marker analysis (rs1868790 and rs12503398); three-marker analysis (rs1868790, rs6813183 and rs12503398) and four-marker analysis (rs1868790, rs6813183, rs12503398 and rs734644)

	Combined ADHD			Inattentive ADHD			All ADHD		
Marker* haplotype	Global *P*-value	Best haplotype *P*-value (adjusted *P*-value)	Risk haplotype OR	Global *P*-value	Best haplotype *P*-value (adjusted *P*-value)	Risk haplotype OR	Global *P*-value	Best haplotype *P*-value (adjusted *P*-value)	Risk haplotype OR
21 29	0.0038	5.1 e-04 (0.0036)	1.86 (1.36–2.55)	0.066	—	—	0.0095	0.0011 (0.0055)	1.68 (1.26–2.24)
21 28 29	**8.3e-04**	**7.5 e-05 (0.001)**	**2.06 (1.46–2.90)**	**0.36**	—	—	**0.0028**	**1.9 e-04 (0.0013)**	**1.80 (1.31–2.48)**
21 28 29 31	7.6e-04	6.2 e-05 (6.0e-04)	2.00 (1.41–2.84)	0.36	—	—	0.0036	4.1 e-04 (0.0022)	1.78 (1.28–2.46)

	Combined ADHD			Inattentive ADHD			All ADHD		
Marker* haplotype	Cases *n* (%)	Controls *n* (%)	Haplotype-specific *P*-value; OR (CI)	Cases *n* (%)	Controls *n* (%)	Haplotype-specific *P*-value; OR (CI)	Cases *n* (%)	Controls *n* (%)	Haplotype-specific *P*-value; OR (CI)
21 29									
A-A	242 (55.5)	398 (59.6)	—	119 (57.7)	398 (59.6)	—	377 (56.6)	398 (59.6)	—
A-G	62 (14.2)	111 (16.6)	—	31 (15.1)	111 (16.6)	—	96 (14.4)	111 (16.6)	—
T-A	98 (22.5)	90 (13.5)	5.1 e-04; 1.86 (1.36–2.55)	35 (17.1)	90 (13.5)	—	138 (20.8)	90 (13.5)	0.0011; 1.68 (1.26–2.24)
T-G	34 (7.8)	69 (10.3)	—	21 (10.1)	69 (10.3)	—	55 (8.2)	69 (10.3)	—
Global *P*-value	$\chi^2 = 13.41$; df = 3; $P = 0.0038$			$\chi^2 = 1.56$; df = 3; $P = 0.67$			$\chi^2 = 11.46$; df = 3; $P = 0.0095$		

	Combined ADHD			Inattentive ADHD			All ADHD		
Marker* haplotype	Cases *n* (%)	Controls *n* (%)	Haplotype-specific *P*-value; OR (CI)	Cases *n* (%)	Controls *n* (%)	Haplotype-specific *P*-value; OR (CI)	Cases *n* (%)	Controls *n* (%)	Haplotype-specific *P*-value; OR (CI)
21 28 29									
A-C-A	214 (56.7)	357 (61.9)	—	103 (58.1)	357 (61.9)	—	334 (57.8)	357 (61.9)	—
A-G-G	50 (13.1)	100 (17.4)	0.018; 1.38 (0.96–2.00)†	27 (14.9)	100 (17.4)	—	79 (13.7)	100 (17.4)	0.028; 1.33 (0.96–1.83)†
T-C-A	87 (22.9)	73 (12.8)	7.5e-05; 2.06 (1.46–2.90)	31 (17.2)	73 (12.8)	—	120 (20.8)	73 (12.8)	1.9e-04; 1.80 (1.31–2.48)
T-G-G	27 (7.3)	46 (7.9)	—	17 (9.8)	46 (7.9)	—	45 (7.7)	46 (7.9)	—
Global *P*-value	$\chi^2 = 16.67$; df = 3; $P = 8.3e-04$			$\chi^2 = 3.24$; df = 3; $P = 0.36$			$\chi^2 = 14.07$; df = 3; $P = 0.0028$		

	Combined ADHD			Inattentive ADHD			All ADHD		
Marker* haplotype	Cases *n* (%)	Controls *n* (%)	Haplotype-specific *P*-value; OR (CI)	Cases *n* (%)	Controls *n* (%)	Haplotype-specific *P*-value; OR (CI)	Cases *n* (%)	Controls *n* (%)	Haplotype-specific *P*-value; OR (CI)
21 28 29 31									
A-C-A-C	208 (58.3)	340 (64.9)	—	98 (59.3)	340 (64.9)	—	322 (59.3)	340 (64.9)	—
A-G-G-T	37 (10.5)	77 (14.7)	0.03; 1.48 (0.98–2.25)†	23 (13.6)	77 (14.7)	—	63 (11.5)	77 (14.7)	—
T-C-A-C	84 (23.7)	70 (13.3)	6.2 e-05; 2.00 (1.41–2.84)	30 (17.8)	70 (13.3)	—	117 (21.4)	70 (13.3)	4.1 e-04; 1.78 (1.28–2.46)
T-G-G-T	27 (7.5)	37 (7.1)	—	15 (9.3)	37 (7.1)	—	42 (7.8)	37 (7.1)	—
Global *P*-value	$\chi^2 = 16.85$; df = 3; $P = 7.6e-04$			$\chi^2 = 3.18$; df = 3; $P = 0.36$			$\chi^2 = 13.54$; df = 3; $P = 0.0036$		

In bold, the best allelic combination (highest OR).

*21-rs1868790; 28-rs6813183; 29-rs12503398; 31-rs734644.

†Under-represented in ADHD cases.

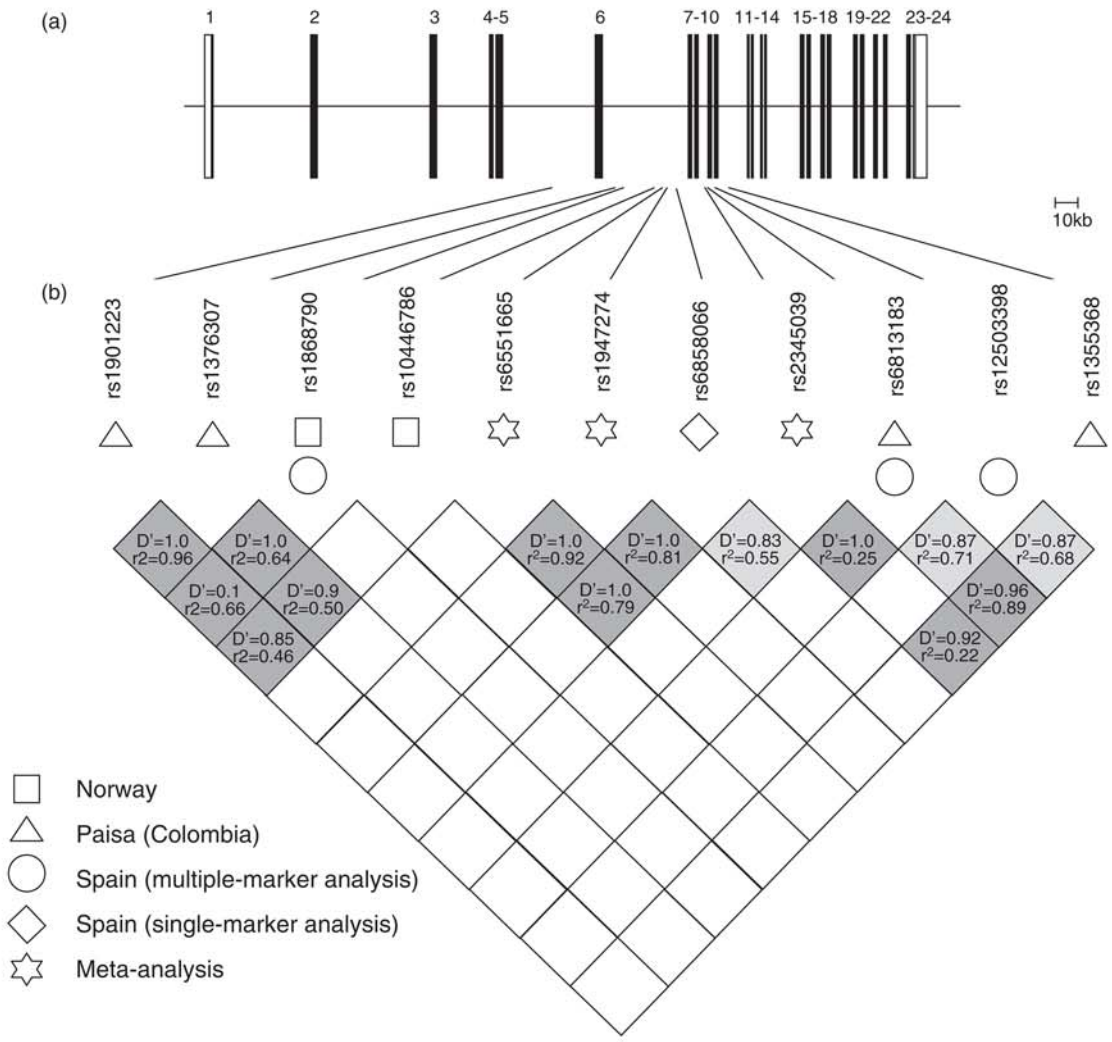

Figure 2: Association study of the *LPHN3* gene in adulthood ADHD. (a) Diagram of the *LPHN3* gene (NM_015236; chr4: 62045434-62620762; http://www.hapmap.org; release 24). Black and white boxes correspond to coding and non-coding exonic regions, respectively. Exon numbering is indicated above. (b) LD patterns between *LPHN3* SNPs found associated with ADHD in different datasets. Symbols below the SNP codes indicate SNPs associated with ADHD in different cohorts: Norway (adulthood ADHD), Paisa, Colombia (childhood ADHD), Spain (adulthood ADHD, present study) and meta-analysis carried out in seven independent datasets from five populations (Paisa, United States, Germany, Norway and childhood patients from Spain).

linkage of the *LPHN3* locus with conduct disorder, nicotine dependence and alcohol abuse/dependence (Jain *et al*. 2007) is consistent with a differential association between *LPHN3* and 'antisocial ADHD' (Christiansen *et al*. 2008; Faraone *et al*. 1998, 2000c). Alternatively, it is also possible that the limited sample size of the inattentive clinical sample may account for the absence of association observed when this ADHD group was considered and, thus, further studies in larger samples are required.

In conclusion, our findings replicate the association between *LPHN3* and ADHD described by Arcos-Burgos *et al*. (in press) specifically with the persistent form of the disorder, and further highlight a new neuronal pathway as a common susceptibility factor for ADHD in childhood and adulthood. As all the associated variants identified are intronic, further functional studies and possibly deep sequencing of *LPHN3* will be required to identify causal variants that will allow delineating the pathogenesis of this complex phenotype.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1: Description of the 48 SNPs in the *LPHN3* gene (NT_022778.15) initially selected for the SNPlex analysis.

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Table S1. Description of the 48 SNPs in the *LPHN3* gene (NT_022778.15) initially selected for the SNPlex analysis

ID	Chromosome position	Location	P-value HWE*	Exclusion Criteria	Other SNPs within the BIN
rs335286	62051357	Intron 1	0,73		-
rs11131328	62054501	Intron 1	1,00		-
rs10029192	62063506	Intron 1	0,21		-
rs335314	62068712	Intron 1	1,00		rs335306, rs335312, rs335315, rs335317
rs6813884	62081533	Intron 1	-	Failed	-
rs904243	62089040	Intron 1	0,77		-
rs1565901	62089922	Intron 1	1,00		-
rs867711	62089994	Intron 1	0,33		rs17090504
rs1565902	62091215	Intron 1	0,74		rs335322, rs335305, rs10008945, rs10015239
rs13121223	62098391	Intron 1	0,70		rs7656189, rs10866120, rs6812571, rs6813628, rs13122170, rs2132074
rs10011995	62101253	Intron 1	0,88		rs186750, rs11734047, rs12498776, rs12499587, rs6841637, rs17828396, rs10019669, rs723120, rs10517542, rs9996810, rs6820887
rs2343249	62102021	Intron 1	0,3		rs9312078, rs1497914, rs13350977, rs1113996, rs7688741, rs7693763
rs958862	62117443	Intron 1	0,15		rs11131329, rs1497907, rs1391321, rs1497906, rs7669283, rs6817476, rs17239080, rs10866124, rs11131334, rs1497901, rs6551634, rs6835374, rs1497916, rs13123888, rs6551637, rs12504607, rs6851424, rs6551640, rs13123468, rs7656051, rs1497921, rs11939481, rs1497909, rs6843311
rs2172802	62135804	Intron 2	0,78		rs10018746, rs12642315
rs10001410	62156824	Intron 2	0,78		rs1846161, rs1948616, rs11131337, rs10004368, rs4484334
rs1497897	62226650	Intron 3	0,45		rs6551636, rs7675385, rs12642037, rs6820960, rs4860422, rs11931258, rs1542834, rs2036199, rs1391320, rs12507326, rs12233859, rs11131341, rs1497913, rs996208, rs12510774
rs4552500	62232898	Intron 3	0,47		rs7690118, rs7690472
rs11735774	62239467	Intron 3	0,42		-
rs4860429	62272065	Intron 3	0,58		rs4860427, rs4351047, rs4241640, rs6551649, rs12646895, rs2343577, rs4495065, rs2343579
rs10434219	62281284	Exon 5	0,18		rs6551645, rs12649170, rs4283700, rs10022833, rs7670950, rs4860428, rs10021572, rs9998713, rs7684100, rs6840548, rs9997427, rs4312783, rs4585337, rs7678046, rs9312082
rs2122643	62337533	Intron 5	0,81		rs2345049, rs2345047, rs10015258, rs12509742, rs2345045, rs2345044, rs2345043, rs2122640, rs4860434, rs1901223, rs4493573, rs1817052, rs7656882, rs1376307
rs1868790	62377312	Intron 6	0,55		rs17090543, rs2881027, rs9942184, rs11734607
rs7695134	62387447	Intron 6		Failed	-
rs10446786	62409469	Intron 6	0,66		-
rs13110933	62416032	Intron 6	-	Failed	-
rs6551665	62422136	Intron 6	0.0067	No HWE	rs1947274
rs1947275	62427140	Intron 6	0,25		-
rs9683662	62430979	Intron 6	0,54		-
rs6858066	62436915	Intron 6	0,10		-
rs11131347	62441865	Intron 7	0,23		rs1376309, rs1450900, rs2345039, rs7695892, rs1376310, rs7690962
rs1470724	62444465	Intron 8	0,22		rs2011468, rs7691112, rs1349408
rs6813183	62451726	Intron 8	0,78		rs1355368, rs13128833, rs10517549, rs6843945
rs12503398	62460910	Intron 9	0,41		rs6551666, rs5012949, rs6551667, rs10020948, rs4860442
rs12648453	62470706	Intron 10	0,21		-
rs734644	62483323	Exon 11	1,00		rs10023425, rs2122646, rs17082493, rs2305339
rs7667199	62523535	Intron 14	0,55		rs10027079, rs10031103, rs10008326, rs12505782, rs1510923, rs1397544, rs1397548
rs10030755	62527003	Intron 14	0,73		rs1510925, rs1397543
rs1397547	62527983	Exon 15	0,42		-
rs4860106	62533117	Intron 16	0,05		rs1510918
rs17226398	62544536	Intron 16	0,07		rs954645, rs11131352, rs2271338, rs2271339
rs13115125	62550048	Intron 18	0,04		rs13115125
rs997407	62560911	Intron 18	0,68		rs997407
rs1510921	62578187	Intron 19	0,57		rs17292170, rs17226412, rs9998041
rs12509655	62578623	Intron 19	0,38		-
rs6827266	62584757	Intron 20	0,36		rs6827266
rs11736598	62596533	Intron 22	-	No SNPlex	Not genotyped due to design incompatibilities
rs1397546	62597619	Intron 22	0,11		rs11931728
rs1510924	62602302	Intron 21	0,45		rs1510924

* Hardy-Weinberg equilibrium in controls

Case-Control Study of Six Genes Asymmetrically Expressed in the Two Cerebral Hemispheres: Association of *BAIAP2* with Attention-Deficit/Hyperactivity Disorder

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Background: Attention-deficit/hyperactivity disorder (ADHD) is a childhood-onset neuropsychiatric disease that persists into adulthood in at least 30% of patients. There is evidence suggesting that abnormal left-right brain asymmetries in ADHD patients may be involved in a variety of ADHD-related cognitive processes, including sustained attention, working memory, response inhibition and planning. Although mechanisms underlying cerebral lateralization are unknown, left-right cortical asymmetry has been associated with transcriptional asymmetry at embryonic stages and several genes differentially expressed between hemispheres have been identified.

Methods: We selected six functional candidate genes showing at least 1.9-fold differential expression between hemispheres (*BAIAP2*, *DAPPER1*, *LMO4*, *NEUROD6*, *ATP2B3,* and *ID2*) and performed a case-control association study in an initial Spanish sample of 587 ADHD patients (270 adults and 317 children) and 587 control subjects.

Results: The single- and multiple-marker analysis provided evidence for a contribution of *BAIAP2* to adulthood ADHD ($p = .0026$ and $p = .0016$, respectively). We thus tested *BAIAP2* for replication in two independent adult samples from Germany (639 ADHD patients and 612 control subjects) and Norway (417 ADHD cases and 469 control subjects). While no significant results were observed in the Norwegian sample, we replicated the initial association between *BAIAP2* and adulthood ADHD in the German population ($p = .0062$).

Conclusions: Our results support the participation of *BAIAP2* in the continuity of ADHD across life span, at least in some of the populations analyzed, and suggest that genetic factors potentially influencing abnormal cerebral lateralization may be involved in this disorder.

Key Words: ADHD, attention-deficit hyperactivity disorder, *BAIAP2*, brain asymmetry, case-control association study

Attention-deficit/hyperactivity disorder (ADHD) is a common childhood-onset disorder characterized by impaired attention, hyperactivity, and impulsivity that affects 8% to 12% of children and 3% to 5% of adults (1–4). At least 30% of ADHD patients diagnosed in childhood continue to suffer from the disorder into adulthood and several evidences suggest a stronger genetic component in persistent than in remitting ADHD (5–9). Although its pathophysiology is unknown, brain imaging and neuropsychological studies support that impairment of various brain regions may account for ADHD symptoms (10–14) and growing evidence points toward underlying disrupted anatomical (15–22) and functional (23–31) hemispheric brain asymmetries. In this regard, ADHD has been associated with a right hemisphere dysfunction, mainly based on abnormal right-sided fronto-striatal-pallidal activity (15,18,23,25,32–38). This deviation from the common pattern of cerebral lateralization may be involved in a variety of impairments in ADHD individuals, including their core symptoms of attention and impulsivity, as well as executive functions.

Although the exact mechanisms underlying brain laterality are unknown, cerebral asymmetry is considered a complex and highly heritable phenotype (39–41). Interestingly, the gene expression pattern in the central nervous system displays asymmetries that overlap with those in the brain's functional organization, suggesting that multiple genes are likely to interact to

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determine adult cerebral structure (41). In addition, left-right cortical asymmetry in humans has been associated with transcriptional asymmetry between hemispheres at early embryonic stages (42). In this regard, Sun *et al.* (42) identified 27 differentially expressed genes in left and right human embryonic 12-weeks cortex and suggested that their asymmetric expression is related to asymmetric cortical development (42).

Since the asymmetry of human cerebral hemispheres appears to have a molecular basis, we reasoned that altered gene expression may be involved in the abnormal brain lateralization observed in ADHD subjects and, consequently, may contribute to the genetic predisposition to this neurodevelopmental disorder. We selected six functional candidate genes from those described by Sun *et al.* (42) that showed 1.9- to 8-fold differential expression between hemispheres (brain-specific angiogenesis inhibitor 1-associated protein 2 [*BAIAP2*]; Dapper antagonist of beta-catenin homolog 1 [*DAPPER1*]; LIM domain only 4 [*LMO4*]; Neurogenic differentiation 6 [*NEUROD6*]; ATPase, Ca++ transporting plasma membrane 3 [*ATP2B3*]; and inhibitor of DNA binding 2 [*ID2*]) (42) and conducted an initial case-control study in 587 ADHD patients (270 adults and 317 children) and 587 sex-matched unrelated control subjects from Spain. The observed results were then tested for replication in two additional independent case-control samples from Germany and Norway (639 and 417 adult ADHD patients and 612 and 469 control subjects, respectively).

Methods and Materials

Subjects

Table S1 in Supplement 1 shows the clinical description of the 1643 ADHD Caucasoid patients included in the study.

Spain. Five hundred eighty-seven patients with ADHD were recruited: 270 adults (65.9% combined, 29.7% inattentive, and 4.4% hyperactive-impulsive) and 317 children (73.5% combined, 21.8% inattentive, and 4.7% hyperactive-impulsive). Seventy-eight percent of patients were male. The control sample consisted of 531 unrelated Caucasoid blood donors matched for sex with the ADHD group in which DSM-IV ADHD symptomatology was excluded under the following criteria: 1) not having previously been diagnosed with ADHD and 2) answering negatively to the life-time presence of the following DSM-IV ADHD symptoms: 1) often has trouble keeping attention on tasks, 2) often loses things needed for tasks, 3) often fidgets with hands or feet or squirms in seat, and 4) often gets up from seat when remaining in seat is expected. The average age at assessment was 30.2 years (SD = 12.1) for adult patients, 9.3 years (SD = 2.6) for child patients, and 39.9 years (SD = 17.0) for control subjects.

Replication Population 1: Germany. The German sample consisted of 639 adult ADHD patients (67.1% combined, 25.4% inattentive, and 7.5% hyperactive-impulsive) and 612 sex-matched unrelated control subjects. Three hundred thirty-two of those were extensively interviewed for absence of ADHD and did not fulfill DSM-IV ADHD criteria (43,44), while the remaining control subjects consisted of unscreened blood donors and University staff not explicitly screened for absence of psychiatric disorders, although the scope of the study was explained to these individuals. Fifty percent of patients were male (n = 321). The average age at assessment was 34.3 years (SD = 10.4) for patients and 31.2 years (SD = 10.3) for control subjects.

Replication Population 2: Norway. The clinical sample consisted of 417 adult ADHD subjects (75.5% combined, 10.8% inattentive, 3.4% hyperactive-impulsive, and 10.3% subthreshold) with an age at assessment of 35.4 years (SD = 11.5). Fifty-two percent of patients were male (n = 218). The control sample included 469 sex-matched unrelated subjects. Two hundred sixty-nine of them, with an average age at assessment of 29.1 years (SD = 6.5), had no previous ADHD diagnosis and were recruited using a random selection of persons born in Norway from 1967 to 1989, whereas the rest were healthy blood donors for whom no information about ADHD symptoms was available.

Clinical Assessment

Diagnosis was blind to genotype. The study was approved by the ethics committee of each institution and informed consent was obtained from all subjects. A more detailed description of the different diagnostic instruments used was published previously (45).

Spanish Population: Adult ADHD. The ADHD diagnosis was based on the Structured Clinical Interview for DSM-IV Axis I and Axis II Disorders (SCID-I and SCID-II) and the Conners' Adult ADHD Diagnostic Interview for DSM-IV (CAADID). Severity of ADHD symptoms was evaluated using the long version of the Conners' ADHD Rating Scale (self-report [CAARS-S:L] and observer [CAARS-O:L]), the ADHD Rating Scale (ADHD-RS), the ADHD Screening Checklist, and the Wender Utah Rating Scale (WURS) for retrospective symptoms. The level of impairment was measured by the Clinical Global Impression (CGI) included in the CAADID Part II and the Sheehan Disability Inventory (46).

Spanish Population: Childhood ADHD. Patients were evaluated with the Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (K-SADS-PL) reported by parents. Attention-deficit/hyperactivity disorder symptoms were assessed using the Conners' Parent Rating Scale and the Conners' Teacher Rating Scale. Exclusion criteria for the adult and childhood Spanish populations were IQ <70; pervasive developmental disorders; schizophrenia or other psychotic disorders; the presence of mood, anxiety, dissociative, or personality disorders that might explain ADHD symptoms; adoption; sexual or physical abuse; birth weight <1.5 kg; and other neurological or systemic disorders that might explain ADHD symptoms. For additional information see Ribasés *et al.* (9).

German Population. Patients were extensively examined using an open interview by an experienced psychiatrist (C.P.J.), as well as SCID-I and SCID-II (47). Personality assessment was done using the Revised NEO Personality Inventory (NEO PI-R) and Tridimensional Personality Questionnaire (TPQ) (48,49); severity of ADHD was measured with the WURS interview (50). When available, chart reviews were performed and information from relatives and school reports was considered. Eligibility criteria for the study were ADHD according to DSM-IV, onset before the age of 7 years via retrospective diagnosis, lifelong persistence, current diagnosis and age at recruitment between 18 and 65 years. Exclusion criteria for patients were the restricted appearance of ADHD-like symptoms (lack of concentration, hyperactivity, or impulsivity) during episodes of Axis I disorders, as well as a current diagnosis of not withdrawn drug/alcohol abuse/dependence; a lifetime diagnosis of bipolar I disorder, schizophrenia, or any other psychotic disorder; and mental retardation (IQ level <80; multiple-choice word test [MWT-B] <13 points).

Norwegian Population. Patients were recruited using a National Registry of adults diagnosed with ADHD in Norway during 1997 to 2005. Diagnosis was made according to the ICD-10 research criteria (51), with two modifications: allowing

for the inattentive subtype in DSM-IV to be sufficient for the diagnosis and allowing for the presence of comorbid psychiatric disorders, as long as the criteria for ADHD were present before the appearance of the comorbid disorder. This diagnostic strategy was chosen as a compromise between the fact that ICD-10 is the official diagnostic system in Norway and the need to have an assessment comparable with the DSM-IV criteria. Until May 2005, this diagnostic assessment was mandatory for adult patients in Norway who were to be considered for treatment with stimulant drugs. All patients were formally diagnosed with ADHD before inclusion, but subtype data were not systematically available at the time of the primary diagnosis. Thus, the ADHD clinical subtype was assessed using the Adult ADHD Self-Report Scale (ASRS) (52) with a cutoff of 17 or more on each subscale (10.3% were diagnosed as subthreshold). Severity of past and current ADHD symptoms in patients and control subjects was evaluated using the WURS (53) and the ASRS.

DNA Isolation

DNA samples were isolated either from saliva using the Oragene DNA Self-Collection Kit (DNA Genotek, Kanata, Ontario, Canada) or from blood by the salting-out procedure or using magnetic bead technology with the Chemagic Magnetic Separation Module I and the Chemagic DNA Kit (Chemagen, Baesweiler, Germany). DNA concentrations were determined using the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, Oregon).

Genes and Single Nucleotide Polymorphisms

From the 27 differentially expressed genes previously described (42), we considered their involvement in brain functions, the degree of asymmetry in their expression (from 1.9- to 8-fold), as well as SNPlex (Applied Biosystems, Foster City, California) design constraints, and finally genotyped six of them: *LMO4*, *BAIAP2*, *DAPPER*, *NEUROD6*, *ATP2B3,* and *ID2* (Table S2 in Supplement 1). For single nucleotide polymorphism (SNP) selection, we used information on the Centre d'Etude du Polymorphisme Humain (CEPH) panel from the HapMap database (Release 20; http://www.hapmap.org). We evaluated, with the linkage disequilibrium (LD) select software (ldSelect, http://droog.gs.washington.edu/ldSelect.html), the LD pattern of each candidate gene plus 3- to 5-kilobase (kb) flanking sequences. Tagging single nucleotide polymorphisms (tagSNPs) were selected at an r^2 threshold of .85 from all SNPs with minor allele frequency (MAF) >.15 for genes with fewer than 15 tagSNPs (*DAPPER1, LMO4, NEUROD6*, *ATP2B3,* and *ID2*) and MAF >.25 for *BAIAP2*, which had more than 15 tagSNPs. Thirty-one tagSNPs were chosen with these criteria. An additional nonsynonymous SNP, rs17832998, located within exon 4 of *DAPPER1*, was included in the analysis. To detect population admixture, 48 unlinked anonymous SNPs located at least 100 kb distant from known genes were also genotyped (54).

Genotyping

Spanish and German Populations. We assessed the 32 selected SNPs with an automated assay design pipeline (http://ms.appliedbiosystems.com/snplex/snplexStart.jsp). A proper design could not be achieved for two SNPs (Table S2 in Supplement 1). All SNPs were genotyped using the SNPlex platform. Two HapMap samples (NA11992 and NA11993) were included in all assays and a concordance rate of 100% was obtained.

Norwegian Population. Genotyping was carried out by the multiplex MassARRAY *iPLEX* System (SEQUENOM, San Diego, California). A total of 12 *BAIAP2* SNPs passed the assay design requirements and 11 SNPs were successfully genotyped with a final genotyping call rate of 98.6%. Genotype concordance rate was 100% for internal control individuals (n = 253 genotypes) and duplicates (n = 88 calls).

Statistical Analyses

We performed a two-stage association study. We first carried out a case-control association analysis in a Spanish sample that included adult and child ADHD patients and control subjects. Genes showing positive signals were then tested for replication in two independent case-control samples from Germany and Norway.

The analysis of minimal statistical power was performed post hoc using the Genetic Power Calculator software (http://pngu.mgh.harvard.edu/~purcell/gpc) (55), assuming an odds ratio (OR) of 1.5, prevalence of .05, significance level of .05, and the lowest MAF of .16. We tested genetic stratification in the Spanish and German samples by analyzing the SNPs in Hardy-Weinberg equilibrium (HWE) (10) from the 48 anonymous SNPs set with two different approaches: 1) the F-statistics (Fst) coefficient calculated by the Weir and Cockerham approach with the FSTAT software (http://www2.unil.ch/popgen/softwares/fstat.htm); and 2) the method of Pritchard and Rosenberg (9). These data were not available for the Norwegian sample.

Single-Marker Analysis. The analysis of HWE ($p < .01$) and the comparison of genotype and allele frequencies were performed using the SNPassoc R package (http://www.cran.r-project.org/web/packages/SNPassoc) (56). Dominant and recessive models were considered for SNPs displaying nominal association when either genotypes under a codominant model or alleles were taken into account. Genotype frequencies of SNPs within chromosome X were examined in female subjects, whereas in the comparison of allele frequencies, both male and female subjects were analyzed. Bonferroni correction in the initial association study, considering 30 SNPs, two age groups, and the comparison of genotype and allele frequencies, corresponds to a significance threshold of $p < 4.2e\text{-}04$, whereas for the replication study, where 13 SNPs and genotype and allele frequencies were considered, significance was set at $p < .0019$.

Multiple-Marker Analysis. To minimize multiple testing and type I errors (α), we decided a priori to restrict the haplotype-based association study to genes nominally associated with ADHD in the single-marker analyses. The best two-marker haplotype from all possible combinations was identified. Likewise, additional markers (up to four) were added in a stepwise manner to the initial two-SNP haplotype and subsequently assigned specific estimated haplotypes to individuals with the PHASE software (http://www.stat.washington.edu/stephens/software.html) (57). Significance was estimated using 10,000 permutations with the UNPHASED software (http://www.mrc-bsu.cam.ac.uk/personal/frank) (58). Since the expectation-maximization algorithm does not accurately estimate low haplotype frequencies (59), haplotypes with frequencies <.1 were excluded. To avoid bias and ensure accurate haplotype estimations, we confirmed results with the PLINK program (http://pngu.mgh.harvard.edu/purcell/plink) (60). We also tested those allelic combinations showing positive association in the overall ADHD sample in the two diagnostic groups of combined and inattentive ADHD. The hyperactive-impulsive group was not considered due to its small sample size.

Table 1. Association Study in 270 Adult ADHD Patients (178 Combined ADHD, 80 Inattentive ADHD, and 10 Hyperactive-Impulsive ADHD Patients) and 270 Sex-Matched Unrelated Control Subjects from Spain and 639 Adult ADHD Patients (429 Combined ADHD, 162 Inattentive ADHD, and 48 Hyperactive-Impulsive ADHD Patients) and 612 Sex-Matched Unrelated Control Subjects from Germany

		Genotypes								Alleles	
		Genotypes				Genotypes 11 Versus 12 + 22		Genotypes 11 + 12 Versus 22		Allele 2 Versus Allele 1	
Gene	SNPs	11	12	22	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*
Spanish Population											
BAIAP2	rs8079781	177 (66.0) 144 (53.3)	83 (31.0) 115 (42.6)	8 (3.0) 11 (4.1)	.011	1.69 (1.20–2.44)	.0026	—	.49	1.49 (1.12–2.00)	.0061
	rs4969385	171 (63.4) 139 (51.5)	90 (33.3) 120 (44.4)	9 (3.3) 11 (4.1)	.020	1.64 (1.15–2.27)	.0053	—	.65	1.43 (1.07–1.89)	.014
German Population											
BAIAP2	rs8079626	311 (49.2) 258 (42.3)	250 (39.6) 278 (45.6)	71 (11.2) 74 (12.1)	.048	1.32 (1.05–1.67)	.015	—	.62	1.19 (1.01–1.41)	.039

ADHD, attention-deficit/hyperactivity disorder; CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism.

Results

We considered tagSNPs in six functional candidate genes differentially expressed in the right and left human embryonic cortex (*BAIAP2*, *DAPPER1*, *LMO4*, *NEUROD6*, *ATP2B3,* and *ID2*) (42) in a Spanish sample of 587 ADHD cases (270 adults and 317 children) and 587 control subjects. Of the 32 SNPs initially selected, two were discarded because they did not pass through the SNPlex design pipeline. Thus, a total of 30 SNPs with an average genotype call rate of 99.5% (SD = .48) were finally used (Table S2 in Supplement 1). The minimal statistical power was 59.5% and 66.4% when adult or childhood samples were considered, respectively.

Once we excluded evidence for population stratification (Fst coefficient: Theta = .000, 95% confidence interval [CI] = .000–.001; Pritchard and Rosenberg: $p = .32$ for adults and $p = .57$ for children), we compared genotype and allele frequencies between adult or childhood Spanish ADHD patients and their sex-matched unrelated control subjects. The single-marker analysis identified two SNPs in *BAIAP2* displaying nominal association with ADHD in the adult dataset: rs8079781 ($p = .0026$; OR = 1.69 [1.20–2.44]) and rs4969385 ($p = .0053$; OR = 1.64 [1.15–2.27]; Table 1 and Table S3 in Supplement 1), differences that did not remain statistically significant after Bonferroni correction. No association, however, was observed in the childhood ADHD sample.

We further considered *BAIAP2* for a haplotype-based analysis only in the adult dataset. All the associations described below remained significant once adjusted for multiplicity. The study of the 13 *BAIAP2* SNPs revealed a four-marker haplotype (rs8079626/rs11657991/rs7503597/rs7210438) associated with adult ADHD (global *p* value = .0052; Figure 1, Table 2). The analysis of the contribution of individual haplotypes to the phenotype showed overrepresentation of the A-G-G-C allelic combination ($p = .0016$; OR = 1.64 [1.20–2.22]) and a trend toward underrepresentation of the A-C-G-C haplotype in the adult sample ($p = .014$; OR = 1.59 [1.09–2.32]; Table 3). We then considered the frequency of the A-G-G-C risk haplotype carriers and confirmed the association between *BAIAP2* and adult ADHD ($p = .0092$, OR = 1.60 [1.13–2.25]). Interestingly, these differences were specific to the combined ADHD subgroup (global *p* value = 6.6e-04; Table 2) with overrepresentation of the same risk haplotype ($p = 4.2e-04$, OR = 1.87 [1.33–2.64]; Table 3) and an increased frequency of carriers of this allelic combination in this clinical dataset ($p = .0035$; OR = 1.77 [1.21–2.60]). No evidence of association between *BAIAP2* and the inattentive clinical subset was observed.

Replication Studies

The 13 SNPs within the *BAIAP2* gene were selected for follow-up in replication adult cohorts from Germany and Norway. Taking the *BAIAP2* SNP with the lowest MAF (.23), the minimal statistical power was 92.7% and 82.0% in the German or Norwegian populations, respectively.

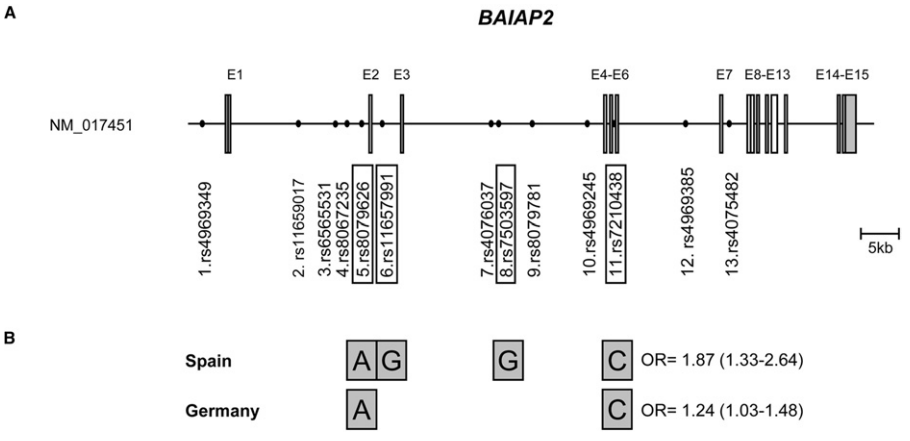

Figure 1. Haplotype analysis of the *BAIAP2* gene in adult ADHD. **(A)** Diagram of the *BAIAP2* gene with all the tagSNPs included in the present study. Boxes correspond to exons. Coding and noncoding exonic regions are indicated in white and gray, respectively. In bold and boxed, SNPs that conform the risk haplotype associated with adult ADHD in the Spanish dataset. **(B)** Allelic combinations associated with adult ADHD in the Spanish and German samples. ADHD, attention-deficit/hyperactivity disorder; SNP, single nucleotide polymorphism; tagSNPs, tagging single nucleotide polymorphisms.

Table 2. Haplotype Analysis of 13 *BAIAP2* SNPs in a Clinical Sample of 270 Adult ADHD Patients, 178 Combined ADHD Adult Subjects, and 270 Control Subjects from Spain Using the UNPHASED Software

	ADHD			Combined ADHD		
Marker[a] Haplotype	Global *p* Value	Best Haplotype *p* Value (Adjusted *p* Value)	Risk Haplotype-OR (95% CI)	Global *p* Value	Best Haplotype *p* Value (Adjusted *p* Value)	Risk Haplotype-OR (95% CI)
5 6	.0046	.0011 (.0051)	1.55 (1.19–2.01)	—	—	—
5 6 8	.0032	.0015 (.0096)	1.40 (1.09–1.79)	—	—	—
5 6 8 11	**.0052**	**.0016 (.0079)**	**1.64 (1.20–2.22)**	**6.6e-04**	**4.2e-04 (.0018)**	**1.87 (1.33–2.64)**

In bold the best allelic combination (highest OR); 5-rs8079626, 6-rs11657991, 8-rs7503597, and 11-rs7210438.
ADHD, attention-deficit/hyperactivity disorder; CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism.

The single-marker analysis was first performed in the German sample. We found no evidence of population substructure (Fst coefficient: Theta = .000, 95% CI = .000–.001; Pritchard and Rosenberg: $p = .32$) and detected a nominal association between rs8079626 and ADHD (Table 1 and Table S4 in Supplement 1), one of the SNPs identified in the multiple-marker analysis of the Spanish sample. The multiple-marker approach showed evidence of association between adult ADHD and a two-marker haplotype (rs8079626/rs7210438) that contains two of the four SNPs present in the *BAIAP2* risk haplotype of the Spanish sample (global p value = .019; Figure 1, Table 4). Consistently with the results in the Spanish cohort, we observed an overrepresentation of the A-C allelic combination in the German ADHD sample ($p = .030$; OR = 1.21 [1.03–1.42]), whereas the G-C haplotype was downrepresented ($p = .0062$; OR = 1.28 [1.07–1.53]; Figure 1, Table 5). As in the Spanish series, once we subdivided patients according to the ADHD subtypes, the association between *BAIAP2* and adult ADHD remained significant only in the combined ADHD sample (global p value = .0031; Table 4).

No evidence of association between adult ADHD and the *BAIAP2* gene was detected in the Norwegian cohort in the analysis of single or multiple markers (Table S4 in Supplement 1).

Discussion

The purpose of this study was to analyze the involvement of brain asymmetry-related genes in the susceptibility to ADHD through a population-based association study. There are consistent data that support the existence of functional asymmetry in the brain (39,40,42). This segregation of human brain functions between hemispheres is associated with asymmetries in anatomical structures (42,61). In addition to environmental effects, growing evidence supports that genes play an essential role in the development of the human brain (39–41). Although little is known about genetic factors underlying brain lateralization, several genes are differentially expressed in the two hemispheres, some of which could be involved in the development of right-left asymmetries (42). As patients with ADHD show deviations from the typical pattern of cerebral asymmetry that may account for a large number of ADHD-related symptoms (12, 62,63), we suggest a relationship between genes differentially expressed in brain hemispheres and the vulnerability to this neurobehavioral disorder.

We performed the first comprehensive screen of common variants in six functional candidate genes showing at least 1.9-fold differential expression between hemispheres (42), conducted an initial case-control study in a Spanish sample, and provided preliminary evidence for the contribution of *BAIAP2* to adult ADHD. Subsequently, SNPs within this ADHD-associated gene were tested for replication in two additional independent adult samples from Germany and Norway. Despite the well-characterized and large case-control populations, the *BAIAP2* gene showed evidence for replication in the German but not in the Norwegian cohort. These results point to reconsider the robustness of this finding and raise several considerations:

1. Discrepancy could be attributed to clinical heterogeneity either within or across European populations. However, all patients fulfilled DSM-IV criteria for ADHD, and the Spanish and German ADHD samples were evaluated using a common set of diagnostic instruments.
2. Different frequencies of clinical subtypes or comorbid disorders that co-occur with ADHD could also explain the lack of association in one of the three populations considered (Table S5 in Supplement 1).
3. The study design ensured a high level of genetic coverage in *BAIAP2* (86.7%) and the replication cohorts were well-powered (>80%) to detect a nominal effect of the magni-

Table 3. Haplotype Distributions of rs8079626, rs11657991, rs7503597, and rs7210438 *BAIAP2* SNPs in 270 Adult ADHD Patients, 178 Combined Adult ADHD Subjects, and 270 Control Subjects from Spain

	ADHD			Combined ADHD		
Marker[a] Haplotype	Cases ($n = 270$)	Control Subjects ($n = 270$)	Haplotype-Specific *p* Value; OR (95% CI)	Cases ($n = 178$)	Control Subjects ($n = 270$)	Haplotype-Specific *p* Value; OR (95% CI)
5 6 8 11						
G G G C	99 (27.2)	124 (31.5)	—	64 (27.4)	124 (31.5)	—
A G G C	139 (38.2)	108 (27.4)	.0016; 1.64 (1.20–2.22)	97 (41.4)	108 (27.4)	4.2e-04; 1.87 (1.33–2.64)
A C G C	53 (14.6)	84 (21.3)	.014; 1.59 (1.09–2.32)[b]	28 (12.0)	84 (21.3)	.0017; 1.99 (1.25–3.17)[b]
A C T T	73 (20.0)	78 (19.8)	—	45 (19.2)	78 (19.8)	—
$\chi^2 = 12.8$; $df = 3$; $p = .0052$				$\chi^2 = 17.1$; $df = 3$; $p = 6.6e\text{-}04$		

ADHD, attention-deficit/hyperactivity disorder; CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism.
[a]5-rs8079626, 6-rs11657991, 8-rs7503597, and 11-rs7210438.
[b]When odds ratio < 1, the inverted score is shown.

Table 4. Haplotype Analysis of 13 *BAIAP2* SNPs in 639 Adult ADHD Patients, 429 Combined ADHD Adult Patients, and 612 Control Subjects from Germany Using the UNPHASED Software

Marker[b] Haplotype	ADHD			Combined ADHD		
	Global *p* Value	Best Haplotype *p* Value (Adjusted *p* Value)	Risk Haplotype-OR (95% CI)	Global *p* Value	Best Haplotype *p* Value (Adjusted *p* Value)	Risk Haplotype-OR (95% CI)
5 11	**.019**	**.0062 (.018)**	**1.21 (1.03–1.42)**	**.031**	**.014 (.038)**	**1.24 (1.03–1.48)**
4 5 11	.035	.0074 (.029)	1.21 (1.02–1.46)	—	—	—
4 5 8 11	.071	.033 (ns)	—	—	—	—

In bold the best allelic combination (highest OR); 4-rs8067235, 5-rs8079626, 8-rs7503597, and 11-rs7210438.
ADHD, attention-deficit/hyperactivity disorder; CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism.

tude estimated in the Spanish adult ADHD population. However, population-specific effects such as different LD patterns cannot be discarded.

4. Furthermore, it is possible that the true effect is less than the point estimate (OR = 1.50, 95% CI = 1.12–2.0) in the initial finding due to the winner's curse effect (64). This could mean that the real risk in Northern European populations is closer to an OR of 1.2 (95% CI = 1.00–1.45), as estimated in the German sample, implying that larger samples are required to detect significant association.
5. The absence of association in one of the replication populations might also indicate that common *BAIAP2* SNPs of moderate effect may not play a major role in ADHD. Further genetic analyses in other large datasets are required to confirm these results and disclose the functional variants involved.
6. Finally, we have previously conducted a pooled genome-wide association analysis using an Affymetrix 500K SNP chip in 343 adult ADHD cases and 250 control subjects from Germany, >90% of which have also been examined in the present study (44). When reanalyzing these data, it became evident that two SNPs in *BAIAP2* were nominally significant: rs8080815 (p = .024) and rs8066330 (p = .002). These SNPs are located 950 and 1902 base pair (bp) proximal from and in strong LD (D′ = 1) with rs7210438, one of the variations contained in both the German and Spanish risk haplotypes. Furthermore, several SNPs in a putative isoform of *BAIAP2*, *BAIAP2L1* (*BAIAP2-like 1*), were also nominally significant: rs13232181 (p = .004), rs7812180 (p = .0006), and rs6465675 (p = .028).

BAIAP2 is expressed at higher levels in the left human cerebral cortex (42) and participates in neuronal proliferation, survival, and maturation (65–68). It encodes the insulin receptor tyrosine kinase substrate protein of 53 kDa (IRSp53) (69), a member of a group of downstream signaling molecules that participate in the signal transduction pathways of insulin and insulin-like growth factor 1 (IGF-1). Interestingly, IRSp53 selectively localizes at synapses (70) and although its specific function is unknown, it may participate in the insulin and/or IGF-1 dependent signaling pathways at the postsynaptic apparatus of excitatory synapses (71) and might be involved in insulin receptor-dependent learning and cognitive behavior in adult rats (72). Interestingly, several groups have suggested the involvement of abnormal cerebral glucose metabolism in ADHD, although results are controversial (73–75). *BAIAP2* expression in rat cerebral cortices is enhanced by treatment with methamphetamine, a drug that has successfully been used to treat ADHD (76). A potential role for the insulin receptor signaling pathway in the pathogenesis of ADHD is also suggested by our previously conducted genome-wide association study (GWAS), where not only *BAIAP2* and its isoform *BAIAP2L1* were nominally significant, but also *GRB10*, which encodes a protein known to bind to and regulate the insulin receptor (44).

The fact that the association between ADHD and *BAIAP2* was only observed in the adult group is in agreement with previous results and suggests a distinct genetic load between persistent and remitting ADHD (5,6,8,9). In this regard, alterations in this gene might contribute to the maintenance of ADHD symptoms in the subgroup of children in whom the disorder will persist throughout the life span. Alternatively, confounding factors, such as environmental influences, comorbidities, or IQ, could also contribute to these differences and should be considered in further analyses. Follow-up studies of child ADHD patients may allow us to discern between individuals with and without

Table 5. Haplotype Distributions of rs8079626 and rs7210438 *BAIAP2* SNPs in 639 Adult ADHD Patients, 429 Combined ADHD Adult Patients, and 612 Control Subjects from Germany

Marker[a] Haplotype	ADHD			Combined ADHD		
	Cases (n = 639)	Control Subjects (n = 612)	Haplotype-Specific *p* Value; OR (95% CI)	Cases (n = 429)	Control Subjects (n = 612)	Haplotype-Specific *p* Value; OR (95% CI)
5 11						
A C	590 (51.2)	537 (46.4)	.030; 1.21 (1.03–1.42)	406 (51.8)	537 (46.4)	.030; 1.24 (1.03–1.48)
G C	315 (27.4)	376 (32.6)	.0062; 1.28 (1.07–1.53)[b]	214 (27.3)	376 (32.6)	.014; 1.28 (1.05–1.57)[b]
A T	247 (21.4)	243 (21.0)	—	164 (20.9)	243 (21.0)	—
χ^2 = 7.9; df = 2; p = .019				χ^2 = 7.0; df = 2; p = .031		

ADHD, attention-deficit/hyperactivity disorder; CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism.
[a]5-rs8079626 and 11-rs7210438.
[b]When odds ratio < 1, the inverted score is shown.

symptomatic remission and their genotype data could be analyzed back to determine the genetic influence on the stability of ADHD symptoms.

Methodological Limitations

In the present study, we carried out a quite conservative two-stage statistical approach to address multiple testing. We first performed an association study considering single markers. However, as this strategy may neglect information about the joint contribution of different SNPs, for the single gene nominally associated with ADHD, we further moved on to a multiple-marker analysis. Although this approach allowed us to limit the number of tests, it may also increase the probability of false negative results for those genes in which the multiple-marker approach was not performed (77) and we do not know a priori whether individual SNPs and/or a combination of markers confer susceptibility to ADHD. However, we cannot overlook the fact that, although haplotype differences were significant after adjustment for multiplicity, the *BAIAP2* individual SNPs identified under the single-marker approach did not remain significant after the Bonferroni correction. As this multiple correction is often overconservative, particularly when the dependence between statistical tests is high (78), further studies are required to gain more insight into the involvement of individual *BAIAP2* SNPs, as well as specific haplotypes, in ADHD.

Because SNPs were selected to ensure genetic coverage according to LD criteria and all four *BAIAP2* SNPs that make up the ADHD risk haplotype are intronic and located far from any splice site or branch point, the *BAIAP2* sequence variants associated with ADHD may not have functional implications by themselves but rather be in LD with the causative variants. This idea is also supported by the fact that the Spanish and German risk haplotypes overlap (Figure 1). Further sequencing of *BAIAP2* may allow the identification of genetic variants directly involved in the predisposition to this complex phenotype.

Our findings provide tentative evidence for the contribution of *BAIAP2* to adult ADHD, support its participation in the persistence of the disorder, and suggest that genetic factors influencing abnormal cerebral lateralization may be involved in the predisposition to this neurodevelopmental disorder. To our knowledge, this is the first association study showing that a gene potentially involved in cerebral asymmetry may be considered a good candidate for ADHD. However, further investigation is required to replicate our results and to establish the participation of brain asymmetry-related genes in the adult outcome of the disorder.

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Supplemental Tables

Table S1. Description of the four cohorts included in the association study.

	Cases							Controls		
		Clinical subtype N (%)				Gender N (%)			Gender N (%)	
Cohort	**Total**	**Combined**	**Inattentive**	**Hyperactive-Impulsive**	**Unknown**	**Male**	**Female**	**Total**	**Male**	**Female**
Spain	270 adulthood ADHD	178 (65.9)	80 (29.6)	12 (4.5)	-	195 (72.2)	75 (27.8)	270 controls	195 (72.2)	75 (27.8)
	317 childhood ADHD	233 (73.5)	69 (21.8)	15 (4.7)	-	261 (82.3)	56 (17.7)	317 controls	261 (82.3)	56 (17.7)
Replication cohort	**Total**	**Combined**	**Inattentive**	**Hyperactive-Impulsive**	**Unknown**	**Male**	**Female**	**Total**	**Male**	**Female**
Germany	639 adulthood ADHD	429 (67.1)	162 (25.4)	48 (7.5)	-	321 (50.2)	318 (49.8)	612 controls	307 (50.2)	305 (49.8)
Norway	417 adulthood ADHD	315 (75.5)	45 (10.8)	14 (3.4)	43 (10.3)*	218 (52.3)	199 (47.7)	469 controls	244 (52.0)	225 (48.0)

* Subjects that scored below threshold.

Table S2. Description of the 32 SNPs initially selected for the SNPlex analysis within six brain asymmetry-related candidate genes for ADHD.

Gene	Contig reference	Location	Length (kp)	Num. exons	SNPs	Tag SNPs	ID	Location	Exclusion Criteria	Gene Coverage**	Genotype Call Rate	Other SNPs within the bin
BAIAP2	NM_017451	17q25	82.27kb	17	22	15	rs4969349	5'		0.86	99.7%	-
							rs11659017	I1			99.8%	rs11869351
							rs6565531	I1			99.9%	-
							rs8067235	I1			99.8%	-
							rs8079626	I1			99.5%	-
							rs11657991	I2			99.8%	rs3934492, rs9901648
							rs9903763	I3	No SNPlex design			-
							rs4076037	I3			99.8%	-
							rs7503597	I3			99.9%	-
							rs8079781	I3			99.8%	rs4969382
							rs4969245	I3			98.0%	-
							rs7210438	I5			99.8%	rs12601810, rs4969387
							rs4969385	I6			99.5%	-
							rs4075482	I7			99.5%	rs4076427
							rs11664	I13	No SNPlex design			-
DAPPER1	NT_026437.11	14q23.1	10.2kb	4	6	4	rs167481	I3		1	99.7%	-
							rs863091	E4			99.8%	rs11541, rs221327
							rs17832998	E4			99.5%	
							rs160472	3'			99.7%	-
LMO4	NM_006769	1p22.3	17.1kb	5	1	1	rs10873830	I2		1	99.7%	-
NEUROD6	NM_022728	7p15.1	3.46kb	1	10	5	rs2191888	5'		1	99.2%	-
							rs3807602	5'			99.5%	rs3807603
							rs2233404	I1			99.4%	-
							rs10238918	3'UTR			99.5%	rs10487721
							rs9655313	3'			99.7%	rs11767648, rs2233402, rs2215617
ATP2B3	NM_021949	Xq28	46.8kb	21	11	6	rs2980017	I8		1	99.9%	rs2285034, rs594514
							rs2239682	I10			99.7%	rs4898415
							rs2239683	I16			99.7%	-
							rs4898359	I17			99.5%	-
							rs4898421	I19			98.1%	rs5945307, rs6643626
							rs4898360	I20			99.5%	-
ID2	NM_002166	2p25	2.47kb	3	1	1	rs4669330	3'		1	98.8%	-

** Analyzed tagSNPs/total tagSNPs within the gene

Table S3. Nominal P-values observed when genotype and allele frequencies of SNPs within the *BAIAP2* gene were considered in 270 Spanish adults with ADHD, 317 Spanish children with ADHD and their sex-matched unrelated controls.

	Spanish Adulthood ADHD (n=270)								Spanish Childhood ADHD (n=317)							
	Genotypes						Alleles		Genotypes						Alleles	
	Cases N (%)			Controls N (%)					Cases N (%)			Controls N (%)				
SNP	11	12	22	11	12	22	P	P	11	12	22	11	12	22	P	P
rs4969349	146 (54.1)	106 (39.3)	18 (6.6)	155 (57.4)	90 (33.3)	25 (9.3)	0.26	0.89	181 (57.3)	114 (36.1)	21 (6.6)	191 (60.3)	104 (32.8)	22 (6.9)	0.69	0.58
rs11659017	160 (59.3)	93 (34.4)	17 (6.3)	153 (56.7)	98 (36.3)	19 (7.0)	0.82	0.52	180 (56.8)	113 (35.6)	24 (7.6)	175 (55.2)	120 (37.9)	22 (6.9)	0.83	0.85
rs6565531	77 (28.5)	137 (50.7)	56 (20.7)	73 (27.0)	148 (54.8)	49 (18.1)	0.61	0.85	94 (29.7)	158 (49.8)	65 (20.5)	90 (28.4)	158 (49.8)	69 (21.8)	0.90	0.65
rs8067235	135 (50.0)	107 (39.6)	28 (10.4)	127 (47.0)	111 (41.1)	32 (11.9)	0.75	0.43	136 (42.9)	146 (46.1)	35 (11.0)	135 (42.6)	149 (47.0)	33 (10.4)	0.95	0.95
rs8079626	122 (45.7)	121 (45.3)	24 (9.0)	107 (39.6)	139 (51.5)	24 (8.9)	0.33	0.30	144 (45.4)	135 (42.6)	38 (12.0)	131 (41.3)	148 (46.7)	38 (12.0)	0.54	0.44
rs11657991	112 (41.5)	123 (45.6)	35 (13.0)	99 (36.7)	125 (46.3)	46 (17.0)	0.31	0.13	112 (35.4)	157 (49.7)	47 (14.9)	121 (38.2)	138 (43.5)	58 (18.3)	0.26	0.90
rs4076037	154 (57.0)	97 (35.9)	19 (7.0)	144 (53.3)	103 (38.1)	23 (8.5)	0.64	0.33	189 (59.6)	102 (32.2)	26 (8.2)	170 (53.6)	129 (40.7)	18 (5.7)	0.06	0.48
rs7503597	124 (46.3)	117 (43.7)	27 (10.1)	132 (48.9)	114 (42.2)	24 (8.9)	0.79	0.50	166 (52.5)	125 (39.6)	25 (7.9)	153 (48.3)	134 (42.3)	30 (9.5)	0.52	0.25
rs8079781	177 (66.0)	83 (31.0)	8 (3.0)	144 (53.3)	115 (42.6)	11 (4.1)	0.01	0.01	189 (59.8)	107 (33.9)	20 (6.3)	192 (60.6)	106 (33.4)	19 (6.0)	0.97	0.82
rs4969245	123 (47.1)	116 (44.4)	22 (8.4)	134 (50.0)	109 (40.7)	25 (9.3)	0.67	0.72	140 (45.2)	142 (45.8)	28 (9.0)	159 (50.2)	126 (39.7)	32 (10.1)	0.31	0.45
rs7210438	157 (58.1)	92 (34.1)	21 (7.8)	155 (57.4)	99 (36.7)	16 (5.9)	0.62	0.83	190 (60.3)	108 (34.3)	17 (5.4)	187 (59.0)	110 (34.7)	20 (6.3)	0.87	0.64
rs4969385	171 (63.3)	90 (33.3)	9 (3.4)	139 (51.5)	120 (44.4)	11 (4.1)	0.02	0.01	186 (59.0)	108 (34.3)	21 (6.7)	186 (58.7)	109 (34.4)	22 (6.9)	0.99	0.89
rs4075482	90 (43.3)	89 (42.8)	29 (13.9)	96 (36.2)	128 (48.3)	41 (15.5)	0.29	0.17	112 (35.7)	152 (48.4)	50 (15.9)	136 (43.2)	139 (44.1)	40 (12.7)	0.13	0.05

Table S4. Nominal P-values observed when genotype and allele frequencies of 13 SNPs within the *BAIAP2* gene were considered in 639 German adults with ADHD, 417 Norwegian adults with ADHD and their sex-matched unrelated controls.

	German Adulthood ADHD (n=639)								Norwegian Adulthood ADHD (n=417)							
	Genotypes							Alleles	Genotypes							Alleles
	Cases N (%)			Controls N (%)					Cases N (%)			Controls N (%)				
SNP	11	12	22	11	12	22	P	P	11	12	22	11	12	22	P	P
rs4969349	339 (55.1)	241 (39.2)	35 (5.7)	348 (58.0)	216 (36.0)	36 (6.0)	0.51	0.46	230 (58.4)	136 (34.5)	28 (7.1)	276 (59.7)	159 (34.4)	27 (5.8)	0.75	0.53
rs11659017	350 (55.6)	234 (37.1)	46 (7.3)	351 (58.5)	209 (34.8)	40 (6.7)	0.58	0.31	206 (49.5)	175 (42.1)	35 (8.4)	238 (51.0)	183 (39.2)	46 (9.9)	0.59	1.00
rs6565531[&]	216 (34.2)	298 (47.2)	117 (18.5)	186 (30.5)	287 (47.1)	136 (22.3)	0.18	0.06	-	-	-	-	-	-	-	-
rs8067235	288 (45.6)	281 (44.5)	62 (9.8)	287 (47.0)	264 (43.2)	60 (9.8)	0.89	0.72	179 (43.3)	185 (44.8)	49 (11.9)	197 (42.6)	202 (43.7)	63 (13.6)	0.73	0.59
rs8079626	311 (49.2)	250 (39.6)	71 (11.2)	258 (42.3)	278 (45.6)	74 (12.1)	0.05	0.04	184 (44.9)	181 (44.1)	45 (11.0)	213 (46.1)	204 (44.2)	45 (9.7)	0.82	0.58
rs11657991	200 (31.8)	302 (48.0)	127 (20.2)	208 (34.0)	285 (46.6)	118 (19.3)	0.70	0.43	121 (30.0)	200 (49.6)	82 (20.3)	123 (27.0)	238 (52.2)	95 (20.8)	0.60	0.46
rs4076037	316 (50.5)	272 (43.5)	38 (6.1)	321 (52.8)	241 (39.6)	46 (7.6)	0.29	0.81	222 (53.4)	161 (38.7)	33 (7.9)	240 (51.6)	177 (38.1)	48 (10.3)	0.46	0.33
rs7503597	312 (50.0)	254 (40.7)	58 (9.3)	318 (52.1)	241 (39.5)	51 (8.4)	0.71	0.40	203 (49.2)	175 (42.4)	35 (8.5)	252 (54.0)	173 (37.0)	42 (9.0)	0.27	0.32
rs8079781	369 (58.9)	220 (35.1)	38 (6.1)	329 (54.3)	243 (40.1)	34 (5.6)	0.19	0.24	221 (53.0)	165 (39.6)	31 (7.4)	237 (50.6)	197 (42.1)	34 (7.3)	0.74	0.61
rs4969245	312 (50.2)	252 (40.6)	57 (9.2)	299 (50.1)	239 (40.0)	59 (9.9)	0.92	0.82	224 (54.5)	159 (38.6)	29 (7.0)	255 (54.5)	180 (38.5)	33 (7.1)	1.00	0.98
rs7210438	360 (57.5)	220 (35.1)	46 (7.3)	358 (58.7)	217 (35.6)	35 (5.7)	0.52	0.42	223 (54.1)	159 (38.6)	30 (7.3)	273 (58.6)	155 (33.3)	38 (8.2)	0.26	0.39
rs4969385	344 (55.1)	233 (37.3)	47 (7.5)	315 (52.2)	252 (41.7)	37 (6.1)	0.24	0.66	218 (53.4)	159 (39.0)	31 (7.6)	239 (51.3)	191 (41.0)	36 (7.7)	0.81	0.60
rs4075482 [&*]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[&] Not genotyped in the Norwegian population; * Genotype call < 90% in the German sample

Table S5. Comorbidity rates of the three adulthood ADHD cohorts included in the association study.

	Spain (n=270)	Germany (n=639)	Norway* (n=417)
Comorbid Axis I Disorder	63.70%	71.04%	76%
Mood Disorder	26.67%	51.84%	69%[&]
Anxiety Disorder	35.55%	28%	69%[&]
Substance Use Disorder	40.74%	37.12%	38%
Eating Disorder	1.48%	8.48%	NA
Bipolar Disorder	0.74%	3.68%	11%
Borderline Personality Disorder	3.33%	16.8%	NA

*Self-reported; [&]Depression and/or Anxiety; NA: Data not available.

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ORIGINAL INVESTIGATION

Candidate pathway association study in cocaine dependence: The control of neurotransmitter release

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Abstract

Objectives. Cocaine is the second most used illegal drug in Europe. The transition from use to dependence involves both genetic and environmental factors. Genetic variation in neurotransmitter systems is involved in the susceptibility to cocaine dependence. We examined the possible contribution to cocaine dependence of 16 genes involved in the cellular machinery that controls neurotransmitter release: genes encoding proteins of the SNARE complex (*STX1A*, *SNAP25*, *VAMP1* and *VAMP2*), fusion control elements (*SYT1, SYT2*, *CPLX1*, *CPLX2*, *CPLX3* and *CPLX4*) and regulatory elements (*STXBP1*, *SYP, SNPH*, *NSF, NAPA* and *RAB3A*). *Methods.* We genotyped 121 SNPs, selected according to genetic coverage criteria, in 360 cocaine-dependent patients and 360 controls from Spain. *Results.* Single and multiple-marker analyses revealed a strong association between cocaine dependence and the *NSF* gene, encoding the *N*-ethylmaleimide-sensitive factor (P = 5.1e-04, OR = 2.44 (1.45–4.00) and P = 0.001, OR = 1.82 (1.28–2.59), respectively). The presence and absence of psychotic symptoms were also studied. Interestingly, when we considered the time between initial consumption and the onset of cocaine dependence, we observed that the association was mainly restricted to the group of patients that rapidly developed drug dependence (≤2 years; P = 2.98e-06, OR = 1.33 (1.20–1.47)). *Conclusions.* Our data show preliminary evidence that *NSF* may predispose not only to cocaine dependence, but also to an early onset of the dependence.

Key words: *Cocaine dependence, NSF, SNARE complex, case-control association study, synaptic exocytosis*

Introduction

Cocaine is the second most used illegal drug in Europe, with around 13 million consumers (3.9% of adult Europeans) (EMCDDA Annual Report 2009; European Monitoring Centre for Drugs and Drug Addiction). Cocaine is a powerful addictive drug with almost 16% of cocaine users developing cocaine dependence within 10 years after the first cocaine use (Wagner and Anthony 2002), and 5–6% within the first 2 years (O'Brien and Anthony 2005). The transition from use to dependence is not a fixed pharmacological property of cocaine, since both environmental and genetic factors influence cocaine dependence. Heritability studies have estimated that 60–70% of an individual's risk for developing cocaine dependence is due to genetic factors (Kendler and Prescott 1998; Tsuang et al. 1998; Kendler et al. 2000), although the underlying genetic susceptibility factors are poorly understood.

Cocaine binds to the dopamine, serotonin and norepinephrine transporters (DAT1, SERT and NET, respectively), inhibiting the reuptake of these neurotransmitters and increasing their levels at the neuronal synapses (Kuhar et al. 1991; Kalivas 2007).

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Interestingly, the dopamine (DA) neurotransmission and the indirect activation of DA receptors have been established as central mediators of cocaine response (Woolverton and Johnson 1992; Volkow et al. 1996, 1999, 2002). Other neurotransmitters such as serotonin or glutamate also play an important role in cocaine effects (Spealman 1993; Walsh and Cunningham 1997; Filip 2005; Filip et al. 2001, 2004). Thus, cocaine indirectly influences glutamate transmission in the limbic system producing persistent changes in neuronal function that alter the behavioral effects of cocaine (Gass and Olive 2008; Kalivas and O'Brien 2008; Thomas et al. 2008; Uys and LaLumiere 2008).

In addition, animal models and also pharmacological and association studies support an essential role of these neurotransmitter systems in cocaine dependence. The rewarding effects of cocaine were abolished in homozygous *DAT1*(–/–)*SERT*(–/–) and heterozygous *DAT1*(–/–)*SERT*(+/–) double knockout mice (Sora et al. 2001; Hall et al. 2002, 2004; Uhl et al. 2002). Interestingly, *NET*(–/–), *SERT*(–/–) and *NET*(–/–)/*SERT*(–/–) knock-outs showed an even increased rewarding cocaine effect (Sora et al. 1998; Xu et al. 2000; Hall et al. 2002). Other knockout studies also revealed an important role of the endocannabinoid system in cocaine self-administration and in the consolidation of the psychostimulant addictive process (Soria et al. 2005).

Pharmacological studies also provide insights into the role of neurotransmitter systems in cocaine dependence. Dopamine receptor D3 antagonists block cocaine acquisition and place preference and reduce cocaine induced reinstatement of self-administration (Vorel et al. 2002; Di Ciano et al. 2003). Glutamate agonists reduce the euphoric effects of cocaine and withdrawal symptoms (Dackis and O'Brien 2003; Dackis et al. 2003; Malcolm et al. 2006; Hart et al. 2008). Cannabinoid receptor agonists attenuate relapse induced by environmental cocaine-associated cues or cocaine re-exposure and antagonists induce relapse to cocaine seeking after a prolonged withdrawal period (DeVries et al. 2001; DeVries and Schoffelmeer 2005). In addition, some promising medications that may prevent cocaine relapse (such as gamma-vinyl GABA "GVG", tiagabine and topiramate) are related to GABA neurotransmission (Dewey et al. 1997; Morgan and Dewey 1998; Cornish and Kalivas 2000; Gonzalez et al. 2003).

Finally, positive associations have been described between cocaine dependence and polymorphisms in genes of the dopaminergic (Noble et al. 1993; Comings et al. 1999; Ballon et al. 2007; Guindalini et al. 2006; Fernàndez-Castillo et al. 2010), serotoninergic (Patkar et al. 2001; Mannelli et al. 2005), noradrenergic (Cubells et al. 2000; Guindalini et al. 2008), endocannabinoid (Ballon et al. 2006; Zuo et al. 2009) and cholinergic neurotransmitter systems (Grucza et al. 2008).

All these neurotransmitter systems are candidates for being involved in cocaine dependence and depend on mechanisms that control neurotransmitter release at the synapse, including synaptic vesicle docking, fusion and recycling. The process is complex and involves different proteins such as the *N*-ethylmaleimide sensitive factor (NSF), the soluble NSF attachment proteins (SNAPs), the SNAP receptors (SNAREs), synaptobrevins (VAMP1, VAMP2), syntaxin-1 and SNAP-25, the SM protein Munc18-1 (STXBP) and small GTPases from the RAB3 family (Rizo and Rosenmund 2008). Interestingly, cocaine induces expression changes of some genes encoding proteins involved in this neurotransmitter release machinery, such as synaptotagmin, synaptobrevin (VAMP1), syntaxin-1, synaptophysin and RAB3A (Freeman et al. 2002; Yuferov et al. 2003; Ahmed et al. 2005).

Based on previous data that link different neurotransmission systems with cocaine dependence, we hypothesized that alterations in the neurotransmitter release machinery may be involved in the genetic susceptibility to this disorder, as well as cocaine induced psychotic symptoms and time between initial consumption and the onset of cocaine dependence. We performed a case–control association study in 360 cocaine-dependent patients and 360 sex-matched controls, with SNPs covering 16 candidate genes that encode proteins of the neurotransmitter release machinery: SNARE complex formed by syntaxin 1A, SNAP-25 and synaptobrevins (*STX1A*, *SNAP25*, *VAMP1* and *VAMP2*), the fusion control elements synaptotagmins and complexins (*SYT1*, *SYT2*, *CPLX1*, *CPLX2*, *CPLX3* and *CPLX4)* and the regulatory elements Munc18.1 (*STXBP1*), synaptophysin (*SYP*), syntaphilin (*SNPH*), *NSF*, αSNARE (*NAPA*) and *RAB3A*.

Methods and materials

Subjects

The patient sample consisted of 360 cocaine dependent patients (mean age 34.6 ± 7.6 years and 83% males (n = 299)) recruited and evaluated at the Psychiatry Department of the Hospital Universitari Vall d'Hebron (Barcelona, Spain) according to DSM-IV TR criteria (Diagnostic and Statistical Manual of Mental Disorders, 4th ed., text revision). The Structured Clinical Interview (SCID) (First et al. 1997) was administered and volunteers with current DSM-IV diagnosis of cocaine dependence were included in the study. Other drug dependences were assessed in 334 patients (92.8%): alcohol dependence was present in 22.7% of the patients (n = 76), cannabis

dependence in 26% (n = 87), opiate dependence in 13.8% (n = 46), benzodiazepine dependence in 5.1% (n = 17), and amphetamine or methamphetamine dependence in 2.1% (n =7). Seventy-six percent of the patients were evaluated for the presence (n =149) or absence (n = 124) of psychotic symptoms, and 71.4% (n =257) reported age at the initial consumption as well as age at dependence onset. Three hundred and sixty sex-matched unrelated controls (mean age 54.9 ± 16.6 years) were recruited at the Blood and Tissues Bank of the Hospital Universitari Vall d'Hebron. None of them had injected drugs intravenously. Both patients and controls were Spanish and Caucasian. The study was approved by the Ethics Committee of Hospital Universitari Vall d'Hebron, and written informed consent was obtained from all the participating individuals.

DNA isolation and quantification

Genomic DNA samples were obtained either from peripheral blood lymphocytes by the salting-out procedure (Miller et al. 1988) or from saliva using the Oragene DNA Self-Collection Kit (DNA Genotek, Kanata, Ontario, Canada). The double-stranded concentrations of all samples were determined on a Gemini XPS fluorometer (Molecular Devices, Sunnyvale, CA, USA) using the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR, USA), following the manufacturer's instructions.

Gene and SNP selection

Sixteen candidate genes involved in the synaptic vesicle fusion and neurotransmitter release at the synapse were selected for this study: *STX1A*, *SNAP25*, *VAMP1*, *VAMP2* (SNARE complex), *SYT1*, *SYT2*, *CPLX1*, *CPLX2*, *CPLX3* and *CPLX4* (synaptotagmins and complexins) and *STXBP1*, *SYP*, *SNPH*, *NSF*, *NAPA* and *RAB3A* (regulatory elements). SNP selection was based on genetic coverage parameters in terms of linkage disequilibrium (LD). Genotyping data of CEU population for each candidate gene plus 5-kb flanking sequences upstream and downstream were downloaded from the HapMap database (HapMap data release 22/phase II Apr07, dbSNPb126) (Thorisson et al. 2005). To minimize redundancy, LD was evaluated using the Haploview software (Barrett et al., 2005) setting a maximum r^2 threshold at 0.85 for all SNPs with minor allele frequency (MAF) of 0.15 or 0.25 for those genes with more than 20 tagSNPs (*SNAP25*, *SYT2*, *CPLX2*, *SNPH*). A total of 141 tagSNP (72 in multi-loci bins and 69 singletons) were selected with these criteria. Three additional SNPs were included: rs2293485 in exon 3 of *STX1A*, rs1968583 in exon 2 of *SYT2* (both exonic and synonymous) and rs2293945 in intron 6 of *SYP* (previously studied in ADHD (Brookes et al. 2005)).

Plex design, genotyping and quality control

From the initial selection of 145 SNPs, a VeraCode assay of 141 SNPs was designed (four SNPs did not pass the pipeline). SNPs were genotyped using the Illumina BeadXpress platform and the GoldenGate Genotyping Assay (Illumina, San Diego, CA, USA). This technology is based on allele-specific primer extension and highly multiplexed PCR with universal primers. Raw hybridization intensity data processing, clustering and genotype calling were performed using the genotyping module in the Illumina GenomeStudio package. The genotype cluster plots generated by GenomeStudio were visually inspected for quality of calls and edited when necessary. A total of 21 HapMap individuals including 7 trios were genotyped and used to help in the clustering and as a control of the genotyping process.

Statistical analysis

The minimal statistical power was estimated *post hoc* using the Genetic Power Calculator software (http://pngu.mgh.harvard.edu/~purcell/gpc/cc2.html), assuming an odds ratio (OR) of 1.5, prevalence of 0.0062 (16% developing dependence of 3.9% consumers), significance level of 0.05 and the lowest MAF value of 0.126. The analysis of Hardy–Weinberg equilibrium (threshold set at $P < 0.01$) and the comparison of genotype frequencies between cases and controls was performed using the SNPassoc R package (Gonzalez et al. 2007). Only when a SNP displayed nominal association under a codominant model, the dominant (11 vs. 12+22) and recessive (11+12 vs. 22) were considered. Genotype frequencies of SNPs within the genes located on chromosome X (*SYP*) were only considered in the female sample. For the multiple testing correction we used the Q-value R package (Storey 2002), considering all the tests performed and assuming a false discovery rate (FDR) of 10%, which corresponded to a significance threshold of $P \leq$ 5.1e-04. Additionally, we also corrected the significant P values that overcame the 10% FDR threshold by performing a permutation test using 10,000 permutations with the PLINK software (Purcell 2007). Significant P values after multiple testing corrections were adjusted for age. The haplotype-based association study was restricted to the single gene that was found associated with cocaine dependence in the single-marker analysis

after correction for multiple comparisons. The best two-marker haplotype from all possible combinations was identified in the whole sample and additional markers (up to four) were added to the initial two-SNP haplotype in a stepwise manner. Significance was estimated by a permutation procedure using 10,000 permutations with the UNPHASED software (Dudbridge 2003). Haplotypes with frequencies <0.05 were excluded. Specific estimated haplotypes were assigned to individuals with the PHASE 2.0 software (Stephens et al. 2001). The comparison of the risk haplotype carriers in cases and controls as well as the effect of this risk haplotype in the presence of cocaine-induced psychotic symptoms, age at initial consumption and the time between initial and regular consumption were evaluated using the statistical package SPSS 15.0 (SPSS Inc., Chicago, IL, USA). For the age at the first consumption and the lapse between initial consumption and the onset of cocaine dependence, normality was rejected using a Kolmogorov–Smirnov test and the comparison of medians was performed using the non-parametric Mann–Whitney *U*-test. Additionally, time between initial consumption and onset of cocaine dependence was dichotomized into early (≤2 years) or late (>2 years) dependence onset and the comparison of the risk haplotype carriers between the two groups was performed with two-tailed Fisher's exact test. In the multiple-marker analysis *P* values were also adjusted for age and, considering cocaine dependence as well as related phenotypes, the significance threshold was set at $2P < 0.01$ after the multiple testing correction of Bonferroni considering five comparisons (cocaine dependence, presence or absence of psychotic symptoms, and early or late dependence versus controls).

Results

We examined tagSNPs in 16 candidate genes encoding proteins of the neurotransmitter release machinery in 360 cocaine dependent patients and 360 controls. Of the 145 SNPs initially selected, 23 were discarded (four did not pass the Veracode pipeline design, 19 had genotype calls <90% and one had a significant departure from Hardy–Weinberg equilibrium in the control group). A total of 121 SNPs within 15 genes (the two SNPs of the *NAPA* gene failed) with an average call rate of 99.2% (SD = 1.8) were considered for the analysis (Table SI). The minimal statistical power, considering the SNP with the lowest MAF (0.126), was 57.9% assuming a codominant model, 68.3% considering a dominant model and 8.4% under a recessive model of inheritance.

The comparison of genotype frequencies between cocaine dependent patients and controls allowed identification of nominal differences for eight SNPs located in six genes: *NSF*, *SYT1*, *SYT2*, *CPLX1*, *CPLX2* and *CPLX4* (Table I, Table SII). However, after correcting for multiple comparisons applying a FDR of 10% ($P \leq$ 5.1e-04), only rs183211 in the *NSF* gene (P = 5.1e-04, OR = 2.44 (1.45–4.00)) remained associated with cocaine dependence, with a higher frequency of carriers of the common G allele in cases (93.6%) than in the control group (85.9%). Consistently, the G allele is present in 71.5% of cases and in 65.7% of controls. The rs183211 SNP in *NSF* remained significantly associated with cocaine dependence after adjusting for age and correcting by permutation (Table I).

The analysis of all the possible SNP combinations within *NSF* revealed a two-marker haplotype (rs183211–rs1769817), that includes the SNP identified in the single-marker analysis, associated with cocaine dependence (global P = 0.031; Figure 1), which remained significant after correcting by permutation (P-adjusted = 0.039). The evaluation of the contribution of individual haplotypes to the phenotype showed an over-representation of the G-T allelic combination (P = 0.013, OR = 1.3 (1.06–1.60)) and an under-representation of the A-T haplotype (P = 0.017, OR = 1.3 (1.05–1.64)) in the cocaine dependence sample (Table IIb). Consistently, we also identified an increased frequency of individuals carrying the G-T risk haplotype in this clinical sample, result that remained significant after adjusting by age (P-adjusted = 0.001, OR = 2.16 (1.38–3.83; Table III).

When patients were subdivided based on the presence/absence of psychotic symptoms, we did not identify differences between these two subgroups (P = 0.627) and observed an over-representation of G-T carriers in the two clinical samples when they were compared to the control sample (patients with psychotic symptoms: P-adjusted = 0.002, OR = 2.51 (1.4–4.5); patients without psychotic symptoms: P-adjusted = 0.0055, OR = 2.3 (1.28–4.14); Table III), and remained significant after the Bonferroni correction (Table III).

We then focused on the time between first consumption and onset of cocaine dependence and observed an earlier dependence onset among carriers of the G-T *NSF* risk haplotype than in non-carriers (Z = –3.15, P = 0.0015). Interestingly, the main differences were clearly observed in the group of patients whose dependence onset started within two years after the initial drug use (Figure 2). When patients were subdivided in two subgroups, early (≤2 years) and late (>2 years) dependence onset, we identified a higher frequency of carriers of the

Table I. Single-marker analysis: nominal associations identified in 360 cocaine dependent patients and 360 controls.

		Genotypes										
		Cases *N* (%)				Controls *N* (%)				Genotype 11 vs. 12+22		Genotype 22 vs. 11+12	
Gene	SNP	11	12	22	Sum	11	12	22	Sum	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
NSF	rs183211&	178 (49.4)	159 (44.2)	23 (6.4)	360	164 (45.6)	145 (40.3)	51 (14.2)	360	2.4e-03	–	NS	2.44 (1.45–4)	5.1e-04&
SYT1	rs10861941	145 (42.9)	160 (47.3)	33 (9.8)	338	105 (32.9)	171 (53.6)	43 (13.5)	319	0.023	1.54 (1.11–2.08)	8.3e-03	–	NS
SYT2	rs10800855	162 (45.0)	141 (39.2)	57 (15.8)	360	124 (34.6)	175 (48.9)	59 (16.5)	358	0.012	1.54 (1.14–2.08)	4.5e-03	–	NS
	rs4400672	167 (46.4)	147 (40.8)	46 (12.8)	360	161 (45.0)	169 (47.2)	28 (7.8)	358	0.048	–	NS	1.73 (1.05–2.83)*	0.028
CPLX1	rs11722977	133 (38.0)	181 (51.7)	36 (10.3)	350	163 (48.8)	133 (39.8)	38 (11.4)	334	6.4e-03	1.56 (1.15–2.11)*	4.3e-03	–	NS
CPLX2	rs4868539	133 (36.9)	182 (50.6)	45 (12.5)	360	140 (38.9)	153 (42.5)	67 (18.6)	360	0.029	–	NS	1.61 (1.06–2.44)	0.023
CPLX4	rs1914321	279 (77.9)	78 (21.8)	1 (0.3)	358	271 (77.0)	72 (20.5)	9 (2.6)	352	0.022	–	NS	9.09 (1.17–100)*	5.9e-03
	rs640401	245 (68.1)	107 (29.7)	8 (2.2)	360	233 (64.7)	106 (29.4)	21 (5.8)	360	0.042	–	NS	2.7 (1.19–6.25)	0.012

NS, not significant.
*When odds ratio <1, the inverted score is shown.
&Statistically significant *P* value after applying a false discovery rate of 10% ($P < 5.1e-04$), *P* value adjusted for age = 7e-04, *P* value corrected by a permutation test = 0.00224).

Table II. (a) Haplotype analysis of four *NSF* SNPs in a clinical sample of 360 cocaine-dependent patients and 360 controls using the UNPHASED software; (b) haplotype distributions of the rsrs183211 and rs17698176 *NSF* SNPs.

(a)

	NSF		
Marker* haplotype	Global *P* value	Best haplotype-specific *P* value (Adjusted P-value)	Haplotype-specific OR
14	0.031	0.013 (0.039)	1.3 (1.06–1.60)

(b)

Marker* haplotype	Cases	Controls	Haplotype specific *P* value; OR (CI)
14			
A T	205 (28.5)	247 (34.3)	0.017; 1.3 (1.05–1.64)**
G G	111 (15.4)	116 (16.1)	NS
G T	404 (56.1)	357 (49.6)	0.013; 1.3 (1.06–1.60)

NS, not significant.
*1-rs183211; 4-rs17698176.
**Inverted odds ratio score is shown.

G-T haplotype in the group of patients showing an early regular cocaine consumption ($P = 2.2e-04$, OR = 1.85 (1.4–2.4)). These differences were also observed when this group of patients, but not those showing late regular drug consumption, was compared with controls (*P*-adjusted = 5.77e-05, OR = 3.90 (2.01–7.57); Table III), and was still significant after the Bonferroni correction. No significant differences were observed when we compared the average age at the first cocaine consumption

Figure 1. (A) Diagram of the *NSF* gene (NM_006178). Black boxes indicate exons. The four tagSNPs included in the study are shown on top, with the two SNPs that conform the risk haplotype associated with cocaine dependence boxed. (B) Linkage disequilibrium plot of the four SNPs analyzed in the *NSF* gene, according to Haploview. Considering the Confidence Interval algorithm (Gabriel et al. 2002), the four SNPs are located in the same LD block in our control sample.

Table III. Distribution of carriers of the G-T (rs183211–rs1769817) allelic combination within the *NSF* gene.

	Haplotypes, *n* (%)				Adjusted for age	
	G-T carriers	Other haplotypes	OR (95%CI)	*P*	OR (95%CI)	*P*
Cocaine dependence	295 (81.9)	65 (18.1)	1.82 (1.28–2.59)	**0.001**	2.16 (1.38–3.83)	**0.001**
Cocaine dependence with psychotic symptoms	126 (84.6)	23 (15.4)	2.20 (1.33–3.62)	**0.0015**	2.51 (1.40–4.50)	**0.002**
Cocaine dependence without psychotic symptoms	102 (82.3)	22 (17.7)	1.86 (1.11–3.11)	0.017	2.30 (1.28–4.14)	**0.0055**
Early cocaine dependence (≤2 years*)	134 (89.9)	15 (10.1)	3.58 (2.00–6.41)	**2.98e-06**	3.90 (2.01–7.57)	**5.77e-05**
Late cocaine dependence (>2 years*)	77 (71.3)	31 (28.7)	–	NS	–	NS
Controls	257 (71.4)	103 (28.6)				

NS, not significant.
In bold, significant *P* values after Bonferroni correction ($P < 0.01$).
*Time between initial consumption and dependence onset.

between carriers and non-carriers of the G-T haplotype (*P*-adjusted = 0.372). Interestingly, the individual analysis of rs183221, the only SNP displaying positive signals in the single-marker analysis, also showed association with cocaine dependence when those cases with early dependence were considered (*P*-adjusted = 6.9e-04, OR = 2.42 (1.45–4.04)).

Discussion

The present case–control association study aims at covering an entire candidate pathway or functional network rather than focusing on single candidate genes. To our knowledge, this is the first association study in cocaine dependence focused on genes coding for the main components of the neurotransmitter release machinery and have found a significant association with *NSF*, mainly in the group of patients that rapidly develop drug dependence (≤2 years from the initial cocaine consumption). These results suggest that genetic factors may contribute to the neurobiological mechanisms underlying not only cocaine dependence but also an early development of this dependence. No relationship was observed between *NSF* and age at the first cocaine use or the presence of cocaine-induced psychotic symptoms.

The *NSF* gene encodes the *N*-ethylmaleimide sensitive factor, which participates in the SNARE complex recycling, ensuring that sufficient amounts of free SNAREs are available for the maintenance of intracellular membrane trafficking (Barszczewski et al. 2008). NSF is essential for the synaptic vesicle turnover as it modulates the kinetics of neurotransmitters release and the integrative properties of synapses (Schweizer et al. 1998; Littleton et al. 2001;

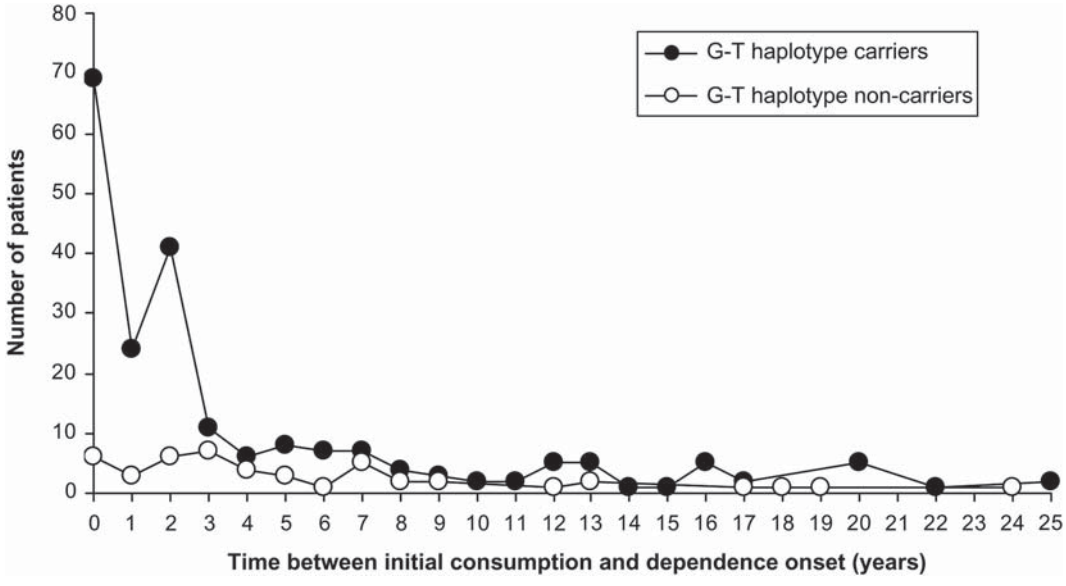

Figure 2. Time between initial consumption and cocaine dependence onset (years) in carriers and non-carriers of the *NSF* G-T (rs183211-rs1769817) risk haplotype in cocaine-dependent patients.

Malsam et al. 2008). It also has an essential role in the modulation of the trafficking between the plasma membrane and endosomes and in the binding of several cell-surface signalling receptors, such as the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPAR), the beta-2 adrenergic receptor (β2-AR), the DA receptors (more strongly D1 and D5), the adrenomedulin (AM) receptor and the γ-amino-butyric acid (GABA) receptor (Nishimune et al. 1998; Osten et al. 1998; Song et al. 1998; Cong et al. 2001; Heydorn et al. 2004; Bomberger et al. 2005; Pontier et al. 2006).

Altered NSF function may modulate the activity of the neurotransmitter systems involved in cocaine's effect and dependence. Thus, in agreement with the "reward deficiency syndrome", hypothesis that postulates that hypodopaminergic activity predisposes to cocaine addiction (Comings and Blum 2000), malfunction of NSF could have an effect on the turnover and availability of DA vesicles, altering the DA release to the synaptic cleft.

Some methodological considerations, however, should be taken into account in the present case–control association study: (i) in order to avoid sample heterogeneity that may bias the results in association studies, our sample consisted of patients and controls recruited in the same geographical area around Barcelona (Spain), all of them were Spanish, Caucasian and sex-matched; (ii) although significant after 10% FDR corrections for multiple testing, *NSF* did not remain associated with cocaine addiction under the most restrictive Bonferroni correction, considering 121 SNPs; (iii) the *NSF* risk haplotype associated with cocaine dependence consists of two SNPs located within introns, so it is possible that they do not cause functional effects by themselves, but rather are in LD with other sequence variants directly involved in the genetic susceptibility to cocaine dependence; (iv) the modest sample size (360 patients versus 360 controls) may have prevented from detecting subtle phenotypic effects; (v) cocaine dependence could not be discarded in the control sample, which may potentially dilute positive findings in the association study; and (vi) although the SNP selection was designed to cover 16 genes, gaps still exist in eight genes due to experimental constraints. Specifically, *NAPA* could not be tested for association as the two tagSNPs covering this gene failed in the genotyping assay.

To sum up, our study suggests that *NSF* contributes to the genetic susceptibility to cocaine dependence and, more specifically, to an early development of dependence. These results, however, need to be replicated in other samples. Also, further genetic and functional studies of the *NSF* gene are necessary to identify those functional variants directly involved in this psychiatric disorder.

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Statement of Interest

None to declare.

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Supplementary material available online

Table SI. Description of the VeraCode genotyping assay within 16 candidate genes encoding proteins involved in vesicle fusion for neurotransmitters release (data from HapMap Phase II_April07_dbSNP126_NCBI B36).

Table SII. Nominal *P* values observed when genotype frequencies of 121 SNPs within 15 candidate genes were considered in 360 cocaine-dependent patients and 360 controls.

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Table S1. Description of the VeraCode genotyping assay within 16 candidate genes encoding proteins involved in vesicle fusion for neurotransmitters release (data from HapMap Phase II_April07_dbSNP126_NCBI B36).

Gene	Contig reference	Location	Length (kp)	N° of exons	SNPs	Tag SNPs	SNP ID	Location	Exclusion criteria	Gene Coverage#	Other SNPs within the bin
SNAP25	NM_003081	20p12.2	88.59	8	40	19	rs1889189	5′		1	–
							rs6039769	5′			–
							rs363039	Intron 1			–
							rs363043	Intron 1			rs6077699, rs6514092
							rs12626080	Intron 1			rs363016, rs6039792, rs6104571
							rs6074113	Intron 1			rs363021
							rs362547	Intron 1			rs362569, rs362548, rs363018, rs362562, rs6039795, rs362569, rs362564, rs363022
							rs362570	Intron 1			–
							rs6039806	Intron 3			rs6039807, rs6108461, rs362549, rs362987
							rs3025873	Intron 4			–
							rs362988	Intron 7			rs6039820
							rs6108464	Intron 7			–
							rs3787283	Intron 7			–
							rs4813925	Intron 7			rs8636
							rs6074121	3′			–
							rs6032845	3′			rs4813927
							rs3025879	3′			rs362599, rs362600
							rs6032846	3′			–
STX1A	NM_004603	7q11.23	20.45	10	8	4	rs6951030	Intron 1	Failed	0.75	–
							rs941298	Intron 1			rs4717806, rs867500, rs941299
							rs2293485*	Exon 3			–
							rs3793243	Intron 3			–
							rs4363087	Intron 6			rs1001220
VAMP2	NM_014232	17p13.1	3.83	5	4	1	rs8067606	5′		1	rs2278637, rs1150, rs9899533
VAMP1	NM_014231	12q13.31	8.4	5	33	8	rs2534724	5′	Failed	0.71	rs2534722, rs10849466, rs2532485, rs7390, rs1045452, rs2534712, rs1045548
							rs10492096	5′			rs10849462, rs12422771, rs11064210, rs1045546, rs12426815
							rs11064213	5′	Failed		rs2072377, rs7298570
							rs2072376	Exon1 5'UTR			–
							rs2240867	Intron 1			–
							rs2534717	Intron 4			rs2534715, rs1034969, rs1017101, rs2240866, rs2058288, rs2072375, rs2534713, rs2243750, rs2243977, rs2244083, rs2534721, rs2534711
							rs12964	Exon 5			–

(Continued)

Table SI. *(Continued)*

Gene	Contig reference	Location	Length (kp)	N° of exons	SNPs	Tag SNPs	SNP ID	Location	Exclusion criteria	Gene Coverage[#]	Other SNPs within the bin
SYT1	NM_005639	12q21.2	233.6	8	132	14	rs2177963	Intron 1		0.85	rs2177963, rs10861698, rs1918193, rs2037743, rs1918195, rs7968716, rs1918188, rs1918190, rs7311549, rs8181778, rs7967265, rs1918191, rs4842447, rs10861717, rs1405498, rs1465054, rs1358248, rs6539317, rs1245803, rs1245804, rs1245806, rs1245810, rs1245814, rs1245819, rs1245820, rs1268463, rs1245824, rs1312846, rs1245825, rs1245828, rs1245835, rs1245837, rs1245840, rs1245841, rs1245842, rs10778564, rs10778565, rs10861753, rs10861755, rs10861757, rs2141701, rs1921026, rs10861758, rs10861799, rs6539315, rs1245807, rs1245831, rs1245832, rs10861769, rs2293650, rs11113673, rs1465053, rs1245805, rs12578266, rs7296233, rs10746105, rs11609774, rs10746106, rs10746114, rs7967411
							rs6539344	Intron 1	No Veracode		rs1245829, rs7963801
							rs11113425	Intron 1			–
							rs2400393	Intron 1			rs11113410, rs10861762, rs11113435, rs10778573, rs10778575, rs7300645, rs2272500, rs10861795, rs10861796, rs10861797, rs6539373, rs4612885, rs1526952, rs10746103
							rs1732664	Intron 6			–
							rs10861941	Intron 6			–
							rs1245775	Intron 6	Failed		–
							rs6539445	Intron 6			rs11113980
							rs11114027	Intron 6			–
							rs7308297	Intron 6			rs1245765, rs1245778, rs1465051, rs2400395, rs9788036, rs7966962, rs1245769, rs1245766, rs10861976, rs10778657, rs10861975, rs1465044, rs1245767
							rs1465046	Intron 6			rs17005544, rs12581451, rs10506815, rs1405493, rs11608808, rs7303658, rs1465048, rs1465049, rs1405495, rs1526955, rs17005561, rs1358243, rs1405497, rs7315638, rs7295888, rs17005588, rs11612225, rs2701566, rs1465046
							rs17294719	Intron 6			rs7954927, rs1526963, rs17005594, rs11833031, rs11114107, rs7135647, rs11114122, rs7315113, rs11114116
							rs2251214	Intron 6			–

SYT2	NM_177402	1q32.1	114.7	9	85	23	rs10800856	5’	Failed	0.77	–
							rs12564274	5′			–
							rs12409197	Intron 1	Failed		rs3923371, rs4325195
							rs12141884	Intron 1			rs4364933
							rs4400672	Intron 1			rs4072140, rs10920457, rs11590011, rs11588808, rs4950738, rs4950788, rs4362037, rs11578029, rs12040487, rs12046408
							rs10920459	Intron 1	Failed		rs4418670, rs4572021
							rs6673562	Intron 1			rs6698441, rs11585345, rs6693751, rs4453099
							rs10800855	Intron 1			rs4950786, rs4950865, rs4113203, rs6661284, rs6662116, rs10920451, rs10920452, rs4950866, rs4950867, rs2095981, rs12038816, rs6696612, rs4372314, rs16850204
							rs7550433	Intron 1			rs867316, rs12047572, rs10920440, rs10920445, rs6669326, rs1417160, rs7517181, rs4950785, rs12728991, rs10800847
							rs10733069	Intron 1			rs867313, rs867314, rs867315, rs946857, rs12404969, rs10920442, rs4950863, rs4537626, rs10737582
							rs12121078	Intron 1			–
							rs11585565	Intron 1			–
							rs7534078	Intron 1			–
							rs1934663	Intron 1			–
							rs12739678	Intron 1			rs11585137, rs12739678, rs12354333
							rs10920427	Intron 1			–
							rs7552201	Intron 1			–
							rs578816	Intron 1	Failed		–
							rs12566757	Intron 1	No Veracode		–
							rs1968583*	Exon 2	Failed		–
							rs907697	Intron 7			rs4520471
							rs9633344	Intron 8			rs6427958, rs504261, rs10494834, rs6666325, rs907698, rs1467120, rs1040010
							rs6427957	3′			–
CPLX1	NM_006651	4p16.3	41.2	4	27	16	rs11248047	5′		0.81	rs936551
							rs7375209	Intron 2			rs9328757, rs6842106, rs2276906
							rs6832751	Intron 2			rs7377207
							rs7376690	Intron 2	Failed		–
							rs11248043	Intron 2			–
							rs11248042	Intron 2			–
							rs9328758	Intron 2			–
							rs7677766	Intron 2			rs10004482
							rs10004297	Intron 2	Failed		–
							rs11722977	Intron 2			–
							rs2306251	Intron 2			rs1052595

(Continued)

Table SI. *(Continued)*

Gene	Contig reference	Location	Length (kp)	N° of exons	SNPs	Tag SNPs	SNP ID	Location	Exclusion criteria	Gene Coverage[#]	Other SNPs within the bin
							rs3816676	Intron 3			rs3775140, rs6840352
							rs4690313	Intron 3			–
							rs2242237	Exon 4 3′UTR			–
							rs3733358	3′			rs17165034
							rs6811804	3′	Failed		rs6816868
CPLX2	NM_006650	5q35.2	87.41	5	62	27	rs2243404	Exon 1 5′UTR		0.85	rs2443541, rs2434237, rs890736, rs2382120, rs7727183
							rs10866688	Intron 2	Failed		–
							rs7718856	Intron 2			–
							rs6874025	Intron 2			rs17065511, rs883188
							rs12520557	Intron 2			rs2382123, rs688724, rs10475623
							rs11134932	Intron 2			rs2382121
							rs4242187	Intron 2	Failed		–
							rs10476170	Intron 2			–
							rs17065535	Intron 2			–
							rs6556225	Intron 2			–
							rs1560035	Intron 2			rs6887620
							rs10072860	Intron 2	No Veracode		–
							rs12188152	Intron 2	Failed		–
							rs4867806	Intron 2			rs4868537, rs1560036
							rs11134935	Intron 2			–
							rs4868538	Intron 2			rs10866690, rs4867807
							rs10866691	Intron 2			rs10076867, rs999065
							rs4868539	Intron 2			–
							rs890737	Intron 2			rs7705543, rs1820646, rs1366116, rs12522368
							rs11134938	Intron 2			rs6860661, rs871853, rs1560033, rs4867808, rs3892909, rs4077871, rs3180249, rs12656972
							rs1006101	Exon 5 3′UTR			rs4867809, rs9313730, rs3822674
							rs10866692	Exon 5 3′UTR			rs1560034
							rs930047	Exon 5 3′UTR			–
							rs13166213	3′			–
							rs11134942	3′			–
							rs2114968	3′			rs2382114, rs7718100
CPLX3	NM_001030005	15q24.1	5.19	3	4	2	rs7162232	5′		1	rs11634474, rs9210
							rs6495122	3′			–
CPLX4	NM_181654	18q21.32	23.23	3	27	9	rs609209[$]	Intron 1	No HWE	0.89	–
							rs10503024	Intron 2			–
							rs509886	Intron 2			rs561894, rs668992, rs654802
							rs640401	Intron 2			rs537048, rs536113, rs508858
							rs12232757	Intron 2			rs4940456
							rs12456930	Intron 2			–

							rs7228681	Intron 2			rs4940455, rs10871768, rs7243416, rs12232751, rs599535, rs583241, rs11662475, rs1980176, rs4108696
							rs499824	Exo 3 3′UTR			rs606952
							rs1914321	Exo 3 3′UTR			rs17696543
NSF	NM_006178	17q21.32	166.6	21		4	rs183211	Intron 13		1	rs199438, rs199536, rs199438, rs142167, rs199455, rs199454, rs199452, rs199449, rs199442, rs199536, rs199530, rs199529, rs199439, rs199456, rs199448, rs199445, rs199535, rs199534, rs199533, rs538628, rs199443
							rs17692129	Intron 15			–
							rs7224296	Intron 15			–
							rs17698176	Intron 17			–
NAPA	NM_003827	19q13.32	27.61	11	2	2	rs3786521	Intron 1	Failed	0	–
							rs830146	Intron 1	Failed		–
STXBP1	NM_003165	9q34.11	80.43	19	8	3	rs2039204	5’		1	rs1573178
							rs7852204	5’			rs4837172, rs10819303, rs3824523, rs4836575
							rs2241167	Intron 9			–
SNPH	NM_014723	20p13	43.01	6	22	12	rs11905792	Intron 2	No Veracode	0.64	–
							rs6109320	Intron 3			–
							rs2317645	Intron 4	Failed		rs3795141, rs3818331
							rs912105	Intron 4	No HWE		–
							rs7354385	Intron 5			rs6033370, rs6078505, rs7466
							rs3764715	Exon 6 3′UTR			rs966846, rs912108
							rs6134520	Exon 6 3′UTR			rs11449
							rs3803947	Exon 6 3′UTR	Failed		–
							rs4814106	3′			–
							rs1048621[$$]	3′	Monomorphic		–
							rs2281711	3′			rs2273959, rs2273958, rs3795137
RAB3A	NM_002866	19p13.2	7.2	5	10	5	rs874628			1	rs1044821
							rs2271881				–
							rs17683539				rs17683509, rs2049050
							rs2049051				–
							rs2271882				rs1667476, rs16982263
SYP	NM_003179	Xp11.23	12.4	7	1	1	rs5906754	5′		1	–
							rs2293945*	Intron 6			–

HWE Hardy-Weinberg equilibrium, MAF minimum allele frequency (in the control sample).

[$]The SNP rs609209 of the *CPLX4* gene was not included in the assay because was not in HWE as described by Corominas et al. (in preparation).

[$$]The SNP rs1048621 of the *SNPH* gene was not included in the assay because was monomorphic as described by Corominas et al. (in preparation).

*SNPs included in the assay not according to genetic coverage parameters.

**MAF: Minor allele frequency in the control sample.

Table SII. Nominal P-values observed when genotype frequencies of 121 SNPs within 15 candidate genes were considered in 360 cocaine dependent patients and 360 controls.

GENE	SNP	P-value
SYT2	rs6427957	0.15212
	rs9633344	0.46772
	rs907697	0.90325
	rs7552201	0.86890
	rs10920427	0.75248
	rs12739678	0.06504
	rs1934663	0.57648
	rs7534078	0.96311
	rs11585565	0.75220
	rs12121078	0.08003
	rs10733069	0.16372
	rs7550433	0.34683
	rs10800855	**0.01254**
	rs6673562	0.15972
	rs4400672	**0.04832**
	rs12141884	0.32292
	rs12564274	0.20735
VAMP1	rs12964	0.15651
	rs2534717	0.34739
	rs2240867	0.22587
	rs2072376	0.75251
	rs10492096	0.39987
SYT1	rs2177963	0.62265
	rs11113425	0.64354
	rs2400393	0.39748
	rs1732664	0.35062
	rs10861941	**0.02279**
	rs6539445	0.83461
	rs11114027	0.37482
	rs7308297	0.18092
	rs1465046	0.57448
	rs17294719	0.36636
	rs2251214	0.32359
CPLX3	rs7162232	0.67135
	rs6495122	0.60608
VAMP2	rs8067606	0.80588
NSF	rs183211	**0.00238**
	rs17692129	0.83791
	rs7224296	0.05305
	rs17698176	0.38155
CPLX4	rs1914321	0.02163
	rs499824	0.81498
	rs7228681	0.13420
	rs12456930	0.08485
	rs12232757	0.42805
	rs640401	**0.04187**
	rs509886	0.20299
	rs10503024	0.13737
RAB3A	rs874628	0.73628
	rs2271881	0.77492
	rs2271882	0.25798
	rs17683539	0.70461
	rs2049051	0.05203
SNPH	rs6109320	0.98512
	rs7354385	0.58227
	rs3764715	0.23104
	rs6134520	0.32681
	rs4814106	0.25008
	rs2281711	0.97015
SNAP25	rs1889189	0.15589
	rs6039769	0.15654
	rs363039	0.38271
	rs363043	0.87723
	rs12626080	0.54758
	rs6074113	0.32190
	rs362547	0.16478
	rs362570	0.28418
	rs6039806	0.05962
	rs3025873	0.75712
	rs362988	0.78774
	rs6108464	0.35760
	rs3787283	0.33002
	rs4813925	0.57665
	rs6074121	0.45345
	rs6032845	0.67322
	rs3025879	0.24403
	rs6032846	0.14444
CPLX1	rs3733358	0.30114
	rs2242237	0.46714
	rs4690313	0.11870
	rs3816676	0.50524
	rs2306251	0.69868
	rs11722977	0.00644
	rs7677766	0.75547
	rs9328758	0.97542
	rs11248042	0.17946
	rs11248043	0.16668
	rs6832751	0.65893
	rs7375209	0.33853
	rs11248047	0.93505
CPLX2	rs2243404	0.76688
	rs7718856	0.07444
	rs6874025	0.72218
	rs12520557	0.76957
	rs11134932	0.92888
	rs10476170	0.53281
	rs17065535	0.11211
	rs6556225	0.36353
	rs1560035	0.47697
	rs4867806	0.65625
	rs11134935	0.49431
	rs4868538	0.16514
	rs10866691	0.94357
	rs4868539	**0.02956**
	rs890737	0.90204
	rs11134938	0.57680
	rs1006101	0.88312
	rs10866692	0.99481
	rs930047	0.51534
	rs13166213	0.58418
	rs11134942	0.27905
	rs2114968	0.45165
STX1A	rs4363087	0.90163
	rs3793243	0.86838

(Continued)

Table SII. *(Continued)*

GENE	SNP	P-value
	rs2293485	0.66049
	rs941298	0.76395
STXBP1	rs2039204	0.71640
	rs7852204	0.84553
	rs2241167	0.62985
SYP	rs2293945	0.67915
	rs5906754	0.94017

In bold, nominally significant p-values.