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Peripheral blood biomarkers in Psychiatric Diseases

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PERIPHERAL BLOOD BIOMARKERS IN PSYCHIATRIC DISEASES

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PERIPHERAL BLOOD BIOMARKERS IN PSYCHIATRYC DISEASES

MÒNICA SEGURA CASTELL

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Memòria de la tesi doctoral presentada per Mònica Segura Castell per optar al títol de Doctora per la Universitat Internacional de Catalunya.

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A vatros,

Mama, Papa i Antonio

La verdadera ciència ensenya, sobretot, a dubtar i ser ignorant

La verdadera ciencia enseña, sobre todo, a dudar y a ser ignorante

True science teaches, above all, to doubt and be ignorant

(Miguel de Unamuno)

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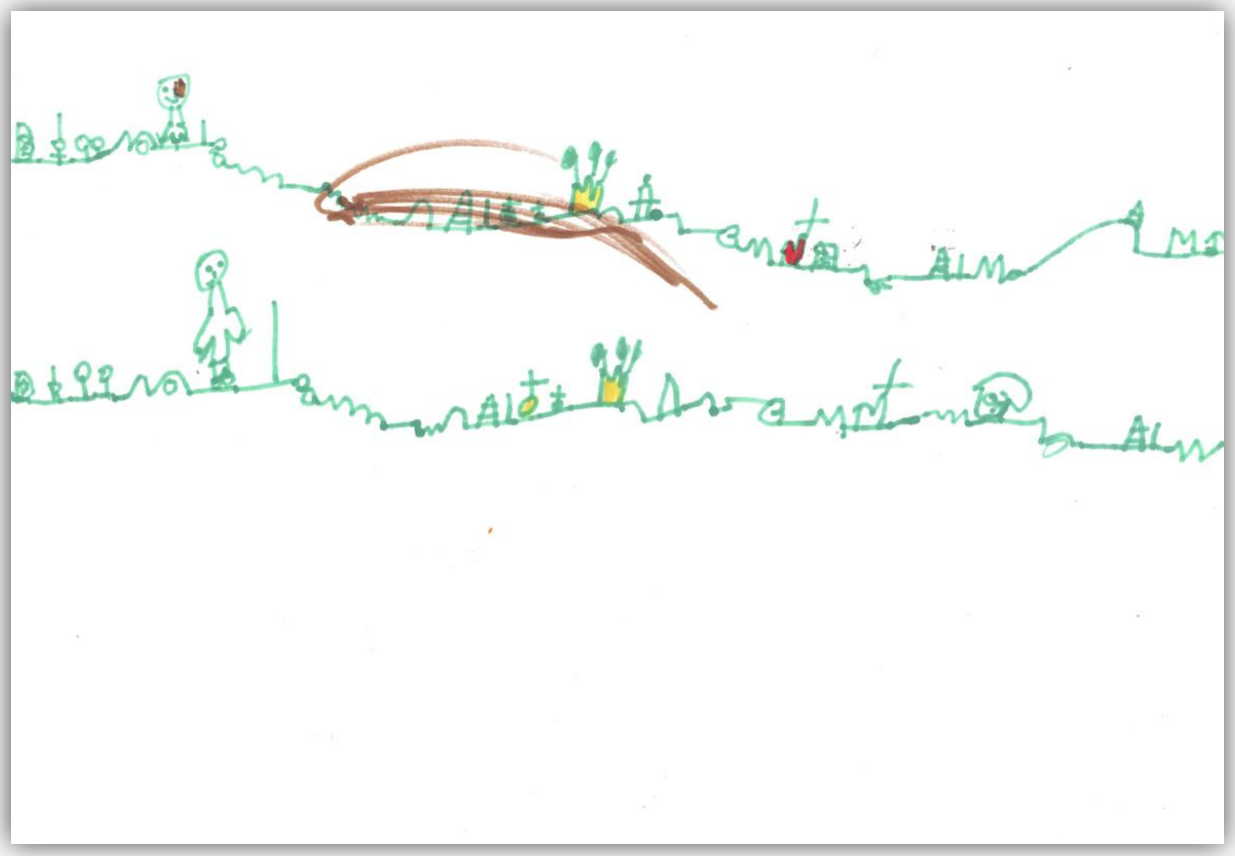

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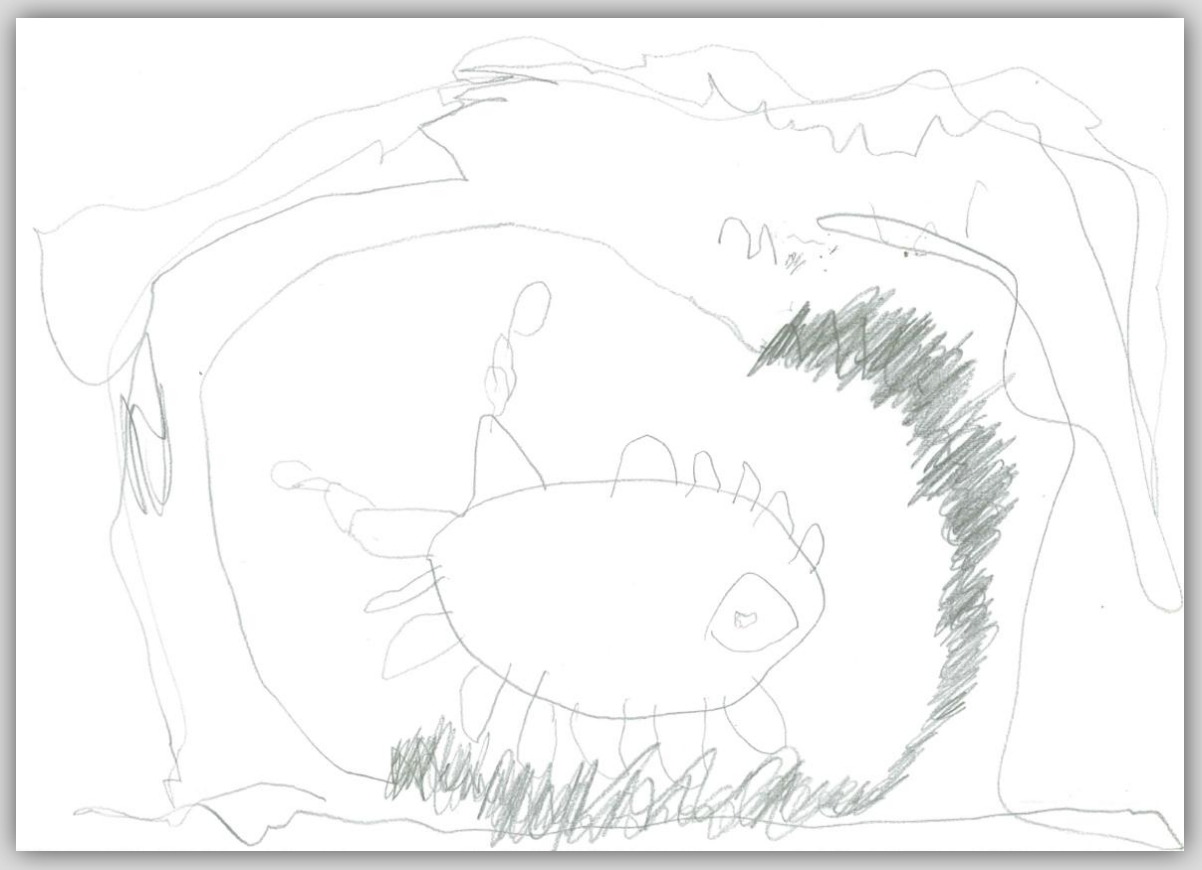
A	**A**denine
ACTB	**Act**in β
ADHD	**A**ttention **D**eficit **H**yperactivity **D**isorder
ADI-R	**A**utism **D**iagnostic **I**nterview-**R**evised
ANK	**A**nkyrin
ADOS	**A**utism **D**iagnostic **O**bservation **S**chedule
ANK3	**Ank**yrin-**3**
AS	**A**sperger **S**yndrome
ASD	**A**utism **S**pectrum **D**isorders
ASD-MR	**A**utism **S**pectrum **D**isorders-**M**ental **R**etardation
BC	**B**efore **C**rist
BDWG	**B**iomarkers **D**efinition **W**orking **G**roup
BDNF	**B**rain-**D**erived **N**eurotrophic **F**actor
BM	**Bio**marker
CDD	**C**hildhood **D**isintegrative **D**isorder
CIDI	**C**omposite **I**nternational **D**iagnostic **I**nterview
CNS	**C**entral **N**ervous **S**ystem
DALY	**D**isability-**A**djusted **L**ife-**Y**ears
DNA	**D**eoxyribo**n**ucleic **a**cid
DSM-IV	**D**iagnostic and **S**tatistical **M**anual, **4**th edition
EPHA	**Eph**rin-receptor-**A**
EPHA4	**Eph**rin-receptor-**A4**
EPHB	**Eph**rin-receptor-**B**
G	**G**uanine
GPCRs	**G p**rotein-**c**oupled **r**eceptors
GWA	**G**enome **W**ide **A**ssociation
HFA	**H**igh **F**unctioning **A**utism
HWE	**H**ardy **W**einberg **E**quilibrium
ICD10	**I**nternational **C**lassification of **D**iseases, **10**th revision
Ig	**I**mmuno**g**lobulin
LFA	**L**ow **F**unctioning **A**utism
LPHN	**L**atro**ph**ili**n**
LPHN1	**L**atro**ph**ili**n**-**1**
LPHN2	**L**atro**ph**ili**n**-**2**
LPHN3	**L**atro**ph**ili**n**-**3**
Met	**Met**ionine
MAF	**M**inor **A**llele **F**requency
NGF	**N**erve **G**rowth **F**actor
NS	**N**ervous **S**ystem
NT-3	**N**euro**t**rophin-**3**
NT-4/5	**N**euro**t**rophin-**4/5**
p75^{NTR}	**p75 N**eurotrophic **R**eceptor
PANSS	**P**ositive **A**nd **N**egative **S**yndrome **S**cale
PBMC	**P**eripheral **B**lood **M**ononuclear **C**ell
PDD	**P**ervasive **D**evelopmental **D**isorders
PDD-NOS	**P**ervasive **D**evelopmental **D**isorders-**N**ot **O**therwise **S**pecified
PNS	**P**eripheral **N**ervous **S**ystem
RFLP-PCR	**R**estriction **F**ragment **L**ength-**P**olymorphism-**P**olymerase **C**hain **R**eaction
RNA	**R**ibo**n**ucleic **a**cid
RRN18S	**18S r**ibosomal **RN**A
SNP	**S**ingle **N**ucleotide **P**olymorphism
TMR	**T**rans**m**embrane
TrkA	**T**yrosine **K**inase receptor **A**
TrkB	**T**y**r**osine **K**inase receptor **B**
TrkC	**T**y**r**osine **K**inase receptor **C**
Val	**V**aline
WHO	**W**orld **H**ealth **O**rganization



II. *RESUM EN CATALÀ*

A. HIPÒTESI I OBJECTIUS DE LA TESI

Els desordres psiquiàtrics com ara els trastorns de l'espectre autista (TEA), la esquizofrènia o els desordres bipolars són patologies severes i inhabilitants, essent en molts casos la major causa de morbiditat en nens i adolescents. A diferencia de la clàssica herència mendeliana, els desordres mentals es consideren complexos, pel seu caràcter poligenètic i sovint desconegut, a nivell dels mecanismes patològics i les vies biològiques que els desencadenen. En la majoria dels casos, es tracta d'una interacció entre múltiples gens que, modulats per diferents factors ambientals, desencadenaran un fenotip psiquiàtric. Així doncs, és ben sabut que es tracta d'unes patologies fenotípicament i etiològicament heterogènies. Les fronteres entre les diferents desordres mentals romanen poc delimitadess, lo que fa evident una urgent increment de la recerca i el coneixement sobre aquestes.

Des de fa temps es coneixen alteracions arquitectòniques en el sitema nerviós dels pacients psiquiàtrics, encara que els ponts d'unió entre la distribució anatòmica i els processos moleculars que la desencadenen romanen desconeguts. Així, atenent a aquestes evidencies morfològiques, el treball realitzat es basarà en la teoria del neurodesenvolupament com a causa de les patologies psiquiàtriques, de maenra que les molècules estudiades estan relacionades amb processos que es desencadenen durant el desenvolupament del sistema nerviós (tant a nivell prenatal com postnatal).

Els estudis es realitzaran a partir de mostres de sang perifèrica, amb la finalitat de trobar marcadors biològics en mostres fàcils d'obtenir i que, per tant, puguin resultar eines útils per milloar el diagnòstic i la monitorització dels pacients.

L'oobjectiu principal de l'estudi és trobar marcadors biològics potents en sang perifèrica de pacients psiquiàtrics i, per tant, contribuir a la millora del diagnòstic i les teràpies personalitzades. Per dur a terme aquest objectiu principal, es varen marcar els següents objectius específics:

Capítol 1
Investigar el paper de les neurotrofines i els seus receptors en els trastorns de l'espectre autista.

Capítol 2
Investigar l'associació del polimorfisme d'un sol nucleòtid (SNP) rs4680079 de la Latrofilina-3 en els trastorns de l'espectre autista.

Capítol 3
Investigar l'associació de nou SNPs (rs2952834, rs2392936, rs2248489, rs2056290, rs2052940, rs1864461, rs1897120, rs3087584, rs3770181) del receptor EPH A4 en l'esquizofrènia i els desordres bipolars.

Capítol 4
Confirmar l'associació de tres SNPs (rs9804190, rs10761482) de la Ankirina-3 en esquizofrènia i desordres bipolars.

B. INTRODUCCIÓ

L'Organitzacio Mundial de la Salut (OMS) estimà l'any 2008 que aproximadament un 13% de la càrrega global de malalties eren desordres neuropsiquiàtrics (World Health Organization, 2008). Tot i que l'extensió d'aquesta càrrega varia entre països, és cert que en tots hi ocupen un lloc important, doncs aproximadament 450 milions de la població total pateix algun tipus de patologia mental o neurològica; així com també de problemes psicosocials com ara l'alcohol o l'abús de drogues (World Health Organization, 2001). L' afectació és igual en dones que en homes, excepte an casos com els desordres relacionats en l'alcohol o l'abús de substancies on la prevalença és més elevada en homes, o en els desordres depressius que més freqüents en dones (World Health Organization, 2001). A més, s'ha vist que les patologies mentals són força comuns en persones afectades per algun altre tipus de patologia física, com ara en l'infart de miocardi amb un 22%, en la diabetis mellitus amb un 27% i en un 33% de les persones afectades per algun tipus de càncers (World Health Organization, 2003).

A nivell europeu, s'estima que uness 100 milions de persones d'uns 870 milions totals pateixen ansietat i depressió, 21 milions de persones pateixen desordres pel consum d'alcohol, més de 7 milions de persones pateixen Alzheimer i altres demències i, aproximadament uns 4 milions de persones pateixen esquizofrènia i desordres bipolars o del pànic (World Healt Organization, 2005). També és important destacar que els pacients psiquiàtrics són aproximadament el 24 % de les persones ateses per l'atenció primària (Goldberg DP & Lecrubier Y, 1995) i que representen un 19.5% de les causes de discapacitat (Murray CJ & Lopez AD, 1997); es preveu un augment del 40% en l'inidència d'aquestes discapacitats entre els anys 1990 i 2020.

Els costos econòmics i socials de les patologies mentals per a la societat són elevats, estimant que a Europa costa un 3-4% del producte nacional brut (Gabriel P & Liimatainen MR, 2000). La mitjana de despeses per càpita és 1.63 $, però amb una àmplia variació entre regions, essent el rang entre 0.20 $ en els països més pobres i arribant a uns 44.84 $ en els països més rics (World Health Organization, 2011). A aquestes despeses s'hi ha de sumar els costos dels serveis socials, la poca ocupació laboral i la baixa productivitat, com ara un 35-40% d'absentisme laboral (World Organization, 2001). En els països europeus és disposen de pocs recursos, comptant amb pressupostos només d'un 5.8% de mitjana del total de les despeses (World Health Organization, 2005). Als Estats Units la situació és molt semblant, disposant d'un 7% del total de les despeses en salut, per les patologies mentals i amb un 2% del producte nacional brut (The-wei H, 2004).

B.1.DESORDRES NEUROPSIQUIÀTRICS

Ja des de l'antiga Grècia, al segle IV a.C. han anat sorgint i adaptant teories sobre les patologies psiquiàtriques. Tot començà amb Hippocrates, qui postulà el primer sistema de classificació (Parke-Davis AP, 1966), més tard Rhazas establí la primera secció especialitzada en psiquiatria a l'Hospital de Bagdad, i on aplicava una psicoteràpia primitiva (Alexander FG & Selensnik ST, 1996). No fou fins passada la 2a Guerra Mundial que es desenvolupà el primer instrument diagnòstic: l'Entrevista de Diagnostic Internacional Composta (CIDI – Composite International Diagnostic Interview-) (World Health Organization, 2007). Arrel d'aquesta, sorgiren els dos manuals de diagnòstic emprats internacionalment: ICD-10 (Classificació Internacional de les Malalties, 10a revisió) i el DSM-IV (Manual de Diagnòstic i Estadística, 4a revisió) (Kessler RC & Ustun TB, 2004).

Són molts els avenços que s'han fet per entendre la patofisiologia dels desordres psiquiàtrics i neurològics, tot i així són molts els buits que hi ha en el coneixement d'aquestes. Un dels majors problemes amb el que es conviu, és el fet que el diagnòstic és basa majoritàriament en la categorització de senyals i símptomes; aixi,

l'actual classificació, en realitat, limita la capacitat d'identificar la causa biològica i el desenvolupament de tractaments específics (Turck CW et al., 2005). Tant el ICD-10 com el DSM-IV identifiquen diferents patologies mentals d'acord a un llistat de símptomes però no amb la causa subjacent de la patologia.

Trastorns de l'espectre autista

Els trastorns de l'espectre autista responen a unes condicions de neurodesenvolupament complexes, caracteritzades per tres blocs: deficiència en la comunicació, interaccions socials recíproques alterades i restringides i, finalment comportaments i interessos estereotipats i repetitius. Aquestes mancances varien tant en rang com en severitat. A més, s'ha vist que, el diagnòstic precoç i la conseqüent intervenció ràpida és força important, millorant d'aquesta manera l'efectivitat i disminuint l'impacte en la família (Matson JI et al., 2007). Les eines de diagnòstic emprades són: entrevista diagnòstica de l'autisme-revidasa (ADI-R) i programa d'observació de diagnòstic de l'autisme (ADOS) realitzades a pacient i familiars respectivament, que es basen en interpretacions logarítmiques dels manuals de diagnòstic ICD-10 i DSM-IV (Le Couter A et al., 2003; Lord C et al., 1994). Estudis epidemiològics recents indiquen que una de cada 100 persones pateixen algun tipus de TEA (Kim YS et al., 2011). L'afectació és desigual entre sexes, essent el ratio de 4 home per 1 dona (Gillbert C & Wing L, 1999).

Esquizofrènia

L'esquizofrènia és un desordre psiquiàtric devastador caracteritzat pels anomenats símptomes positius (al·lucinacions i il·lusions) i negatius (aïllament social, emocions reduïdes, dèficits cognitius, disminució en l'execució de funcions, déficits en l'atenció i la memòria de treball (American Psychiatric Association, 1994). La patologia s'inicia en l'adolescència o en els inicis l'edat adulta (Lieberman JA et al., 2001; Perkins DO et al., 2005). El diagnòstic es basa en l'observació del personal psiquiàtric professional que, d'acord amb les pautes marcades en els manuals ICD-10 i DSM-IV decidirà la resolució diagnòstica (American Psychiatric Association, 1994). Estudis epidemiològics descriuen una incidència de 15.2 per 100000 persones (McGrath J et al., 2004), que és 1.4 vegades major en homes que en dones, i un 4.6 vegades major en immigrants respecte els que no ho són (Saba G et al., 2006).

Desordres bipolars

Els desordres bipolars són uns dels desordres psiquiàtrics més importants, i s'han descrit en moltes cultures al llarg del temps (Goodwin FK, 1990). També es coneixen com patologia maniaco-depressiva (Craddock N & Jones I, 1999). Es caracteritzen per canvis d'humor amb fases recurrents de mania, provocant un aïllament social (Nakatani N et al., 2006). Els símptomes i la severitat varia notablement, arribant a l'hospitalització en els casos més severs. El diagnòstic també es dona d'acord als manuals internacionals ICD-10 i DSM-IV, els quals marquen com a requisit que la persona pateixi un o mes episodis de mania, amb o sense episodis de depressió durant el temps d'història clínica (Craddock N & Jones I, 1999). Estudis epidemiològics estimen una prevalença d'entre 0.2% - 2% per la manifestació més severa de la patologia, amb depressió (Goodwin FK, 2007).

B.2. ETIOLOGIA DELS DESORDRES PSIQUIÀTRICS: ÉS L'ALTERACIÓ DEL NEURODESENVOLUPAMENT LA CAUSA D'AQUESTES PATOLOGIES?

El desenvolupament estructural, funcional i de comportament del sistema nerviós sorgeix d'un estret diàleg entre l'herència genètica i els factors ambientals. Entendre com aquests factors interaccionen en diferents estadis durant el desenvolupament, pot ajudar a identificar com i quan intervenir en les patologies del neurodesenvolupament (Lenroot RK & Giedd JN, 2008). D'altra banda, la recerca dels desordres del neurodesenvolupament complexos com l'esquizofrenia i l'autisme ha demostrat que determinats símptomes clínics poden estar associats a aquesta varietat de factors genètics de risc, suggerint que poden haver múltiples rutes provinents d'un mateix comportament (Merikangas AK et al., 2009; Abrahams BS & Geschwind DH, 2008; Owen MJ et al., 2005; Samaco RC et al., 2005; Walsh P et al., 20011). Així doncs, els desordres del neurodesenvolupament estarien causats per un ampli rang de mutacions genètiques modulades per factors ambientals, com per exemple infeccions, disfuncions immunològiques, intoxicacions, disfuncions metabòliques i endocrines, factors nutricionals, traumes, etc. A més, l'heretabilitat estimada indica que hi ha un gran component genètic alhora de desenvolupar aquestes patologies (Ehninger D et al., 2008).

Pel que fa a l'esquizofrènia i l'autisme, són dues entitats diferenciades en quant a simptomatologia, edat de desenvolupament i curs de la patología; tot i això, ambdues comparteixen disminucions en el funcionament social (American Psychiatric Association, 1994) que estaria relacionada en les disminucions de la cognició social (Hughes C et al., 1997; Klin A et al., 2002; Pinkham AE et al., 2003). Estudis recents demostren que les disfuncions cognitives estarien causades per la disfunció dels sistemes neuronals (Pinkham AE et al., 2008). Tot i així, el coneixement sobre aquests dèficits i la potencial semblança entre aquests desordres romanen incomplets, ja que els processos genètics i de desenvolupament que organitzen les xarxes neuronals estan encara per determinar (Courchesne E et al., 2007).

Pel que fa a la etiologia dels TEA cal destacar la important càrrega genética, evidenciada per una concordança d'entre un 60% i un 90% en bessons monozigots. Encara així, és necessària la contribució de factors ambientals, tant a nivell prenatal com postnatal, jugant un paper important en la modulació d'aquets substrat genètic, portant a desencadenar anomalies en l'organització neuronal (Pardo CA & Eberhart CG, 2007).

En quant a l'etiologia de l'esquizofrènia, aquesta també roman desconeguda, encara que gràcies a estudis de bessons, familiars i d'adopció s'ha vist que tant important és la genètica com els factors ambientals (Gottesman II, 1991, però en aquest cas, els factors ambientals estan més descrits que en l'autisme. S'ha vist que poden contribuir al desenvolupament de la patologia: les complicacions obstètriques (Clarke MC et al., 2006), dèficits en la nutrició prenatal (Ludvigsson JF et al, 2007), infeccions maternes (Buka SL et al., 2001), traumes infantils (Morgan C & Fisher H, 2007), interaccions familiars (McGuffin P, 2004), sensibilitat al gluten (Kalaydijan AE et al., 2006) i l'ús de cànnabis (Arseneault L et al., 2002; Dean K & Murray RM, 2005).

B.3. POTENCIALS BIOMARCADORS PER LES PATOLOGIES PSIQUÀTRIQUES

S'han realitzat importants avenços amb l'objectiu d'entendre la patofisiologia de les patologies mentals, tot i així encara hi ha mancances importants en aquest coneixement. L'objectiu preferent és la identificació de marcadors que permetin categoritzar els pacients en subgrups d'una manera consistent (Turck CW et al., 2005). Així, tal i com varen postular Schwarz i Bahn en el seu moment, només el coneixement dels mecanismes

patològics facilitaran el descobriment de biomarcadors (BM) per fer un diagnòstic objecti i permetre la identificació de la susceptibilitat individual, la predicció de la resposta a tractaments i la revolucionaria recerca de noves aproximacions terapèutiques (Schwarz E and Bahn S, 2008). Atenent això, es defineix com a BM "una característica que és objectivament mesurada i avaluada com a indicador de processos biològics normals, processos patològics, o respostes farmacològiques a una intervenció terapèutica" (Biomarkers Definitons Working Group, 2001). A més, els BM haurien de ser fàcilment mesurables, precisos i econòmics. També, haurien de tenir potència predictiva, estant disponibles per assajos rutinaris i permetent la identificació del risc individual (Stöber G et al., 2009).

Gottesman II va dir que donada l'elevada complexitat dels desordres psiquiàtrics, els quals es caracteritzen per ser multifactorials i poligenètics, el cervell és el més complex de tots els òrgans (Gottesman II & Gould TD, 2003). Però és evident la dificultat d'obtenir mostres representatives de pacients per realitzar els estudis (Sullivan PF et al., 2006). Per tant, és interessant trobar teixits on els patrons d'expressió gènica siguin raonablement correlacionats amb els de l'expressió del sistema nerviós central, resultant un gran avenç per la recerca dels mecanismes patològics de les malalties mentals. El grup de Sullivan i companys va demostrar que patrons d'expressió del sistema nerviós, eren equitatius als patrons d'expressió que es trobaven en sang perifèrica (Sullivan PF et al., 2006).

Després de tot això, és evident que l'objectiu preferencial de trobar BM és la detecció de molècules que és correlacionin amb les patologies, per tal d'emprar-los en un diagnòstic objectiu. El primer pas per fer la recerca d'aquests és l'adquisició d'un nombre raonable de mostres, així com el processament i emmagatzematge amb la màxima qualitat possible (Akbarian S et al., 1996). Com a curiositat dir que avui dia hi ha de l'ordre de 10 bancs de cervell, encaminats a reunir gran nombre de mostres psiquiàtriques (Bunney WE et al., 2003). És imprescindible un protocol estandarditzat entre els biobancs i els col·lectors de les mostres, així com l'acompanyament de la mostra amb informació extensament detallada sobre la seva procedència (Bunney WE et al., 2003). Un cop es te la mostra, hi ha diferents estratègies a seguir per la recerca d'aquests nous marcadors biològics, per això és essencial fer un rigorós disseny de l'estudi que és vol portar a terme. Un cop s'ha identificat un possible gen candidat per una patologia en concret, pot ser que aquest ja sigui conegut per la comunitat científica, però pot donar-se el cas de trobar algun gen desconegut fins al moment. Donant-se aquest fet, un estudi molecular del gen en qüestió seria necessari. També serien importants els estudis encaminats a conèixer la seqüencia gènica d'aquesta molècula, ja que possibles canvis en una sola base podrien ser la causa del mal funcionament del mateix (Pennacchio LA et al., 2001). Els estudis més populars actualment per la recerca de nous biomarcadors són els cas-control, ja que han permès testar múltiples hipòtesis sobre el paper que juga una determinada molècula en una patologia concreta (Bunney WE et al., 2003).

Un factor limitant alhora de fer l'estudi és el temps disponible per estudiar un gran nombre de gens a la vegada, ja que ha quedat evidenciat que les patologies psiquiàtriques estan causades per un conjunt d'anomalies genètiques (Sawa A & Snyder SH, 2002),. Els recents avenços tecnològics estan permetent l'estudi de milers de mostres a la vegada (Shoemaker DD & Linsley PS, 2002), determinant patrons característics d'expressió de mRNA, proteïnes o metabòlits. Aquesta tipologia d'estudis "omics" està fent una gran aportació al coneixement sobre possibles marcadors biològics en les patologies psiquiàtriques i per tant, serien les direccions a seguir en un futur. També l'estudi de l'epigenètica és força comú en aquests últims temps, ja que s'ha vist que canvis no codificats en el DNA, podrien influir en el desenvolupament de les patologies mentals (Szyf M et al., 2007).

C. CAPÍTOL 1. PAPER DE LES NEUROTROFINES I ELS SEUS RECEPTORS EN ELS TEA

Tal i com s'ha comentat en l'apartat anterior, l'etiopatologia dels TEA és poc coneguda, tot i això, hi ha evidències científiques per referir-se a ells com a desordres del neurodesenvolupament. Aquests evidències científiques de les quals es parla, són associacions entre la malaltia i alteracions en el desenvolupament de les persones que la pateixen (Belmonte MK et al., 2004; Polleux F & Lauder JM, 2004). És per això que les neurotrofines són uns bons candidats per veure's involucrats en els TEA; doncs les seves funcions principals recauen en la guia del desenvolupament i l'organització cortical del sistema nerviós central. A més, estudis previs han mostrat anomalies en els patrons d'expressió d'aquestes molècules en autistes (Pardo CA & Eberhart CG, 2007).

La família de les neurotrofines està formada bàsicament per 4 membres: factor neurotròfic derivat del cervell (BDNF), factor de creixement del nervi (NGF), neurotrofina-3 (NT-3) i neurotrofina-4/5 (NT-4/5). Tot i així, altres membres com la neurotrofina-6 (NT-6) i la neurorofina-7 (NT-7), han estat descrits gràcies a la clonació en teleostis, però no en altres vertebrats (Götz R et al., 1994; Lai KO et al., 1998). L' estructura de totes elles està força conservada, a excepció de la NT-4/5 que només comparteix un 50% d'homologia de la seqüència proteica (Hallböök F, 1999; Shooter EM, 2001). D'aquesta estructura tant conservada és important destacar la presència de 6 residus cisteïna localitzats en les mateixes posicions, i que per tant proporcionen una estructura terciària semblant en totes elles. Les neurotrofines són sintetitzades com a prepro-neurotrofines, tant en cèl·lules neuronals com en d'altres (Thoenen H, 1995; Seidah NG et al., 1996). Durant el seu processament intracel·lular, el domini pre- és eliminat quedant-se llavors, com a pro-neurotrofina. Una vegada es troba en la seva forma pro-, la proteïna pot seguir essent processada i eliminant aquest domini, o per contra, quedar-se com a pro-neurotrofina (Suter U et al., 1991; Kolbeck R et al., 1994; Ibanez CF, 2002). Ambdues estructures són funcionals, i s'ha descrit que tindrien funcions oposades, conseqüència de la unió preferent a diferents receptors.

Les neurotrofines es poden unir a tres tipus de receptors diferents: receptors tirosina quinasa –Trk- (TrkA, TrkB i TrkC), receptor de les neurotrofines p75 ($p75^{NTR}$) i a la sortilin. Les formes madures de les neurotrofines i que, per tant, han eliminat el domini pro, s'uneixen preferencialment als receptors del tipus Trk. Per contra, les pro-neurotrofies s'unirien preferencialment al receptor $p75^{NTR}$, que utilitzant com a co-receptor la sortilin (Lee R et al., 2001; Nykjaer A et al., 2004; Teng HK et al., 2003). El NGF s'uneix preferencialment a TrkA, el BDNF i la NT-4/5 s'uniria al TrkB i, la NT-3 s'uniria al TrkC.

El BDNF és la neurotrofina més àmpliament distribuïda pel sistema nerviós central, és per això que ha estat més estudiada en el present estudi. Doncs es sap que BDNF juga un paper important en la mediació de processos tals com l'aprenentatge i la memòria (Korte M et al., 1995; Patterson S et al., 1996, Desai NS et al., 1999). També s'ha vist relacionada amb la susceptibilitat a desencadenar dèficits cognitius, tant característics dels TEA; aquest fet suggeriria que variacions en aquest gen, juguessin un paper important en el desenvolupament dels desordres neuropsiquiàtrics, on hi ha una alteració del funcionament del sistema nerviós.

En el present estudi, els pacients autistes es varen separar en grups d'acord amb el seu coeficient intel·lectual (IQ), en autistes de baix funcionament o LFA (IQ < 70) i autistes d'alt funcionament o HFA (IQ > 70). Primer que tot, es va realitzar el genotipat de la variació genètica val66met, també coneguda com rs6265, en pacients diagnosticats com a TEA. Així doncs, Els resultats no mostraren una associació entre el polimorfisme i els

subgrups d'autisme. A més, es va voler analitzar si l'expressió del mRNA del gen BDNF era genotip-dependent. Segons els resultats, els nivells d'expressió de mRNA no mostraven diferències entre els diferents genotips presentats pels individus.El polimorfisme val66met ha estat prèviament associat amb desordres obsessivo-compulsius (Hall D et al., 2003), trastorns d'hiperactivitat (TDH) (Kent L et al., 2005) i trastorns d'ansietat (Lang UE et al., 2005). S'ha postulat que aquest polimorfisme juga un paper important en l'hipocamp i el còrtex prefrontal, regions cerebrals importants per les funcions de memòria i aprenentatge en humans (Egan MF et al., 2003; Hariri AR et al., 2003). També s'han descrit alteracions corticals a nivell de l'àrea de superfície (Raznahan A et al., 2009). Per contra, els resultats del present estudi no mostres associació entre val66met i el autisme. Doncs l'estudi es veu limitat per l'heterogeneïtat i el nombre de mostres de les diferents cohorts de TEA. És per això que seria important incrementar el nombre de pacients en cadascun dels subgrups delimitats.

D'altra banda, es va examinar l'expressió de mRNA tant de les neurotrofines com dels seus receptor, ja que està descrit que un increment de l'expressió de BDNF podria perjudicar el desenvolupament normal del sistema nerviós.Doncs donant suport a la hipòtesi que postula que el BDNF és un factor decisiu en el desenvolupament, Nelson i col·laboradors varen observar nivells de mRNA de BDNF elevats en mostres de sang neonatal arxivada, provinent de pacients autistes, comparat amb controls normals (Nelson KB et al., 2001). Aquestes observacions, varen reafirmar-se per estudis tals com els que descrivien un augment de la concentració de BDNF en encèfal (Perry EK et al., 2001) i en mostres de sèrum de pacients TEA (Connolly AM et al., 2006; Miyazaki F et al., 2004). Seguint aquest mateix camí, alteracions en l'expressió de les neurotrofines es varen descriure en altres desordres psiquiàtrics. Otsuki K i col·laboradors, varen observar alteracions en l'expressió de les neurotrofines de pacients afectats per depressió major, concretament varen trobar nivells reduïts de mRNA en les cèl·lules blanques de la sang.

Per tant, en concordança en tots aquests estudis descrits, els resultats del present estudi mostraven alteracions en l'expressió d'algunes de les neurotrofines i els seus receptors en els pacients autistes. Concretament, es van veure alterats l'expressió de BDNF, NT-3 i NT-4 en els pacients del grupLFA, comparats amb els controls. Aquests resultats suggereixen que els nivells d'expressió de mRNA de les neurorofines poden ser dependents del nivell de funcionament de l'individu. En detall es va veure que els pacients LFA tenien un increment dels nivells de mRNA de BDNF en el plasma, la qual cosa donaria suport als estudis prèviament descrits. Per contra, el mRNA de NT-3 i NT-4/5 es trobaria en menor representació, suggerint un paper compensatori entre les neurotrofines, tal i com ha estat descrit en altres estudis de malalties no psiquiàtriques (Kyin R et al., 2001).

A més, els nivells de mRNA de $p75^{NTR}$ s'observaren reduïts en els pacients LFA respecte els controls. La principal funció d'aquest receptor, quan interacciona amb les pro-neurotrofines, és l'activació de vies apoptòtiques (Cassaccia-Bonnefil P et al., 1996; Coulson EJ et al., 1999; Frade JM et al., 1996; Friedman WJ, 2000; Beattie et al., 2002; Lee et al., 2001; Volosin et al., 2006). Així doncs, i basant-nos en aquesta informació, els resultats suggeririen que els nivells baixos d'expressió de $p75^{NTR}$ portar a una disminució de la mort cel·lular programada, procés important per un correcte desenvolupament (Graaf-Peters VB & Hadders-Algra M, 2006). Finalment, nivells elevats de l'expressió de TrkA varen ser observats en pacients LFA. És ben sabut que les vies activades per la interacció neurotrofina-receptor Trk estan involucrades en supervivència, així com també en la diferenciació i el creixement cel·lular (Friedman WJ & Greene LA, 1999; Kaplan DR & Miller FD, 2000; Patapoutina A & Reichardt LF, 2001). Això, podria portar a pensar que, un increment de l'expressió d'aquests receptors resultaria beneficiós per la cèl·lula i, en conseqüència, per un millor desenvolupament del sistema nerviós. Per contra, un desenvolupament correcte consisteix en un conjunt de processos que succeeixen de manera ordenada i en un determinat moment (Graaf-Peters VB & Hadders-Algra M, 2006). Així, considerant

aquest fet, es podria postular que un increment dels nivells dels receptors Trk, en aquest cas TrkA, causarien alteracions en aquest procés tant precís com és el desenvolupament dels sistema neuronal i, per tant causaria anomalies arquitectòniques tals com les observades en la patologia dels TEA.

D'altra banda, aquest mateix anàlisi es va fer en mostres de pacients adolescent i adults. En aquest cas, no es varen trobar diferències entre el grup d'adolescents i adults respecte els controls. Aquest fet podria explicar-se perquè els pacients d'aquest estudi eren tots HFA i, tal i com s'ha pogut observar anteriorment, l'expressió de les neurotrofines és probablement depenent dels nivells de funcionament.

Per acabar, es va marcar com objectiu veure si aquestes alteracions del mRNA es veien reflectides en els nivells de proteïna. Cal destacar, per tal de fer una correcta interpretació dels resultats obtinguts, que la majoria de mostres incloses en l'estudi provenien del grup dels pacients adolescents i adults. Així, els resultats obtinguts en l'anàlisi dels nivells de proteinai donarien suport als resultats observats en l'expressió del mRNA prèviament descrit, ja que no hi havia alteracions significatives en les mostres de plasma, de cap de les neurotrofines. A més, si els perfils d'expressió es miren amb detall, es pot observar com els nivells de proteïna segueixen la mateixa tendència que l'expressió de mRNA. També, es varen estudiar els nivells de proteïna de BDNF i TrkB en cèl·lules de sang perifèrica polimorfonucleades (PBMC) i, sorprenentment, en comparar-los amb els nivells observats en plasma, si que s'observaven diferències. Doncs els nivells proteïna de la forma preproBDNF i proBDNF eren diferents i, a més, associats positivament amb la patologia dels TEA. Tot i això, l'estudi tenia certes limitacions ja que el nombre de mostres estudiades era reduït i no estaven perfectament delimitades; també, les mostres de PBMCs no corresponient exactament ales mostres de plasma analitzades. Això vol dir que el grup de TEA analitzat resultava de la combinació de pacients nens i adolescents o adults. Per tant, seria força important incrementar el nombre de mostres de plasma i PBMCs dels dos grups i comprovar si els patrons observats es mantenen o no.

D. ***CAPÍTOL 2. LA LATROFILINA-3 COM A BIOMARCADOR PERIFÈRIC PER ALS TRANSTORNS DE L'ESPECTRE AUTISTA***

Les Latrofilines (LPHN) són una petita família de receptors acoblats a proteïnes-G i conegudes com proteïnes d'adhesió (Volynski KE et al., 2004; Lelianova VG et al., 1997; Rahman MA et al., 1999; Serova OV et al., 2008). La família compren tres isoformes: LPHN1, 2 i 3 les quals estan codificades pels gens LPHN1, LPHN2 i LPHN3 (Ichtchenko K et al., 1999; Sugita S et al., 1998). Els receptors LPHN1 i LPHN2 són els receptors per excel·lència de la α-latrotoxina (Ichtchenko K et al., 1999), a diferència de la LPHN3 que no la reconeix (BIBLIO). A més, és ben coeguda la importància dels receptors LPHN en la regulació de l'exocitosi dels neurotransmissors (Krasnoperov VG et al., 1997; Lelianova VG et al., 1997; Davletov BA et al., 1998; Rahman MA et al., 1999; Silva JP et al., 2009). L'estructura conservada dels tres membres de la familia (amb 48%-63% de similitud) està formada per una regió extracel·lular que conté un pèptid senyal, una regió rica en Ser/Thr, un domini lectina, un domini olfactomedina i un domini selectiu. També tenen una regió de set dominis transmembrana i una cua citoplasmàtica acoblada a les proteïnes G (Ichtchenko K et al., 1998; Sugita S et al., 1998).

L'autisme és, com prèviament s'ha comentat és un desordre del desenvolupament complex, amb múltiples factors etiològics i diferents graus en la severitat dels símptomes (Novaes CM et al., 2008). Aquest desordre es troba fàcilment associat a altres patologies (Holtmann M et al., 2007; Bailey A et al., 1998; Kent L et al., 1999; Reiersen AM & Todd RD, 2008) com els trastorns d'hiperactivitat (TDH) (Gadow KD et al., 2006; Goldstein S & Schwebach AJ, 2004; Hattori et al., 2006; Holtmann M et al., 2007; Reiersen AM et al., 2007). Recentment, gràcies a uns estudis realitzats per Arcos Burgos M i companys, es va observar l'associació entre el TDH i variants genètiques del gen LPHN3, a la població Paisa, Colòmbia (Arcos Burgos M et al., 2010). Aquesta troballa fou replicada en diferents poblacions (Paisa, Estats Units, Alemanya, Noruega i Espanya) (Arcos Burgos M et al., 2010; Ribasés M et al., 2011). Donada la importància de la coexistència clínica entre els pacients diagnosticats amb TDH i els TEA, i els recurrents estudis sobre el paper de les alteracions del gen LPHN3 en aquests TDH, sembla obvi la importància de realitzar un anàlisi de la possible associació entre aquestes alteracions genètiques i els TEA.

Els resultats obtinguts en el present estudi mostren l'associació entre els pacients HFA i el polimorfisme rs4860079 (p = 0.039). A més, la variant genotípica "rara" (AA) és probablement la variant que fa augmentar la probabilitat de presentar aquest fenotip (p = 0.058). Tot i això, és important tenir en compte les limitacions de l'estudi. D'una banda, és cert que hi ha una associació positiva entre la variant genètica i els TEA d'alt rendiment, però aquest resultats podrien esta emmascarats com a conseqüència d'una possible comorbiditat dels pacients TEA inclosos en l'estudi, amb TDH. Aquest fet no resultaria tant estrany si tenim en compte que els pacients no varen estar discriminats entre si patien o no, a més del TEA, de TDH; tots els pacients foren inclosos sota les mateixes condicions, agafant només com requisit indispensable ser diagnosticat com a TEA i respondre a un QI de més de 70. És per això, que com a futures direccions de l'estudi caldria apuntar cap a un àmpliament de l'estudi amb tres grups distintius: TEA d'alt rendiment, TEA d'alt rendiment en comorbiditat amb TDH i TDH. Només en aquesta direcció seria possible confirmar o no, la possible contribució del gen LPHN3 en l'etiologia dels TEA.

D'altra banda, el grup TEA de baix rendiment era molt reduït i, per tant, resultaria prioritari el fet d'augmentar el nombre de pacients. Llavors, si en augmentar el nombre de pacients de l'estudi, els resultats obtinguts fossin els mateixos és a dir, no associació, podrien quedar confirmats aquests resultats més preliminars. En aquest

cas, cap dels pacients inclosos en l'estudi es trobava en comorbiditat amb TDH, la qual cosa justificaria que els resultats no es troben emmascarats per aquest fenotip d'hiperactivitat.

Tan importat com la causa genètica de la patologia, ho és la necessitat d'analitzar l'expressió d'aquests potencials marcadors en mostres fàcils d'obtenir. Aquest fet fou el motiu que portà a realitzar l'anàlisi de l'expressió de mRNA de la LPHN3. D'acord amb estudis realitzats prèviament en altres laboratoris (Lelianova VG et al., 1997; Sugita S et al., 1998; Matsushita H et al., 1998; Ichtchenko K et al., 1999) en els que es demostrava com l'expressió d'aquest gen era preferencialment a cervell, els resultats del present estudi no mostraren expressió del mRNA de la LPHN3 en les mostres de sang perifèrica dels pacients.

Per acabar i tenint en compte els resultats obtinguts, sembla que hi ha una importància en el rol de la família de les Latrofilines en les desordres psiquiàtrics i generen nous aspectes sobre la possible associació de variants genètiques del gen LPHN3, concretament en HFA. A més, seguint la mateixa línia que els resultat anteriors, no s'ha trobat expressió del mRNA de la LPHN3. Aquest resultat deuria de ser complementat amb l'anàlisi de l'expressió en cervells post-morter de pacients autistes, ja que és el teixit més representatiu per les patologies mentals. Llavors, en el cas que s'observessin alteracions en aquesta expressió, s'hauria de procedir a la realització d'estudis més rigorosos sobre l'expressió de la LPHN3 en algun teixit perifèric, amb l'objectiu de poder utilitzar el gen LPHN3 com a potencial marcador genètic dels TEA.

E. ***CAPÍTOL 3. EL RECEPTOR DE L'EFRINA A4 COM A BIOMARCADOR PERIPHERIC DE LES PATOLOGIES PSIQUIÀTRIQUES***

Els receptors EPH són una amplia família de receptors tirosina kinasa, que promouen el contacte directe entre cèl·lula-cèl·lula, quan interaccionen amb els seus lligands directes, les efrines. La interacció entre receptors EPH i efrines regulen diferents processos durant el desenvolupament embriogènic, en molts casos afectant el citoesquelet, el patró neuronal així com el sistema vascular (Pasquale EB, 2008; Himanen JP & Nikolov DB, 2003; Pasquale EB, 2005). També, s'han vist implicats en processos tals com migració cel·lular, guia axonal, cartografia topogràfica, proliferació, formació de sinapsis i formació i estabilització de les espines (O'Leary DD & Wilkinson DG, 1999; Wilkinson DG, 2001; Essmann CL et al., 2008; Klein R, 2009). Recentment, s'ha vist també que tant els receptors EPH com les efrines es troben expressades en nínxols de cèl·lules mare adultes, com ara en cervell, pell i intestí i que regulen la proliferació de les cèl·lules mare progenitores (Holmberg J et al., 2005; Holmberg J et al., 2006).

En aquest estudi es varen analitzar nou SNPs del receptor EPH A4 (EPHA4) en una població psiquiàtrica total de 1555 pacients, diagnosticats segons la classificació del ICD10, com a esquizofrènia (n=929) o com a desordres bipolar (n=626). A més els pacients també es varen separar, atenent a la classificació de Leonhard, en: psicosi cíclica, esquizofrènia no sistemàtica i esquizofrènia sistemàtica.

Les efrines i els receptors EPH són reguladors del desenvolupament del sistema nerviós central, incloent la guia axonal, la migració i els patrons neuronals, la formació de sinapsis i la morfogènesi vascular (Drescher U, 1997; Tessier-Lavigne M & Goodman CS, 1996; Flanagan JG & Vanderhaeghen P, 1998; Palmer A & Klein R, 2003; Eichmann A et al., 2005). Carmona MA et al. (Carmona MA et al., 2009) va descriure l'important paper de les interaccions ephrin-A4/EphA4 en la regulació del transportador de glutamat a la glia i el transport d'aquest mateix neurotansmissor, del qual se sap que es troba alterat en desordres psiquiàtrics com ara l'esquizofrènia (Danbolt NC, 2001; Beart PM & O'Shea RD, 2007). També, en aquest mateix estudi es va descriure que l'activació de la EPHA4 induïa l'escurçament i la retracció de les espines dendrítiques. Així doncs, aquests resultats eren consistents amb experiments previs que demostraven la presència d'unes espines dendrítiques més llargues de lo normal en l'hipocamp de ratolins Knockout per el el gen de la EPHA4 (Murai KK et al., 2003).

Tal i com va proposar el grup del Dr. Carmona, aquestes observacions prèviament detallades podien estar involucrades en processos patològics tals com els desordres psiquiàtrics, ja que anomalies en les espines dendrítiques s'han observat tant en pacients autistes com esquizofrènics; així com també un increment dels nivells de transportador del glutamat en l'esquizofrènia (Fiala JC et al., 2002; Danbolt NC, 2001; Beart PM & O'Shea RD, 2007). Totes aquestes evidencies portaren a pensar en la possible implicació del la EPHA4 en les patologies mentals i, per tant amb possibles alteracions en la seqüència genètica. Així doncs, petits canvis comuns en la seqüencia de la EPHA4 podrien desencadenar una funció anòmala de la proteïna.

Per això, es varen seleccionar 9 SNPs amb una MAF (freqüència al·lèlica minoritària) d'entre el 30% i el 50%. D'acord amb la informació obtinguda del HanMap, els marcadors es troben en un moderat desequilibri de lligament, per la qual cosa es segreguen de forma independent. La població escollida per fer l'estudi es trobava en equilibri de Hardy-Weinberg, indicant que era representativa de la població total. Tot i així, en contra a la primera hipòtesi formulada, cap dels nou marcadors (rs2952834, rs2392936, rs2248489, rs2056290, rs2052940, rs1864461, rs1897120, rs3087584, rs3770181) es va veure associat ni amb l'esquizofrènia ni amb

els desordres bipolars (p > 0.05). Així mateix, tampoc es va veure una associació significativa aplicant la classificació Leonhard, que aten a una divisió dels pacients d'acord amb la simptomatologia. Aquest estudi tenia una important potència, d'un 87.5% per detectar la possible associació dels gens de susceptibilitat amb els desordres bipolars, i d'un 91.4% en l'esquizofrènia (alpha = 0.05%).

Com a conclusió d'aquest capítol, es podria dir que els resultats obtinguts recolzen suport la hipòtesi postulada, on la EPHA4 juga un paper important en la patofisiologia de l'esquizofrènia i els desordres bipolars. Tot i així, caldria replicar l'estudi en diferents poblacions així com també altres tipus d'estudis genètics, a fi de descriure aquesta possible implicació de les variants genètiques de la EPHA4 en les patologies psiquiàtriques.

F. CAPÍTOL 4. L'ANKIRINA-3 COM A BIOMARCADOR PERIFÈRIC DE LES PATOLOGIES PSIQUIÀTRIQUES

La recerca d'un gran nombre de gens de susceptibilitat per l'esquizofrènia i els desordres bipolars plantegen la idea de canviar el diagnòstic tradicional d'aquestes patologies, així com també els límits entre aquestes entitats. El present estudi tenia com objectiu principal replicar prèvies associacions genètiques del gen de la Ankirina-3 (ANK3) com a possibles factors de risc, tant per l'esquizofrènia com per als desordres afectius, en un estudi del tipus cas-control de més de 2000 individus de procedència alemanya.

ANK3 és una proteïna integral de membrana subjacent al citoesquelet d'espectrina-actina, localitzada en els segments inicials de l'axó i en els nodes de Ranvier, tant en el sistema nerviós central com en el perifèric (Lambert S et al., 1997). S'ha proposat que el producte gènic de la ANK3 juga un paper important en la regulació dels canals de sodi-potassi, adhesió cel·lular i desenvolupament neuronal (Bennett V & Lambert S, 1999; Dzhashiashvili Y et al., 2007; Kizhatil K et al., 2007; Jenkins SM & Bennet V, 2002; Jenkins SM & Bennett V, 2001).

Els polimorfismes que en aquest cas es van estudiar es trobaven en diferents blocs de desequilibri de lligament, dos d'ells localitzats en regions intròniques (rs9804190 i rs10761482), i un altre 30 kb seqüència avall de la ANK3 (rs10994336). Com a resultat destacat, es trobà l'associació del polimorfisme rs10761482 amb els desordres bipolars (p = 0.015, OR 1.304), però no amb l'esquizofrènia. Els resultats donarien suport a un estudi previ d'associació de tot el genoma (GWA) en mostres europees, on tampoc es va veure associació (Jenkins SM & Bennett V, 2001). Contràriament, l'estudi va fallar en la confirmació de l'associació dels polimorfismes rs9804190 i rs10994336 amb els desordres bipolars, que si que s'havien descrit en estudis de GWA, ni tampoc per l'associació amb els trastorns de depressió major o esquizofrènia (Dzhashiashvili Y et al., 2007; Kizhatil K et al., 2007). La fallada en la replicació d'aquests estudis podria ser conseqüència de la falta de mostra. Tot i així, la potència de l'estudi era d'un 55.1% de replicació de l'associació descrita per als desordres bipolars i d'un 69.1% amb l'esquizofrènia (alpha = 0.05%) (Kizhatil K et al., 2007; Jenkins SM & Bennett V, 2001). A favor de l'estudi cas-control realitzat, cal dir que tots els subjectes inclosos tenien la mateixa base genètica (tots provinents de descendència alemanya), minimitzant d'aquesta manera l'heterogeneïtat genètica.

També destacar, que la força de l'estudi recau en el fet de la combinació de ambdues classificacions, la IDC10, és a dir, la clàssica i la classificació categòrica de Leonhard. En la recerca de factors de risc per l'esquizofrènia i els desordres bipolars, no es va trobar associació amb l'espectre esquizofrènic, així com tampoc amb l'esquizofrènia sistemàtica ni amb els subgrups amb combinacions dels desordres bipolars i símptomes psicòtics; particularment de l'esquizofrènia no sistemàtica i la definició estricta de la depressió maníaca (Franzek E & Beckmann H, 1998; Pfuhlmann B et al., 2004; Stöber G et al., 1995). Altres entitats patològiques, segons la classificació de Leonhards, tampoc es varen veure associades amb cap dels marcadors.

Els resultats obtinguts en aquest treball donen suport a estudis previs de GWA on es buscaven variants genètiques de la ANK3 relacionades amb l'esquizofrènia i els desordres bipolars: un estudi que combinava meta-anàlisi amb el genotipat de desordres bipolars i unipolars, de mostres procedents del Regne Unit, Irlanda i els Països Baixos, no trobaren associació de les variants de la ANK3 amb la depressió unipolar. Un altre meta-anàlisi sobre els estudis de GWA basats en l'esquizofrènia i els desordres bipolars, en mostres procedents del Regne Unit, tampoc observaren significança en l'associació. Això si, tots dos suggerien un efecte de la ANK3 en els desordres bipolars (Liu Y et al., 2011; Williams HJ et al., 2011).

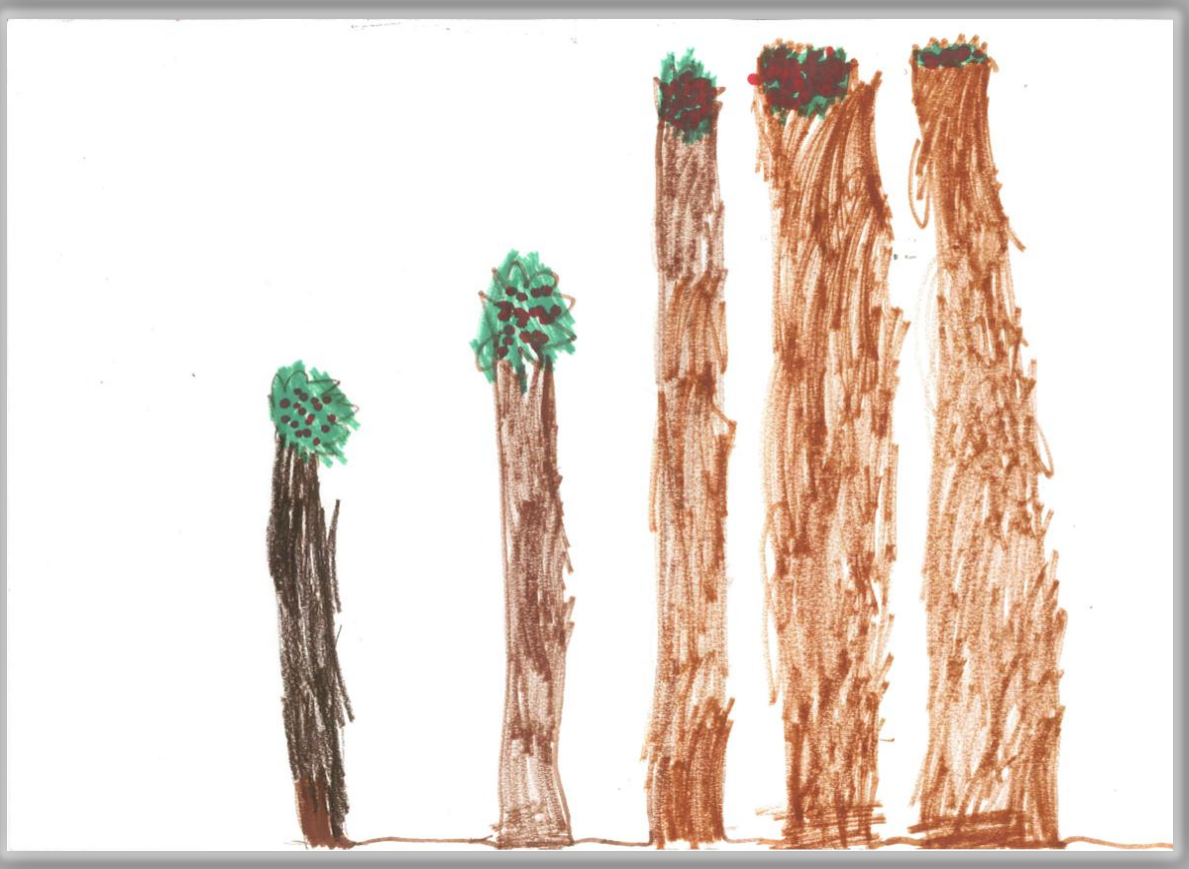
III. HYPOTHESIS OF THE THESIS

Psychiatric disorders such as autisms spectrum disorders, schizophrenia, bipolar disorders, mood and anxiety disorders and substance abuse are severe and disabling diseases, and some of them are major causes of morbidity even in childhood and adolescence. In contrast to diseases adhering to strict Mendelian inheritance, mental disorders are complex, polygenetic and often poorly understood with regards to pathomechanisms and biological pathways. In most cases, it is likely that the interplay of multiple gene products with environmental factors produces a given psychiatric phenotype. Then, it is well know that psychiatric diseases are phenotypically and etiologically heterogeneous, aside from genetic links between them. The frontiers between different neuropsychiatric disorders are not well delimitated and further research and pathophysiological knowledge is urgently needed.

For years evident alterations in the morphology of psychiatric brains as well a large number of molecules that have been identified as a possible cause of these diseases have been known. However, the bridging between the anatomic distribution and regional molecular events remains unknown. The probable heterogeneity and lack of major gene influence in these disorders severely limits the understanding to date of signaling pathways underlying these changes. Thus, focusing on these cytoarchitectural abnormalities and the environmental factors risk, which can be occur in prenatal and postnatal periods, our researcher group supports at the neurodevelopmental hypothesis as a cause of the neuropsychiatric diseases in neurodevelopmental processes. Thereby, the molecules that have been analyzed along this work, are associated with different processes that success in the development of nervous system (NS), in both, early intervention to form the correct morphology of NS, and in subsequent normal function.

Moreover, to date few revolutionary tests have been developed to differentiate between similar phenotypes and states of psychiatric diseases, to monitor therapeutic progress or to assess the prognosis of individual patients, because in most cases in the studies postmortem brains were used for analyses. However, although brain is the best tissue to represent psychiatric pathologies, it is necessary to also use peripheral tissue that can be analyzed when the patient is alive. Knowing disease specific pattern of mRNA, protein or metabolite expression in peripheral tissue would help to improve diagnostics, but also monitoring the patients to identify the current disease state. Since time ago, it is known the positive correlation between brain expression and peripheral blood, which allow to advance in research on the mechanisms of disease for its readily available. Therefore, our group thought whit peripheral blood as a sample easily to obtain and reasonably representative of what might be happening in the central nervous system of subjects.

As a conclusion, the general hypothesis of the present thesis is to identify potential biological markers in peripheral blood of patients diagnosed with different psychiatric diseases, and thus contribute to an improvement of diagnostic opportunities and a personalized therapy.



IV. GENERAL OBJECTIVES

In absence of clinical objective characteristics the identification of biomarkers in neuropsychiatric disorders is highly relevant for the diagnostic process, in order to categorize subsets of subjects in a consistent manner, and for an individualized therapy. The search of peripheral biomarkers has been underway for more than 20 years, but there are no diagnostic tests available yet and this is largely due to the multi-factorial nature of psychiatric disorders.

Thus, the main objective of this thesis is to identify and validate potential peripheral biomarkers for psychiatric disorders by using different molecular techniques.

Specific aims:

Chapter 1

Investigation of the role of neurotrophins and their receptors in autism spectrum disorders.

Chapter 2

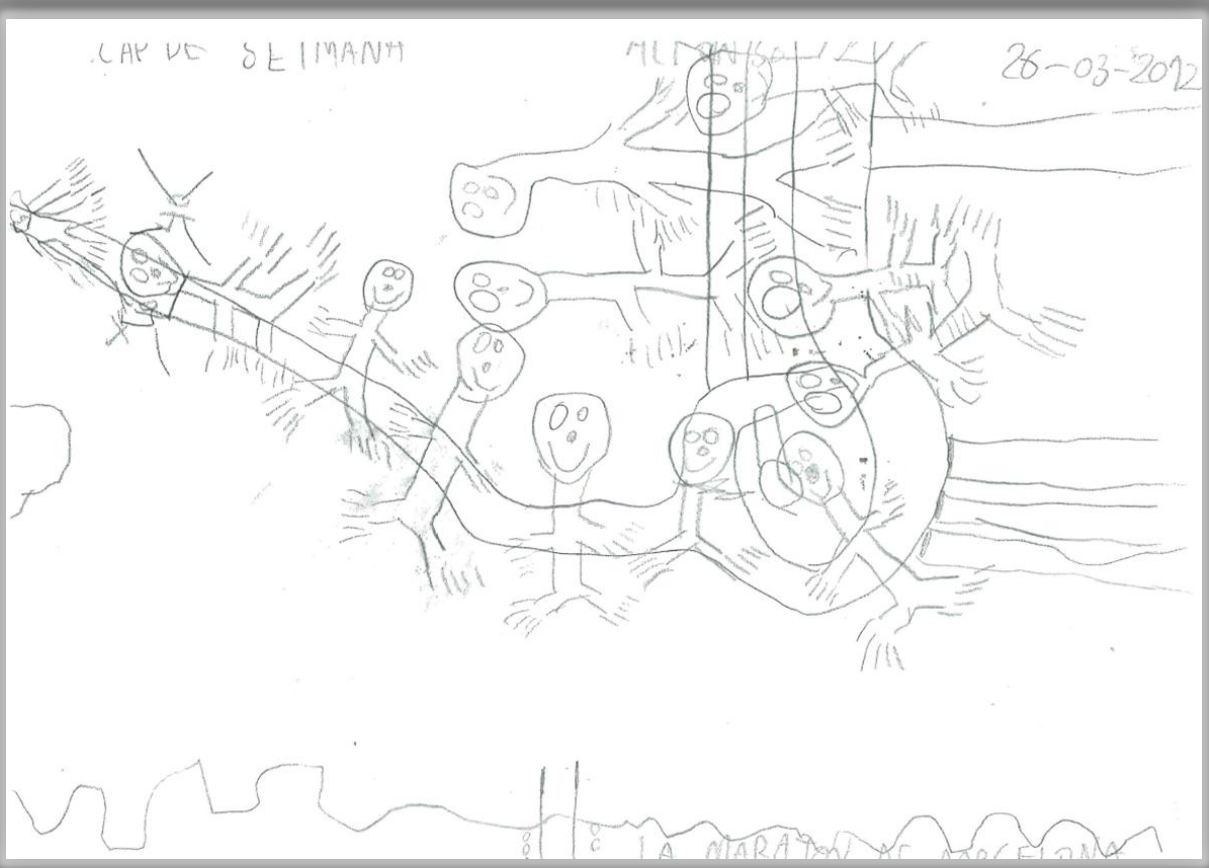
Investigation of the association of one single nucleotide polymorphism of Latrophilin3 and Autism Spectrum Disorders.

Chapter 3

Investigation of the association of nine single nucleotides polymorphisms of Eph-receptor-4-type A and schizophrenia as well as bipolar disorder.

Chapter 4

Confirmation of the reported association of three single nucleotides polymorphisms of Ankyrin-3 and schizophrenia and bipolar disorder.



V. GENERAL INTRODUCTION

A. CURRENT SITUATION OF PSYCHIATRIC DISORDERS

The World Health Organization (WHO) reported in 2008 that neuropsychiatric disorders are estimated to contribute to 13% of the global burden disease (World Health Organization, 2008). Though the extent of the burden varies between countries, neuropsychiatric disorders account for a substantial amount of the disease burden in every country of the world. Furthermore, 450 millions (10%) of the total population suffer from mental or neurological disorders or from psychosocial problems such as alcohol or drug abuse (World Health Organization, 2001), in other words, one in four people will be affected some time in their life (World Health Organization, 2005). Effects are equal on men and women with some exceptions, such as a higher prevalence of alcohol and substance abuse disorders in men and of unipolar depressive disorder in women (World Health Organization, 2001). Moreover, it is well known that mental illness is also common in people with physical illness, for example 22% of people with myocardial infarction, 27 % of diabetics and 33 % of people affected by cancer suffer from major depression (World Health Organization, 2003). Focusing on the incidence in Europe, 100 millions of 870 millions of people are estimated to suffer from anxiety and depression; 21 millions from alcohol use disorders; over 7 millions from Alzheimer's disease and other dementias; about 4 millions each from schizophrenia, bipolar affective disorder and panic disorder (World Health Organization, 2005).

There is a high prevalence of mental disorders among people who attend primary health care (PHC), in detail 24 % all patients seeking help (Goldberg DP & Lecrubier Y, 1995). Neuropsychiatric disorders are the second greatest cause of the burden of disease after cardiovascular diseases, within 19.5% of all disability-adjusted life-years (DALYs) – years lost to ill health and premature death (World Health Organization, 2005). Importantly, projections based on the study of Murray and Lopez (Murray CJ & Lopez AD, 1997) of the 1990 WHO Global Burden of Disease Study report that there will be observed a 40% increase (from 10.5% to 14.7%) of DALY caused by neuropsychiatric disorders from 1990 to 2020. An update of these projections predicts that unipolar depressive disorders will be ranked as the second leading cause (5.7%) of DALY in 2030 (Mathers CD et al., 2006). It is alarming that a high proportion of individuals affected remain untreated (Kohn R et al., 2004). In a European Union survey published in 2003, 90% of people with mental problems did not receive care or treatment; only 2.5% of the affected individuals had seen a psychiatrist or psychologist. Even in developed countries with well-organized health care systems, between 44% and 70% of patients with mental health disorders do not receive treatment (World Health Organization, 2005).

The social and economic costs of mental illness for societies are wide ranging, long lasting and enormous, being the estimated cost of 3–4% of gross national product in the Member States of the European Union (EU) (Gabriel P & Liimatainen MR, 2000). Median mental health expenditures per capita are 1.63 $ with large variations among regions, ranging from 0.20 $ in low incomes countries to 44.84 $ in high incomes countries (World Health Organization, 2011) (Figure 1), and 67% of financial resources are directed towards mental hospitals. An associated problem is the economic burden caused by the health and social services costs, lost employment and reduced productivity, as 35-45% of absenteeism from work is due to mental health problems; also the burdening impact on families and caregivers, levels of crime and public safety, and the negative impact of premature mortality are many other immeasurable cost factors that have not been taken into account (World Health Organization, 2001).

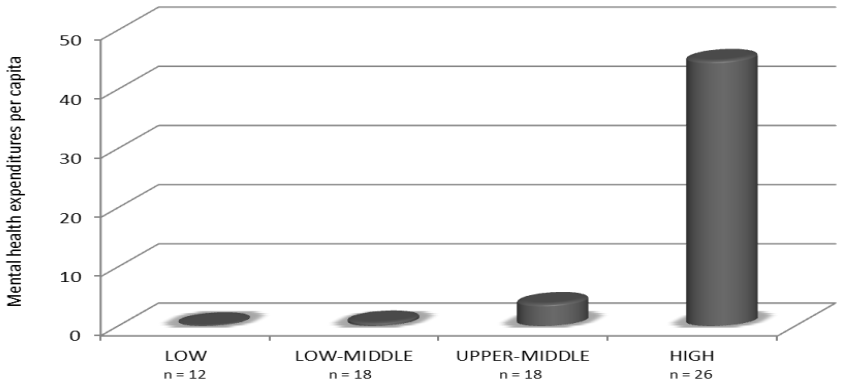


Figure 1. Median mental health expenditures per capita by World Bank incomes group. The graphic represents the mental health expenditures per capita of 73 evaluated countries for the World Bank. Global median mental health expenditures per capita is US$ 1.63 per year, it been more than 200 times greater in high income countries compared with low income countries. *Adapted from World Health Organization, 2011.*

All countries in the European Region must work with limited resources, and their mental health budgets constitute on average only 5.8% of their total health expenditure, ranging from about 0.1% to 12%. A large proportion of these budgets are allocated to services, and only negligible amounts are invested in promotion and prevention (World Health Organization, 2005). In U.S.A. the situation is similar, where only 7% of total health care expenditures are spent on mental illness and the total costs of mental disorders are about 2% of gross domestic product (The-wei H, 2004). Thus, the economic costs of mental health problems are substantial. In contrast to the situation for other health issues, most of these economic costs are incurred outside the health care system (Knapp M et al., 2009). Figure 2, about the study of costs caused by childhood conduct disorders in England that shows how only 16% of costs account for the heath care systems.

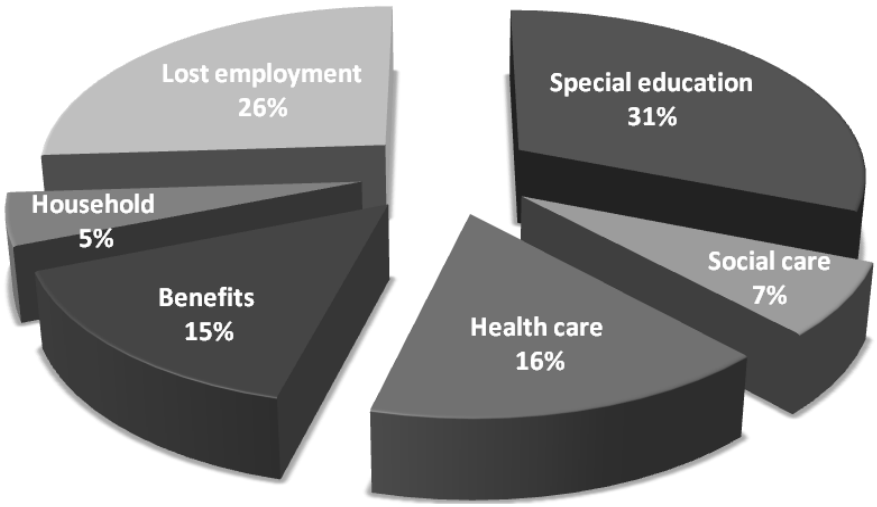


Figure 2. Comprehensive costs of childhood conduct disorder in England. Graphic shows different entities that take a care of the expenditures of the mental health, as well their relative contribution in percentages. *Adapted from Knapp M et al., 2009*.

B. NEUROPSYCHIATRIC DISORDERS

In the 4th century before Christ (BC), in ancient Greece, Hippocrates (460 BC-370 BC) theorized that physiological abnormalities may be the root of mental disorders and postulated the first classification system for mental illness (Parke-Davis AP, 1966). Another important personage in the history of psychiatry was Rhazes (865-925) who in 10th century, guided by the principles of Hippocrates, combined psychological methods and physiological explanations; he used psychotherapy in a primitive but dynamic fashion and, as director of the Baghdad's Hospital, established a special section for the treatment of mental illnesses (Alexander FG & Selesnik ST, 1966); this being considered nowadays as the first recorded reference to psychiatric aftercare. Leaving behind the beliefs that were formed around people affected by mental illness, a more scientific view on the causes and symptoms was adapted by Cotton Mather (1663-1728) who broke with former superstitions (mentalwellness.com). He was one of the first to advance physical explanations for mental illnesses in Renaissance Europe (mentalwellness.com). However, it was not until 1808 when Cristhian Reil (1759-1813) coined the term "Psychiatry" (Marneros A, 2006).

Although psychiatric epidemiological surveys have been carried out since after World War II (Cooper B, 1987), absence of a common format for diagnosis hampered cross-national syntheses. The situation was changed in the early eighties, with the development of fully structured research diagnostics (Robins LN et al., 1981) and the implementation of large-scale psychiatric epidemiological surveys in many countries (Weissman MM, 1997; Weissman MM et al., 1996). The WHO developed a diagnostic instrument: the WHO Composite

International Diagnostic Interview (CIDI) (World Health Organization, 1993) which generated both the International Classification of Diseases, 10th revision (ICD-10) (World Health Organization, 2007) and the Diagnostic and Statistical Manual, 4th revision (DSM-IV) (American Psychiatric Association, 1994) diagnoses (Kessler RC & Ustun TB, 2004).

Great advances have been made in our understanding of the pathophysiology of psychiatric and neurological diseases, although significant gaps remain in our knowledge about their causes. A major problem is the fact that current diagnoses are mainly based on categorizing the signs and symptoms of the syndrome, which limits the ability to reliably identify biological causes and develop specific treatments (Turck CW et al., 2005). Both, ICD-10 and DSM-IV identify different mental illnesses according to a list of symptoms and do not address the underlying cause of the disease.

B.1. Autism Spectrum disorders

B.1.1. Definition

Autism and other pervasive developmental disorders (PDD) were described in 1943 by Kanner (Kanner L, 1968). Since then, its criteria and ways of assignment have changed considerably. The term "Autism Spectrum disorders" (ASD) describes complex neurodevelopmental conditions, characterized by three core deficits: impaired communication, altered reciprocal social interaction and restricted, repetitive and stereotyped patterns of behaviors or interests. These impairments are variable in range and severity and often change with the acquisition of competences.

ASD include the diagnoses of autism, PDD-not otherwise specified (PDD-NOS) and Asperger syndrome (AS). Rett's syndrome and Childhood disintegrative disorder (CDD) are associated with significant developmental regression, which makes them more distinct than the other disorders in the PDD group (American Psychiatric Association, 1994). It is important to know that the DSM-V edition, that will be published in 2013, will consider ASD as a new category which includes autistic disorder, AS, CDD and PDD-NOS (dsm5.org). As comorbid condition, mental retardation is often present in PDD patients (in 30-70%), furthermore seizures in 25% and abnormalities in encephalogram in 20-50% of patients (Schaill L, 2005).

B.1.2. Diagnosis and classification

Early diagnostics and intervention is important, because is more effective in children with autism than in children with other developmental disabilities and it help minimizing the impact on the family (Matson JL et al., 2007). Currently, the diagnostic process typically includes a clinical developmental history, assessments of speech, language and intellectual abilities, and of educational or vocational attainment. Additional medical assessments are used to exclude other internal medical and neurological pathologies, as well as genetic syndromes or to assess for co-morbid conditions (Johnson CP et al., 2007). Standardized and semi-standardized procedures for conducting developmental interviews with caregivers and for observing and assessing social, communicative and repetitive behaviors, that are characteristic of autism, have been developed to aid and improve clinical diagnosis (Johnson CP et al., 2007). However, these so-called "gold-standard diagnostic tools" have not been widely adopted in community services because they are quite laborious, expensive and resource intensive; in fact, in many communities, autism-specific services have yet to be developed (Walsh P et al., 2011).

Diagnostic tools widely accepted for research are algorithms know as the "Autism Diagnostic Interview-Revised" (ADI-R) (Le Couteur A et al., 2003; Lord C et al., 1994) and the "Autism Diagnostic Observation Schedule" (ADOS). The ADI-R is a standardized, semi-structured 2-3 h interview with caregivers in individuals with autism or PDD. It yields summary scores in the following domains: qualitative impairments in reciprocal social interactions, communication, and repetitive behaviors and stereotyped patterns (Seltzer MM et al., 2003). The ADOS is a semi-structured, standardized assessment of children in which the examiner observes the social interaction, communication, plays, and imaginative use of materials. The ADOS requires approximately 30-45 min. and includes four possible modules; the examiner chooses the one that best matches the expressive language level of the individual child to prevent a relatively low level of language ability from impeding accurate measurement. This test provides measures in the following domains: reciprocal social interaction, communication, stereotyped behaviors and restricted interests and play. Both diagnostic algorithms are available for autism or for broader ASD/PDD phenotype (Seltzer MM et al., 2003).

B.1.3. Epidemiology and economic costs

Recent epidemiological studies, conducted in different regions of the world, have indicated that at least 1 in every 100 people has some form of ASD (Kim YS et al., 2011). This is a much higher prevalence than previously estimated in the early reports with prevalence rates of 4-5 per 10000 births (Fombonne E, 1999) or in reports of the last few years, where it was suggested that autistic disorders occur in 1-2 per 1000 births, the broader phenotype of autism spectrum in 4-6 per 1000 (Chakrabarti S & Fombonne E, 2005; Yeargin-Allsopp M et al., 2003). The difference between prevalence approximations may reflect an increase in the number of diagnoses owing to improved methods of detection and to a shift away from understanding autism as a narrowly defined, categorical disorder to understanding it as a spectrum of conditions that affect differently (Wing L, 1996).

The gender ratio male : femal is about 4 : 1, but is approaches 1 : 1 among individuals with severe cognitive impairments (Gillberg C & Wing L, 1999). A large proportion of individuals with autism manifest abnormal development from birth, a subset of at least 20-30% experience a regression with onset between 18 and 24 months of age after a period of apparently normal development (Lainhart JE et al., 2002).

The information about prevalence rates is important because the societal economic costs, including education and treatments for children with ASD, amount to approximately 35 billions $ per year (Ganz ML, 2007). Total expenditures per 10.000 covered lives associated with ASD increased 142.1% over a 5-years period (Leslie DL & Martin A, 2007), because of a 20% increase in average health care expenditures from the year 2000 to 2004 and rising prevalence rates.

B.2. Schizophrenia

B.2.1. Definition

Schizophrenia is a devastating psychiatric disorder characterized mainly by positive and negative symptoms, affect dysregulation and psychomotor symptoms. Positive symptoms include delusions and hallucinations, negative symptoms blunted emotions, social isolation, and cognitive deficits such as impairments in executive functions, attention and working memory (American Psychiatric Association, 1994). The disease onset usually is in adolescence or early adulthood and follows an episodic and deteriorative course where the prognosis might become worse with each episode (Lieberman JA et al., 2001; Perkins DO et al., 2005). Although

outcomes are variable, even with treatment, the typical course is one of relapses followed by only partial remission as well as a marked reduction in social and occupational function such that sufferers are often the most vulnerable, isolated, and disadvantaged individuals in society (Kirov G et al., 2005).

There is growing support that schizophrenia should not be seen as discrete entity, but rather as a disease with continuous variance (Kendell R & Jablensky A, 2003). It is true that onset of schizophrenia occurs in the 2nd or 3rd decade of life, however cognition abnormalities, impairments in social interaction, impairments in motor function and physical morphology are frequently observed more later, which leaves to suggest a developmental vulnerability (Niemi LT et al., 2003).

B.2.2. Diagnosis and classification

Unlike other illness such as diabetes, intoxication, etc. where the diagnostics is clearly established, and the causes can be detected or ruled out by methods such as clinical examinations or blood tests, schizophrenia, as well as other psychiatric disorders, not has any validated marker that allow an objective diagnostic. Thereby, diagnosis of schizophrenia is done using either the DSM-IV (American Psychiatric Association, 1994) or ICD-10 (World Health Organization, 2007) criteria, and definitive diagnosis is often assigned during hospital admission for a psychotic episode (American Psychiatric Association, 1994). These classification systems objectively define symptoms and characteristic impairments of schizophrenia in a similar way and the reliability of diagnosis between the two systems is high (Peralta V & Cuesta MJ, 2003), even though a more narrow definition of the disorder is used in the DSM-IV. Schizophrenia disease can be subdivided into different groups, according to the most predominant symptoms of the patients. Thereby, classification of schizophrenic entity include: paranoid schizophrenia, hebephrenic schizophrenia, catatonic schizophrenia, undifferentiated schizophrenia, residual schizophrenia (World Health Organization, 1994). On the other hand, a classification named Leonhard classification wich is based on symptomatology of patients is used. Thereby, according to the Leonhard classification (Leonhard K, 1999), psychoses exhibiting schizophrenic symptoms (DSM-IV criterion A) can be divided into three distinct clinical and nosological subgroups: cycloid psychoses, unsystematic schizophrenia, and systematic schizophrenia, taking into account symptoms patterns occurring during the long-term course of the diseases (Franzek E & Beckmann H, 1998). Therefore, cycloid psychoses run a phasic and prognostically favorable long-term course that is similar to maniac-depressive disease; schizophrenic symptoms are frequently present during the acute phases, producing a complete remission and thus, the total absence of residual symptoms. In the other hand, unsystematic schizophrenia is a subtype that generally leads to residual states of varying severity. Last subgroup distinguished in a Leonhard's classification is a systematic schizophrenia, which usually begins insidiously and run a chronic, progressive course without remission; in this case, the pathology is irreversible (Franzek E & Beckmann H, 1998; Leonhard K, 1999). Systematic schizophrenia can be subdivided into three groups: systematic catatonia (schizophrenia, catatonic type in the DSM-IV), systematic hebephrenia (schizophrenia, disorganized type in the DMS-IV), and systematic paraphrenia (schizophrenia, paranoid type in the DMS-IV).

Scales, such as "the positive and negative syndrome scale" (PANSS), might be used to assess positive and negative symptoms (Kay SR et al. 1987). The positive symptoms are more incident during a psychotic episode, and include delusions and hallucinations; in contrast, the negative symptoms are more persistent during the course of diseases, and include loss of interest and motivation, inability to experience pleasure from normal activities and diminished speech (McGlashan TH & Fenton WS, 1992).

B.2.3. Epidemiology and economic costs

Epidemiological systematic reviews about approximation of incidence and prevalence of schizophrenia show a median incidence rate of 15.2 per 100.000, with the 10% to 90% quartiles between 7.7 to 43.0 per 100.000 (McGrath JA et al., 2004). The median lifetime prevalence estimates are 4.0%; furthermore, the estimation of incidence show that males have a 1.4 times higher risk for this pathology than females and that migrants have a 4.6 times higher risk than native born individuals (Saba G et al., 2006). Another etiological factor is living in an urban area, as the risk is increased comparing to living in mixed urban/rural sites (McGrath JA et al., 2004). Developing nations showed a lower prevalence of schizophrenia than developed nations, but this finding should be interpreted with caution, because the country's status of development was only based on a single crude economic variable (per capita gross national product).

The estimated economic costs of schizophrenia, taking into account expenditures of direct medical, non medical and indirect costs, in Europe is 35 billions € (Andlin-Sobocki P & Rössler W, 2005) and 32.5 billions $ in the U.S. (Rice DP, 1999) per year, which is primarily due to the early onset in adulthood and the fact that 2/3 of affected individuals have persistent and/or fluctuating symptoms despite optimal treatment (American Psychiatric Association, 1994). Cost-effectiveness studies are necessary to maintain mental health care accessible for large groups of patients.

B.3. Bipolar disorders

B.3.1. Definition

Bipolar disorders or manic depressive illness was described in antiquity by Hippocrates (460-370 BC) and Aristotle. Aretaeus of Cappadochia (2nd century AD) seems to have been the first to bring the symptoms of melancholia and depression together. Emil Kraepelin built the first modern diagnosis system for psychiatric disorders and differentiated manic-depressive Insanity from dementia praecox (later to be renamed schizophrenia by Bleuler) in his textbook "Depressive Insanity and Paranoia" (Kraepelin E, 1921). He also pointed out the necessity of making long-term observation of manic-depressive illness, and made incisive clinical observations greatly influencing what we know today about bipolar disorder. Kraepelin was succeeded by Karl Leohnard, Jules Angst and Carlo Perris, all of whom made seminal contributions to our understanding of the long-term course of bipolar disorders (sleepmedicine.org/karl-leonhard.html).

Bipolar disorder, classified as a mood disorder (American Psychiatric Association, 1994) is one of the most important syndromes in psychiatry and has been described by many cultures along history (Goodwin FK, 1990). This disorder, also known as a manic depressive illness (Craddock N & Jones I, 1999) is characterized by mood swings with recurrent phases of mania, depression and euthymia, and causes immense personal and social losses (Nakatani N et al., 2006). The severity and duration of episodes, and the signs and outcome of the illness vary considerably. There is a spectrum of bipolar disorders from the most severe form comprising severe episodes necessitating hospitalization to less severe forms often needing no treatment at all.

B.3.2. Diagnosis and classification

The nosological relationship between schizophrenia, bipolar disorder and mixed forms of illness (particularly schizoaffective disorder) have been the subject of substantial interest and debate since Kraepelin proposed his

well-known dichotomy at the end of the 19th century (Marneros A et al., 2006; Crow TJ, 1990; Kendell RE, 1987). However, the dichotomy continues to be reflected prominently in recent operational descriptive classifications, including the DSM-IV (American Psychiatric Association, 1994) and ICD10 (World Health Organization, 2007).

In modern classifications (DSM-IV and ICD10), the diagnosis of bipolar disorder requires that a person has suffered one or more episodes of mania with or without episodes of depression at other times during the life history. This requirement for the occurrence of an episode of mania at some time during the course of illness distinguishes bipolar disorder from unipolar disorder, in which subjects suffer one or more episodes of depression without ever experiencing episodes of pathologically raised mood (Craddock N & Jones I, 1999).

The early symptoms of bipolar disorder can be discrete and the diagnosis is therefore difficult to make. According to earlier studies, 40-90% of the cases are misdiagnosed or are not diagnosed at all (Regier DA et al., 1988; Das AK et al., 2005). On average, there is a period of five years between onset and the first consultation, and the delay from onset to the introduction of mood stabilizers is ten years or more (Bryant-Comstock L et al., 2002;). This delay may be ominous, since early diagnosis and the instigation of pharmacological treatment may be crucial to avoid suffering from several illness episodes with serious social, psychological and cognitive consequences.

B.3.3. Epidemiology and economic costs

Bipolar disorder is one of the most common and severe psychiatric disorders. Prevalence estimates range from 0.2% to 2.0% for the more severe illness with depressions and full-blown manias (i.e. Bipolar disorder type 1), and 1% to 3% for bipolar illnesses with depressions and milder forms of mania (i.e. Bipolar disorder type 2) (Goodwin FK, 2007). The mean age at onset of illness is 21 years; similar in men and women, and more than 80% of patients having a first episode will suffer a recurrence (Winokur G et al., 1994). In most cases, the course of the disease is progressive and the suicide frequency is high, ranging from 11 to 19% (Tondo L et al., 1999; Ösby U et al., 2001).

C. ETIOLOGY OF PSYCHIATRIC DISEASES: IS NEURODEVELOPMENTAL DISRUPTION THE CAUSE OF THESE PATHOLOGIES?

Human structural, functional, and behavioral brain development emerges as an ongoing dialogue between genetic heritage and environment. Understanding how these factors interact at different points during development may help to identify how and when to intervene to help children grow to their fullest potential (Lenroot RK & Giedd JN, 2008). However, establishing the links between risk factors and developmental outcomes has proven to be extremely challenging. Research of complex neurodevelopmental disorders such as schizophrenia and autism (Merikangas AK et al., 2009) has shown that particular clinical syndromes may be associated with a wide variety of genetic risk factors, suggesting that there may be multiple routes from nucleotide to behavior (Abrahams BS & Geschwind DH, 2008; Owen MJ et al., 2005; Samaco RC et al., 2005; Walsh P et al., 2011).

Then, based on the belief that some psychiatric disorders have their origin in neurodevelopment, it would be appropriate to do a brief description of what is understood of normal development. So, development is described as a process that occurs in the normal human brain as a function of age. Hence, any pathology or deviance is closely entwined with the age-associated stage of development or degeneration of brain. Some important facts about normal developments are necessary to have present: (a) different brain regions are generated at different times during development, thus the timing of the insult to the growing fetus in intrauterine life is a major determinant of the subsequent anomality observed, (b) minor abnormalities in early events can produce large differences in subsequent stages, (c) specific molecular signals play specific roles at various stages during neurodevelopment (Gupta SK et al., 2010). For example, Brain Derived Neurotrophic Factor (BDNF) and Insulin Like Growth Factor (IGF) are the major signals to proliferation (Johnson-Farley NN et al., 2007). Proteins such as reelin cause migration of the growing neurons to the appropriate positions in the brain (Weeber EJ et al., 2002), and are controlled by genes. Once development is understood, the term “neurodevelopmental disorders” implies that the brain is not formed normally from the beginning (Gupta SK et al., 2010), and encompasses a large group of disorders that share the fact that disease onset is during periods of ongoing maturation and development (Ehninger D et al., 2008). Then, abnormal regulation of fundamental neurodevelopment processes may occur, or there may be disruption by insult that may comprise various forms (Gupta SK et al., 2010). These disorders are often associated with complex neuropsychiatric features including intellectual disability, specific learning disabilities, ADHD, autism, and epilepsy, among others (Ehninger D et al., 2008; Gupta SK et al., 2010).

Neurodevelopmental disorders are caused by a wide range of genetic mutations and environmental factors (e.g., infections, immune dysfunction, intoxication, endocrine and metabolic dysfunction, nutritional factors, trauma, etc.). Heritability estimates indicate that genetic factors play an important role in these disorders (Ehninger D et al., 2008). One approach to disentangle these complex interactions is to use stepping stones such as brain structure to help bridge the gap between genetic and environmental risk factors and behavior, because genes do not code for behaviors but for the building block of the cells whose interaction eventually gives rise to those behaviors; conversely, the translation of environmental input into persistent behavioral changes occurs through alterations in brain systems and even structures (Lenroot RK & Giedd JN, 2008). Figure 6 shows how different factors may contribute to phenotype expression.

Although schizophrenia and autism have different presentations of symptoms, age of onset, and development courses, impaired social functioning is a hallmark characteristic of both disorders (American Psychiatric Association, 1994) and these social deficits are related to impairments in social cognition (Hughes C et al.,

1997; Klin A et al., 2002; Pinkham AE et al., 2003). Recent research suggests that social cognitive deficits in both disorders may arise from dysfunctions in the neural systems that underlie social cognition (Pinkham AE et al., 2008). However the knowledge of these deficits, and potential similarities between these disorders, remain incomplete because genetic and development processes, that organize developing brain networks and presumably aid in disorganizing brain networks, are largely unstudied in the early developmental time period in humans (Courchesne E et al., 2007). Thus the original alterations in brain architecture, that produce dysfunction in people with schizophrenia and autisms, remain a mystery.

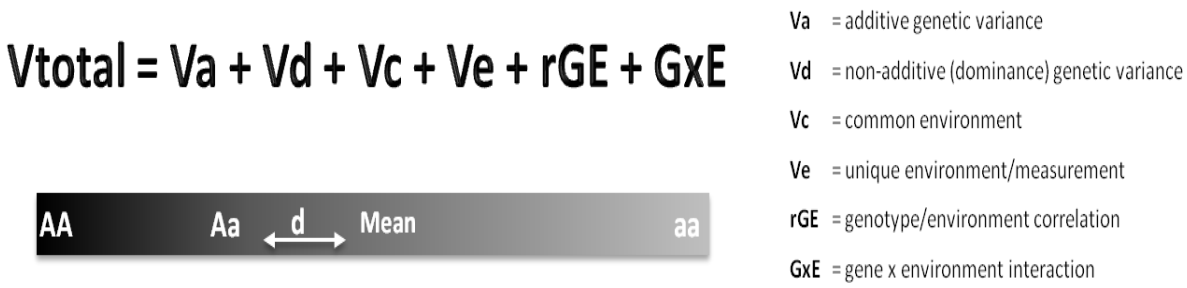


Figure 3. Factors contributing to variance. If the genotypic effects were completely additive, the phenotype of the heterozygous allele Aa would be the mean value between them. The degree to which the heterozygote departs from this is the degree of dominance (d). *A: dominant allele; a: recessive allele..Adapted from Lendroot RK and Giedd JN, 2008.*

C.1. Etiology of Autism Spectrum Disorders

ASD are the most devastating conditions in the broad range of developmental abnormalities known as "pervasive developmental disorders" (Rapin I, 2002). The stereotypic behaviors and marked delay or disruption of communication and social behavior trajectories that characterize ASD indicate, that crucial neuroanatomic structures and neurodevelopmental pathways may be affected during intra-uterine and/or early postnatal brain development (Pardo CA & Eberhart CG, 2007). Several lines of research indicate that ASD are associated with disarrangement of neuronal organization, cortical connectivity and neurotransmitter pathways. However, the cause of these abnormalities is still unknown, but it is generally believed that genetic as well as environmental factors are involved in the pathogenesis of ASD (Herbert MR et al., 2006; Minshew NJ & Williams DL, 2007; Persico AM & Bourgeron T, 2006).

There are different approaches (i.e. clinical assessment, neuroimaging and neurophatological studies) that have been used to assess the structural and morphological brain abnormalities. One consistent finding in ASD is the alteration of two phases of brain growth: a reduced head size at birth, then a sudden and excessive increase between 1-2 months and 6-14 months of age (Courchesne E et al., 2004; Courchesne E & Pierce K, 2005). Furthermore, it has been observed, that an abnormal brain overgrowth also occurs in area of the frontal lobe, cerebellum and limbic structures between 2 and 4 years of age, a pattern that is followed by abnormal slowness in brain growth (Courchesne E et al., 2004; Courchesne E & Pierce K, 2005; Courchesne et al., 2007; Schumann CM et al., 2004). The interest of these features lies in the fact that these regions are involved in the development of social, communication and motor abilities that are impaired in ASD. Other neuroimaging studies also showed an overall enlargement of brain volume associated with increased subcortical white matter in the frontal lobe, and abnormal patterns of growth in the cerebral cortex, amygdale and hippocampus formation (Herbert MR, 2005) as well as inter-regional disconnectivity (Herbert MR, 2005; Herbert MR et al., 2003; Herbert MR, 2004), potentially resulting in poor integration within and across neurobehavioral developmental domains (Courchesne E & Pierce K,, 2005; Koshino H et al., 2005). Post-mortem studies also showed disturbances in neuronal and cortical organization. Indeed, cytoarchitectural organizational abnormalities of the cerebral cortex, cerebellum, and other subcortical structures appear to be the most prominent neurophatological changes in autism (Bailey A et al., 1998; Kemper TL & Bauman M, 1998).

These neurobiological alterations likely affect the developmental trajectory of social behavior and communication during early stages of childhood (Landa RJ et al., 2007) and appear to be influenced by both genetic and environmental factors such as advanced parental age (Durkin MS et al., 2008) and possibly prenatal pesticide exposure (Roberts EM et al., 2007). Figure 7 shows how environmental risk factors may contribute to alter neurodevelopment trajectories.

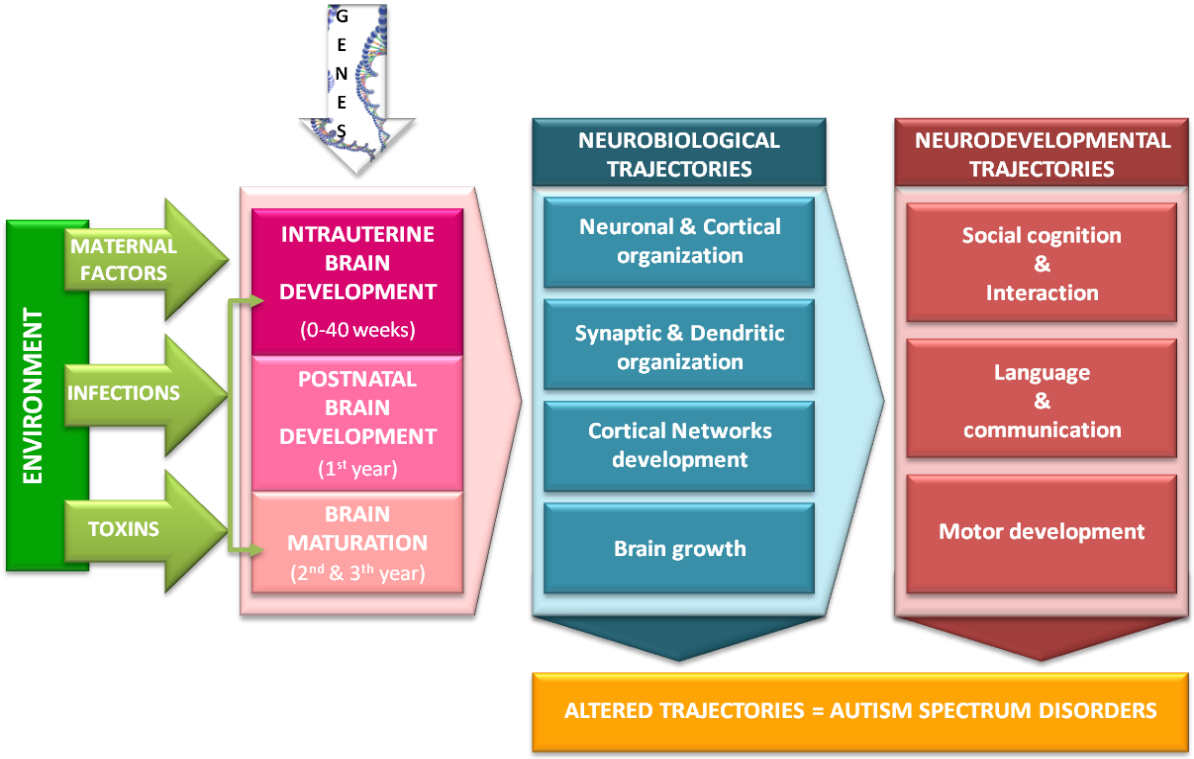


Figure 4. Genetic and environmental factors that influence intrauterine and early postnatal brain development likely alter neurobiological and neurodevelopmental trajectories that determine the clinical core of ASD. *Adapted from Pardo CA & Eberhart CG, 2007*

Even knowing the crucial role of development, the major role of genetics in ASD is well known because there is a concordance rate of 60% to 90% in monozygotic twins (Bailey A et al., 1995) versus 4.5% of siblings (Jorde LB et al., 1991)). Many recent studies showed the genetic complexity of ASD, it is considered as polygenetic disorder (Nishimura Y et al., 2007; Schellenberg GD et al., 2006; Sebat J et al., 2007; Autism Genome Project Consortium et al., 2007; Yang MS et al., 2007). Taking these studies into account, the involvement of molecular pathways with the potential to disrupt neurodevelopment becomes evident in the pathogenesis of ASD. Even so, environmental factors including both intrauterine and postnatal influences, play a crucial role and may modify the underlying genetic substrate and lead to great abnormalities in neuronal organization and cortical network development (Pardo CA & Eberhart CG, 2007).

Thus in attention of genetic burden that has been reported until now, is important to put emphasis in the fact, that many molecular pathways implicated in autism have effects on multiple central nervous system (CNS) processes. One of these molecular pathways that has been implicated in ASD is directed by growth factors such as hepatocyte growth factor (HGF) and its receptor MET (Campbell DB et al., 2006; Campbell DB et al., 2007), signaling proteins such as reelin (Persico AM et al., 2001; Fatemi SH, 2005; Serajee FJ et al., 2006; Skaar DA et al., 2005) and neurotrophic factors such as BDNF that are good candidates for involvement in ASD because of their fundamental roles in guiding CNS development and cortical organization (See chapter 1, Role of neurotrophins in ASD). Figure 8 represents how multiple genes associated with ASD are influencing the neurodevelopment at different stages.

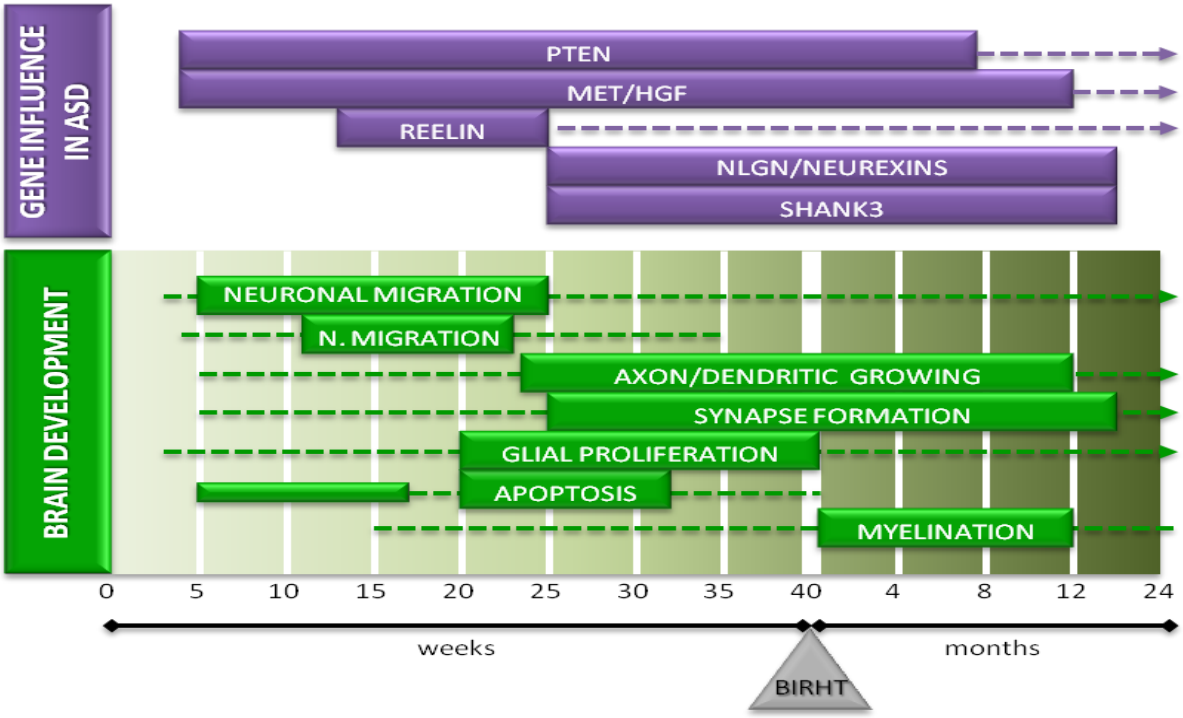


Figure 5. Representation of the steps in neuronal development and important genes in this process, which has association with ASD. A multiple number of genes that has an important role in NS development have been associated with ASD physiopathology. Green boxes represent the specific periods of the processes that can be longed in the time (represented by discontinuous green lines). During this stages, genes that has been associated with ASD has an specific (violet boxes), although it can be extended (represented by discontinuous violet lines). *Adapted from Graaf-Peters VB & Hadders-Algra M, 2006.*

C.2. Etiology of Schizophrenia

The etiology of schizophrenia is also unknown, but twin, family and adoption studies showed that complex interactions between genetic and environmental factors are involved in its etiology (Gottesman II, 1991). In contradistinction to ASD, the theories are controversial because there are many hypotheses about the origin of pathology. The theory that schizophrenia might be a developmental disorder was proposed at the first time by Thomas Clouston (Clouston T et al., 2005) who called it “neurodevelopment insanity” and posits that abnormalities of early brain development increase the risk for the subsequent emergence of clinical symptoms (Marenco S & Weinberger DR, 2000). However, according to Gupta and Kulhara´s review (Gupta SK et al., 2010), this theory left questions unanswered, as how a neurodevelopmental disorder could manifest for the first time in an adolescent or an adult. Some of these gaps could be answered by a neurodegenerative hypothesis that has its beginnings in the Kraepelin’s decryption that popularizes the term “dementia praecox” (Gupta SK et al., 2010). There is no unique hypothesis for schizophrenia etiology, because many other researchers are betting on theories such as the dopaminergic or glutamatergic hypothesis These hypothesis are supported by the presence of obvious alterations in neurotransmitters systems; meaning that, some patients present a hyperdopaminergic state (Stöber G et al., 2009; Carlsson A, 1988) or hypoglutamatergic state (Stöber G et al., 2009; Coyle JT, 1996). Even so, these alterations can be explained the abnormal development. Then there is many evidence of this abnormalities contributes to etiology of schizophrenia, such as the following: (a) abnormalities of early motor and cognitive development and histories of obstetrical adversity, (b) absence of evidence of neurodegeneration in postmortem tissue studies, and (c) association of developmental pathological conditions with adult emergence of psychosis and related phenomena in animal and neurological models (Marenco S & Weinberger DR, 2000; Cannon TD et al., 1999). Thereby, the cytoarchitectural abnormalities, such as neuronal disarray, heterotopias and malpositioning, suggest disruption of proliferation or migration at the gestational period (Miyamoto S et al., 2003). Accordingly, brains of schizophrenic patients exhibit a 30% to 50% reduction in the expression of reelin, a glycoprotein that acts as

a “stop” signal for neuronal migration during development in the prefrontal cortex and hippocampus (Curran T & D’Arcangelo G, 1998). Furthermore, in many studies abnormalities in synaptic, dendritic, axonal, and white matter organization, and abnormalities of glutamatergic neurotransmission have been described, which are consistent with defective connectivity between brain regions, including the midbrain, nucleus accumbens, thalamus, temporo-limbic, and prefrontal cortices (Coyle JT, 1996; Arnold SE, 1999; Selemon LD & Goldman-Rakic PS, 1999; Lim KO et al., 1999). In addition, changes in adhesion molecules (i.e. neural cell adhesion molecule), cytoskeletal proteins, neurotrophins (i.e. BDNF) and other cell-cell signal molecules have been observed in the schizophrenic brains (Maynard TM et al., 2001). Postmortem brains also show cellular aberrations, such as decreased neuronal size, increased cellular packing density, and distortions in neuronal orientation (Arnold SE, 1999).

Environmental risk factors are more definitive for schizophrenia than for ASD; however, their effects on disease susceptibility are likely to be small and, therefore, difficult to incorporate into analyses (Merikangas AK et al., 2009). For schizophrenia, some factors under investigation include obstetric complications (Clarke MC et al., 2006), prenatal nutritional deficiencies (Ludvigsson JF et al, 2007), maternal infections and cytokines (Buka SL et al., 2001), childhood trauma (Morgan C & Fisher H, 2007), family interactions (McGuffin P, 2004), gluten sensitivity (Kalaydijan AE et al., 2006), and cannabis use (Arseneault L et al., 2002; Dean K & Murray RM, 2005). Figure 6 shows pre and postnatal risk factors for schizophrenia and their approximate effect sizes.

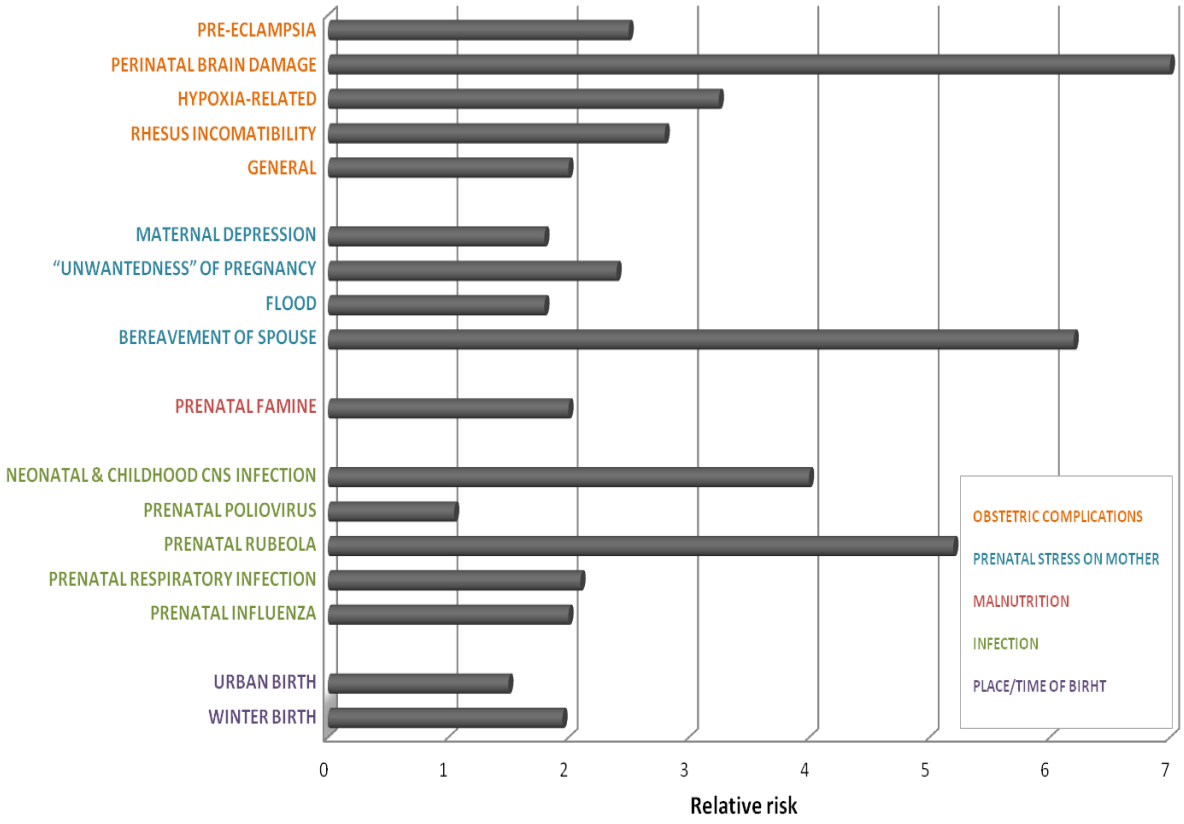


Figure 6. Approximation of effect size for pre- and postnatal risk factors for schizophrenia. *Adapted from Cannon M et al., 2003.*

After revising the consistent findings that give support for a neurodevelopmental hypothesis of schizophrenia and how the development may be influenced by environmental factors, it is necessary to emphasize the importance of genetic burden. Schizophrenia has one of the highest heritability estimates of psychiatric diseases, as seen in figure 10, adapted from Gottesman II, where the lifetime morbid risks are shown from

combined study results published between 1921 and 1987 (Gottesman II, 1991). The heritability of liability to develope schizophrenia is estimated to be approximately 80% (Riley B & Kendler KS, 2006). The relative importance of genes and environmental liability have been estimated by twin studies, showing a consistently higher concordance rate in monozygotic (50% approximately) than dizygotic (17% approximately) twins (Cardno AG & Gottesman II, 2000).

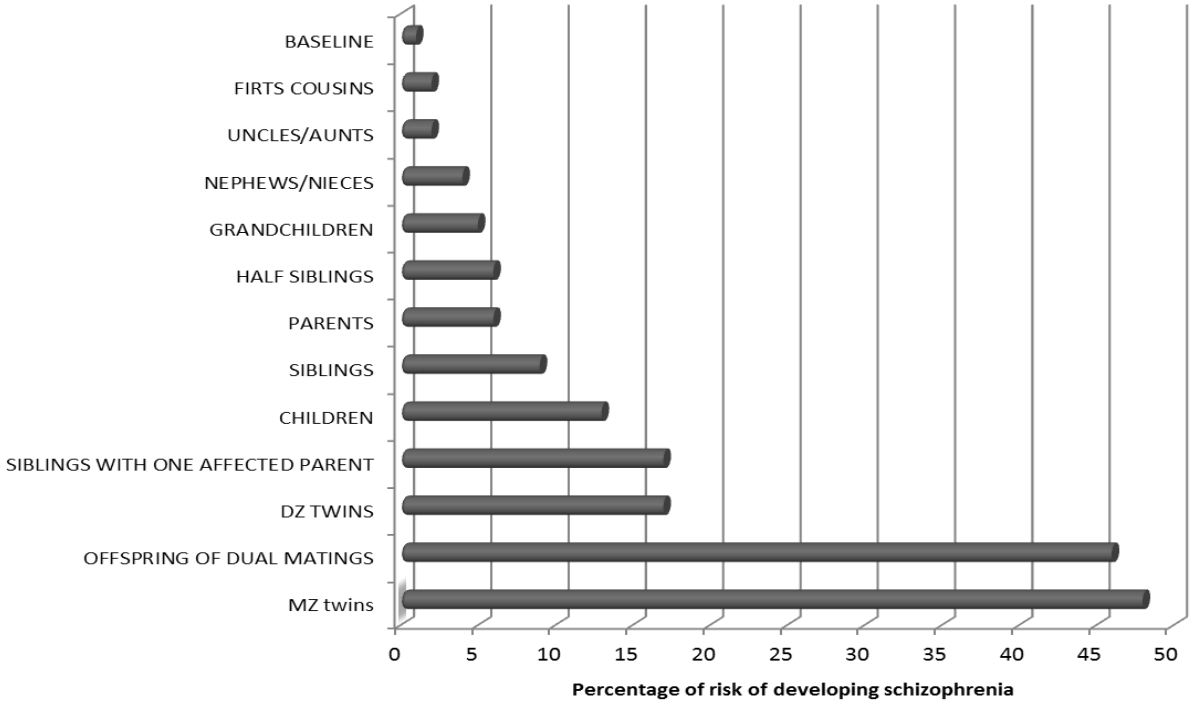


Figure 7. Risk of developing schizophrenia in relatives of schizophrenic relatives. *Modified from Gottesman II, 1991.*

D. POTENTIAL BIOMARKERS FOR PSYCHIATRIC DISORDERS

Great advances have been made in our understanding of the pathophysiology of psychiatric diseases; however significant gaps remain in our knowledge. Even so, the identification of genetic bases and assessment of dynamic disease related alterations will hopefully come to a new stage in the complex field of psychiatric research (Maher BS et al. 2008). A major goal in the area of psychiatric disorders is therefore the identification of markers that can categorize subsets of subjects in a consistent manner (Turck CW et al., 2005). Accordingly, Schwarz and Bahn told that only the knowledge of disease mechanism will facilitate the discovery of biomarkers (BM) that will help with objective diagnosis, allow the identification of individual susceptibility, predict treatment response and revolutionize drug discovery approaches (Schwarz E and Bahn S, 2008). This present interest reflects a degree of dissatisfaction with the limited success of genetic studies of psychiatric diseases, defined on the basis of DSM and ICD. There is an overall agreement that phenotypic variation and pathogenic heterogeneity is not adequately captured by current clinical classification systems (Stöber G et al., 2009). The Biomarkers Definitions Working Group (BDWG) of the National Institute of Health defines a BM as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001). Another definition of a BM is "measurable indicator of a specific biological state, particularly one relevant to the risk of contraction, the presence or stage of disease" (Rifai N et al., 2006). Nowadays, the term BM is often used to refer to a molecular biological marker but it should also be considered for physical traits or physiological metrics (Rifai N et al., 2006).

BM should be measured easily and with high precision, they should be inexpensive and Knowing BM could result in faster decision making and reduced sample size requirements for clinical studies. As already mentioned, it is important that BM may be measured easily and non-invasive as a biosample (e.g. blood, urine or tissue), physical examination (e.g. electrocardiograph) or imaging test (e.g. electroencephalogram or fMRI). An important aim of BM discovery is the detection of disease correlates that can be used as diagnostic tools. For this reason, BM should have predictive power, should be available during routine assays and allow the identification of individual risk (Stöber G et al., 2009). Thus, there are many valuable applications of BMs in neurological diseases for: (a) diagnosis and differential diagnosis, (b) screening of potential new therapies, (c) measuring severity, progression of disease and responses to therapy, (d) predicting prognosis, and (e) measuring toxicity (Feigin A, 2004). Then, BM would help to identify individuals with increased risk for devastating mental disorders and contribute to early diagnosis and intervention. They furthermore could contribute to more individualized treatment strategies with increased efficacy and reduced side effects and also possibly be used as prognostic predictors.

The identification of credible BM would probably be the optimal measure for refining the disease phenotype, so genetic research will benefit from a better disease classification by BM and studies could include samples with more homogenous and narrowly defined phenotypes (Sher L, 2006). According to classic test by Sturtevant, phenotype is often an imperfect indicator of the genotype, because on the one hand the same genotype may give arise to a wide range of phenotypes ("Pleiotropy") and on the other hand the same phenotype may have arisen from different genotypes. A particular subtype of BM was described by Gottesman and Shields, who told about "endophenotype" as internal phenotype, discoverable by a "biochemical test or microscopic examination" (Gottesman II & Shields J, 1971 & 1973). Then, it may be defined as a discrete, genetically determined trait, which should co-segregate with clinical illness in pedigrees and may also be expressed in clinically unaffected family members (Stöber G et al., 2009); furthermore, it is often part of the illness, but which is not really discerned by routine clinical examination of the patient (Braff

DL et al., 2007). Ideally, an endophenotype should be "state-independent"; this means, it should be detectable whether or not the patient is acutely ill, though sometimes a "change test" may be necessary to bring out the endophenotypic trait (Doran CM, 2008). The identification of endophenotypes, which do not depend on what was obvious to the unaided eye, could help to resolve questions about etiological models. Therefore, endophenotypes are being seen as viable and perhaps necessary tools for overcoming the barriers to progress (Lenox RH et al., 2002, Merikangas KR, 2002).

D.1. "From the brain to peripheral systems": blood expression profiles and biomarker research

Gottesman said that to tie inherent complexity of psychiatric diseases, which have multifactorial and polygenetic origins, the brain is the most complex of all organs (Gottesman II & Gould TD, 2003). Furthermore, this problem is compounded by the fact that obtaining tissue from the relevant brain regions is essentially impossible for reasonably large and representative patient sample sets (Sullivan PF et al., 2006). The comprehensive dissection of a complex trait ideally requires assessment of individual differences at all steps along the canonical path (i.e. DNA → RNA → protein). Access to disease and control tissue in organic diseases, such as cancer, is relatively straight-forward for many complex traits (i.e. sample malignant and normal breast tissue from the same individual). An unknown issue is whether gene expression in a more accessible tissue (i.e. peripheral blood lymphocytes) is a useful surrogate for gene expression in the central nervous system (CNS). The existence of a tissue whose pattern of gene expression was reasonably correlated with CNS gene expression could advance research on the mechanisms of disease in neuropsychiatric disorders.

Lymphocyte gene expression profiling is an interesting area of research of peripheral biomarkers, so that there are a lot of studies that compare human blood gene expression profiling between case-controls and cross match with human postmortem brain gene expression data. Sometimes, brain gene expression is also studied in animal models and cross-validated in human fresh blood; these studies are tied to illness state, and aim to provide genetic linkage data as well as information of biological pathways (Le-Niculescu H et al, 2007). The availability of an accessible tissue whose gene expression profile is similar to more inaccessible CNS tissues has the potential to advance research in psychiatric disease and - according to the Sullivan et al. studies (Sullivan PF et al., 2006) of transcriptomic profiling data of the Genomics Institute of the Novartis Research Foundation (symatlas.gnf.org) - whole blood shares significant gene expression similarities with multiple CNS tissue. The expression levels of many classes of biological process were statistically similar between whole blood and CNS, furthermore about half of a set of candidate genes relevant to schizophrenia were expressed on both whole blood and CNS (Sullivan PF et al., 2006).

D.2. Biomarkers for diagnostics and monitoring of mental disorders

BM to use for diagnostic tests for psychiatric diseases should fulfill eight criteria (Lakhan SE, 2006): (a) detect a fundamental feature of disease with high sensitivity and specificity, (b) be validated in confirmed post-mortem cases, (c) be standardized with proper bioinformatics and proper statistics, (d) be specific for the disease compared with related disorder, (e) be preferably non-invasive, (f) be simple to perform, and (g) be inexpensive. Early diagnosis of autism, schizophrenia or bipolar disorders is important. In case of autism, early intervention in children is likewise associated with a better prognosis (Matson JL et al., 2007). In schizophrenia, the early intervention, already in the prodromal phasereduces the period of untreated psychoses and sometimes it even prevents or delays the onset of psychosis in high-risk individuals (Killackey E & Yung AR, 2007); furthermore, early adequate intervention improves social and functional outcomes and is cheaper than standard care models that start treatment once a psychosis is manifest (Killackey E & Yung AR,

2007). But the current diagnostic systems (DSM-IV and ICD-10) are neutral with respect to theories of etiology, because only limited insight is known which therapy, either pharmacological or psychosocial, is most effective.

BM identification approaches are used as research and development tools accompanied by some preliminary clinics evidence. Their main purpose is to support the generations of new hypotheses (e.g. gene expression profiling). Biomarkers that may serve as a surrogate end point are most interesting in psychiatry, since clinical endpoints of mental disorders are diverse and not well defined. The most important indicators of recovery from a mental disorder are: having paid work fit to the patient's educational background, having normal interpersonal relationships and being devoid of any symptoms that impair daily living.

D.3. Biomarkers discovery

An important aim of BM discovery is the detection of molecular disease correlates that can be used as diagnostic tools. They should furthermore have predictive power and allow the identification of at-risk individuals.

D.3.1. Strategy to discover candidate vulnerability genes in psychiatric disorders

The first step in the process involves the acquisition characterization, processing and storage of highest quality tissue (Akbarian S et al., 1996). As important as the quality of sample, is the number of these which leaves to suggest the magnitude of biobanks. As an example, more than 10 brain banks focused on psychiatric disorders have been established in the Unites States and the United Kingdom (Bunney WE et al., 2003). Collaboration between biobanks and standardized collections of samples as well as extensive accompanying information is a key of the exist of these institutions. Thereby, it is indispensable to develop standardized procedures for diagnostic, clinical characterization and reliable sources of tissues (Bunney WE et al., 2003).

After that, there are many strategies to search possible biomarkers and it is essential to design of the study that would carry out. Then, approaches to look candidate genes could have a major magnitude such as microarrays, however their results must be validated for other techniques such as real-time PCR. As interesting as these macro-studies are other type approaches. In this way, candidate genes form knowledge of pathophysiology of the diseases remains important, because is most focused on symptoms and alterations observed in the diseases. Figure 11 summarizes a strategy for discovering candidate vulnerability genes.

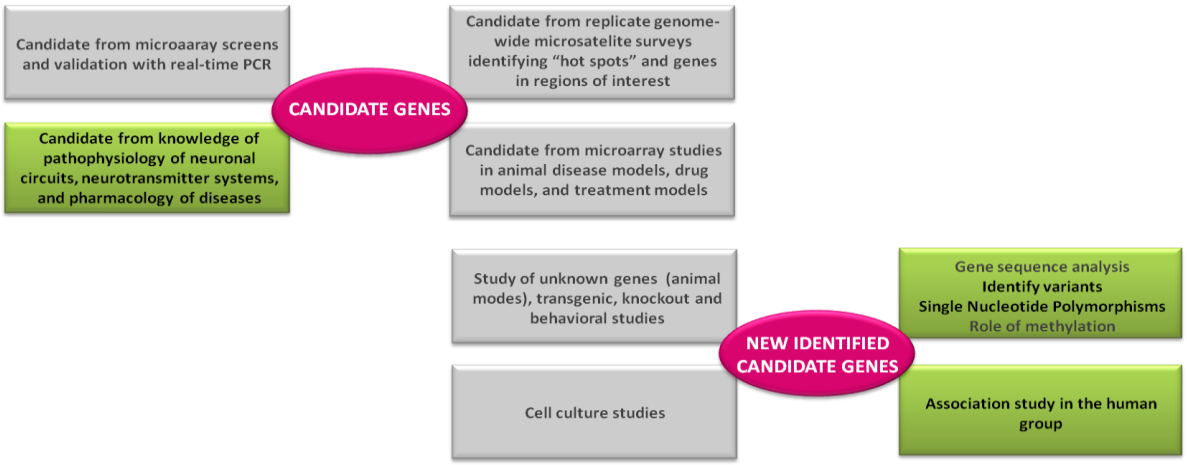


Figure 8. Strategy to discovery of candidate vulnerability genes. The figure summarizes a strategy for discovering candidate vulnerability genes in psychiatric diseases. When a gene is identified as a possible biomarker, this should be validated by different techniques and groups of patients. Green boxes (with a black letters) represent the techniques that are employed in the present thesis. *Adapted prom Bunney WE et al., 2003.*

When a gene is identified as a candidate biomarker, it is possible that the gene in question is well known, and then the problem is whether it is contributing to the diseases process. However the gene is totally unknown and it is necessarily to investigate it in experimental models such as cell culture. Gene-sequence analysis can be conducted for identify SNPs in the codding or in the promoter domain of the gene, splice variants or actual mutations. This information may help identify important functions of the genes (Pennacchio LA et al., 2001).

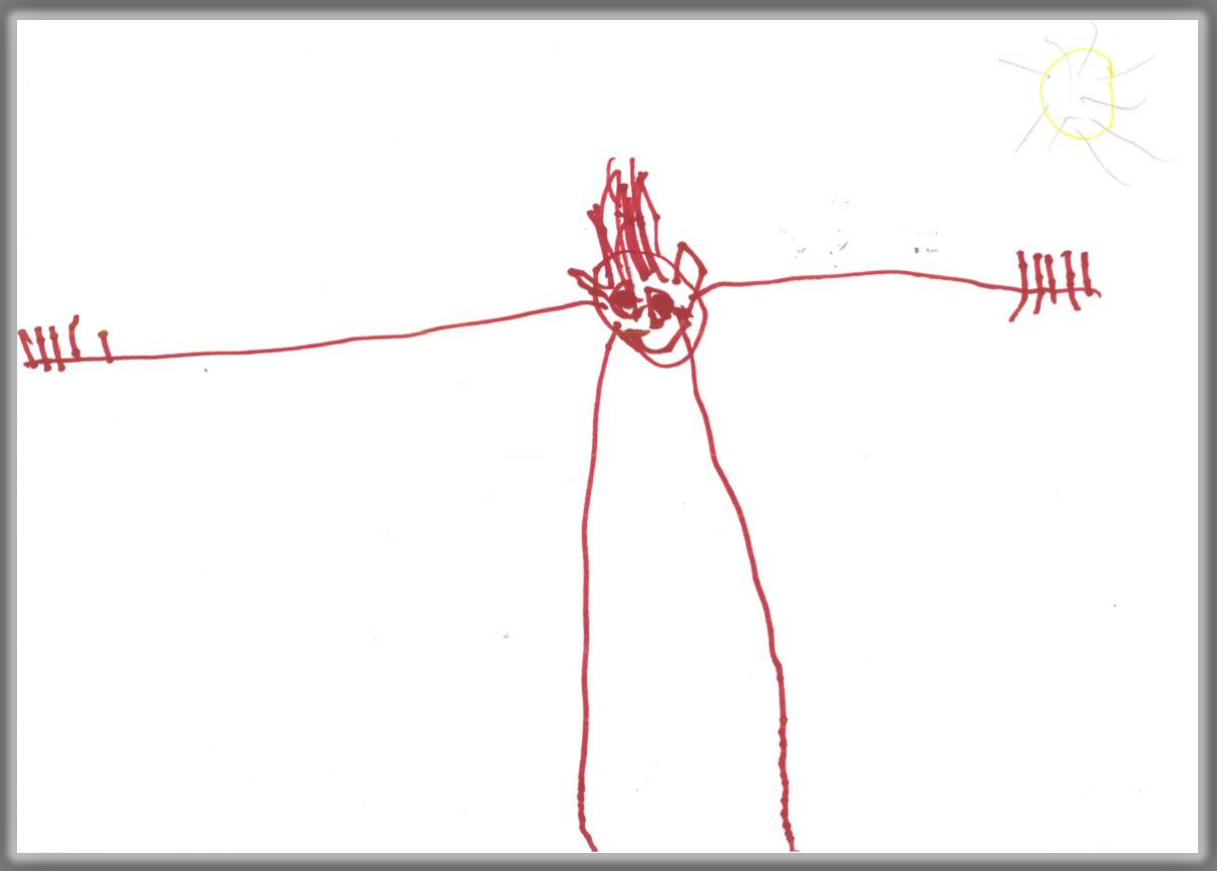
Association studies with candidate genes remain conceptually the simplest genetic studies. They are popular because specific biological hypotheses can be tested in a design similar to classical case-control study.

D.3.2. Future directions: *"omics" technologies in biomarker research*

It is now clear that psychiatric disorders are caused by a set of abnormal genes (Sawa A & Snyder SH, 2002), however one of the limitation factors is the time required to screen large number of genes, furthermore it is also a critical element the analysis of a large number of samples in studies aiming to define BM for complex psychiatric disorders. Recent advances in technology include high-throughput methods which allow the screening of tens of thousands of genes (Shoemaker DD & Linsley PS, 2002). Profiling at the level of mRNA, proteins or metabolites has not been possible on a large scale until recently, either. Therefore, BM discovery experiments based on profiling approaches facilitated by recent technical development are likely to make a great contribution to uncovering disease mechanisms, such as transcriptomics, proteomics and metabolomics and could be important tools in the future.

Subsequently, bioinformatic analysis of data will be useful to extract essential information from large amount of data that are generated by these techniques. Generally, datasets used contain a low number of samples (10's-100's) and a large number of variables (1000's-10000's), referred to as the high-dimensionality small-sample-size (HDSS) problem (Horvatovich P et al., 2006). The analysis and well-characterization of collected samples and subsequent data analysis, by appropriate bioinformatics approaches, enable the extraction of valuable information of a diagnostic or therapeutic biomarker.

Another new research area is the epigenomics that is the study of heritable changes in gene expression not coded in the DNA sequence itself. Epigenetics involves three interacting molecular mechanisms as follows: DNA methylation, modifications of histones and RNA-mediated gene silencing (Peedicayil J, 2007). The problem in studying epigenetics is the possibility that alterations, such as DNA methylation and histone modification involving gens in the brain, may not be reflected in genes in peripheral tissues (Preedicayil J, 2007); this fact is supported by the study of Sullivan, who compared gene expression in the brain and the blood in humans, and found that the median non-parametric correlation to be about 0.5, suggesting only partial correlation (Sullivan PF et al, 2006). In contrast, protein and metabolites have dynamics properties that are very valuable not only because they may be closely associated to an organism's phenotype, but also because they reflect the influence of environmental factors and allow for monitoring of disease progressing; however, epigenetic mechanisms are also thought to be dynamically regulated in response to environmental.



VI. EXPERIMENTAL PROCEDURES

A. SUBJECTS

A.1. Recruitment

The study design was approved by local ethics committees of the involved institutions:

- ✓ The Ethical Committee of the University of Wurzburg.
- ✓ *Comité Ètic de la Investigació Clínica* (CEIC) of *Hospital Universitari Psiquiàtric Institut Pere Mata*
- ✓ CEIC of *Corporació Santitària Parc Taulí* (annex 1)
- ✓ CEIC of *Fundació Altahia Manresa* (annex 2)
- ✓ *Escola Carrilet* (annex 3).

The candidate patients were asked to participate through invitation by telephone or during therapeutic sessions and after complete description of the study procedure. All subjects, parents or guardians signed the written informed consent before inclusion in the study (Annex 4). In the table 1EP all the patients are shown that were recruited from each collaborating institution. Age, comorbidity and contemporary medication were taken from the hospital records.

Table 1EP: Collaborating institutions

COLLABORATING INSTITUTION	City	Country	PATIENTS (number)
Hospital of Psychiatry, Psychosomatic and Psychotherapy	Würzburg	Gemany	920
Hospital of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy	Würzburg	Gemany	56
Salut Mental-Parc Taulí	Sabadell	Spain	23
Centre de Salut Mental de Manresa-Althaia	Manresa	Spain	9
Escola Carrilet	Barcelona	Spain	18
Universitat Rovira I Virgili	Reus	Spain	85

The 720 volunteer control subjects were recruited from the blood donor center at University of Würzburg and *Hospital Parc Taulí* of *Sabadell.*

A.2. Subjects with Autism Spectrum Disorder

One-hundred and ninety-one of subjects with autism spectrum disorders (ASD) were diagnosed as broader spectrum of pervasive development disorders (PDD) according to the DSM-IV, by The Autism Diagnostic Interview-Revised (ADI-R) (Le Couter A et al., 2003), that it is a standardized, semi-structured diagnostic interview for the use talking with the parents or caregivers of people with autism or other PDD; and The Autism Diagnostic Observation Schedule (ADOS) (Lord C et al., 2006) that it is a semi-structured, standardized assessment of communication, social interaction and play. It has been designed to assist in the diagnosis of autism and PDD and is suggested as a complementary instrument to the ADI-R.

Both tests produce an algorithm which is linked to DSM-IV and ICE-10 diagnostic criteria and exactly, the patients were include on autistic disorders, AS and PDD-NOS. Figure 1EP shows distribution of the subjects according to the Intellectual coefficient wich was used to classify the subjects included on the studies.

Intellectual coefficient (IC) was determined for each participant mostly by the Wechsler Intelligence Scale for Children (WISC) or the Wechsler Adult Intelligence Scale (WAIS) or by similar standardized scales. WISC is the test that evaluates intellectual abilities (Sagiv SK et al., 2012) of children between the ages of 6 years through to 16 years 11 months. WAIS is the most popular scale to measure intelligence in adults and older adolescents (Kaufman AS et al., 2001). According to scores obtained from WISC and WAIS, ASD patients were subdivided in two subgroups: Low Functioning Autism (LFA) when the IC is lower than 70, and High Functioning Autism (HFA) when the IC is higher than 70.

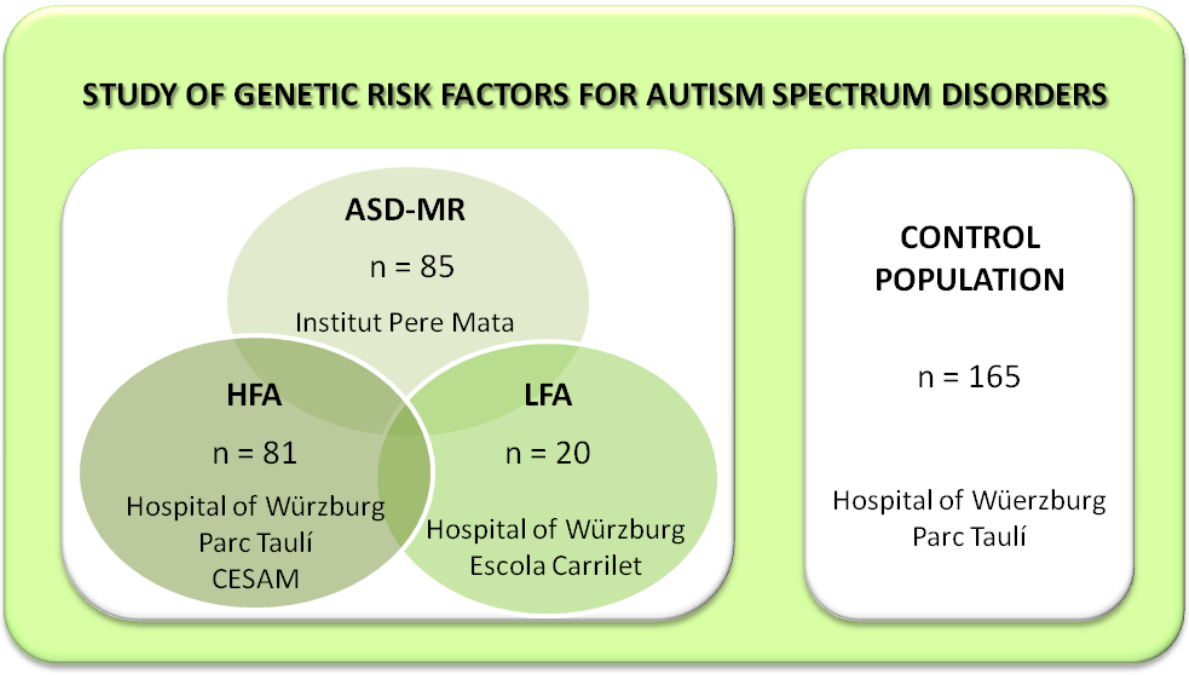


Figure 1EP. Distribution of the participants in the study on autism spectrum disorders and the collaborating institutions. *LFA (Low Functioning Autism; HFA (High Functioning Autism); ASD-MR (Autism Spectrum Disorders-Mental Retardation).*

A.3. Subjects with Schizophrenia, Bipolar and Monopolar Depression disorders

The samples, recruited from the Department of Psychiatry, Psychosomatic and Psychotherapy at the University of Würzburg, encompassed 920 cases with psychosis according to ICD10 for schizophrenia (n=280), bipolar disorders (n=400) and unipolar depression (n=220).

The sample was further subdivided according to Leonhard's classification: systematic schizophrenia (n=228), unsystematic schizophrenia (n=635), cycloid psychosis (n=309), manic depression (n=284) and monopolar depression (n=90). The figure 2EP shows distribution of the subjects according to the ICD 10 (World Health Organization, 2007) and Leonhard's classifications (Leonhard K, 1999).

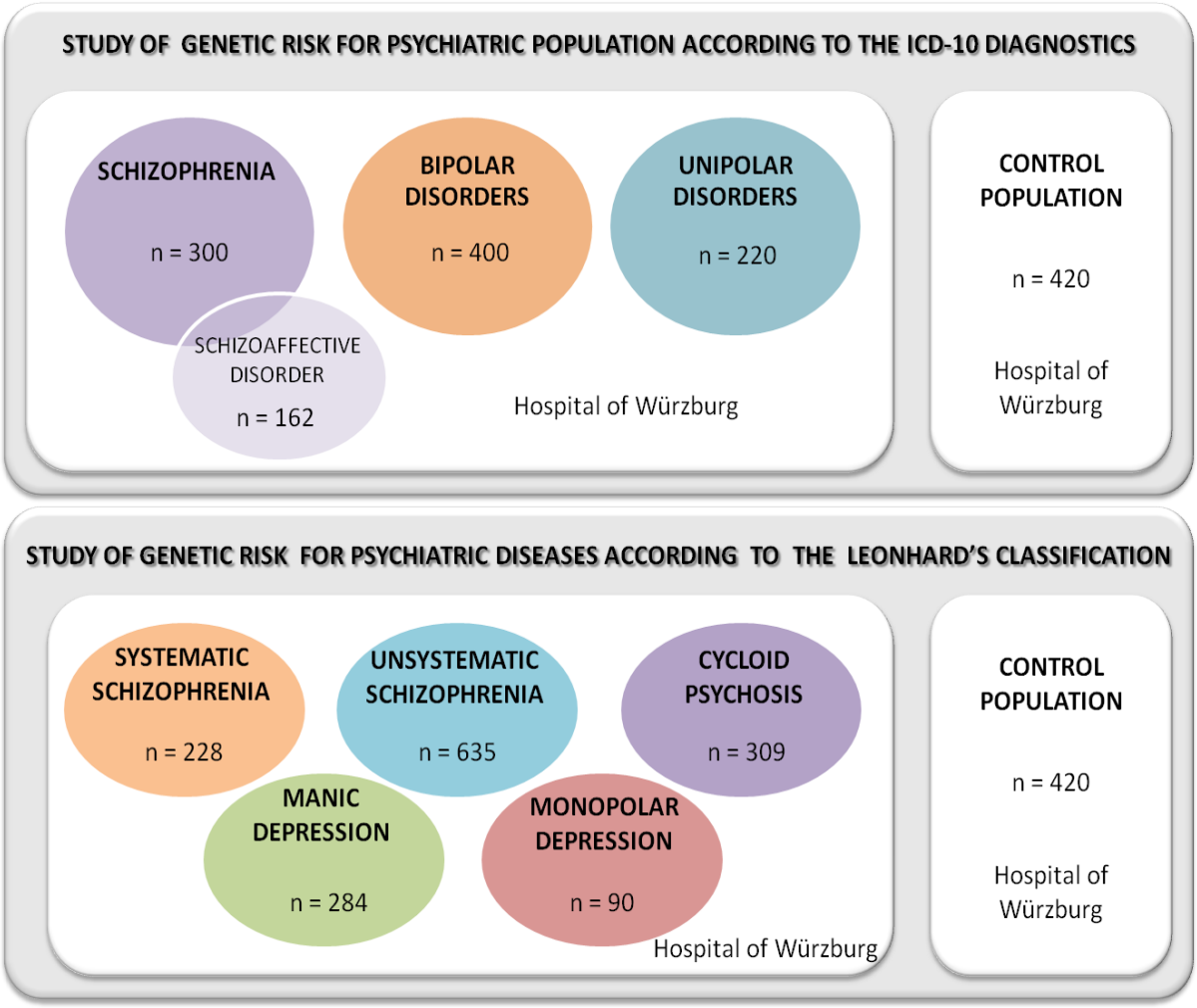


Figure 2EP. Distribution of participants in the study on genetic risk of psychiatric diseases according to ICD-10 and Leonhard's classifications.

A.4. Peripheral blood collection

Venous whole blood was collected using different tubes according to the destination of this blood; three different tubes were used to isolate DNA, RNA or plasma and peripheral blood mononuclear cells (PBMCs).

To isolate DNA, 10 ml of whole blood were drawn using Vacutainer tubes containing ethylenediaminetetracetic acid (EDTA) (Vacutainer, Ref.:367525). EDTA tubes with blood were kept at 4ºC until DNA isolation was performed within 24 h after drawing blood (See B.1.1.1).

To isolate RNA, 2.5 ml of whole blood were drawn using PAXgene tube (Quiagen, Ref.; 762165) and the sample was stored at - 20ºC until RNA isolation (See part B.2.1).

To isolate plasma and PBMCs, 6 ml of whole blood was drawn using vacutainer tubes containing acid citrate dextrose (ACD-B) (Vacutainer, Ref.:367756). ACD tubes that contain blood were kept at 4ºC until plasma and PBMcs isolation, that was performed within 24 h after drawing blood (See C.2.1).

B. MOLECULAR BIOLOGY METHODS

B.1. Genotyping assays

B.1.1. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

A variant of the PCR technique, described at the first time in 1885 by Saiki (Saiki RK, 1985), was used to determine the genotype of subjects, in relation to BDNF (rs6265) and LPHN3 (rs4860079) polymorphisms. RFLP is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples with specific restriction endonucleases.

B.1.1.1. DNA isolation

To isolate DNA, Lysis buffer [155 nM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4] was added to 10 ml of whole blood (EDTA) until a final volume of 40 ml and was shaken gently and incubated for 15 minutes on ice. The falcon was centrifuged at 350 x *g* for 15 min. at 4ºC. Followed supernatant was removed, 500 µL of Pronase (Applichem, Ref.: A3459), 660 µL of SDS 10% and 10 ml Nucleus-lysis buffer [10mM Tris-HCl, 400 mM NaCl, 2 mM EDTA, pH 7.4] were added. The pellet was resuspended gently and incubated overnight at 37ºC.

After 24 h, 3.5 ml of 6 M NaCl was added and shaken gently. The mix was centrifuged at 2500 x *g* for 20 min. at 18ºC. The supernatant was transferred into a new falcon and 20 ml of isopropanol was added and shaken by hand until the DNA precipitated. The DNA collected was transferred and dissolved into the cryotube with 500 µL TE buffer [10 mM Tris-Cl, 1 mM EDTA, pH 8].

The DNA concentration was measured at absorbance of 260 nm (A_{260}), adjusting the (A_{260}) measurement for turbidity (measured by absorbance at 320 nm), multiplying by the dilution factor, and by 50, because 1.0 unite of A_{260} is equivalent at 50 µg/ml of pure DNA (see formula 1).

$$\textbf{DNA concentration (µg/ml)} = (A_{260} - A_{320}) \times \textbf{dilution factor} \times 50\ \text{µg/ml} \quad (1)$$

The DNA purity was estimated from the A_{260}/A_{280} ratio; value between 1.7 and 2.0 generally represents a high-quality DNA sample. The ratio could be calculated after correcting for turbidity (absorbance at 320nm) (see formula 2).

$$\textbf{DNA Purity } (A_{260}/A_{280}) = (A_{260} - A_{320}) / (A_{280} - A_{320}) \quad (2)$$

Finally, total yield was obtained by multiplying the DNA concentration by the final total purified sample volume (see formula 3).

$$\textbf{DNA yield (µg)} = \text{DNA (µg/ml)} \times \textbf{total sample volume (ml)} \quad (3)$$

B.1.1.2. DNA amplification

To detect the coding variants of BDNF(rs6265) and LPHN3(rs4860079), fragments of 274 bp and 451 bp respectively were amplified using PCR. The following table (Table 2EP) shows the conditions that PCRs were performed.

Table 2EP. Reagents, primers and PCR conditions for BDNF and LPHN3 amplification.

GENE	PRIMERS	REACTION MIX		PCR CONDITIONS	
BDNF	5'-AAAGAAGCAAAGATCCGAGGACAAG-3 5'-ATTCCTCCAGCAGAAAGAGAAGAGG-3	DNA genomic	50 ng	95 ºC x 3 min.	
		$MgCl_2$	2.5 mM	95 ºC x 45 sec. 56 ºC x 45 sec. 72 ºC x 45 sec.	35 cycles
		Fordware primer	400 mM		
		Reverse primer	400 mM		
		dNTP	2.5 mM		
		Taq Polymerase	1 U	72 ºC x 3 min.	
LPHN3	5'-CAT CATACAGTAAGCATTTTT-3' 5'-TTCAAACCTATCTTTCCATAC-3	DNA genomic	50 ng	95 ºC x 3 min.	
		$MgCl_2$	1.5 mM	95 ºC x 45 sec. 62 ºC x 45 sec. 72 ºC x 45 sec.	35 cycles
		Fordware primer	200 mM		
		Reverse primer	200 mM		
		dNTP	2.5 mM		
		GeneCraft Buffer	1 X		
		Taq Polymerase	1 U	72 ºC x 3 min.	

BDNF (Brain-Derived Neurotrophic Factor); LPHN3 (Latrophilin 3); dNTP (deoxyribonucleotides).

Finally, the amplification was checked by DNA electrophoresis, where 10 µL of PCR product were resolved in 3% agarose gel (see B.1.1.4).

B.1.1.3. DNA digestion

PCR products were digested by restriction enzymes and restriction fragment length polymorphisms were obtained. In case of BDNF (rs6265), the presence of G or A alleles was determined by *Nla*III restriction enzyme (New England BioLabs, Ref.:R0125L) and in case of LPHN3 (rs4860079), the presence of both alleles was determined by *BsaBI* restriction enzyme (New England bioLabs, Ref.:R0537S). In the following table reagents and conditions of digestion (Table 3EP) are shown according to the interest gene.

Table 3EP. Reagents, restriction enzymes and temperature conditions for digestion o BDNF and LPHN3 amplicons.

GENE	RESTRICTION ENZYME	REACTION MIX		RESTRICTION CONDITIONS
BDNF	NlaII	PCR product	11 µL	2.5 h x 37 ºC
		NlaIII	1 U	
		BSA	1 g/L	
		NEB Buffer 4	1 X	
LPHN3	BsaBI	PCR product	10 µL	3 h x 60 ºC
		BsaBI	1 U	
		NEB Buffer 4	1 X	

BDNF (Brain-Derived Neurotrophic Factor); LPHN3 (Latrophilin 3); BSA (Bovine Serum Albumin).

After digestion, 10 µL of the products were separated with 3% agarose gel (see B.1.1.4). Figure 3EP shows a scheme of possible restriction fragments according to the genotype of the subject.

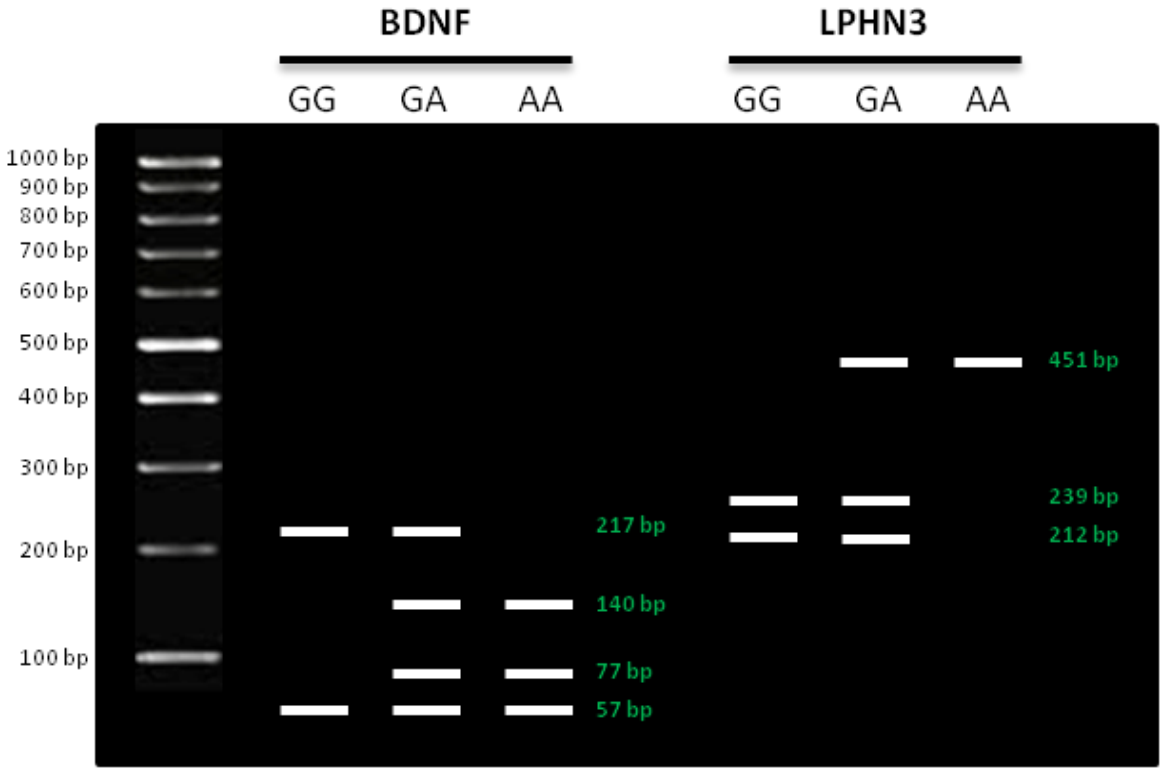


Figure 3EP. Diagram of three possible genotypes for rs6265 BDNF SNP and rs4860079 LPHN3 SNP. BDNF has three possible genotypes: homozygote with GG alleles and AA alleles, and heterozygote with GA alleles. LPHN3 has three possible genotypes, too: homozygote with GG alleles and AA alleles, and heterozygote with GA alleles. *BDNF (Brain-derived neurotrophic factor), LPHN3 (Latrophilin 3).*

B.1.1.4. Gel electrophoresis of DNA

DNA electrophoresis is a technique to separate fragments by size and reactivity, using an electric field. DNA fragments migrate through a gel matrix electrophoresis, induced toward the anode due to negative charge of the sugar-phosphate backbone of the nucleic acid.

Gels were used to check the amplification and digestion results containing 3% (w/v) of agarose (Pronadisa, Ref.:8065) dissolved on TAE Buffer [40mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8]. Ethidium Bromide as intercalating agent was used as fluorescent tag and was added in final a concentration of 0.005%, before matrix solidification.

The samples were mixed with a 1X Loading buffer [0.021% (v/v) Bromophenol Blue, 0.021% (v/v) Xylene Cyanol, 0.02 M EDTA (pH 8), 50% (v/v) Glycerol].

After loading the samples on the agarose gel, electrophoresis was carried out at 130 V for 45 min.

DNA fragments were visualized using a Gene Flash Syngene Bio Imaging system (Syngene).

B.1.1.5. Epidat Software

Epidat 3.1 is a software developed by the *Servicio de Información sobre Saúde Pública de la Consellería de Sanidade e Servicios Sociais de la Xunta de Galicia (Spain)* and the Health Situation Analysis Program (SHA) of the Pan American Health Organization that gives tools to perform epidemiological analyses. Hardy-Weinberg equilibrium was previously calculated for all participants of study (cases and controls); after population equilibrium was checked, the equilibrium was analyzed for both cohorts separately, as it is important that the control population was in equilibrium. After that, software Epidat 3.1 was used to analyze genotypic distribution.

B.1.2. TaqMan genotyping assay

TaqMan® SNP Genotyping Assays provide optimized technique for detection of polymorphisms within specific SNP alleles in purified genomic DNA samples. The advantage of using this technique is a high degree of reliability with no false positives as well as the high-throughput way of analysis. The method is based on 5' exonuclease activity by cleavage of an allele-specific 5' dye label, which generates fluorescence (Figure 4EP). In the intact probe, nonfluorescent quencher eliminate the background fluorescence associated with traditional quenchers and provides a greater signal for superior assay sensitivity. Both probes have a different fluorescent molecule associated with VIC or FAM dyes depending on nucleotide to unite.

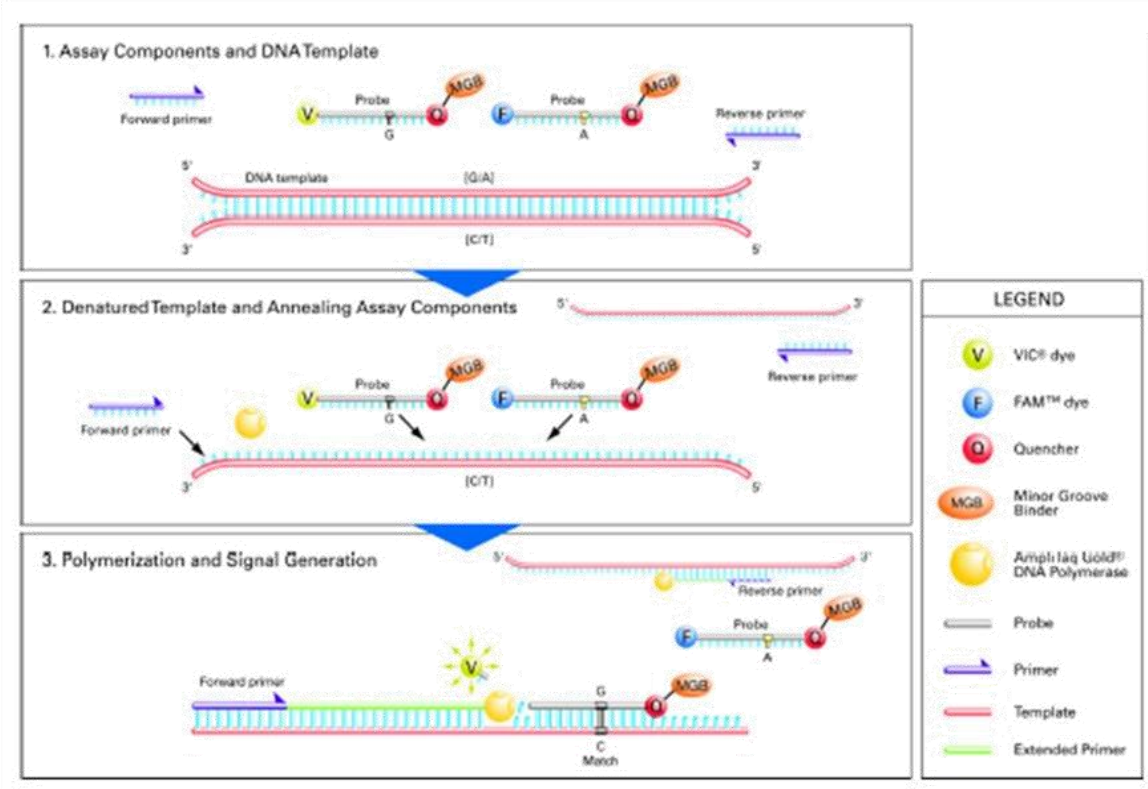


Figure 4EP. Schematic depiction of a TaqMan Genotyping Assay. Specific primers will bind to the site on the DNA strand close to the polymorphism of interest and DNA polymerase will start to elongate the new strand of DNA. At the moment the DNA polymerase reaches the probe, it will cleave the probe with 5' exonuclease activity and thereby remove the quencher from the reporter dye. The fluorescence emitted by VIC or FAM dyes is detected by software.

Different polymorphisms of EPHA4 and ANK3 were analyzed using this method. The reaction mix was composed by 12.5 µL of TaqMan®Genotyping Master Mix (Applied Biosystem, ref.: 4371357), 0.5 µL of SNP probe (40X), 20 ng of DNA and 7 µL of H_2O. Table 4EP shows the SNP probes were used.

Table 4EP. Polymorphisms and references of TaqMan SNP genotyping assays from Applied Biosystems.

GENE	GENE SYMBOL	SNP	PRODUCT REFERENCE
Ephrin type-A receptor 4	EPHA4	rs2052940	2039857
		rs2952834	27153416
		rs2248489	15919479
		rs2056290	1751887
		rs2392936	143672
		rs3087584	2039835
		rs1897120	11469582
		rs1864461	11469576
		rs3770181	11733900
		rs35084379	57896940
Ankyrin 3	ANK3	rs10994336	31344821
		rs9804190	29667085
		rs10761482	1344706

B.1.2.1. Analytical tools

Haplotype Association Analysis Software known as FAMHAP and developed by *Universität Bonn*, was used to perform haplotype analysis. This is a software for single-marker analysis and, in particular, haplotype analysis (http://famhap.meb.uni-bonn.de/documentation_famhap18.pdf) that can be used both for the analysis of case-control data and the analysis of nuclear family data. The program is optimized for the haplotype frequency estimation of many markers and provides linkage disequilibrium (LD) measures, among other things.

Furthermore, the International HAPMAP Project was consulted. The goal of this project is to determine the common patterns of DNA sequence variants in the human genome, by characterizing sequence variants, their frequencies, and correlation between them in DNA samples from populations with ancestry from parts of Africa, Asia and Europe. It is also important to make this information freely available in the public domain. The HapMap project focuses only on common SNPs with alleles that occur in at least 1% of the population (The International HapMap Consortium, 2003).

B.2. Quantitative Real Time- Polymerase Chain Reaction (qRT-PCR)

PCR was developed by Kary Mullis and coworkers in 1983 (Saiki RK et al., 1985). It is a molecular biology technique often used in a medical or biological research. One decade later, in 1992, the limitations of PCR, because only allow to evaluate the final product, were resolved by Higuchi et al. (Higuchi R et al., 1992) who developed of a real-time PCR. qRT-PCR permits quantifying amount of DNA that has been amplifying at the moment by monitoring a fluorescence.

B.2.1. RNA isolation

To isolate RNA from blood, Paxgene blood RNA kit (Quaigen, Ref.:762174) was used according to the manufacturer's instructions (with some modifications) with a DNAse treatment.

The PAXgene Blood RNA Tubes were centrifuged at 3220 x *g* for 15 min. at 20ºC. The supernatant was removed by decanting and 4 ml RNAse-free water was added, homogenized and centrifuged at 3220 x *g* for 15 min at 20ºC. The supernatant was discarded, 350 µL of resuspension buffer (BR1) was added and the mix was homogenized. After that, 300 µl of binding buffer (BR2) and 40 µl of proteinase K were added. The mix was homogenized and incubated at 55ºC for 10 min. at 1400 rpm. Then, the lysate was transferred into a PAXgene Shredder spin column (PRC) and centrifuged for 3 min. at 14000 rpm. Carefully, the supernatant of the flow-through fraction was transferred into a new tube. The next step was to add 350 µl of ethanol at 96-100% purity grade and the sample was transferred into a PAXgene RNA shredder spin column (PSC). The columns with samples were centrifuged for 1 min. at 14000 rpm. In the following, 350 µl of wash buffer 1(BR3) was added and centrifuged at 14000 rpm for 1 min. Then, 10 µl of DNase I was mixed with 70 µl DNA digestion buffer (RDD), and placed on the benchtop for 15 min. After incubation, 350 µl of washing buffer 1 (BR3) was added and centrifuged for 1 min. at 14000 rpm. Then, 500 µl of washing buffer 2 (BR4) was added and centrifuged for 3 min. at 14000 rpm. To continue de procedure, 40 µl of elution buffer (BR5) was added directly on the benchtop and centrifuged for 1 min. at 12000 rpm; the elution step was repeated one more time. Finally, RNA eluted was incubated for 5 min. at 65ºC and was chilled immediately on ice.

The RNA concentration was measured at absorbance of 260 nm (A_{260}), adjusting the (A_{260}) measurement for turbidity (measured by absorbance at 320 nm), multiplying by the dilution factor, and by 40, because 1.0 unite of A_{260} correlate with 40 µg/ml of pure RNA (see formula 4).

$$\textbf{RNA concentration (µg/ml)} = (A_{260} - A_{320}) \times \textbf{dilution factor} \times 40\ \text{µg/ml} \quad (4)$$

The RNA purity was estimated from the A_{260}/A_{280} ratio, between 1.7 and 2.0 generally represents a high-quality RNA sample. The ratio could be calculated after correcting for turbidity (absorbance at 320nm) (see formula 5).

$$\textbf{RNA Purity } (A_{260}/A_{280}) = (A_{260} - A_{320}) / (A_{280} - A_{320}) \quad (5)$$

Finally, total yield was obtained by multiplying the RNA concentration by the final total purified sample volume (see formula 6).

$$\text{RNA yield (µg)} = \text{RNA (µg/ml)} \times \text{total sample volume (ml)} \quad (6)$$

B.2.2. Retro-transcription

Reverse transcription to generate cDNA from isolated RNA (see B.2.1) was done with iScript cDNA Synthesis kit (BioRad, Ref.:170-8891). The procedure was carried out according to the manufacturer's indications (table 5EP).

Table 5EP. Manufacturer's conditions of iScript cDNA Synthesis Kit from BioRad.

REAGENT	REACTION MIX	PCR CONDITIONS
5X i Script reaction mix	4 µL	25 ºC x 5 min.
iScript reverse transcriptase	1 µL	42 ºC x 30 min.
Nuclease- free Water	(until final volume of 20 µL)	85 ºC x 5 min.
RNA template	500 ng	Hold at 4 ºC

The cDNA obtained was diluted with nuclease-free water, to 5 ng/µL and was stored at -20ºC until expression analyses.

B.2.3. DNA amplification by qRT-PCR

The amplification of gens of interest by qRT-PCR was performed with iQ™ SYBR Green Supermix (BioRad, Ref.:170-8825) in 25 µl final volume of reaction, in accordance with the manufacturer's instructions. The primers used for the amplification were standard kits previously proved by Geneglobe manufacturer. Table 6EP shows which reference of all QuantiTect Primer Assay was used to perform gene expression analyses.

Table 6EP. *QuantiTect* primers assays used for expression analyses study

GENE	GENE SYMBOL	REFERENCE	DETECTED TRANSCRIPT	AMPLICON LENGTH (bp)	OTHER DETECTED TRANSCRIPT
18S Ribosomal RNA	**RRN18S**	QT00199367	X03205	149	-
Heat Sock 70KDa protein 8	**HSPA8**	QT00030079	NM 153201	79	NM 006597
Actin β	**ACTB**	QT00095431	NM 001101	146	-
Aminolevulinate Delta-Synthase 1	**ALAS1**	QT00073122	NM 000688	113	NM 199166
Glyceraldehyde-3-phospahte Dehydrogenase	**GAPDH**	QT01192646	NM 002046	119	-
Peptidylprolyl Isomerase A (cyclophilin A)	**PPIA**	QT00052311	NM 203430	121	NM 203431, NM 021130, NM 001008741
Brain-Derived Neurotrophic Factor	**BDNF**	QT00235368	NM 170731	120	NM 170732, NM 170733, NM 170734, NM 170735, NM 001709, NM 001143805, NM 001143806, NM 001143807, NM 001143808, NM 001143809, NM 001143810, NM 001143811, NM 001143812, NM 001143813, NM 001143814, NM 001143815, NM 001143816
Neurotrophin 3	**NTF3**	QT00204218	NM 002527	104	NM 001102654
Neurotrophin 4	**NTF4**	QT00210924	NM 006179	96	-
Nerve Growth Factor (beta polypetide)	**NGF**	QT00001589	NM 002506	73	-
Nerve Growth Factor Receptor	**NGFR**	QT00056756	NM 002507	118	-
Neurotrophic Tyrosine Kinase Receptor Type 1	**NTRK1**	QT00054110	NM 001007204	112	NM 001007792, NM 002529, NM 001012331
Neurotrophic Tyrosine Kinase Receptor Type 2	**NTRK2**	QT00082033	NM 001007097	103	NM 006180, NM 001018064, NM 001018065, NM 001018066
Neurotrophic Tyrosine Kinase Receptor Type 3	**NTRK3**	QT00052906	NM 001007155	143	NM 001007156, NM 002530, NM 001012338
Latrophilin 3	**LPHN3**	**QT00007560**	NM 015236	136	-

The amplification was carried out with a CFX96™ Real-Time PCR Detection System (Bio Rad) using a Multiplate™ 96-wells unskirted PCR plate (BioRad, Ref.:MLP-9601). The reaction was done with the conditions that are shown in the following table (Table 7EP).

Table 7EP. Reagents and temperature conditions of reaction of expression analyses.

REAGENT	REACTION MIX (µL)	PCR CONDITIONS	
SYBR Green	12.5	95 ºC x 10 min	
QuantiTect Primer Assay	2.5	95 ºC x 15 sec 60 ºC x 30 sec 72 ºC x 30 sec	41 cycles
DEPC Water	9		
cDNA (5 ng/µL)	1	Melt Curve: 60 ºC to 95ºC	

DEPC (Diethylpyrocarbonate).

B.2.4. Gene expression analyses method

Gene expression was analyzed by Vandesompele method, which based on the principle that the expression ratio of two proper control genes should be identical in all samples, regardless of the experimental conditions, with increasing ratio variation corresponding to decreasing expression stability of the tested genes (Vandesompele J et al., 2002). The approximation considers that the difference between the minimum and maximum expression levels, or any outlying value, could have an influence on the regression line, and consequently on the value of the correlation coefficient.
VBA applet geNorm was used to calculate the gene expression stability measure (M) for a reference gene as the average pairwise variation (V) for that gene with all other tested reference genes. The housekeeping genes with the highest M of the tested genes were excluded, and according to recommendation of Vanseompele et al. three proper control genes were used for calculating a normalization factor (NF) (see formula 8) from their relative values of expression (Q) (see formula 7).

$$Q = E^{(minCt-sampleCt)} \quad (7)$$

Q = Sample quantity relative to sample with highest expression
E = amplification efficiency (2 = 100%)
minCt = lowest Ct value = Ct value of sample with highest expression

$$NFn = \sqrt[n]{REF\ 1 \times \ldots \times REFn} \quad (8)$$

NF = Sample normalization factor based on n reference genes
REF = Reference gene

After calculation of a normalization factor, the expression level (EL) for each sample was calculated by dividing his Q value of interest genes (IG) with the appropriate normalization factor for each sample (see formula 9).

$$EL = Q_{IG} / NF \quad (9)$$

EL = Expression level of sample
Q_{IG} = Sample quantity relative to sample with highest expression of interest gene
NF = Sample normalization factor

C. BIOCHEMICAL AND CELULAR BIOLOGY METHODS

C.1. Western Blot

The western blot is analytical technique to detect specific proteins. It was described by Towbin in 1979 (Towbin H et al., 1979), based on the specificity of binding between antigen and antibody.

C.2.1. Plasma and peripheral blood mononuclear cells (PBMCs) isolation

The ACD vacutainer tubes with blood samples were centrifuged at 800 x *g* for 10 min. at room temperature (RT). The interface top was transferred by plastic Pasteur sterile, into the cryotubes. Four aliquots of 0.5 ml per subject were kept at -80ºC.

The rest of the blood was transferred into the falcon, and RPMI 1640 with glutamine and hepes medium (PAA, Ref.:E15-842) was added until a final volume of 20 ml. The blood with the medium was homogenized during 30 minutes at room temperature (RT), while shaking. A 15.5 ml of Ficoll (Amersham Biosciences, ref.:17-1440-02) were added in a Leucosep® tube of 50 ml (OxfordImmunotec, Ref.:163290), and was centrifuged at 1000 x *g* for 1 min. In the following, the homogenized blood was decanted on Leucosep® tube and centrifuged at 800 x *g* for 15 min. at 18 ºC; it was important that the centrifuge was programmed with acceleration and deceleration ramps. Afterwards, a gradient of densities was done and the PBMCs top was visible as whitish top up to the leucosep tube membrane; PBMCs top was withdrawn carefully by plastic Pasteur pipette. The PBMCs were washed with 8 ml of RPMI 1640 with glutamine and hepes medium, centrifuged at 600 x *g* for 8 min at RT.

The last step was repeated twice and afterwards PBMCs were counted using Neubauer Chamber. Finally the cells were cryoconserved with Fetal Bovine Serum supplemented with 10% of DMSO, in a density of 10^7 cells for 1 ml approximately.

C.2.2. Lyses of PBMCs

Cells were centrifuged at 300 x *g* for 4 min. at RT. In the following, 200 µl of RIPA buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 1% (v/v) proteases inhibitors, 1% (v/v) phosphatases inhibitors, pH 7.4] was added to dissolve the PBMCs. After that, the lysates were sonicated three times for 10 sec.

C.2.3. Protein quantification

Total protein concentration of each sample was measured using Bradford reagent (BioRad, Ref.:500-0006). The method is based on quantification of absorvance between 465 and 595 nm, gived when Comassie Blue G-250 binds with proteins. Bovine serum albumin (BSA) was employed as a standard with increasing and known concentration of protein (5, 10, 15, 25, 35 and 50 µg/ml).

Duplicates of each sample were performed in a dilution of 1/3000 for plasma samples and 1/200 for PBMCs lysates. The absorbance was read with Synergy HT System (BioTek), at 595 nm and the concentration of samples was calculated from the equation of the standard curve.

C.2.4. Electrophoresis and immunoblotting

Plasma samples in a concentration of 60 µg of total protein were prepared with loading buffer [10 mM Tris-HCl, 0.4% (v/v) SDS, 20 mM DTT, 2% (v/v) glycerol, pH 6.8). On the other hand, PBMCs samples were prepared with loading buffer in a final concentration of 30 µg of total protein. All of them were heated at 95ºC for 10 min.

Samples and standards of molecular weight (Precision Plus Protein Standard dual color, BioRad, Ref.: 161-0374) were separated by polyacrylamide gel electrophoresis (PAGE), using a Criterion™ TGX™ Precast Gel with a gradient density of 4 to 15% (BioRas, Ref.:567-1085), and without SDS. Electrophoresis was carried out in a vertical midi-format electrophoresis cell system (BioRad, Ref.: 465-6001) with of electrophoresis buffer [25mM Tris, 0.2 M Glycine, 0.2% (v/v) SDS, pH 8.3], applying a voltage of 120 V for 15 min., and continuing with a voltage of 200 V.

Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Ref.:IPVH00010), applying constant amperage of 400 mA during 1.5 h in a Criterion™ Blotter System (BioRad, Ref.:170-4070), and with transference buffer [25mM Tris, 0.2 M Glycine, 10% (v/v) Methanol, pH 8.3]. Then, membranes were washed twice with TBS-T [5 mM Tris, 15 mM NaCl, 10 mM KCl, 0.2% (v/v) Tween-20, pH 7.4].

C.2.5. Immunodetection

The membranes were blocked with blocking buffer [5% (w/v) nonfat milk solid, TBS-T], under constant agitation during 1 h, with the exception of the membranes that were incubated with the anti-BDNF that were blocked for 3 h.

After that, membranes were incubated O/N at 4ºC in constant agitation with primary antibody (Table 8EP), diluted in BSA at 5% (w/v) with TBS-T. Afterward, 3 washings for 10 min. were done with TBS-T.

Table 8EP. Primary antibodies.

PRIMARY ANTIBODY	MANUFACTURERS & REFERENCES OF PRIMARY Ab	DILUTION OF PRIMARY Ab
Anti-BDNF	Santa Cruz (sc-546)	1:200
Anti-NTF3	Abcam (ab65804)	1:500
Anti-TrkB	Abcam (ab18987)	1:500
Anti-TrkA	Abcam (ab33870)	1:500
Anti-p75	Abcam (ab38335)	1:500

BDNF (Brain-Derived Neurotrophic Factor); NTF3 (Neurotrophin 3); TrkB (Tyrosine Kinase Receptor Type B); TrkA (Tyrosine Kinse Receptor Type A); p75 (Receptro p75); Ab (Antibody).

When washings were finished, membranes were incubated with anti-rabbit as secondary antibody, conjugated with peroxidase (Chemicon International, Ref.: AB142) and diluted with blocking buffer during 1 h at RT and constant agitation. Finally, membranes were washed three more times with TBS-T and specific proteins were visualized using ECL® reagent (Millipore, ref.: WBLUF0500). As a loading control of samples coomassie blue of membranes was done, and a band of approximately 25 kDa was considered. The detection system was used was SyngeneG:Box with GeneSnap software.

C.2.6. Deshybridization of membranes

To perform deshybridization of membranes, they were incubated with stripping solution (Thermo Scientific, Ref.:21059) for 30 min. at 37 ºC in constant agitation. In the following, membranes were washed three times with TBS-T.

After that, membranes were already to be hybridized with other primary antibody. For it, membranes were blocked with blocking buffer and were incubated with corresponding primary antibody. Then, membranes were also incubated with corresponding secondary antibody and proteins were visualized with ECL®.

C.2.7. Protein specific quantification

The amount of the protein of interest was quantified using Image J software, based on image processing developed by National Institute of Health. Densitometries of specific bands and a loaded control were quantified by the same method, and after that were compared between control subjects and ASD subjects.

D. STATISTICAL ANALYSES

All data were analyzed by the Graph Pad Prism package (San Diego, CA, USA), version 5.0. Statistical tests varied according to the groups included into the studies. Hence, when the study included more than two groups, Kruskal-Wallis test was employed. This is analogue of one-way ANOVA, but does not assume that samples are below normal distribution. In contrast, when only two groups were included into the studies, t-test with Welch's correction was used. This test is analogue to the t-test, but assumes unequal variances.

When $p \leq 0.05$ a significant differences between samples were assumed.

VII. CHAPTER 1

ROLE OF NEUROTROPHINS AND THEIR RECEPTORS IN AUTISM SPECTRUM DISORDERS

INTRODUCTION

A. NEUROTROPHIC FACTORS

The neuronal system has been conserved in evolution, and responsible mechanisms for development and survival of neurons are similar in most species. Thereby, neurotrophic factors are a family of proteins that promote neuronal differentiation and survival, and cause the inition of neuronal growth, developing and the maintenance of matures neurons. These molecules are involved in both central nervous system (CNS) and peripheral nervous system (PNS), some of them even are active in non-neuronal embryonic tissues or re-activated in adulthood in tissue renewal or regeneration (Deister C & Schmidt CE, 2006).

There are different families of neurotrophic molecules, however, the major part of them belongs to one of three following families (Korsching S, 1993):

a) Neurotrophins: Nerve growth factor (NGF), Brain-derived neurotrophic factor (BDNF), Neurotrophin-3 (NT-3), Neurotrophin-4/5 (NT-4/5).

b) Transforming growth factors: Glial cell line-derived neurotrophic factor (GDNF), Neurturin (NTN), Persephin (PSP), Transforming growth factor a (TGFa) Transforming growth factor b (TGFb).

c) Neuropoietic cytokines: Ciliary neurotrophic factor (CNTF), Leukemia inhibitory factor (LIF), Cardiotrophin-1 (CT-1), Oncostatin 1 (OSM), Growth promote activity factor, Tumor necrosis factor (TNF).

A.1. Neurotrophin family

Neurotrophins are classically defined as target-derived survival and differentiating factors for the development of neuronal populations, although many of them have also effects on other cell types (Levi-Montalcini R, 1987; Sariola H et al., 1994; Barbacid M, 1995; Segal RA & Greenberg ME, 1996). In the early 1950s, Levi-Montalcini and Cohen first described NGF (Levi-Montalcini R & Hamburger V, 1951; Cohen S et al., 1954). Afterwards, several novel homologous molecules belonging to the NFG family were termed neurotrophins: BDNF, NT-3, NT-4/5 (Barde YA et al., 1982; Phillips HS et al., 1990; Ibanez CF et al., 2002). Other members of the family, such as neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7), were only cloned from some teleost species but not in other vertebrates (Götz R et al., 1994; Lai KO et al., 1998).

The structure of neurotrophins is highly conserved with the exception of NT-4/5 that shares only about 50% amino acid sequence with other neurotrophins (Hallböök F, 1999; Shooter EM, 2001). An important common feature of all neurotrophins is the presence of six cysteine residues at identical positions. This leads to the formation of disulfide bridges and the similar ternary structure of the molecules. Neurotrophins are synthesized as pre-pro-proteins by both neuronal and non-neuronal cell types (Thoenen H, 1995; Seidah NG et al., 1996). Thereby, the protein is formed by a hydrophobic signal peptide for secretion -known as prepro-protein- (~30-34 KDa), and the precursor protein -known as pro-protein- (~24 KDa) (Suter U et al., 1991; Kolbeck R et al., 1994; Ibanez CF, 2002). Finally, the proneurotrophin is cleaved by furin and proconvertases in the endoplasmic reticulum and Golgi, and is converted to the mature protein (~14 KDa) (Seidah NG et al., 1996). Alternatively, proneurotrophins can be transported to the plasma membrane and released in an unprocessed form –proneurotrophin- (Figure 1.C1) (Seidah NG et al., 1996; Lee R et al., 2001). In this case,

plasmin or another extracellular protease can convert the pro-protein to the mature form, through proteolytic cleavage (Pang PT et al., 2004).

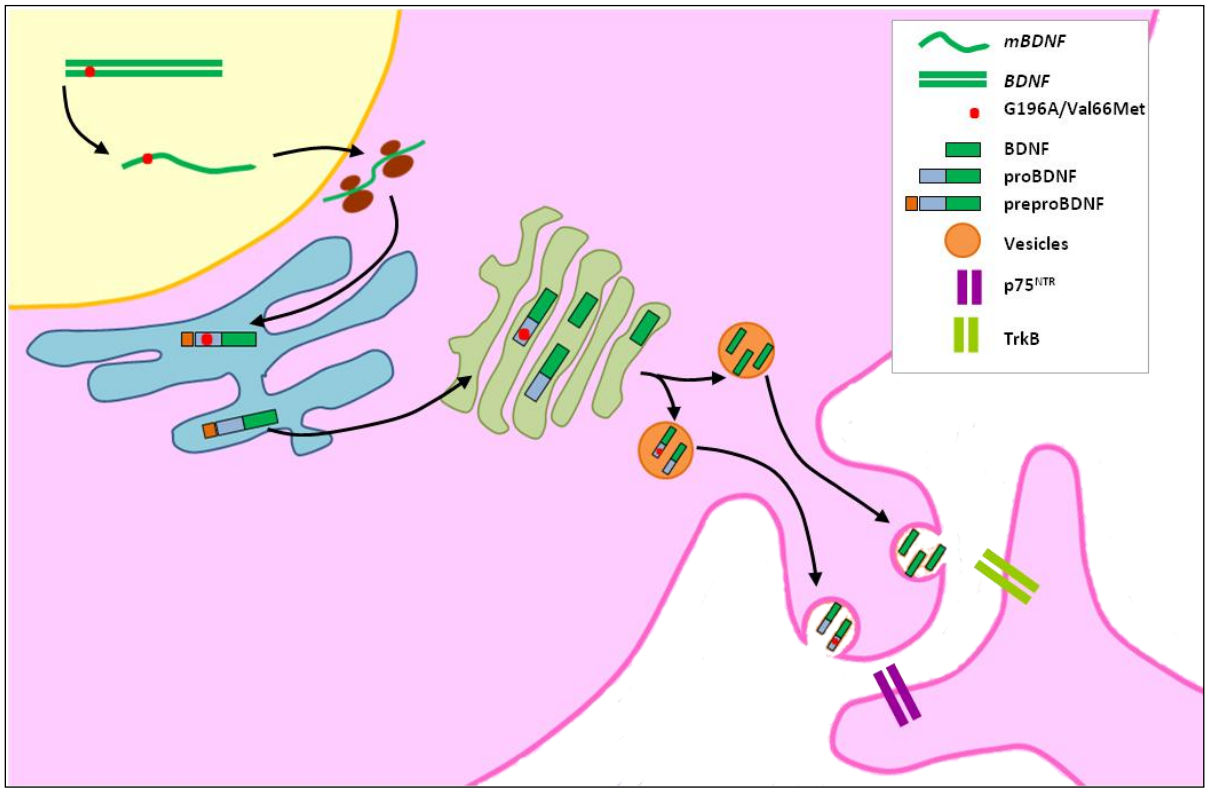


Figure 1.C1. Synthesis of BDNF from preproBDNF and secretion. The *BDNF* gene produced preproBDNF protein, which is cleaved in the endoplasmatic reticulum. ProBDNF binds to intracellular sortilin in the Golgi apparatus to facilitate the folding mature protein; even so, proBDNF can be released as immature form in the synaptic space. When the mature BDNF and proBDNF are in synaptic space, they bind preferentially with TrkB and p75NTR, respectively. The process can be adapted for other members of neurotrophin family. *Adapted from Krishnan V & Nestler EJ, 2008; and Hashimoto K, 2006.*

Neurotrophins in their mature form are secreted as homodimers into the extracellular space. They act in a paracrine and/or autocrine way, controlling many processes in development of the nervous system including proliferation, migration, differentiation, survival, apoptosis and synaptic modulation as well as influence on memory and behavior (Lu J et al., 2005; Glebova NO & Ginty DD, 2005; Zweifel LS et al., 2005; Schecterson LC & Bothwell M, 2008; Lessmann V & Brigadski T, 2009). On the other hand, neurotrophins plays a role in adult statements like synaptic plasticity, and loss of this process can results in cognitive impairments, that can be observed e.g. in ASD (Lim KC et al., 2003).

A.1.2. Brain-derived neurotrophic factor (BDNF)

BDNF is located at chromosome 11p14.1. and is the most abundant neurotrophin in brain (Altar CA et l., 1997). The structure and regulation of *BDNF* have been extensively studied in rodents, although the human gene is also known (Timmusk T et al., 1993; Aid T et al., 2007). Human *BDNF* gene is composed of seven 5' noncoding exons which are associated with different promoters, and one 3' exon that encodes the ~ 32 KDa preproBDNF protein (Liu QR et al., 2006; Timmusk T et al., 1993; Pruunsild P et al., 2007). This prepro-form can be cleaved to produce the ~ 24 KDa proBDNF and ~ 14 KDa mature BDNF protein (Mowla SJ et al., 2001). Eight distinct mRNAs are transcribed as response to distinct environmental and regional demands, getting differential subcellular targeting (Wong J et al., 2009). Transcripts which contain exons I-III are

expressed predominantly in the brain, whereas exon IV is found in lung and heart (Fang H et al., 2003; Liu QR et al., 2006; Aoyama M et al., 2001). As previously described, BDNF can be released into the extracellular space in the forms proBDNF and BDNF (Figure 1.C1). However, Mowla SJ and colleagues postulated that proBDNF is the released form (Mowla SJ et al., 2001), raising the possibility of postsecretory proteolytic processing by membrane-associated or extracellular proteases in the modulation of BDNF action (Lee FS et al., 2001).

BDNF binds to $p75^{NTR}$ and TrkB receptors. TrkB also interacts with NT-4/5. During development, BDNF supports the survival and differentiation in both central and peripheral neuronal populations. The functions of BDNF refer to axonal growth, as well as modulation of dendritic growth and morphology (Binder DK & Scharfman HE, 2004; Bibel M & Barde YA, 2000). This neurotrophin also has a prominent role in the developmental in later stages of the nervous system as well as in adulthood, where it regulates synaptic transmission and plasticity (Pezet S & McMahon SB, 2006). Accumulated data in recent years suggest, that neuronal activity regulates transcription of *BDNF*, transport of mRNA and protein into dendrites, as well as the secretion of BDNF protein. These processes are important for the formation of appropriate synaptic connections and for learning and memory during development and in adults (Lu B, 2003). The antagonist signalling via $p75^{NTR}$ is believed to be important for the maintenance of synaptic homeostasis (Woo NH et al., 2005).

BDNF is a prerequisite for more complex neuronal structures (Chao MW, 2000) and is highly expressed in hippocampus, frontal cortex, amygdala and hypothalamus of mammals (Yan Q et al., 1997). Furthermore, *BDNF* is expressed in immune cells (Kerschensteiner M et al., 1999), endothelial cells (Donovan MJ et al., 2000) and in liver and muscle tissue (Cassiman D et al., 2001; Matthews VB et al., 2009). BDNF is transported in the blood but the source is heavily debated (Gass P & Hellweg R, 2010), although it is believed that CNS is the major contributor to blood BDNF levels (Rasmussen Pet al., 2009).

A.2. Neurotrophin receptors

Neurotrophins bind to three distinct types of receptors: members of the Trk receptor tyrosine kinase family (TrkA, TrkB and TrkC), the p75 neurotrophin receptor ($p75^{NTR}$) and the sortilin (Figure 2.C1). The cleaved mature forms of neurotrophins bind with high affinity to Trk receptors, which may be in a complex with $p75^{NTR}$, whereas proneurotrophins bind preferentially to $p75^{NTR}$ in a complex with the sortilin co-receptor (Nykjaer A et al., 2004). Thereby, NGF preferentially binds TrkA, BDNF and NT-4/5 bind TrkB, and NT-3 binds TrkC. Additionally, all neurotrophins interact with $p75^{NTR}$. On the other hand, proneurotrophins are also active as ligands of Trk receptors, but their binding elicits functional effects opposite to those elicited by binding of mature neurotrophins (Lee R et al., 2001; Teng HK et al., 2005). All proneurotrophins interact with $p75^{NTR}$ or co-receptor complex of $p75^{NTR}$ and sortilin receptors (Lee R et al., 2001; Nykjaer A et al., 2004; Teng HK et al., 2003).

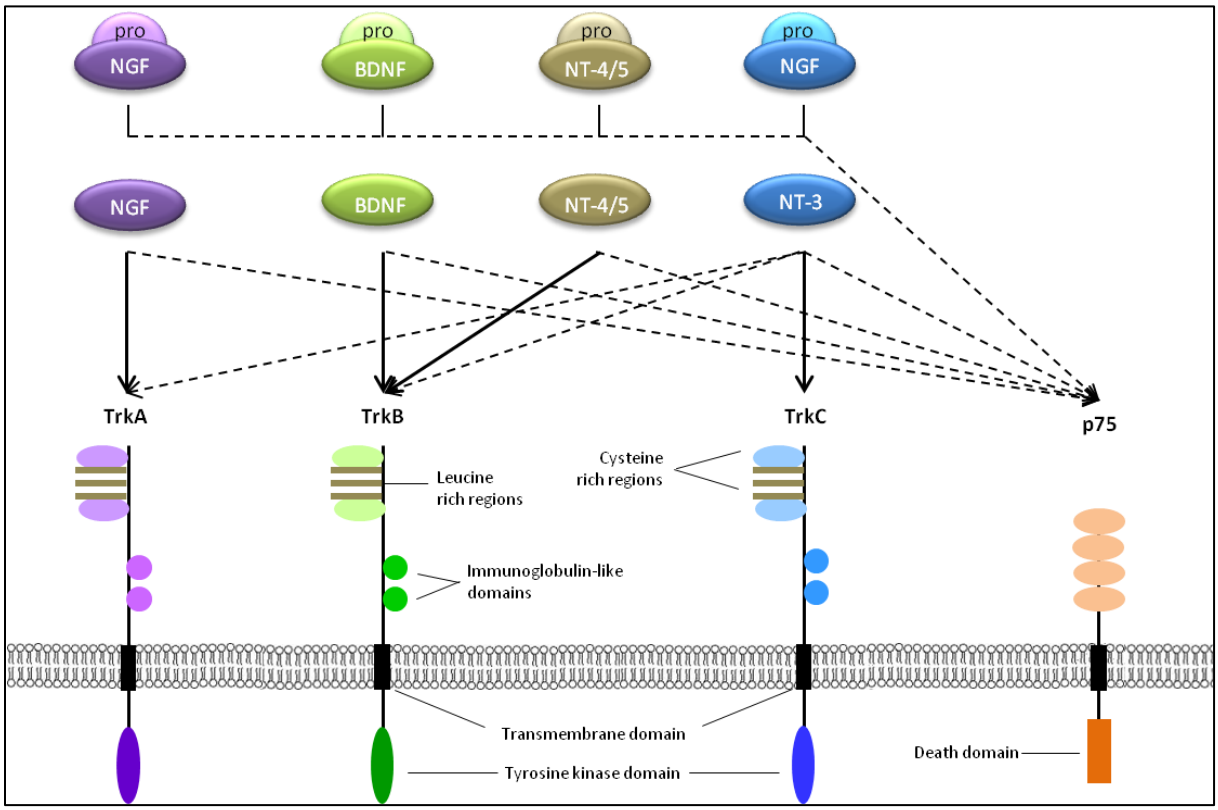


Figure 2.C1. Schematic design of neurotrophin receptors and their specific ligands. Neurotrophins bind to their receptor with high affinity (continue line) or low affinity (discontinuous line). Trk receptors contain a tyrosine kinase domain in their intracellular region; the extracellular regions are formed by two residues of cysteine, leucine rich region and two immunoglobulin-like domains and transmembrane domains. $p75^{NTR}$ receptor encompasses an extracellular domain with four cysteine rich regions, a transmembrane domain and death domain.

A.2.1. Tyrosine kinase receptors (Trk)

The structure of Trk receptors contains three well differentiated regions: an extracellular ligand binding-region, a transmembrane region and the cytoplasmatic region. The cytoplasmatic region has a tyrosine kinase catalytic domain, and the extracellular region of Trk receptors contains two cysteine-rich clusters, three leucine-reach repeats domains and two immunoglobulin-like (Ig) domains. The 2nd Ig domain enables each of Trk receptors to bind selectively with its specific neurotrophins, although Trk catalytic activity is also regulated by other extracellular domains (Arevalo JC et al., 2000). When the neurotrophins binds to Trk receptors, these dimerize (Ohira K et al., 2001), and activate the tyrosine kinase catalytic domain, causing auto-phosphorylation and consequent activation (Huang EJ & Reichardt LF, 2003).

The signal transduction of Trk receptros has been intensively studied and many downstream target molecules have been identified (Kaplan DR and Miller FD, 1997). The role of Trk receptors in mediating neuronal survival, differentiation, and synaptic function has been well defined. The major signalling pathways activated (Figure 3.C1) are similar to those of other tyrosine kinase receptors, including activation of the PI3 kinase-Akt, ras-MAP kinase, and PLCɣ signaling pathways, which have been extensively studied (Friedman WJ & Greene LA, 1999; Kaplan DR and Miller FD, 2000; Patapoutina A & Reichardt LF, 2001).

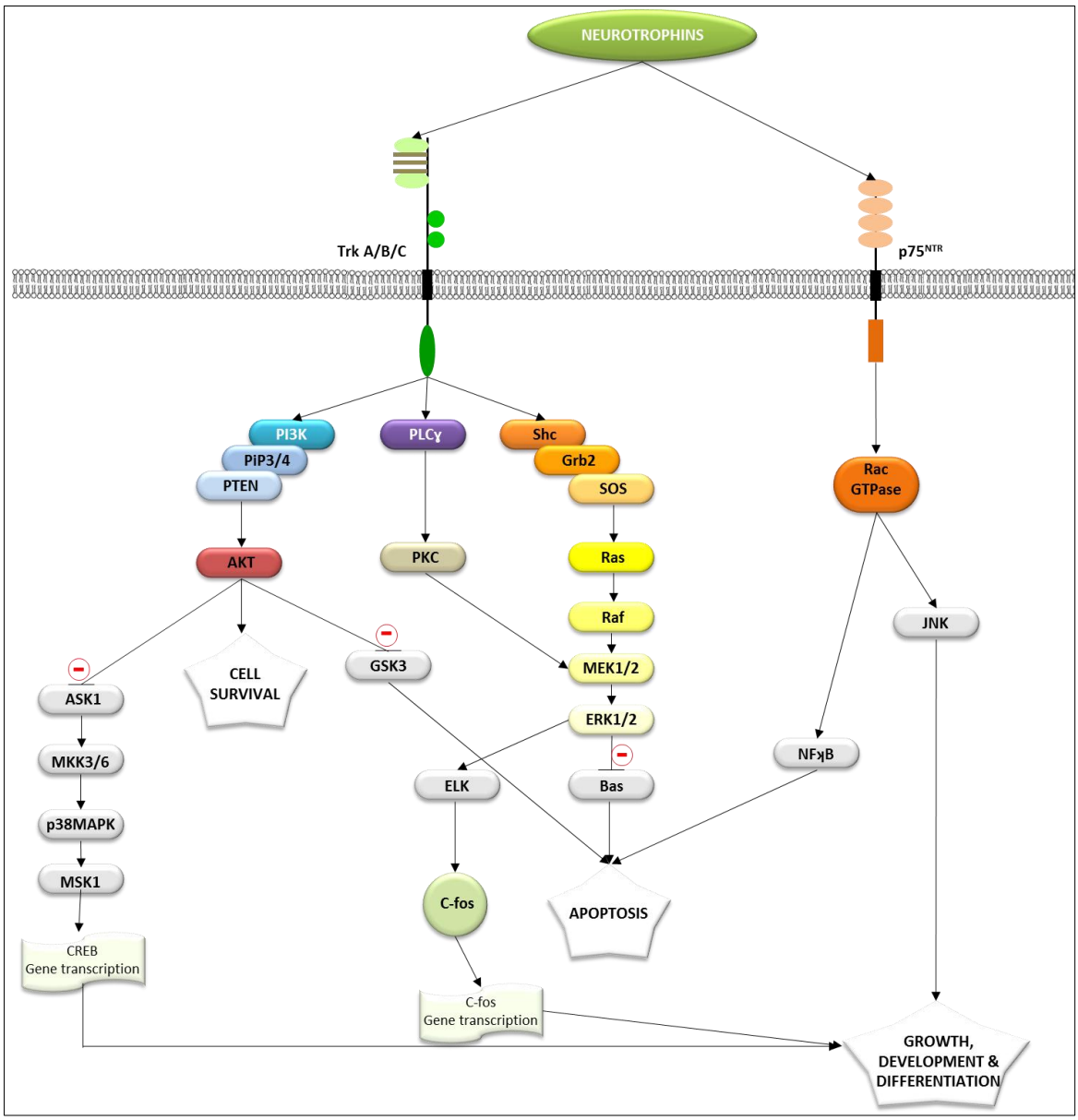


Figure 3.C1. The major signalling pathways activated by neurotrophins when interacts with Trk or p75^{NTR} receptors. *Adapted form abcam.com.*

A.2.2. Neurotrophic p75^{NTR} receptor

The structure of p75^{NTR} receptor is encompassed by an extracellular region with four cystein-rich domains, a transmembrane domain and the intracellular domain (Figure 2.C1). The cytoplasmatic domain, which is named death domain, is characteristic for all members of the tumor necrosis factor family receptors and their activation induces apoptosis (Liepinsh E et al., 1997).

In contrast to Trk receptors, the function of the p75^{NTR} has not been clearly defined. This receptor can form complexes with other co-receptors to mediate many diverse cellular functions, such as regulating survival,

differentiation, synapse plasticity and axonal growth, depending on the cellular context (Underwood CK & Coulson EJ, 2008). The most clearly defined role of $p75^{NTR}$ thus far is in signal apoptosis (figure 3.C1), particularly in response to proneurotrophins (Cassaccia-Bonnefil P et al., 1996; Coulson EJ et al., 1999; Frade JM et al., 1996; Friedman WJ, 2000; Beattie MS et al., 2002; Lee R et al., 2001; Volosin M et al., 2006).

A.3. The role of proneurotrophins versus neurotrophins

It is evident that neurotrophins can regulate opposite functions in the brain, depending on which form of the protein is secreted and which receptor and signal pathway is activated. The pro-domain of neurotrophins is only involved in promoting the folding of the mature protein (Kolbeck R et al., 1994; Rattenholl A et al., 2001; Suter U et al., 1991). However, current data reveal that proNGF may preferentially bind $p75^{NTR}$ whereas the mature form preferentially binds Trk receptors (Lee R et al., 2001). This preferential interaction has an important effect because proNGF-$p75^{NTR}$ interaction elicits apoptotic signalling and it is the most clearly defined role for $p75^{NTR}$ (Lee R et al., 2001; Casaccia-Bonefil P et al., 1996; Frade JM et al., 1996; Friedman WJ, 2000). Additionally, the death of oligodendrocytes following spinal cord injury was mediated mainly by proNGF (Beattie MS et al., 2002). Interestingly proNGF is the dominant form of NGF in the brain and is increased in Alzheimer's diseases (Fahnestock M et al., 2001), spongiform encephalomyelopathy (Stoica G et al., 2008) and Parkinson's diseases progression (Chen LW et al., 2008). Thus, when the balance between the proneurotrophin and mature form is altered, it may have profound implications for neuronal development, synaptic modulation and pathological diseases.

Like NGF, the unprocessed proform of BDNF was originally thought to be a precursor with no biological function of the own. However, multiple studies suggest that proBDNF may escape intracellular processing and be secreted from neurons (Lee R et al., 2001; Pang PT et al., 2004; Woo NH et al., 2005; Bruno MA & Cuello AC, 2006; Yang J et al., 2009). The principal role of proBDNF is modulating the synaptic efficacy during development (Woo NH et al., 2005; Yang J et al., 2009. The pro-region of the neurotrophin may also participate in intracellular synaptic targeting and activity-dependent secretion. This feature is supported by the impairments in hippocampal activity and memory function in human subjects with the Met-BDNF allele, which are located in proregion of BDNF. The regulated secretion of the BDNF Val allele is observed upon depolarization of neurons, whereas the release of BDNF with Met allele is markedly reduced (Hariri AR et al., 2003). These data suggest that the region pro is an important regulator of synaptic targeting and activity-dependent secretion of BDNF.

B. THE ROLE OF NEUROTROPHINS IN AUTISM SPECTRUM DISORDERS (ASD)

Scientific evidence supports the notion that ASD are neurodevelopmental disorders (Belmonte MK et al., 2004; Polleux F & Lauder JM, 2004). Impairments in social interaction, communication and language are characteristic of ASD. For the underlying neurobiology of these symptoms, disturbances in neuronal development and synaptic plasticity have been discussed. Thereby, neurotrophins are good candidates for a pathophysiological involvement in ASD because of their fundamental roles in guiding CNS development and cortical organization, and their abnormal expression patterns in autistic individuals (Pardo CA & Eberhart CG, 2007).

During development, neurotrophins – as already above mentioned- regulate cell proliferation, migration and survival, modulation of axonal and dendritic growth, synapse formation and other neuroplastic processes (Avita A et al., 2003). They induce cellular proliferation, migration, differentiation and integrity, which are also affected in patients with ASD (Nickl-Jockschat T & Michel TM, 2011).

The potential role of neurotrophins has been examined in several studies involving heterogeneous groups of neurodevelopmental disorders (Minshew NJ, 2007; Nelson KB et al., 2001; Riikonen R, 2003). Interestingly, abnormalities in neurotrophins, especially BDNF, have been implicated in the etiology of several brain disorders (schizophrenia and depression) that show altered cortical maturation and plasticity, (Neumeister A et al., 2005; Durany N & Thome J, 2004; Durany N et al. 2001). Neurotrophins have also been studied in ASD (Correia CT et al., 2010; Hashimoto K et al., 2006; Miyazaki K et al., 2004; Sokol DK et al., 2006; Perry EK et al., 2001; Nelson KB et al., 2001). However, until now, the role of neurotrophins in the latter disease has not been confirmed and findings are controversial. Hence, the need for more research about the involvement of these neuronal growth factors in the pathophysiology of ASD is evident.

B.1. Brain-derived neurotrophic factor (BDNF): a peripheral biomarker for autism spectrum disorders?

Decreased levels of BDNF have been linked to various psychiatric disorders including schizophrenia, bipolar disorder, major depression, and ASD (Kozisek ME et al., 2008), although the exact role of this neurotrophin remains unknown. Up-regulation of BDNF and TrkB mRNA has been observed following antidepressant treatment (Nibuya M et al., 1995). Since then, multiple clinical studies have shown decreased serum BDNF levels in patients with major depression (Brunoni AR et al., 2008; Karege F et al., 2005; Lee BH et al., 2007), as well as that the majority of antidepressant treatments can revers the lower serum BDNF levels (Brunoni AR et al., 2008; Sen S et al., 2008).

Genetic studies and expression of BDNF in serum of patients with ASD have pointed out potential links to the pathogenesis of autism. Nelson and colleagues found elevated levels of BDNF in archived neonatal blood samples of ASD patients (Nelson KB et al., 2001). This finding has been supported for many other researchers, as for example in the study on 18 Japanese children with ASD where overall increase of BDNF levels was shown (Miyazaki K et al., 2004). High levels have also been demonstrated with the presence of auto-antibodies against BDNF in a study of American children with ASD (Connolly AM et al., 2006). In contrast to these previous studies, Hashimoto et al showed a lower BDNF concentration in serum of young autistic male adults (Hashimoto K et al., 2006). These results were supported by another study that showed reduced levels of BDNF in children 0-9 years old compared with teenagers or adults, or with age-matched healthy controls, indicating a delayed BDNF increase during development (Katoh-Semba R et al., 2007).
The role of BDNF in the pathogenesis of ASD is still unknown and whether this increase is a primary or secondary mechanism to cortical abnormalities in ASD. Even so, it is suggested that genetic changes in autistic individuals account for altered neurotrophins levels, a finding supporting the hypothesis that BDNF deregulation could be a primary factor in the development of pathology (Pardo CA & Eberhart CG, 2007).

B.1.2. *BDNF* Val66Met polymorphism and psychiatric disorders

The *BDNF* Val66Met is a extensively investigated polymorphism which is relatively frequent in Caucasians (19-25%) and in Asians (45%) (Hwang JP et al., 2006; Shimizu E et al., 2004). This SNP is exclusive of human BDNF, although knock-in mice and transfections *in vitro* are used to study it (Chen ZY et al., 2004; Egan MF

et al., 2003). The replacement of guanine (G) with adenine (A), in 196 position, causes an amino acid shift from a valine (val) to a methionine (met) amino acid in codon 66, which is located in the pro-region of BDNF. The secretion of BDNF is given by two ways: 1) through regulation (activity-dependent), and 2) constitutive pathway. Regulated secretion pathway is modulated by the interaction of proteins in the Golgi apparatus with the proBDNF, where val66met SNP is located (Krishnan V & Nestler EJ, 2008).

Egan and colleagues showed that this change to met produces severe disturbance of BDNF vesicle sorting to the activity-regulated secretory pathway, while the constitutive pathway was unaffected (Figure 4.C1) (Egan MF et al., 2003). These findings were confirmed using knock-in mice with met in either one or both alleles (Chen ZY et al., 2006). Altered activity-dependent secretion of BDNF was observed in primary culture cells of these mice, as well as normal levels of total BDNF in brain.

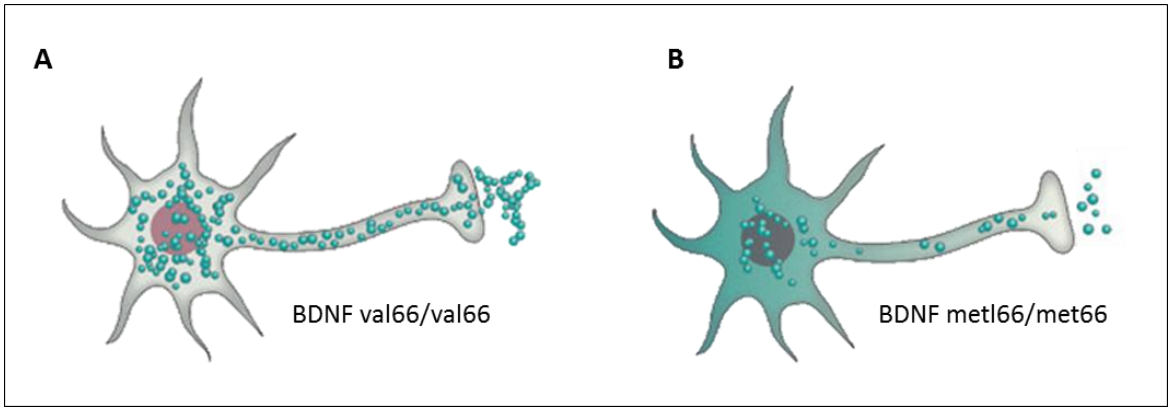


Figure 4.C1. Neuronal secretion of BDNF. **(A)** BDNF val66/val66 vesicular secretion. Vesicles that contain val-66 BDNF are properly distributed and secreted along the neuronal soma and axon. **(B)** BDNF met66/met66 vesicular secretion. In this case, the vesicles that contain this variant have impaired intracellular trafficking. Met-66 BDNF is not properly sorted within the cell; as a consequence there is a distribution throughout the cell body outside the vesicles. Finally, less BDNF is secreted from the nerve terminal. *Adapted from Krishnan V & Nestler EJ, 2008.*

Although there are not differences in total protein levels (Trajkovska V et al., 2008; Tramontina J et la., 2007), some effects caused by altered BDNF secretion have been described. For example, *in vivo* neuroimaging studies of healthy subjects have shown that met-allele is associated with lower prefrontal cortex and hippocampus volumes (Pezawas et al., 2004) and poorer activity in these brain regions involved in human memory and learning (Egan MF et al., 2003; Hariri AR et al., 2003). Moreover, several neuropsychiatric diseases have been associated with the met-allele (Gratacos et al., 2007). These findings are supported by the association of the SNP with obsessive-compulsive disorder (Hall D et al., 2003), attention deficit hyperactivity disorder (ADHD) (Kent L et al., 2005), anxiety-related personality traits (Lang UE et al., 2005; Hünnerkopf R et al., 2007) and bipolar disorder (Xu J et al., 2010).

Controversial results have been reported of the involvement of val66met in ASD. Thereby, there is evidence that the SNP is positively associated with susceptibility of pathology (Raznahan A et al., 2009), but these findings are not supported by other studies (Gadow DK et al., 2009; Nishimura K et al., 2007).

OBJECTIVES

The aims of the present study were to:

⇒ evaluate association between rs6265 ("val66met") SNP and three subgroups of ASD (HFA, LFA and ASD-Mental Retardation)

⇒ evaluate the mRNA expression of neurotrophins (*BDNF*, *NT-3* and *NT-4/5*) and their receptors (*TrkA, TrkB, TrkC* and $p75^{NTR}$) in peripheral blood of ASD patients

⇒ determine levels of neurotrophins (*BDNF* and *NT-3*), TRK (*TrkA* and *TrkB*) and $p75^{NTR}$ receptors in plasma samples of ASD

⇒ compare how Val66Met polymorphism influences mRNA expression of *BDNF* gene.

EXPERIMENTAL PROCEDURES

Technical procedures to perform the present study are detailed in headland VI, experimental procedures.

EXPERIMENTAL PROCEDURE	HEADLAND	PAGE
RFLP-PCR	B.1.1.	72
qRTPCR	B.2.3.	78
Western Blot	C.1.	81
Statistical Analysis	D	84

RESULTS

1. DESCRIPTION of SUBJECTS

A total of 349 subjects were included in the study of neurotrophins as potential biomarkers of ASD. This representative population was divided into two important cohorts: control subjects, meaning that individuals were free of diseases, and ASD population that encompasses a heterogenic autistic population (Figure 5.C1). In turn, control population was separated in a adolescent plus adult subgroup (>16 years; 21.54 ± 5.67 years) and children subgroup (<16 years; 11.38 ± 2.35 years). ASD cohort was also divided in same subgroups according to the age of participants. Then, adults ASD were subdivided into autistics with mental retardation and high functioning autistics (19.79 ± 6.7 years). On the other hand children with ASD were subdivided into high-functioning autism (IQ > 70; 10.47 ± 3.02 years) and low-functioning autisms (IQ < 70; 7.98 ± 2.22 years).

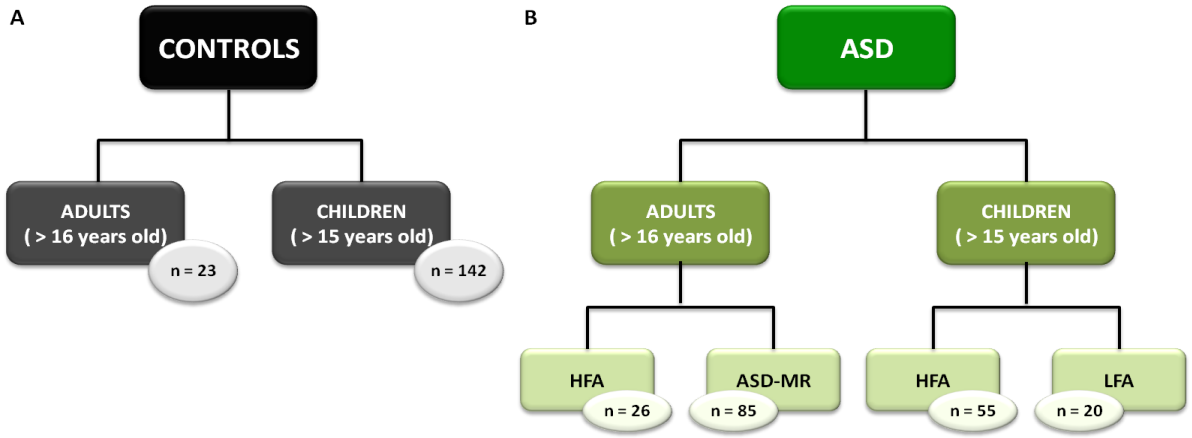


Figure 5.C1. Distribution of the volunteer participants in the study of neurotrophins and their receptors as potential biomarkers for ASD. (A) Control population subdivided in two groups: adolescents plus adults which are older than 16 years. **(B)** ASD population, which was also subdivided into these two groups. Moreover adults ASD were subdivided in HFA and ASD-MR, and children were subdivided in HFA and LFA groups.
ASD: Autism Spectrum Disorders; LFA: Low Functioning Autism; ASD-MR: Autism Spectrum Disorders-Mental Retardation; LFA: Low Functioning Autism.

2. BDNF val66met (rs6265) POLYMORPHISM IN ASD

The Val66Met (rs6265) polymorphism is a functional *BDNF* SNP implicated in higher-order personality traits such as neuroticism as well as the pathogenesis of anxiety, depression, Bipolar disorders and schizophrenia (Hünnerkopf et al., 2007; Jiang X et al., 2006; Xu J et al., 2009; Whalley HC et al., 2009). This implication is also revised in ASD, as for example in order to determine whether rs6265 gene variant can influence anatomy of autistic brains (Raznahan A et al., 2009).

Therefore, the first experimental aim of this study was to determine the relationship between ASD and Val66Met SNP as well as if the potential association can condition the level of functioning of the patients. Thereby, 165 controls and 190 ASD patients were genotyped, obtaining the following genotype *BDNF* percentages: in total population frequences were 61.40% of GG, 30.42% of GA and 6.19% of GA, which did not deviate from Hardy-Weinberg equilibrium (X^2 = 2.87, p > 0.05). After that, patients were analyzed according to the three subgroups above detailed. For all analyses, subjects homozygous for the *BDNF* Val allele, as well as subjects with a presence of this minor allele (GA) were compared to all others (GG). Distribution of genotypes

in the control population was consistent with Hardy-Weinberg equilibrium (X^2 = 0.581, p = 0.446), with the following percentages: 61.21% with a GG genotype, 32.73% with a GA and 6.06% (Table 1.C1).
Firstly, the association between Val66Met and HFA subgroup was tested. Genotype distribution of HFA patients was 64.37% of GG, 27.59% of GA and 8.08% of AA. The met allele frequency was not statistically significant across case and controls groups (p = 0.639) (Table 1.C1).

As shown in table 1.C1., LFA group was compared with controls and no statistical association was found between the presence of rs6265 SNF and ASD pathology (p = 0.357). Genotype distribution for this population was 77.78% of GG, 16.67% of GA and minor representation of AA with 5.56% (Table 1.C1).

Table 1.C1. Comparison of BDNF Val66Met genotype distribution between controls and three differentiated groups of patients.

ASD SUBGROUP	GENOTYPE	HFA (%)	CONTROL (%)	X^2 *p*	X^2 *p (each genotype)*	OR	95 % IC for OR	
							Lower limit	Upper limit
HFA	GG	64.37	61.21	0.634	(ref)	(ref)	(ref)	(ref)
	GA	27.59	32.73		0.455	0.802	0.448	1.433
	AA	8.05	6.06		0.654	1.266	0.455	3.500
LFA	GG	77.78	61.21	0.357	(ref)	(ref)	(ref)	(ref)
	GA	16.67	32.73		0.153	0.401	0.110	1.456
	AA	5.56	6.06		0.763	0.721	0.086	6.072
ASD-MR	GG	67.06	61.21	0.651	(ref)	(ref)	(ref)	(ref)
	GA	28.23	32.72		0.419	0.787	0.441	1.407
	AA	4.70	6.06		0.574	0.709	0.212	2.363

X^2 of Pearson.
HFA: High Functioning Autism; LFA: Low Functioning Autism; ASD-MR: Autism Spectrum Disorders with Mental Retardation;OR: Odd Ratio; IC: Interval Confidence.

Finally, ASD patients with mental retardation were analyzed and compared with control population. In this case, the genotype distribution was 67.06% of GG, 28.23% of GA and 4.7% of AA. For this subgroup, neither association between SNP and ASD-MR was found (p = 0.651) (Table 1.C1).

3. mRNA EXPRESSION OF NEUROTROPHINS AND RECEPTORS IN PERIPHERAL BLOOD

Analyses of gene expression are increasingly important in many fields of biological research. Understanding patterns of expression is expected to provide insight into complex regulatory networks and will most probably lead to the identification of genes relevant to new biological processes, or implicated in a disease (Vandesompele J et al., 2002). Likewise, proteomic techinques are increasingly evolving as additional tools for elucidating the pathophysiology of mental disorders (Taurines R et al., 2010).

3.1. Selection of best housekeeping genes

Internal control genes, known as housekeeping genes, are most frequently used to normalize the mRNA levels of the genes of interest. These housekeeping genes should not vary in their expression in the tissues or cells

under investigation, or in response to experimental treatment. However, the literature shows that housekeeping gene expression can vary considerably (Warrington JA et al., 2000; Thellin O et al., 1999).

Levels of gene expression should be normalized by carefully selected stable internal control genes; gene-stability measure (M) is a practical tool to determine this important stability.

M values were calculated for each housekeeping gene (*ALAS*, *PPIA*, *RRN18S*, *ACTB*, *GAPDH* and *HSPA8*). The most stable genes were *ALAS* and *ACTB* for autistic samples younger than 16 years (M = 0.925), as shown in figure 6.C1.A. Although, in order to measure expression levels accurately; normalization factor (NF) was calculated by three most stable housekeeping (*PPIA*, *ACTB* and *ALAS*) using a geNorm applet for Microsoft Excel.

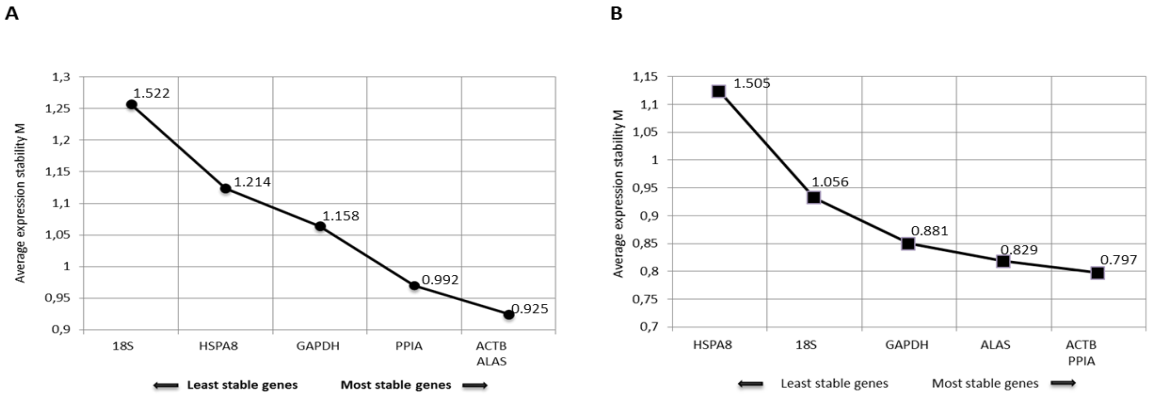


Figure 6. C1. Average expression stability of the remaining control genes. M value is calculated at each step during stepwise exclusion of the least stable reference gene. **(A)** Graphic of M values for young population (< 16 years old). Graphic shows that most stable housekeeping genes are *ALAS* and *ACTB*, and in ascending instability *PPIA*, *GAPDH*, *HSPA8* and *RRN18S* can be observed as the worst stable genes. **(B)** Graphic for M values for adult population (> 16 years). Graphic shows *ACTB* and *PPIA* as most stable control genes and in ascending instability *ALAS*, G*APDH*, *RRN18S* and *HSPA8* can be observed.

In contrast, the most stable genes for autistic patients older than 16 years, meaning those genes with a lower M value (M = 0.797), were *ACTB* and *PPIA* (Figure 6.C1.B). Finally, NF was calculated using *ALAS*, *ACTB* and *PPIA* which were the most stable housekeeping genes.

3.2. The mRNA expression of neurotrophins and their receptors in children

52 control subjects and 68 ASD patients, which were divided into two subgroups according to their functioning level (HFA and LFA), were characterized via quantitative RT-PCR. Thereby, HFA subgroup had a significant overexpression of *NT-3* ($p < 0.0001$) and lower expression of *TrkA* ($p = 0.0015$) versus control subjects. In contrast, no differential mRNA expression was found for *BDNF*, *NT-4*, *TrkB* and $p75^{NTR}$ (Figure 7.C1).

Next, gene expression of LFA was compared with controls. Positive correlation between neurotrophins, receptors and disease was observed. In this case, *BDNF* levels ($p < 0.0001$), *NT-3* ($p < 0.05$) were increased in autistic patients whereas *NT-4* ($p < 0.05$) and $p75^{NTR}$ concentrations ($p = 0.0013$) were decreased. Moreover, *TrkB* and TrkA were no associated with disease (Figure 6.C1).

Finally, no association was observed between the subgroups of ASD for *TrkB* and *NT-3* gene expression. However, *TrkA* and *BDNF* were increased ($p < 0.05$) in LFA to compare with HFA, while NT-4 and $p75^{NTR}$ were decreased ($p < 0.05$) (Figure 7.C1).

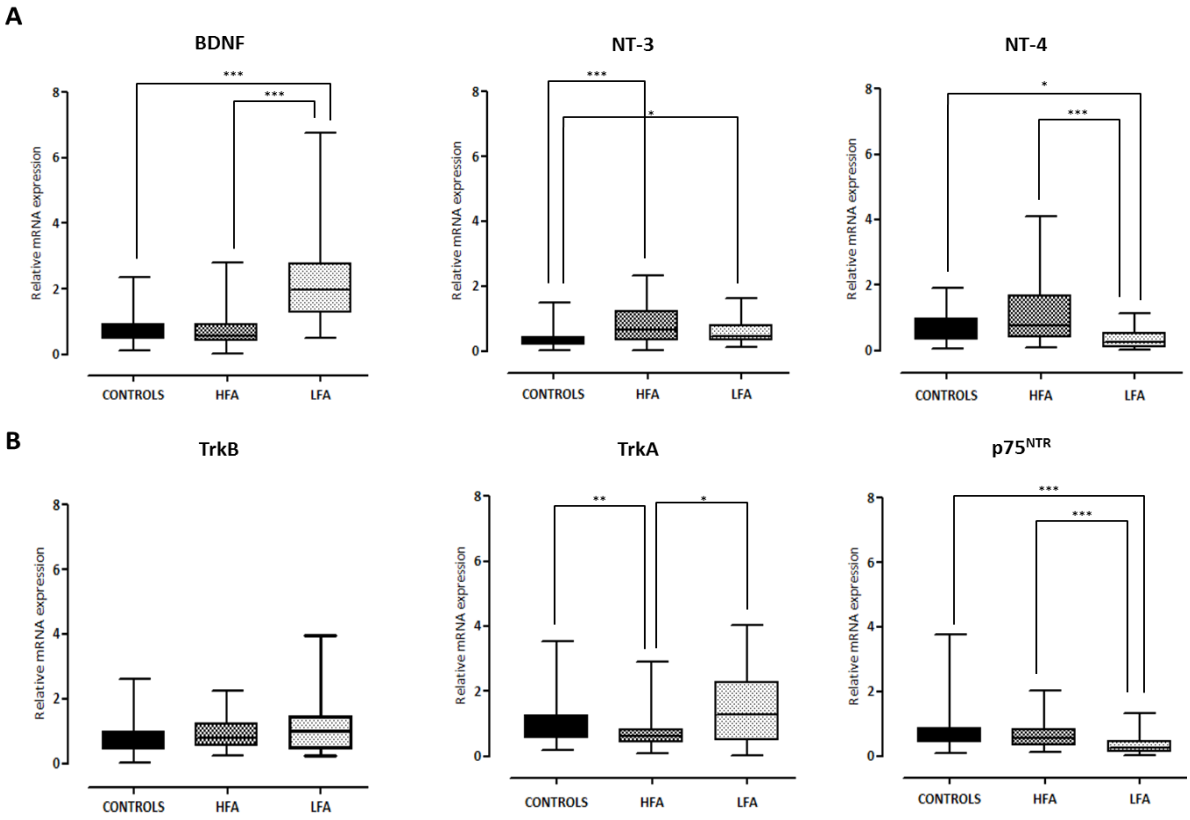


Figure 7.C1. mRNA expression of neurotrophins, Trk receptors and p75NTR in HFA and LFA children patients versus controls. (A) Gene expression of BDNF, NT-3 and NT-4 neurotrophins in both subgroups of ASD and control subjects. **(B)** Gene expression of TrkB, TrkA and p75NTR in both subgroups of ASD and control subjects.

3.3. The mRNA expression of neurotrophins and their receptors in adolescents and adults

23 adult controls and 23 ASD adult patients were characterized via quantitative RT-PCR. No significant association was found between the analyzed neurotrophins (BDNF, NT-3 and NT-4) and the pathology in adult patients ($p > 0.05$) (Figure 8.C1).

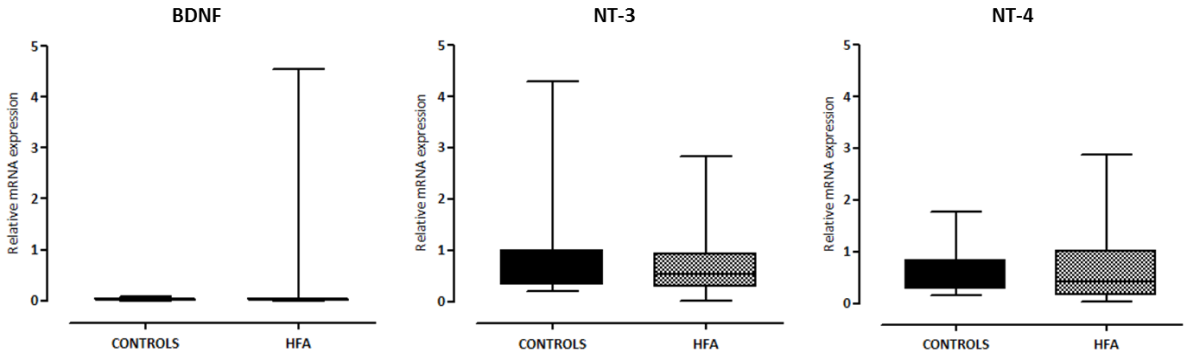


Figure 8.C1. mRNA expression of neurotrophins in adolescents and adults HFA. (A) Gene expression of BDNF, NT-3 and NT-4 neurotrophins in both subgroups of ASD and control subjects.

3.4. Association between *BDNF* genotype and mRNA expression

The effect of Val66Met (rs6265) genotype on the mRNA levels of BDNF in all study population (controls and ASD patients) was analyzed. This SNP, though located in the 5' proBDNF sequence, alters the intrinsic biological activity of the mature protein, affecting intracellular processing and secretion of BDNF (Egan MF et al., 2003). However, nothing is known about the effect of Val66Met SNP on the gene expression.

As shown in figure 9.C1, no correlation between genotype and mRNA expression of BDNF was found neither in the ASD patients younger nor in adolescents and adult ASD, patients.

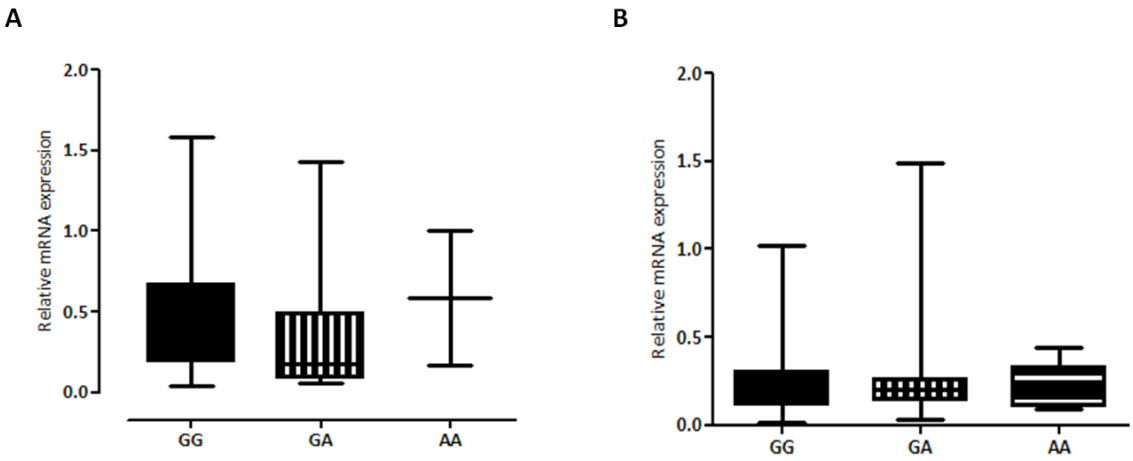


Figure 9.C1. Relative mRNA expression according to the BDNF Val66Met genotype. (A) Representation of mRNA expression distributed according to genotype of BDNF in children. It can be observed that there are no differences between mRNA expressions in different genotypes. **(B)** Representation of mRNA expression of BDNF distributed according to the genotype of the adolescent plus adult population. No difference of mRNA expression is observed.

4. PROTEIN LEVELS OF NEUROTROPHINS AND RECEPTORS IN PERIPHERAL BLOOD

In order to determine the protein levels of neurotrophins and receptors in peripheral blood, Western blot were performed. Samples ofinors and adult patients were combined in this study, although the major sample contribution was from adults. Presence of neurotrophins and their receptors was confirmed in the examined plasma samples. As seen in figure 10.C1 the immunoreactive bands were detected with the anti-human BDNF (that recognize preproBDNF, proBDNF and mBDNF), anti-human NT-3, anti-human-TrkB, anti-human TrkA and anti-humna $p75^{NTR}$ antibodies. Proteins were detected at the predicted molecular mass of ~37 KDa, ~24 KDa, ~14 KDa, ~24 KDa, ~92 KDa, ~88 KDa and ~75 KDa.

4.1. Protein levels in plasma samples

No significant differences were observed in the analyzed protein concentrations (BDNF, NT-3, TrkB, TrkA and $p75^{NTR}$) in plasma samples, in comparison of ASD patients with controls. Even so, it can be seen at figure 9.C1 that TrkA had a tendency of increased levels, whereas TrkB had an opposite trend.

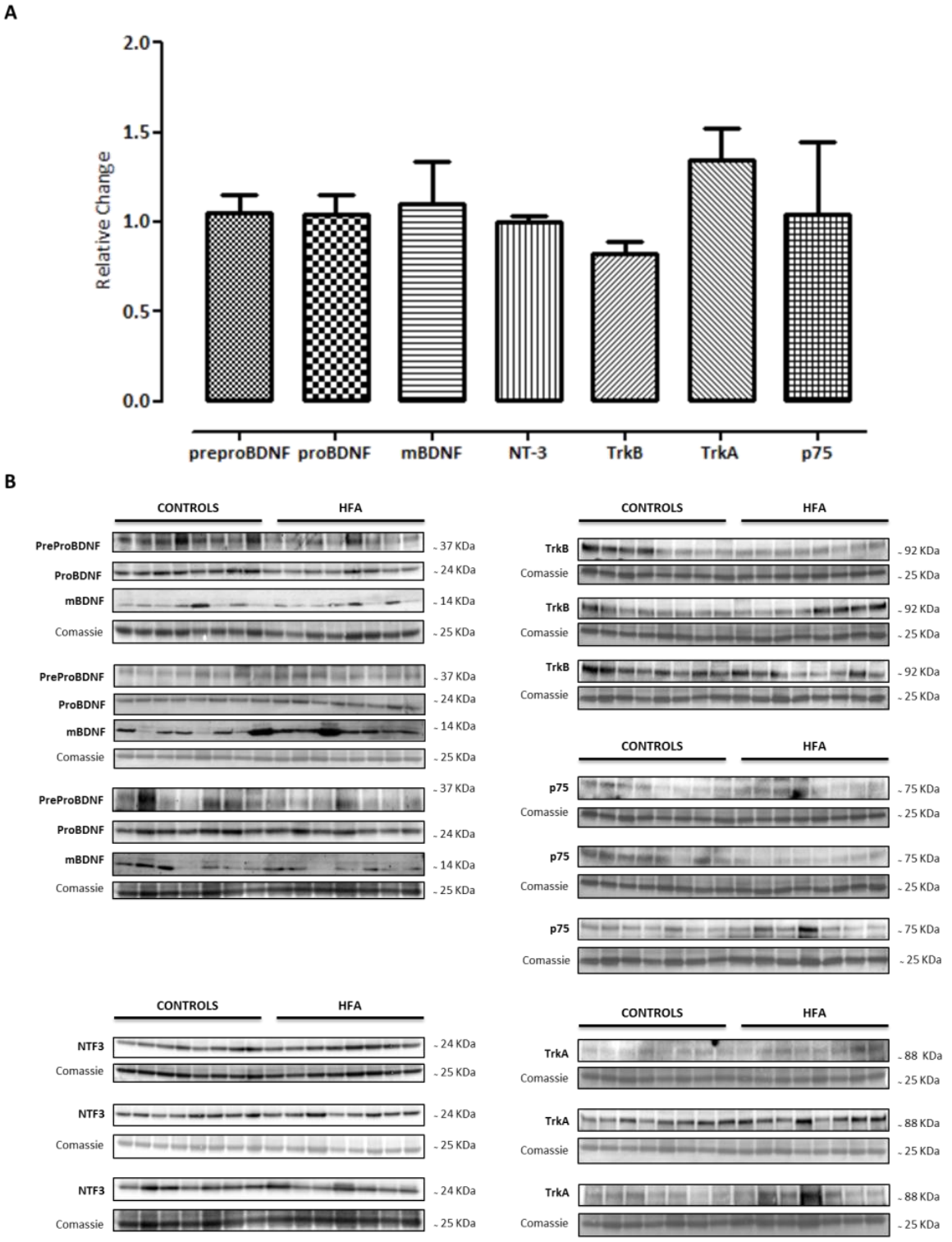


Figure 10.C1. Protein expression of neurotrophins, Trk and p75^{NTR} receptors in plasma of ASD patients. (A) Bands were quantified by densitometry. Proteins are expressed as relative change to the control group (normalized at 1) and presented as mean ± deviation. **(B)** Representative western blots containing both control and ASD cases immuneprobed for the ~37 KDa preproBDNF, ~24 KDa proBDNF, ~14 KDa mBDNF, ~24 KDa NT-3 , ~92 KDa TrkB , ~88 KDa TrkA and ~75 KDa p75^{NTR}.

4.2. Protein levels in PBMCs samples

BDNF and TrkB receptor proteins were also measured in PBMCs. Significant positive correlation was found between preproBDNF and ASD (p = 0.0211), as well as proBDNF (p = 0.0211). In contrast, no significant changes were observed in mature BDNF and TrkB protein levels in plasma of autistic patients in comparison with controls (Figure 11.C1).

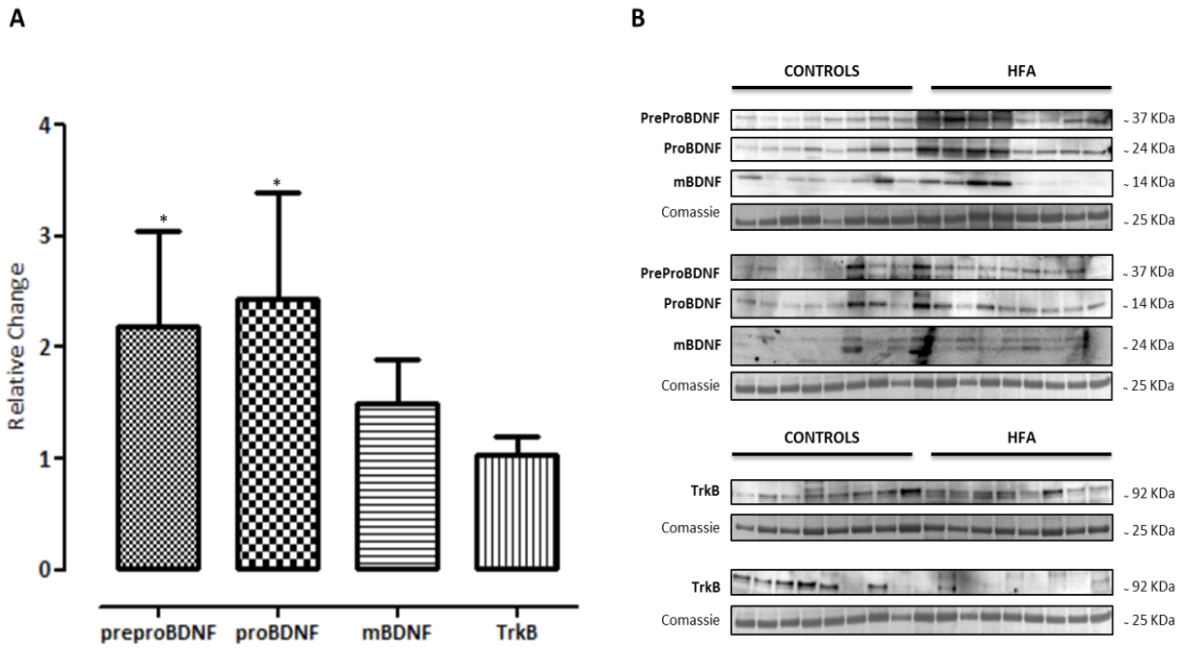


Figure 11.C1. Protein expression of BDNF and TrkB receptor in PBMCs of ASD patients. (A) Bands were quantified by densitometry. Proteins are expressed as relative change to the control group (normalized at 1) and presented as mean ± deviation. Significance between groups was $*p < 0.05$. **(B)** Representative western blots containing both control and ASD cases immuneprobed for the ~37 KDa preproBDNF, ~24 KDa proBDNF, ~14 KDa mBDN and ~92 KDa TrkB.

DISCUSSION

The etiopathogenesis of ASD is poorly understood, but scientific evidence supports the notion of a neurodevelopmental disorder, since autism is often associated with distrubed neural development (Belmonte MK et al., 2004; Polleux F & Lauder JM, 2004), but there has been little effort to date to identify specific developmentally important molecules that might be involved in this disturbances. Neurotrophins are good candidates for a possible involvement in ASD because of their fundamental role in guiding CNS development and cortical organization, and their abnormal expression patterns in autistic individuals (Pardo CA & Eberhart CG, 2007). Thes molecules have been studied previously, however pathophysiological correlation between neurodevelopmental disorder and neurotrophins are not weel explained to date.

It is well known that BDNF has an important role in mediating processes related to learning and memory (Korte M et al., 1995; Patterson S et al., 1996, Desai NS et al 1999), and this increased susceptibility to cognitive impairment, an important feature of ASD, suggests that the variation in BNDF may play a role in the development on neuropsychiatic disorders as well as affect NS functioning. In the present study, genotypic association of *BDNF* val66met gene variant (rs6265) and ASD was analysed. Psychiatric patients were subdivided, according their intellectual coefficient (IQ), in LFA (IQ < 70), HFA (IQ > 70) and ASD-MR. The present results shown no association was found between the SNP and autistic subgroups. Furthermore it was analyzed whether the mRNA expression of *BDNF* was genotype-dependent. The results obtained did not show differences between the expression of *BDNF* gene and genotype of subjects. *BDNF* val66met has been associated with obsessive compulsive disorder (Hall D et al., 2003), ADHD (Kent L et al., 2005) and anxiety-related personality traits (Lang UE et al., 2005). It has also been suggested that this SNP plays a role in the hippocampal and prefrontal cortex which are important in human memory and human learning functions (Egan MF et al., 2003; Hariri AR et al., 2003). Cortical alteration in surface area caused by the val66 met has been reported, too (Raznahan A et al., 2009). However, we did not observe any association of val66 met with autism. The study had an important limitation such as the heterogeneity and samples size of ASD cohort. Therefore, it could be interesting to increase considerably the autistic patients in each of the three delimitate subgroups, because the rare allele is poorly representated and in consequence, the statistical interpretation should make carefully.

On the other hand, the mRNA expression of neurotrophins and their receptors has been examined, because there are several lines of evidence that suggest the important role of these molecules in neurodevelopmental processes. Overexpression of *BDNF*, in this case, can be deleterious for a normal development of nervous system. Given support at the hypothesis of BDNF and the other neurotrophisn have an important factor in neuronal development, Nelson and colleagues reported higher BDNF and NT-4 levels in the archived samples of neonatal blood from autistic children compared to normal controls (Nelson KB et al., 2001). These findings are also supported by other studies, such as one on higher concentrations of BDNF in basal forebrain (Perry EK et al., 2001) and in serum samples of ASD (Connolly AM et al., 2006; Miyazaki K et al., 2004). Miyayaki and colleagues (Miyazaki N et al., 2004) also report overall increment of BDNF in serum samples; even so it is important take into account the age heterogeneity of this samples. Few years later, Nelson and collaborators (Nelson KB et al., 2006) described an over expression of BDNF again. However, NT-4 was lower measured in adult samples that had a lower concentration of this neurotrophin in blood. Finally, they also analyse the NT-3 levels in archieved neonatal blood; in this case, the levels were significantly lower in ASD childrens. In the same way, alterations of neurotrophin expression have been found in other psychiatric disorders. Otsuki K and colleagues reported an alteration of the expression of neurotrophins in patients with major depression,

concretely his group found reduced levels of mRNA in white blood cells. Thus, in accordance with these studies, alterations in the mRNA expression of some neurotrophins and their receptors were observed in the present study. Specifically, alterations in BDNF, NT-3 and NT-4 were found in autistic children patients with low functioning compared to controls. These results suggest that levels of neurotrophin mRNA might be dependent on the level of functioning respectively the subgroup of ASD. LFA patients have an statistically increment of BDNF mRNA levels in their plasma, results that are in concordance with previous studies detailed above. In contrast NT-3 and NT-4/5 are in concordance of second study of Nelson et al., meaning that are in a minor representation. These differences may suggest a compensatory role between the neurotrophins, previously described for other non-psychiatric diseases (Kyin R et al., 2001). Furthermore, not differences of TrkB mRNA levels had been found, in contrast higher levels of TrkA receptor were observed in LFA patients. It is well known that the neurotrophin-Trk pathways activate intracellular signal cascades of survival, as well as differentiation and growing of cells (Friedman WJ & Greene LA, 1999; Kaplan DR & Miller FD, 2000; Patapoutina A & Reichardt LF, 2001). These findings would leave to think, that an increased expression of these receptors could be beneficial for neuronal cells and, in consequence, for a better development of the neuronal system. However, the normal development consists of subsequent processes that occur in orderly manner along the time, during a specific period of development (Graaf-Peters VB & Hadders-Algra M, 2006). Considering this fact, it could be hypothesized that increased levels of Trk receptors, TrkA in this case, would cause an alterations of this precise process such as the development of the neuronal system and then, could be the origin of cytoarquitectural abnormalities found in ASD pathology. Finally, the mRNA levels of $p75^{NTR}$ were reduced in LFA patients compared to controls. The principal role of this receptor, when interacting with proneurotrophins, is to activate the apoptosis pathway (Cassaccia-Bonnefil P et al., 1996; Coulson EJ et al., 1999; Frade JM et al., 1996; Friedman WJ, 2000; Beattie MS et al., 2002; Lee R et al., 2001; Volosin M et al., 2006). Based on these data, it can be suggests that lower levels of $p75^{NTR}$ would trigger a poor cell-programmed death which is important for a normal brain development (Graaf-Peters VB & Hadders-Algra M, 2006).

The mRNA expression levels of three neurotrophins (BDNF, NT-3 and NT-4) had been analysed in adolescent and adult ASD cohort, all of them diagnosed as a HFA. No differences were found between adolescent and adult ASD and controls, a fact that could be explained because all subjects included in the study were autistics with high functioning, and it could be see before, that the mRNA expression of neurotrophins is probably depending on the level of functioning. As it has been comented previously, it could be interesting to increase the samples size; in this case it could be interesting to get adolesctent and adult LFA to compare with the cohort of the study.

Finally we aimed to detect if these alterations of mRNA expression were reflected in protein levels. The major representative patients included in this part of the study were from the adolescent and adult group, even so a few patients near 16 years old had been included. Then, the results obtained support findings from mRNA expression previously reported, because no significant alterations were observed in any of the neurotrophins in plasma samples. Moreover, if the profiles of neurotrophins were accurately evaluated, it can be see that the levels of protein followed the same trend like mRNA expression. Furthermore, it was thought interesting to validate if the plasma protein levels correlates with levels of protein in PBMCs, or in contrast the major aportation of ciculant neurotrophins is from other tissues. When compare the protein levels of plasma with PBMCs, surprisingly, the levels of preproBDNF and proBDNF were different between plasma and PMBCs samples and, furthermore in this case were positively associated with ASD pathology, but not with mBNDF and TrkB. However, this study has some limitations because the number of samples studied was limited and not

ideally delimitated. This means that the ASD group analyzed was the combination of both, children and adults subgroups. Thereby, it could be important to increase the number of plasma samples from two ASD groups and check, whether the patterns were the same.

Then, it is well known that not only mature neurotrophins proteins but also their pro-forms are able to interact with Trk and $p75^{NTR}$ receptors. Due to de affinity of proneurotrophins to the receptor of apoptotic pathways, the ratio between mature and inmature forms might be incluence cellular fate. Consequently, pathomechanims of developmental disorders might not only involve the overall level of the converted proteins, but also the level of the proproteins.

Wheter the neurotrophins and their receptors are part of a causal pathway to ASD remains unclear, even so the results of the present study are in accordance of the importance of these molecules in major behavioral and cognitive disorders. Then, the results encourage hope that further investigation of these molecules and the pathways of their interactions with other potent molecules will lead a better understanding of the pathofisiological mechanisms.

CONCLUSIONS

The conclusions of the present study - where the aim was to determine the pathophysiological influence of neurotrophins and their receptors in ASD - are:

- Ψ The rs6265 gene variant of *BDNF* is not associated with autistic high functioning patients, nor low functioning autism and nor autism spectrum disorders with mental retardation.

- Ψ The mRNA expression of NT-3 neurotrophin is overexpressed in children with high functioning autism, while TrkA mRNa expression is down-regulated in this same subgroup.However, there is no association between BDNF, NT-4, TrkB and p75NTR mRNA expression and autistic children with high function.

- Ψ BDNF and NT-3 neurotrophin mRNA expression is increased in children with low functioning autism, whereas NT-4 and p75NTR are decreased. However, there is no association between levels of mRNA TrkB and TrkA receptors and autistic children with low function.

- Ψ The mRNA expression of BDNF, NT-4, TrkA and $p75^{NTR}$ are differentially expressed in low functioning autism and high functioning autism. Thereby, the expression levels of BDNF and TrkA are higher in LFA than HFA.While, The expression of NT-4 and $p75^{NTR}$ is decreased in children with low functioning autism compared to high functioning autism. However, TrkB and NT-3 expression is equal in both children subgroups.

- Ψ There is no association between val66met genotype of BDNF and relative mRNA expression of this gene.

- Ψ No association between plasma levels protein of preproBDNF, proBDNF, mBDNF, NT-3, TrkB, TrkA and $p75^{NTR}$ and ASD subjects.

- Ψ PreproBDNF and proBDNF protein levels are increased in peripheral blood mononuclear cells.

- Ψ There is no difference between levels of mBDNF and TrkB in peripheral blood mononuclear cells of ASD subjects and controls.

VIII. CHAPTER 2

LATROPHILIN 3 AS A PERIPHERAL BIOMARKER OF AUTISM SPECTRUM DISORDERS

INTRODUCTION

A. LATROPHILIN FAMILY

Latrophilin (LPHN), isolated in 1996 (Davletov BA et al., 1996; Krasnoperov VG et al., 1996), are a small family of G protein-coupled receptors (GPCRs) known as "adhesion GPCRs" (Volynski KE et al., 2004; Lelianova VG et al., 1997; Rahman MA et al., 1999; Serova OV et al., 2008). The family is constituted by three isoforms, LPHN1, 2 and 3, which are encoded by *LPHN1*, *LPHN2* and *LPHN3* genes (Ichtchenko K et al., 1999; Sugita S et al., 1998), located, respectively, on chromosomes 19, 1 and 4 in humans. The latrophilin mRNAs have several sites of alternative splicing (two have been directly identified by cDNA sequencing in *LPHN1*, five in *LPHN2* and four in *LPHN3*) (Sugita S et al., 1998). The exon boundaries in the three *LPHN* genes are essentially the same, with a few exceptions, and many alterative splices sites coincide. LPHN1 and LPHN2 are the major brain receptor for α-latrotoxin (Ichtchenko K et al., 1999), while LPHN3 does not bind the toxin appreciably (Matsushita H et al., 1999). Moreover, the importance of LPHNs receptors for regulation of exocytosis of neurotransmitters is well know (Krasnoperov VG et al., 1997; Lelianova VG et al., 1997; Davletov BA et al., 1998; Rahman MA et al., 1999; Silva JP et al., 2009).

The primary structures of these proteins are 48% - 63% identical with a similar domain organization consisting of seven transmembrane regions (TMRs), together with the intra- and extracellular loops that encompasses 243 residues, unusually long adhesion-like extracellular N-terminal sequences with signal peptides, a serine/threonine-rich glycosylation region lectin, olfactomedin and hormone receptor domains (O'Sullivan ML et al., 2012; Sugita S et al., 1998; Ichtchenko K et al., 1998)). The most prominent feature is the presence of TMRs that are highly homologous to those of the secretin family GPCRs (50-60% sequence similarity and 30 % identity).

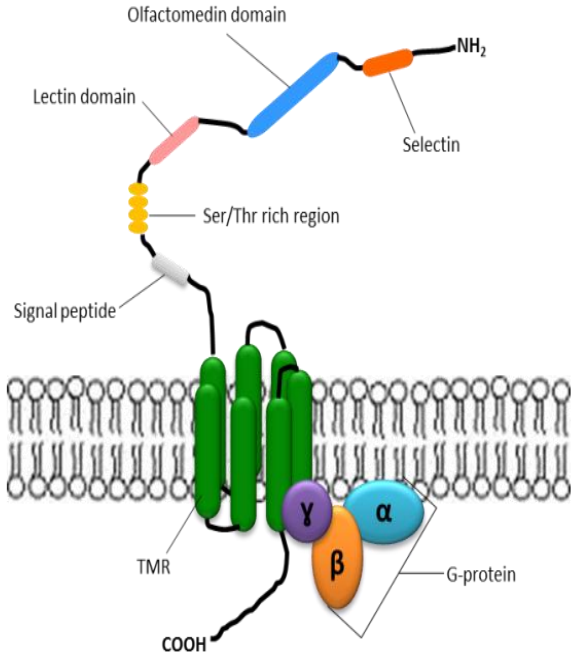


Figure 1.C2. Schematic structure of LPHN3. The structure of LPHN3 is based on GPCRs. LPHN3 receptor encompass a extracellular C-terminal domain formed by a signal peptide, serine/threonine rich region, lectin domain, olfactomedin domain a selectin domain. Such other GPCRs, has a seven transmembrane regions (TMB) and

LPHN has also served as a model to propose the "split personality" hypothesis stating that the fragments of adhesion GPCRs can behave as independent proteins capable of ligands-induced reassociation and concomitant signaling (Volynski KE et al., 2004; Silva JP et al., 2009). Thereby, the "split personality" feature is given by of two independent modules that associate interchangeably by binding their ligands. Binding an agonist, the fragments reassociate and send an intracellular signal. This signal, traduced by a heterotrimeric G protein, causes release of calcium from intracellular signal (Silva JP & Ushkaryov YA, 2010). This signal, traduced by a heterotrimeric G protein, cause release of calcium from intracellular stores and massive release of neurotransmitters (Silva JP & Ushkaryov YA, 2010). This leads to important onclusions that, consistent with Ca^{2+} signaling, latrophilin acts locally, within presynaptic nerve terminals and does not necessarily send signals to the cell body and the nucleus (Ashton AC et al., 2000; Lelyanova VG et al., 2009).

Northern blot analyses performed from different tissues have shown that *LPHN1* and 3 are strictly brain specific, while *LPHN2* is expressed in many tissues (Lelianova VG et al., 1997; Sugita S et al., 1998; Matsushita H et al., 1999; Ichtchenko K et al., 1999). However small levels of *LPHN1* mRNA can be detected outside the brain, in particular in kidneys and pancreas (Volynski KE et al., 2003); mRNA expression of *LPHN1* and 3 has also been found higher than brain in liver and lung of rats (Haitina T et al., 2008).

LPHN3 receptor is localized in the cerebral cortex, cerebellum, caudate nucleus, and amygdala (Choudhry Z et al., 2012), regions of the brain that have been shown to be important in ASD (Bailey A et al., 1998; Kemper TL & Bauman M, 1998). *LPHNs* may play different roles during development and in adult animals: thus, *LPHN1* determines cell fate in early embryogenesis in Caenorhabditis elegans and controls neurotransmitter release in adult nematodes. Studies in the rat model have shown that expression of *LPHN3* is highly regulated during postnatal brain development, with the highest levels observed immediately after birth (Kreienkamp HJ et al., 2000). The large, complex N-terminal fragment of these receptors contains various motifs that have been implicated in cell adhesion. LPHN3 has been shown to be involved in regulated exocytosis of neurotransmitters and hormones. Small G proteins are highly enriched at synapses, playing a critical role for intracellular signaling (Lopez de Maturana R & Sanchez-Pernaute R, 2010). The C-terminal region of LPHNs have also been found to interact with synaptic scaffolding proteins of the ProSAP/SSTRIP/Shank family, which play a vital role of regulating the cytoskeletal architecture at synapses (Kreienkamp HJ et al., 2000). It has been suggested that the interaction between LPHN and these scaffolding proteins contribute the architecture of the cytoskeleton at the synapse, thereby controlling the vesicular fusion/docking process. This diversity suggests that the functions of LPHN may be determined by their interactions with respective ligands (Silva JP & Ushkaryov YA, 2010).

B. BACKGROUND of GENETIC STUDIES

Further evidence for the importance of LPHNs in the proper functioning of neural circuits comes from recent human genetics studies that have linked *LPHN3* mutations to attention deficit hyperactivity disorder (ADHD), a common and highly heritable neurodevelopmental disorder, suggesting a role for latrophilins in human cognitive function. (Arcos-Burgos M et al., 2010; Domene S et al., 2011; Jain M et al., 2011; Ribases M et al., 2011). The association between *LPHN3* and ADHD was first observed from fine-mapping of a region identified in a large, multi-generational linkage study conducted a genetic isolate, Paisa population from Colombia (Arcos Burgos M et al., 2010). The initial finding was confirmed by the same group using both a case-control and family based design from five different populations: Paisa, United States, Germany, Norway and Spain (Arcos-Burgos et al M., 2010). A recent, independent case-control study in a Spanish population confirmed the association between this region of *LPHN3* and ADHD (Ribases M et al., 2011).

It is therefore plausible that polymorphisms that affect the structure of LPHN3 and/or transcription of the gene could affect signal transduction or vesicular trafficking, thereby affecting the concentration of neurotransmitters in the synapse, with down-stream effects on behavior and cognition (Choudhry Z et al., 2012).

LPHN1 and 2 serve as receptors for α-latrotoxin. This α-latrotoxin interacts with neuronal GPCRs to stimulate exocytosis of GABA-containing presynaptic vesicles (Lelianova VG et al., 1997; Matsushita H et al., 1999; Mee CJ et al., 2004; Linets'ka MV et al., 2002). GABA is an inhibitory neurotransmitter. This suggests a possible role of LPHN3 in ASD, the most brain-specific LPHN (Sugita S et al., 1998; Ichtchenko K et al., 1998). In fact, other GPCRs, such as DRD4 and DRD5, have been associated directly with ASD (Taurines R et al., 2011).

To our knowledge, genetic links between the *LPHN* gene and an inheritable disease have not been established yet. This may suggest –in line with our knockout results above- that most mutations in LPHN1, for example, as well as its abolition, are embryonically lethal. Moreover, indirect evidence suggests that *LPHN1* may be associated with mental disorders such as schizophrenia and bipolar disorders. Thus, chronic administration of risperidone, an antipsychotic drug often used to treat schizophrenia, led to an upregulation of LPHN1 in rats (Chen ML & Chen CH, 2005). Furthermore, the lack of latrophilin in mice, despite the compensatory changes, led to behaviors consistent with schizophrenia phenotypes (Kellendonk C et al., 2009).

Although, there are several studies that link up alterations in family of LPHNs and mental disorders, yet there is still much work to do to increase the knowledge of these alterations. Up to now nothing is known about the role of *LPHN*s in ASD, however, considering that ASD and ADHD are often co-morbid, it seems obvious and interesting to analyze LPHNs also in this neurodeveolpmental disorder (Gadow KD et al., 2006; Goldstein S & Schwebach AJ, 2004; Hattori J et al., 2006; Holtmann M et al., 2007; Reiersen AM et al., 2007). So, contrary to the recommendations of manual diagnostics (DSM-IV and ICD10), some authors suggests consideration of comorbidity between ADHD and autism (Holtmann M et al., 2007; Sverd J, 2003; Leyfer OT et al., 2006; Goldstein S & Schwebach AJ, 2004). Many children with ASD show significant deficits in visual attention and impulsivity; however the increased motor activity does not appear to be a specific symptom of ASD (Ponde MP et al., 2010). Furthermore it seems that children who meet diagnostic criteria for ADHD and autism have a greater impairment in activities than children just with autism, suggesting that the presence of both disorders seems to aggravate the functional impairment of the patients.

OBJECTIVES

The aims of the present study were to:

⇒ evaluate the association between SNP rs4860079 of the *LPHN3* and patients diagnosed with ASD

⇒ evaluate if this association is level-functioning dependent, this means whether the SNP is associated with LFA or HFA

⇒ determine mRNA expression of *LPHN3* in peripheral blood.

EXPERIMENTAL PROCEDURES

Technical procedures applied in the present study are described in detail in headland VI, materials and methods.

EXPERIMENTAL PROCEDURE	HEADLAND	PAGE
RFLP-PCR	B.1.1.	72
qRT-PCR	B.2.3.	77
Statistical Analysis	D	84

RESULTS

A. GENOTYPING ASSAY OF LPHN3 IN ASD

In the present study rs4860079 SNP of the *LPHN3* was genotyped that reflects a change of adenine to guanine. A total of 367 samples from Spain and Germany were analyzed, of which 263 were healthy control subjects and 104 were diagnosed with ASD, which in turn were divided in two subgroups, HFA (n=86) and LFA (n=18).

All subjects were in HWE (p = 0.765) for rs4860079 SNP, thus this is a representative cohort for the total population; controls as well were in equilibrium (p = 0.156) indicating that they were well selected, too.

Of a total of 263 controls, 112 (42.59%) had a GG genotype, 127 (48.29%) had GA, and 24 (9.1%) subjects with AA were observed. Of 86 HFA patients the genotype distribution was 37 (43.02%) patients with GG, 33 (38.77%) with a GA genotype and finally, 16 (19.77%) with the rare genotype of AA (Table 1.C2).

Table 1.C2. Association study of rs4860079 SNP in LPHN3 with ASD patients and controls.

ASD SUBGROUP	GENOTYPE	HFA (%)	CONTROL (%)	X^2 p	X^2 p *(each genotype)*	OR	95 % IC for OR	
							Lower limit	Upper limit
HFA	GG	43.02	42.59	0.039	(ref)	(ref)	(ref)	(ref)
	GA	38.37	48.29		0.377	0.786	0.461	1.341
	AA	19.77	9.13		0.058	2.018	0.969	4.203
LFA	GG	38.89	42.59	0.576	(ref)	(ref)	(ref)	(ref)
	GA	44.44	48.29		0.988	1.008	0.357	2.868
	AA	16.67	9.13		0.331	2.000	0.482	8.295

X^2 was calculated using a Chi^2 of Pearson.

HFA: High Functioning Autism; LFA: Low Functioning Autism; OR: Odd Ratio; IC: Interval Confidence

The rs4860079 SNP is associated with the phenotype of HFA (p = 0.039). Furthermore, a trend to association (p = 0.058) between cases-control and genotype could be calculated: the genotype of rare AA alleles increases the probability to be ASD versus GG genotype (OR=2,018; 95% IC = [0,969 – 4,203]) (Table 1.C2). With a confidence level of 95%, taking 3 controls for HFA, the statistical power is 72.4% (by a ratio of 19.77% of AA in HFA, and 9.13% of AA in controls).

A subgroup of LFA has a different genotype distribution so that there is no association (p = 0.576) between this phenotype and the rs4860079 SNP, nor between to be case-control and to have a specific genotype (Table 1.C2). The statistical power of the study, taking 10 controls for 1 LFA and a confidence level of 95 %, is 22.4% (by a ratio of 16.67 % of AA in LFA and 9.13% of AA in controls).

B. *LPHN3* EXPRESSION IN PERIPHERAL BLOOD

LPHN3 is a gene with preferential expression in brain; even so there have been different attempts to determine its expression in other tissues (Ichtchenko K et al., 1999; Sugita S et al., 1998) with contradictory results. For example, mRNAs of lphn3 were absent in mouse lung and liver (Lelianova VG et al., 1997; Sugita S et al., 1998; Matsushita H et al., 1998; Ichtchenko K et al.,1999). However, *LPHN3* appeared to be expressed in liver and lung of rat (Haitina T, et al 2008).

In order to determine whether *LPHN3* mRNA expression can be detected in human peripheral blood, real-time quantitative PCR was performed using a ASD periphral blood and human hippocampus samples as a positive control. As it can be seen in **figure 2.C2**, no expression of *LPHN3* could be measured in a peripheral blood in contrast to the amplification observed in hippocampus sample (Figure 1.C2).

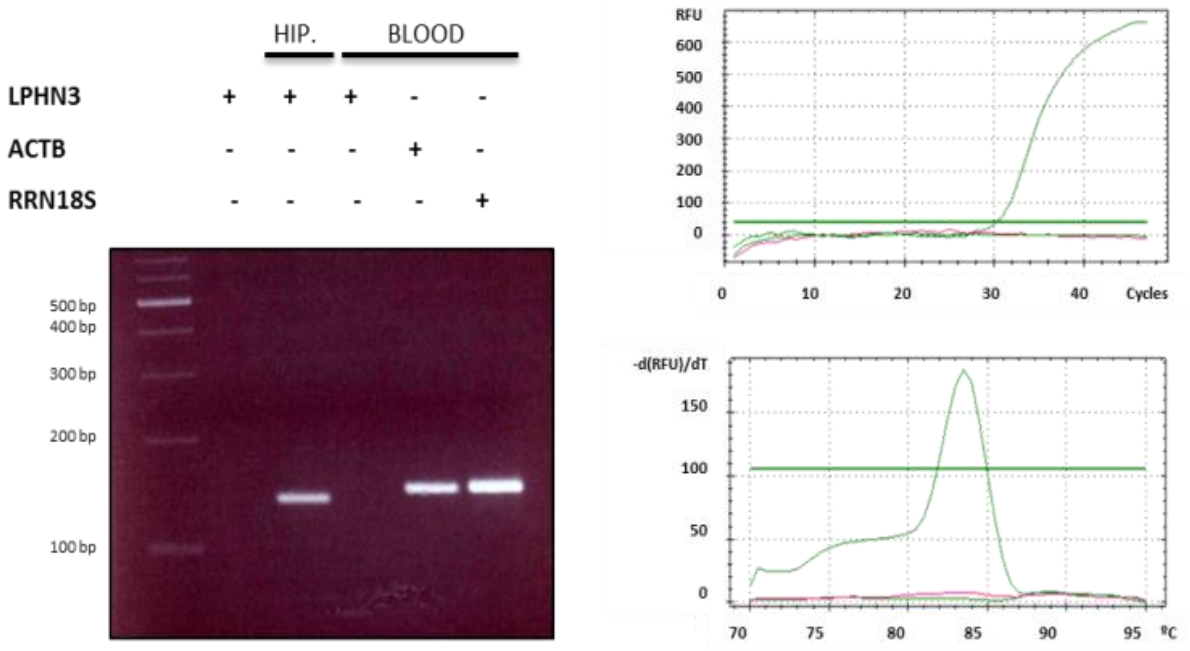


Figure 2.C2. LPHN3 amplification in hippocampus in contrast to lacking expression in peripheral blood. (A) Amplification of *LPHN3* in human hippocampus and *LPHN3*, *ACTB* and *RRN18S* in peripheral blood samples using specific QuantiTect Primer Assays. Real-time PCR products were resolved in agarose gel al 3% and were detected using ethidium bromide. It can be observed the amplification of *LPHN3* in hippocampus. However, lacking amplification of *LPHN3* is shown in contrast to both housekeeping genes (*ACTβ* and *RRN18S*). **(B)** Graphics shown cycles amplification of the real-time PCR and Melting curve: green color represents hippocampus sample, pink color represents LPHN3 in blood samples and finally, blue color represents blank reaction control. *HIP: Hippocamupus; LPHN3: Latrophilin-3; ACTB: Actin-β; RRN18S: 18S Ribosomal RNA; RFU: Relative Fluorescent Unints.*

DISCUSSION

Autism is a complex developmental disorder with multiple etiological factors and different degrees of symptom severity (Novaes CM et al., 2008). This disorder is commonly associated with other pathologies (Holtmann M et al., 2007; Bailey A et al., 1998; Kent L et al., 1999; Reiersen AM & Todd RD, 2008) as ADHD (Gadow KD et al., 2006; Goldstein S & Schwebach AJ, 2004; Hattori J et al., 2006; Holtmann M et al., 2007; Reiersen AM et al., 2007).

Consistently, association between ADHD and genetic variants within the *LPHN3* gene has been reported by different researchers (Arcos-Burgos M et al., 2010; Ribases M et al., 2011) and replicated in different populations (Paisas, United States, Germany, Norway and Spain). Due to the importance of clinical coexistence of ADHD symptoms in ASD patients, and the recurrent findings of the role of *LPHN3* alterations in ADHD, it seems obvious to analyze the possible association of these alterations and ASD pathology.

Results obtained from the present study can corroborate the association between autistic patients with a high level of functioning and rs4860079 gene variants (p = 0.039). Moreover, the rare genotype AA is probably the variant that increases the probability to present with the phenotype. However, the study has some limitations. On the one hand, the HFA cohort has a positive correlation; even so this result could be "masked" with comorbidity of ADHD and ASD. This fact could be possible, because the patients included on the study were not distinguished between them; HFA subjects were included below the same conditions, taking only into account HFA phenotype. As a future direction, could be important to expand the study with the inclusion of three groups: HFA, HFA comorbide with ADHD and ADHD. Only in this way, the possible contribution of *LPHN3* gene in the etiology o ASD could be confirmed or not.

On the other hand, the LFA cohort it was a reduced group and then, increased the number of LFA subjects it should be the priority. After that, whether the results obtained from a bigger LFA population confirmed the negative association, a clear no association would be demonstrated between LPHN3 gene and low functioning phenotype. In this case, any patients were comorbid for ADHD, which would justify that the results were not masked by ADHD phenotype.

As important as unraveling the genetic cause of the pathology of ASD, it is also a need to analyze the expression of these potential markers in samples easy to obtain. For this reason, mRNA expression of *LPHN3* gene was studied in peripheral blood. In line with previous studies (Lelianova VG et al., 1997; Sugita S et al., 1998; Matsushita H et al., 1998; Ichtchenko K et al., 1999), which stated that *LPHN3* is preferentially expressed in brain, our own experiments showed no mRNA expression of *LPHN3* in peripheral blood.

Taken together, these experiments give further hints of a role of LPHN family in psychiatric diseases and provide new aspects of a possible association of *LPHN3* gene variants and ASD, specifically with patients with a high level of functioning. Conversely, more exhaustive experimentations could be necessary to determine whether gene variants of *LPHN3* could be associated with LFA patients. Additionally, in line with former results, no expression of *LPHN3* had been found. Complementing these finding it might be useful to look for changes of expression of these gene in post mortem brain samples of autistic patients and in case of altered expression, rigorous analyses of mRNA expression should be performed in extensive peripheral samples in order to use LPHN3 as a potential biomarker of ASD.

CONCLUSIONS

The conclusions of the present study with the aim to determine the influence of rs4860079 SNP of *LPHN3* in ASD are:

- Ψ The rs4860079 gene variants of *LPHN3* are associated with HFA patients and thus, could be used in the future as genetic marker in a set of several biomarkers to confirm ASD pathology.

- Ψ In the included ASD sample the SNP rs4860079 of *LPHN3* is not associated with LFA; but a larger cohort of LFA subjects should be studied.

- Ψ *LPHN3* gene is not expressed in peripheral blood and thus, possible alterations in its expression could not been determined. Even so, given the importance of the latrophilin family in other mental disorders, potential expression changes should be analyzed in other peripheral tissues.

CHAPTER 3

EPHRIN RECEPTOR A4 AS A PERIPHERAL BIOMARKER OF PSYCHIATRIC DISORDERS

INTRODUCTION

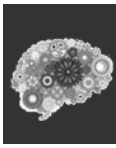
A. EPH-RECEPTOR FAMILY

EPH receptors constitute the largest family of the tyrosine kinase receptors. They interact at direct cell-cell contacts with their membrane-bound ephrin ligands, which are also capable of signaling. Upon ephrin binding, the EPH kinase initiates forward signaling into receptor-expressing cells, and the ephrin cytoplasmatic tail triggers reverse signaling into ligand-expression cells. Ephrins and EPHs regulate many diverse processes during embryonic development, in most cases by affecting the cytoskeleton as well as the patterning of the nervous and vascular system (Pasquale EB, 2008; Himanen JP & Nikolov DB, 2003; Pasquale EB, 2005). Furthermore they have been implicated in processes such as cell migration, axon guidance, topographic mapping, proliferation, synapse formation and stabilization and spine formation (O'Leary DD & Wilkinson DG, 1999; Wilkinson DG, 2001; Essmann CL et al., 2008; Klein R, 2009). More recently, EPHs and ephrins have been found to be expressed in several adult stem cell niches, such as the brain, skin and intestine, and to regulate the proliferation of stem/progenitor cells (Holmberg J et al., 2005; Holmberg J et al., 2006).

The family of EPH receptors as well as their ligands are divided into two subclasses (A and B) on the basis of their affinities for each other. In humans, nine EPHA receptors (EPHA1-8 and EPHA10) are known that bind to A-class ephrins (EPHRIN-A1-A6), although it has the potential to interact with both A-type and B-type ephrins and whose forward signaling reduces spine length (Murai KK et al., 2003). Five EphB receptors (EPHB1-4 and EPHB6) are known that interact with the B-subclass ephrins (EPHRIN-B1-B3) which regulate synapse and spine developing, at least in part by trans-synaptic interaction with ephrin-Bs expressed in axon terminals (Kayser MS et al., 2008).

Given that EPHs and ephrins are membrane-bound, their interaction occurs only at sites of cell-cell contact. In the absence of cell-cell interactions, they exist in loosely associated microdomains, which become more compact and well-ordered when EPH/ephrin complexes assemble to generate clearly defined signaling centers (Pasquale EB, 2005). Moreover, unlike to classical growth factor receptors, EPH/ephrin binding leads to bidirectional signal transduction. The EPH receptors mediating forward signaling in one cell, while ephrin ligands initiating reverse signaling in the adjacent cell. This mechanism is one of the keys of cell-cell contact-dependent pathways, which coordinate developmental processes and normal physiology and homeostasis of mature organs, too (Figure 1.C3.B) (Davy A et al., 1999; Holland SJ et al., 1996). There are a small differences between the two types of ephrins, because in B-ephrin, this active, receptor-like signal transduction involves several highly conserved tyrosine phosphorylation sites in the cytoplasmic domain, C-terminal PDZ motifs, and the binding to several cytoplasmatic adapter and PDZ domain proteins (Figure 2. 3C). In contrast, A-ephrins lack a cytoplasmatic tail, these ligands are still capable of triggering downstream activation of Src family kinases and phosphoinositide 3-kinase (PI3K), which might involve a signal-transducing "co-receptors" or the clustering of plasma membrane microdomains (Davy A et al., 1999; Davy A & Robbins SM, 2000). TrkB and p75 neurotrophin receptors might function as such coreceptors, and it has been shown that their signalling is enhanced by interactions with ephrin-A ligands in *cis* (Lim YS et al., 2008; Marler KJ et al., 2008) (Figure 1.C3.B).

The functions of EPHs/ephrin are understood best as patterning and axon guidance molecules in the nervous system (NS) (Flanagan JG & Vanderhaeghen P, 1998; Kullander K & Klein R, 2002). The motile growth cones at the distal ends of growing axons respond to the environment to ensure the correct connections of the NS. In



these processes, activation of EPH receptors is typical, and leads to a growth cone collapse response and retraction from an ephrin-expressing substrate (Poliakov A et al., 2004; Pasquale EB, 2005; Klein R, 2009). These molecules are expressed in gradient so that neurons, depending on levels of EPH/ephrin interactions, have different response (Klein R, 2004). It is when the axons have reached their targets, when the cone is converted into a presynaptic terminal with neurotransmitters and vesicles. On the other hand, the postsynaptic region in dendrites is highly enriched in neurotransmitter receptors as also scaffolding and signaling proteins. For excitatory synapses, such as glutamatergic synapses (with AMPA, NMDA as well as other glutamate-binding receptors), maturation of these involves actin-rich dendritic spines that form synaptic connections with a single axon terminal. Furthermore, formation of spines and retraction of these is a dynamic process is also linked to synaptic plasticity (Klein R, 2009).

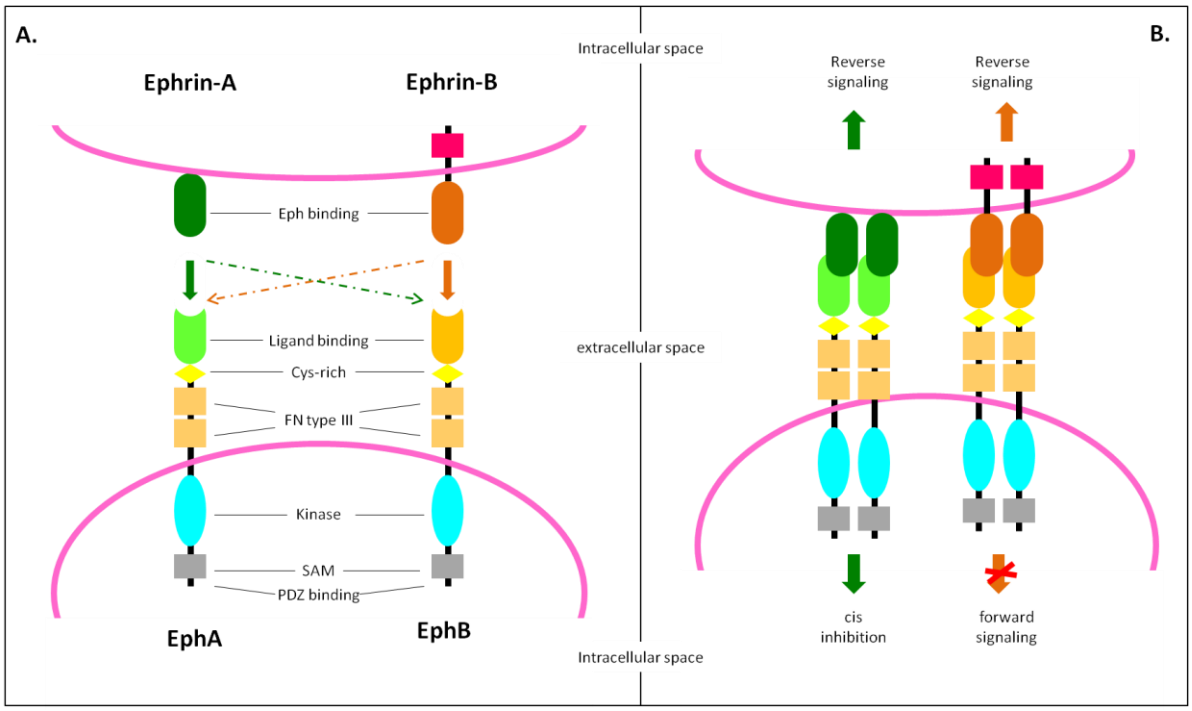


Figure 1.C3. EPH-Receptors structure and bidirectional signal transduction. (A) The structure of both types of EPH receptors contains an extracellular domain as well as a cytoplasmatic part. Thus, the extracellular part of EPH receptors contains a globular ligand-binding domain, adjacent cysteine-rich domains (Lackmann M et al., 1998), followed by two fibronectin type III repeats. The cytoplasmatic EPH region encompasses a short regulatory juxtamembrane region with several conserved tyrosine residues and connect with the kinase domain, a sterile α motif domain protein-protein interaction domain, and a C-terminal PDZ-binding motif (Pitulescu ME & Adams RH, 2010; Himanen JP et al., 2010). All ephrins possess a 20 KDa extracellular receptor-binding domain; B-type ephrins also contain a short cytoplasmic region (Himanen JP et al., 2010). **(B)** Bidirectional signal transduction is taking place, in which Eph receptors mediate forward signaling in one cell, while ephrin ligands initiate reverse signaling in the adjacent cell.

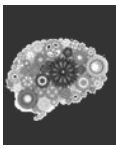
All former findings taken together indicate that EPH/ephrin interactions have diverse roles in the developing and adult NS (Poliakov A et al., 2004). Ephrins and their EPH receptors regulate different stages of neurogenesis and are differentially expressed on distinct cell types in the neurogenic niches (Chumley MJ et al., 2007; Conover JC et al., 2000; Depaepe V et al. 2005; Furne C et al., 2009; Holmberg J et al., 2005). Furthermore, altered Eph/ephrins signaling in these previously described processes can lead to a variety of diseases in humans (Pasquale EB, 2008).

B. BACKGROUND of GENETIC STUDIES

Important study results by Carmona and colleagues leaves to think about a possible role of EPHA4 in psychiatric diseases (Carmona MA et al., 2009). So, in their study the authors revealed the mechanism by which glial glutamate transport is controlled near synapses via EPHA4 and ephrin-A3. It was also described that activation of EPHA4 induces dendritic spine shortening and retraction. Thus, it is well know that dendritic spine abnormalities accompany neurological disorders such as mental retardation, autism and schizophrenia (Fiala JC et al., 2002). Furthermore, increased glial glutamate transporter levels and function are also associated with schizophrenia (Danbolt NC, 2001; Beart PM & O'Shea RD, 2007).

EPHA4 plays a role in the developing and postnatal brain, including axon guidance (Egea J et al., 2005; Gallarda BW et al., 2008; Wegmeyer H et al., 2007), synaptic plasticity (Bourgin C et al., 2007; Deininger K et al., 2008; Fu WY et al., 2007; Inoue E et al., 2009) and proliferation of precursor cells during cortical neurogenesis (North HA et al., 2009). Distribution of Eph4 was studied in mouse development in both embryonic and postnatal period (Greferath U et al., 2002). *EPHA4* expression was shown to be widespread distributed early in central nervous system, when processes as neural differentiation, migration, growth of axon and synapses formation can be observed (Greferath U et al., 2002). In the hippocampus, a dichotomous role of EPHA4 was described for synaptic formation and function; on the one hand, studies revealed that activation of this receptor led to spine retraction *in vivo* (Murai KK et al., 2003; Bourgin C et l., 2007). On the other hand, analysis *in vitro* found that over-expression of the *EPHA4* increases dendritic spine density while activation of this reduces synaptic strength and homeostatic plasticity (Kamioka H et al., 1996). Furthermore, implications of EPHA4 in mediating signalling in synaptic function and neural circuitry have been described in cerebral cortical neurons (Clifford MA et al., 2011).

EPHA4 binds to multiple ligands in vitro and thus may form functional complexes with these ligands *in vivo* (Gale NW et al., 1996), as well as with other EPHs that may have overlapping or shared functions with other ligands. Thus the phenotype of a single gene deletion for *EPHA4* may not inform of all possible functions for this molecule (Greferath U et al., 2002). Even so, the study was focused on EPHA4, that is well-expressed within the developing forebrain and localizes to central synapses (North HA et al., 2009; Bouvier D et al., 2008; Bouvier D et al., 2010).

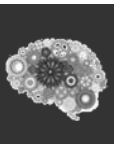


C. SELECTION OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNP)

Correct identification of the most promising SNPs for follow-up is one of the greatest challenges of conducting genome-wide association studies (GWAS) and multiple association tests are conducted across the genome, from which *p*-values or test statistics are obtained that are compared to determine the most promising SNPs. This procedure is conducted regardless of the specific adjustment chosen for multiple testing corrections.

To select the best SNPs susceptible to study, Minor Allele Frequency (MAF) is used by researchers in GWAS SNP arrays as well as other types of genotyping assays. MAF is reported in NCBI dbSNP database from nearly monomorphic (MAF < 0.5%) to very common (MAF ~ 50%) (Tabangin ME et al., 2009). Then, MAF is the minor allele frequency for each reference SNP (rs) included in a default global population. Since this is being provided to distinguish common polymorphism from rare variants, the MAF is actually the second most frequent allele value.

The current default global population is 1000 Genome phase 1 genotype data from 1094 worldwide individuals, released in the May 2011 dataset (www.ncbi.nlm.nih.gov/projects/SNP/docs/rs_attributes.html). The power to detect a given genetic effect with a given study size depends to a great extent on the MAF of the risk allele tested. Specifically, loci with low MAF (<10%) have significantly lower power to detect weak genotypic risk ratios than loci with a high MAP (>40%) (Ardlie KG et al., 2002). Furthermore, previous studies have demonstrated that rare genotypes are more likely to result in spurious findings (Lam AC et al., 2007). Hence, many GWAS have removed SNPs with MAF < 10% (Cupples LA et al., 2007; Florez JC et al., 2008).



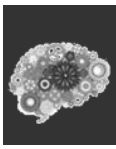
OBJECTIVES

The aims of the study are to:

⇒ valuate *EPHA4* as a biomarker candidate of psychiatric diseases, exactly for schizophrenia and bipolar disorders

⇒ analyses 9 SNPs, previously selected according to MAF (rs2952834, rs2392936, rs2248489, rs2056290, rs2052940, rs1864461, rs1897120, rs3087584, rs3770181) and determine the association between both, schizophrenia and bipolar disorders

⇒ asses the association of two different classifications: current diagnostics of ICD 10 and Leonhard's classification with a specific combination of bipolar and psychotic syndromes



EXPERIMENTAL PROCEDURES

A. GENOTYPING ASSAY

Technical procedure used in the study of *EPHA4* as a biomarker candidate of psychiatric diseases was described in headland VI, materials and methods (see B.1.2. TaqMan Genotyping Assay).

B. SELECTION OF POLYMORPHISMS

MAF of SNPs *EPHA4* were checked from dbSNPs, and after rigorous evaluation, the SNPs with a higher MAF value (30-50%) were selected (Table 1.C3).

Table 1.C3. SNPs chosen for EPHA4 study as a biomarker for psychiatric diseases

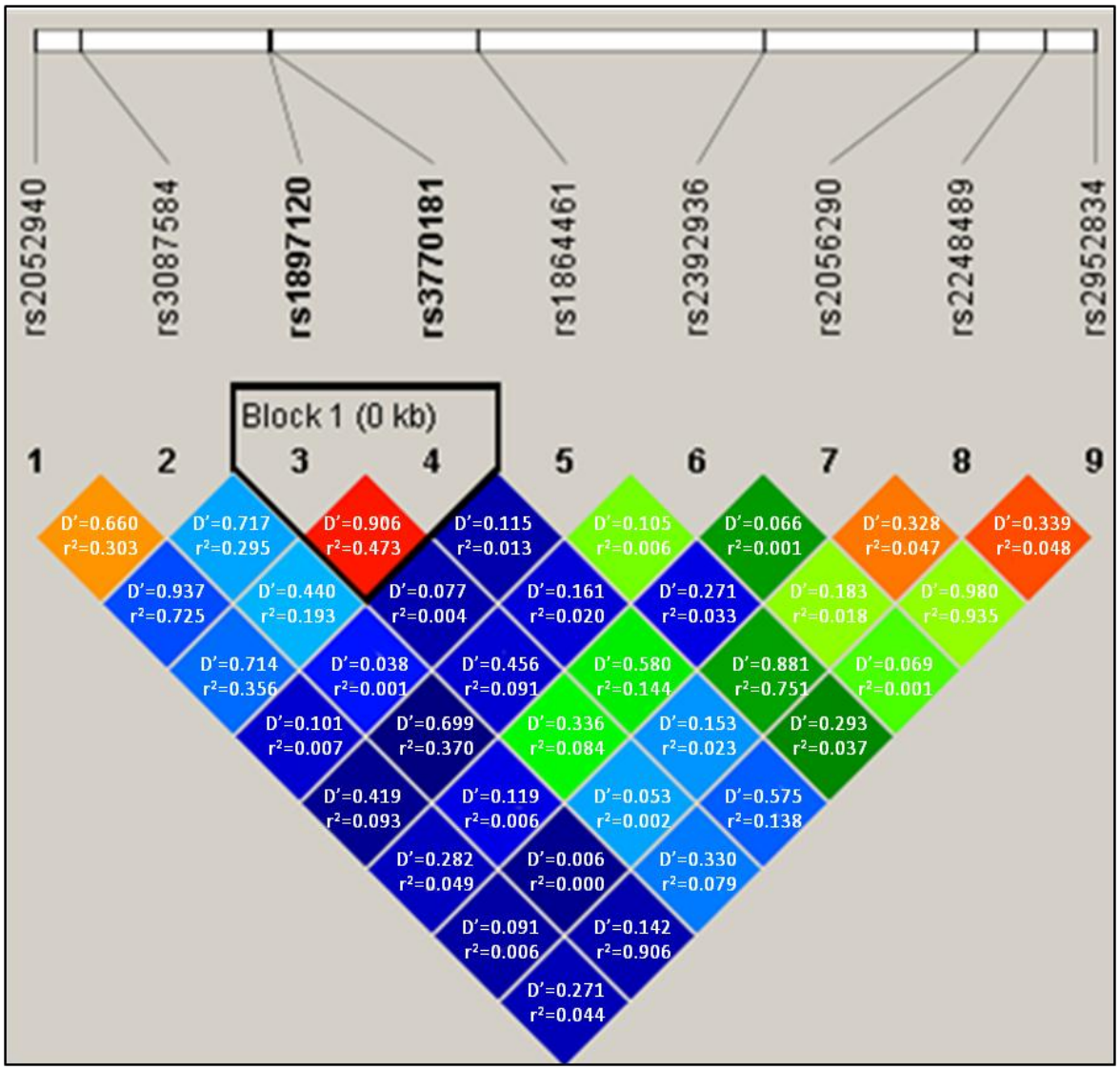
SNP	ALLELES	CHROMOSOME	POSITION	MINOR ALLELE	MAF
rs2952834	**A/G**	2	222446765	**G**	0,4390
rs2392936	**A/G**	2	222393728	**C**	0,4730
rs2248489	**C/T**	2	222438968	**A**	0,4860
rs2056290	**C/T**	2	222427871	**T**	0,3798
rs2052940	**A/T**	2	222275966	**T**	0,4049
rs1864461	**C/T**	2	222347615	**G**	0,4401
rs1897120	**G/T**	2	222313856	**A**	0,4159
rs3087584	**C/T**	2	222283281	**C**	0,4218
rs3770181	**C/T**	2	222314342	**A**	0,3332

SNP (Single Nucleotide Polymorphism); MAF (Minor Allele Frequency)

RESULTS

Genetic evaluation of the *EPHA4* gene was based on 9 SNPs in a German sample that encompassed 1939 subjects, of which 929 were schizophrenic cases and 626 bipolar patients. The LD plot of the 9 SNPs that were considered in the study is shown in figure 2.C3. Corresponding to HAPMAP data, there is only one LD-block were rs1897120 and rs3770181 are located together (in LD-block 1).

Figure 2.C3. Pairwise LD display of 9 SNPs located in the region 222275966-222446765 Kb on chromosome 2.



All SNPs were in Hardy-Weinberg Equilibrium (HWE) for control population according to ICD10 (see table 2.C3 and table 3.C3). No single marker showed evidence of overall association with schizophrenia and bipolar disorder diagnosed according to the ICD classification (see table 2.C3 and table 3.C3). Allele and genotype frequencies were not significantly different between cases and controls (see table 2.C3 and table 3.C3).

Table 2.C3. *EPHA4* cases with schizophrenia according to ICD10.

SNP	Cases n	Controls n	HWE controls *p*	Gen_2DF *p*	Cases AA	Controls AA	Cases AB	Controls AB	Cases BB	Controls BB
rs2952834	929	384	0.843	0.996	0.204	0.206	0.502	0.501	0.294	0.292
rs2392936	929	384	0.543	0.313	0.221	0.193	0.485	0.475	0.294	0.332
rs2248489	929	384	0.130	0.543	0.163	0.151	0.489	0.522	0.348	0.326
rs2056290	929	384	0.135	0.243	0.302	0.285	0.484	0.533	0.214	0.183
rs2052940	929	384	0.898	0.130	0.280	0.321	0.483	0.488	0.236	0.191
rs1864461	929	384	0.755	0.200	0.370	0.414	0.465	0.453	0.165	0.133
rs1897120	929	384	0.400	0.297	0.104	0.134	0.457	0.437	0.439	0.429
rs3087584	929	384	0.600	0.341	0.213	0.184	0.510	0.505	0.277	0.311
rs3770181	929	384	0.560	0.473	0.104	0.128	0.448	0.440	0.448	0.432

HWE: Test for Hardy-Weinberg-Equilibrium.
Gen_2DF: Test for association, calculated with software FAMHAP.
AA, AB, BB: Distribution of genotypes in cases and controls.

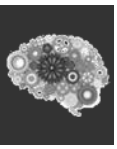
On the other hand, the samples were differentiated according to Leonhard's classification. Analyzing association of SNPs with schizophrenic spectrum divided into subgroups: systematic schizophrenias, unsystematic schizophrenias and cycloid psychosis, provided no significances between any analyzed SNP frequencies and pathologies. No association between markers and any subgroup was observed (data not shown).

Table 3.C3. *EPHA4* cases with bipolar disorders according to ICD10.

SNP	Cases n	Controls n	HWE controls *p*	Gen_2DF *p*	Cases AA	Controls AA	Cases AB	Controls AB	Cases BB	Controls BB
rs2952834	626	384	0.843	0.832	0.212	0.206	0.513	0.501	0.275	0.292
rs2392936	626	384	0.543	0.393	0.204	0.193	0.505	0.475	0.291	0.332
rs2248489	626	384	0.130	0.848	0.165	0.151	0.510	0.522	0.326	0.326
rs2056290	626	384	0.135	0.341	0.302	0.285	0.487	0.533	0.211	0.183
rs2052940	626	384	0.898	0.587	0.305	0.321	0.478	0.488	0.217	0.191
rs1864461	626	384	0.755	0.250	0.362	0.414	0.494	0.453	0.144	0.133
rs1897120	626	384	0.400	0.613	0.118	0.134	0.426	0.437	0.457	0.429
rs3087584	626	384	0.600	0.289	0.226	0.184	0.484	0.505	0.290	0.311
rs3770181	626	384	0.560	0.467	0.117	0.128	0.411	0.440	0.472	0.432

HWE: Test for Hardy-Weinberg-Equilibrium.
Gen_2DF: Test for association, calculated with software FAMHAP.
AA, AB, BB: Distribution of genotypes in cases and controls.

The case-control study, carried out with 1555 psychiatric patients and 384 control subjects, possesses a power of 87.5 % to detect an association with a susceptibility allele in bipolar disorder and 91.4 % in schizophrenia (alpha = 0.05%).



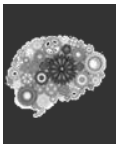
DISCUSSION

In the present study a set of 9 *EPHA4* SNPs was analyzed in a psychiatric population of 1555 patients, diagnosed according to the ICD10 classification, separated into schizophrenic (n=929) and bipolar disorder (n=626) subgroups. Moreover, subjects exhibiting schizophrenic symptoms were divided into three distinct clinical and nosological subgroups, according to Leonhard's classification: cycloid psychoses, unsystematic schizophrenia, and systematic schizophrenia.

Ephrins and EPHs are pivotal regulators of CNS development, including axon guidance, neural migration and patterning, synapse formation and vascular morphogenesis (Drescher U, 1997; Tessier-Lavigne M & Goodman CS, 1996; Flanagan JG & Vanderhaeghen P, 1998; Palmer A & Klein R, 2003; Eichmann A et al., 2005). Carmona MA et al. (Carmona MA et al., 2009) described an important role of ephrin-A4/EphA4 interaction in regulation of glial glutamate transporter expression and glutamate transport, that are known to be altered in psychiatric diseases such as schizophrenia (Danbolt NC, 2001; Beart PM & O'Shea RD, 2007). In the same study, it was also reported that activation of EPHA4 induces dendritic spine shortening and retraction; these findings were consistent with previous research that demonstrated, that the hippocampus spines of *EphA4*-knockout mice were longer than normal (Murai KK et al., 2003).

As Carmona and his workgroup suggested, that their observations could be involved in pathological processes of psychiatric disorders, because abnormalities in dendritic spines of patients with autism and schizophrenia had been described previously as well as increased levels of glial glutamate transporter and function in schizophrenia (Fiala JC et al., 2002; Danbolt NC, 2001; Beart PM & O'Shea RD, 2007). All these findings led to think of a possible implication of *EPHA4* in a psychiatric disorders, and hence with a possible sequence alterations of these gene. Small changes in a common sequence of *EPHA4* gene could trigger an anomalous function of the protein.

Given that, 9 SNPs with a higher MAF (30-50%) were selected and genotyped in psychiatric populations. According to the HanMap information, the markers are in moderate LD and therefore segregate independently. HWE was checked in a control population for each SNPs, and all of them were in equilibrium. In contrast of our own hypothesis, none of markers (rs2952834, rs2392936, rs2248489, rs2056290, rs2052940, rs1864461, rs1897120, rs3087584, rs3770181) was associated with schizophrenia or bipolar disorder (p > 0.05). Equally, no association was observed using Leonhard's classification. Our case-control study has a great powerof 87.5 % to detect an association with a susceptibility allele in bipolar disorder and 91.4 % in schizophrenia (alpha = 0.05%).



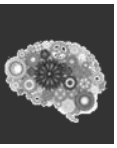
It can be conclude that our findings can not support the hipothesis that *EPHA4* has an important role in the pathophysiology of schizophrenia nor bipolar disorders. Even so, more exhausting studies in other populations as well as more detailed genetic studies could be necessary to describe a possible role of *EPHA4* gene variants in psychiatric diseases.

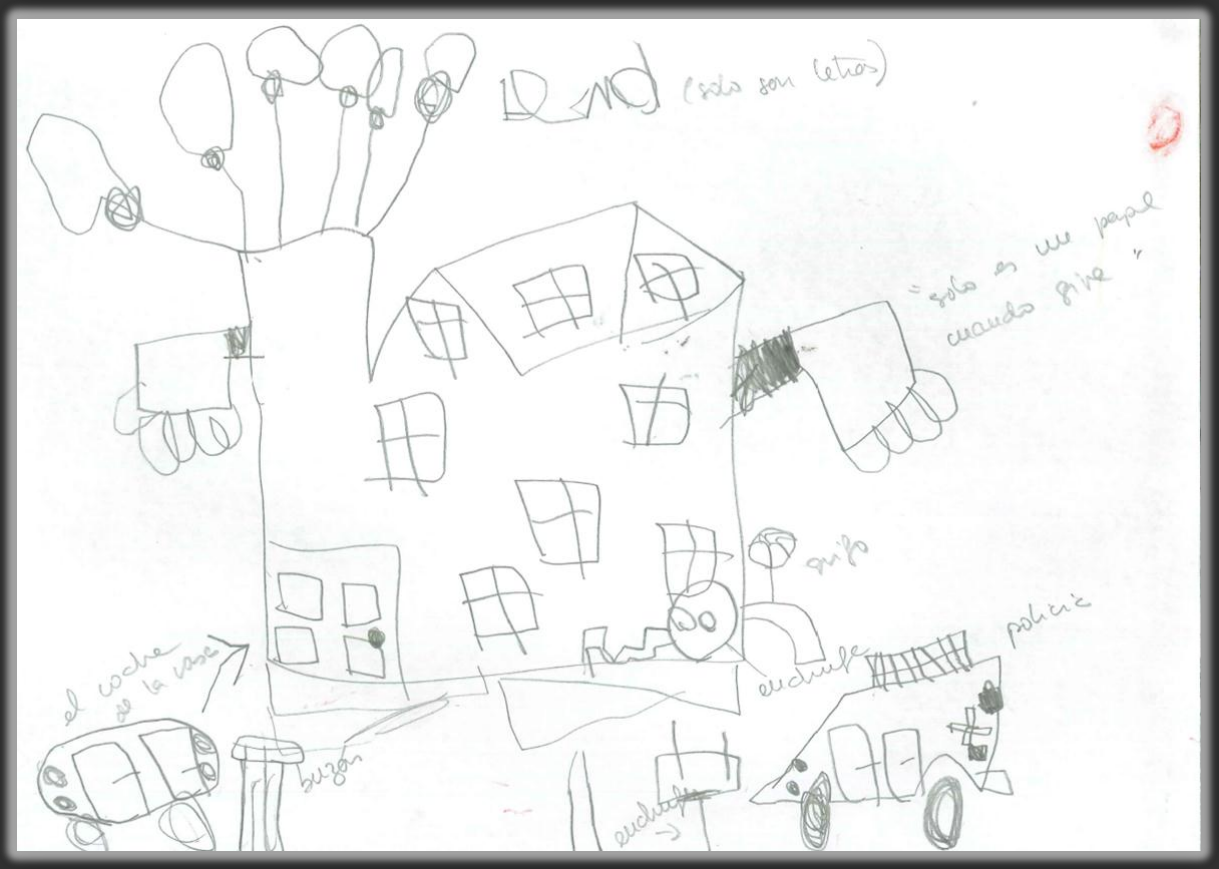
CONCLUSIONS

From the results of the presented study conducted in Würzburg, Germany, where the principal aim has been to evaluate the link between *EPHA4* and psychiatric diseases, specifically with schizophrenia and bipolar disorder, and if their SNPs variants could be risk factors to develop these illnesses, the conclusions are:

- Ψ According to ICD 10 and Leonhard's classifications, there is no genetic contribution of *EPHA4* to bipolar disorder and schizophrenia.

- Ψ Therefore, the study cannot confirm *EPHA4* as a common risk factor for the psychiatric diseases schizophrenia and bipolar disorder.





X. CHAPTER 4

ANKYRIN-3 AS A PERIPHERAL BIOMARKER OF PSYCHIATRIC DISORDERS

INTRODUCTION

A. ANKYRIN FAMILY

Ankyrins (ANK) are a multigene family of integral membrane proteins that posses binding sites for different integral proteins as well as for the spectrin-based membrane skeleton (Lambert S and Bennett V, 1993; Bennett V, 1992) (Figure 1.C4). It was known that ankyrins were associated with the voltage-dependent sodium channel *in vitro* and it are located in the node of Ranvier, axonal initial segments, and neuromuscular junctions (Srinivasan Y et al., 1988; Kordeli E et al., 1990; Kordeli E and Bennett V, 1991; Flucher BE & Daniels MP, 1989). However it was not until 1995, that Kordelli and colleagues (Kordelli E et al., 1995) described a third new subtype of family (Ankyrin-G) expressed in brain and other tissues, that is subject to extensive tissue-specific alternative mRNA processing. Ankyrin-G has two brain specific isoforms that contain a unique stretch of sequence highly enriched in serine and threonine residues immediately following the global head domain.

The interaction between the membrane skeleton and cytoplasmic domains of transmembrane proteins plays a fundamental role in membrane integrity and stability as well as in many cellular processes (Hryniewicz-Jankowska A et al., 2002) (Figure 1.C4). Thereby it is well known, through the knowledge of complete genome sequence, that ANK repeats are present in ~15000 proteins (Al-Khodor S et al., 2010). In these cases, ANK repeat is a 33-residues motif that after occurs in tandem arrays that cooperatively fold in structures that mediate molecular recognition via protein-protein interactions, and it has been considered one of the most important interacting proteins in nature (Bork P, 1993). They are involved in many cellular functions in eukaryotes, such as inhibition (Ink4, 53BP2), or development of tumors (Bcl3), transcriptional regulation (IKB, Mbp1, RFXANK), cell cycle, oncogenesis, signal transductions, and modulation of the inflammatory responses mediated by NF-κB (Mosavi LK et al., 2004; Voronin DA & Kiseleva EV, 2007).

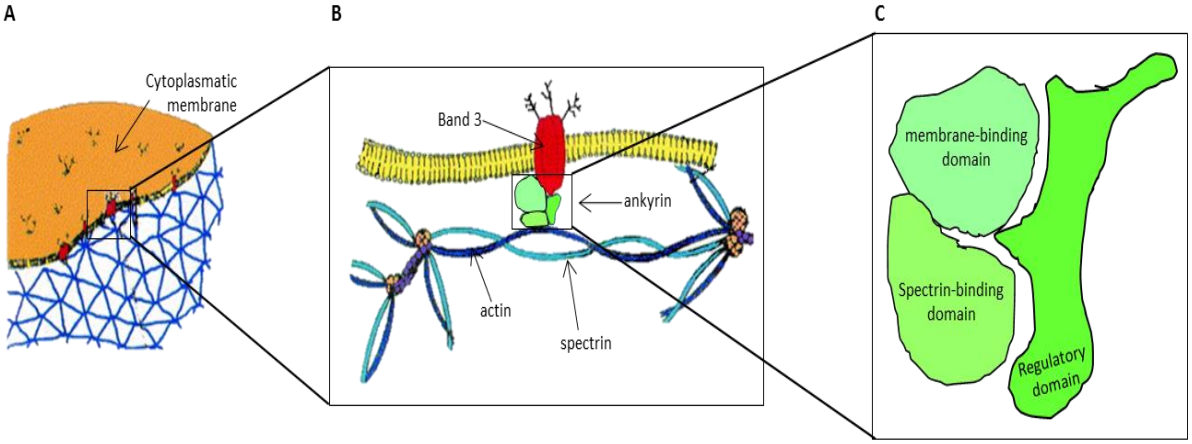


Figure 1.C4. Representative model of components integrated in integrity and stability of sepctrin-based cytoskeleton. (A) Organization of spectrin-based cytoskeleton, (B) Attachment of band 3, integral membrane protein, to spectrin molecule with intervention of ankyrin, (C) Ankyrin domains. *Adapted from Rubtsov AM & Lopina OD, 2000.*

As stated above, Ankyrin was first found in the human erythrocyte and it was subsequently described in a variety of vertebrate cells and tissues, including brain, epithelia and skeleton muscle. Variable cellular localization of these proteins may be due to different affinities of various isoforms for target proteins (Hryniewicz-Jankowska A et al., 2002).

A.1. ANKYRIN-G

ANK3 gene encodes Ankyrin-3 (ANK3) also known as Ankyrin-G (EntrezGene; Kapfhamer D et al., 1995). It is localized at 10q21.2 and consists of 44 exons spanning ~700 Kb on genomic DNA with multiple splicing variants. *ANK3* is an immunological distinct gene product from ANK1 and 2 (EntrezGene), and the gene product of 4377 amino acids was originally found as member of a protein family linking the integral membrane proteins to the underlying spectrin-actin cytoskeleton, located on axonal initial segment and at nodes of Ranvier in the central and peripheral neurons. Alternatively spliced variants may be expressed in other tissues as in epithelial cells, myocytes, hepatocytes and megakaryocytes (Peters LL et al., 1995). Although multiple transcript variants encoding several different isoforms have been found for this gene, the full-length nature of only two has been characterized (EntrezGene).

The gene product *ANK3* is proposed to play a regulatory role on sodium channel function, cell adhesion and neuronal development (Bennett V & Lambert S, 1999; Dzhashiashvili Y et al., 2007; Kizhatil K et al., 2007; Jenkins SM & Bennet V, 2002; Jenkins SM & Bennett V, 2001). The ANK3, in the nervous system, is specifically localized to the axon initial segments and the nodes of Ranvier (Lambert S et al., 1997) (Figure 2.C4); within the nodes of Ranvier where action potentials are actively propagated, ANK3 has long been thought to be the intermediate binding partner to neurofascin and voltage-gated sodium channel (Srinivasan Y et al., 1992). A post mortem study reported reduced immunoreactivity of Ankyrin-G in pyramidal neurons in the superficial cortical layer of the dorsolateral prefrontal cortex in subjects with schizophrenia (Cruz DA et al., 2009).

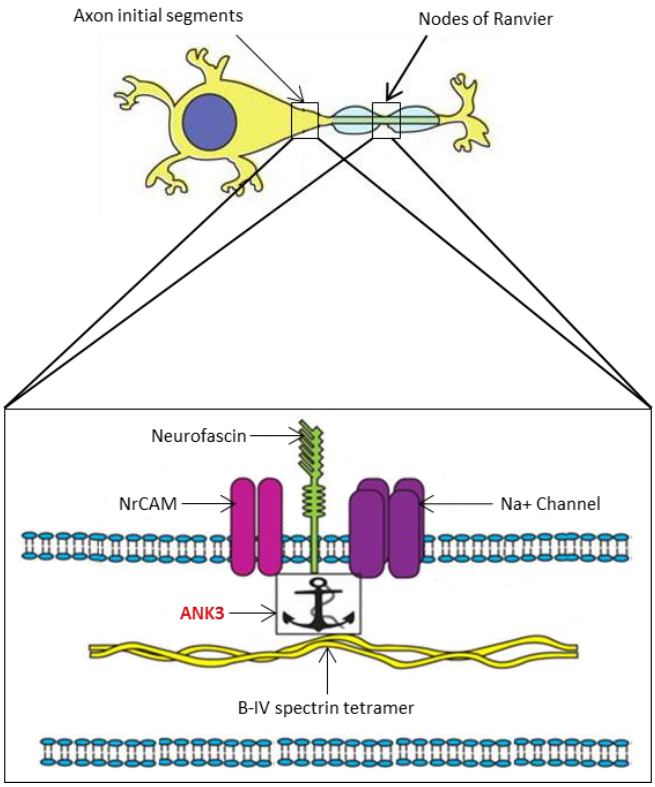


Figure 2.C4. Location of ANK3 in membranes of axon initial segments and nodes of Ranvier of neurons. ANK3 forms a complex with β-IV spectrin, neurofascin, NrCAM (cell adhesion protein), and ion channels. *Adapted from Bennett V & Healy J, 2009; and Simons M & TrajkovicK, 2006.*

The genetic deletions of *ANK3* from multiple neuron types have shown that it is required for the normal clustering of voltage-gated sodium channel at the axon hillock and for action potential firing (Zhou D et al., 1998; Hedstrom KL et al., 2007).

B. BACKGROUND of GENETIC STUDIES

GWA studies entail genotyping hundreds of thousands of common DNA variants in large numbers of individuals with disease and controls (individuals with a low prevalence of illness) (Altshuler D et al., 2008). Over the past two years, this type of studies have made major contributions to advance our knowledge of many common diseases such as diabetes, heart disease, inflammatory bowel disease, various cancer and rheumatoid arthritis (Manolio TA et al., 2008). All data obtained from these studies, provide a powerful resource for exploring the relationship between psychiatric phenotypes and can be used to inform conceptualization, classification, and diagnosis in psychiatry.

Schizophrenia and bipolar disorder are genetically complex diseases with numerous proposed genetic risk factors. These genetic risk factors encompass different pathophysiological pathways of neurotransmission, brain development or synaptic plasticity with each small contribution to diseases risk and inconsistent results among replication studies (Stöber G et al., 2009; Allen NC et al., 2008). Obviously, numerous collaborative studies have started to deliver genome-wide significant genetic association for both psychiatric diseases. Studies of approximately 10.000 samples have shown strong evidence for association with susceptibility to bipolar disorders at variants within two genes: *ANK3* and alpha-1c subunit of the L-type voltage-gated calcium channel (*CACNA1C*) (Ferreira MA et al., 2008); concretely, reported association of rs10994336 about 340 kb distal to rs9804190 located intronic between exon 36 and 37, at 3-UTR. Furthermore, an other GWA study based on pooled DNA found association with bipolar disorder and rs9804190 (Baum AE et al., 2008). A similar study in close to 20.000 individuals has shown strong evidence for association with susceptibility to schizophrenia at a variant within zinc finger transcription factor (*ZNF804A*) on chromosome 2q32 (O'Donovan MC et al., 2008). Other independent data provide further support for the involvement of *ANK3* in bipolar disorders and suggest that each variant (rs10994336 and rs9804190) might contribute independently to the pathophysiology of the disease (Schulze TG et al., 2009). A further SNP located 3'-UTR showed suggestive evidence of genome-wide association in Han Chinese sample (Lee MT et al., 2011). Subsequent studies found a genetic marker at *ANK3* to be associated with schizophrenia as well. GWA studies in a Norwegian discovery sample and a large European replication sample revealed association of rs10761482 (located near 3-UTR between exon 41 and 42) with disease at distance of 84.5 kb to rs9804190 (Athanasiu L et al., 2010). These initials results suggest an important implication of *ANK3* in the etiology of schizophrenia and bipolar disorder, therefore further studies and replication of the findings could be interesting to confirm the implication.

It is already clear that, in general, genetic associations are not specific to one of the traditional diagnostic categories. According to Craddock and colleagues (Craddock N et al., 2009), it is evident that all of these data are not consistent with a simple dichotomous model of functional psychosis and indicate the urgent need for moves toward approaches that better represent the range of phenotypic variation seen in the clinical population and that reflect the underlying biological variation that gives rise to the phenotypes. Then, the conclusion of all these findings is that the traditional dichotomy is not supported by data, some findings are consistent with relatively specific genetic susceptibility for disease with prominent features of both schizophrenia and bipolar illness, and that more genetic and clinical data are needed (Craddock N & Owen MJ, 2005).

OBJECTIVES

The aims of the study are to:

⇒ replicate recent findings of a genetic association of ANK3 with different psychiatric disease entities, in a case-control study

⇒ study the association of the following SNPs: rs9804190, rs10994336, and rs10761482

⇒ assess the association in two different classification systems: current diagnostics of ICD 10 and Leonhard's classification with a specific combination of bipolar and psychotic syndromes

EXPERIMENTAL PROCEDURES

Technical procedures used in the study of ANK3 as a possible biomarker for psychiatric diseases was described in headland VI.

EXPERIMENTAL PROCEDURE	HEADLAND	PAGE
TaqMan Genotyping Assay	B.1.2.	75
Statistical Analysis	D	84

RESULTS

Corresponding to HAPMAP data, rs9804190 locates between LD-block 7 and 8, rs10761482 in LD-block 26 and rs10994336 in a downstream LD block of ANK3. Thus, LD was low between the analyzed markers with LD' 0.018 between rs9804190 and rs10761482, 0.0060 between rs9804190 and rs10994336 and 0.72 between rs10761482 and rs10994336 located at 3'-UTR.

All SNPs were in Hardy-Weinberg Equilibrium (HWE). According to the ICD10 classification, allele and genotype frequencies were analyzed in cases with schizophrenia, bipolar disorder or major depression, and for SNPs rs9804190 and rs10994336 no association was revealed, (Table 1.C4 and Table 2.C4). Furthermore, no significant difference between cases and controls could be observed for the subgroup with schizoaffective disorder.

Table 1.C4. Bipolar disorder according to ICD10: Genotype distribution and test for association.

SNP	Cases n	Controls n	Cases CC	Controls CC	*p*	Cases CT	Controls CT	*p*	Cases TT	Controls TT	*p*
rs9804190 (C/T)	400	480	0.618	0.578	0.237	0.327	0.380	0.101	0.056	0.042	0.337
rs10994336 (C/T)	400	480	0.843	0.874	0.182	0.154	0.119	0.130	0.003	0.006	0.415
rs10761482 (C/T)	400	480	0.652	0.572	0.015	0.300	0.359	0.063	0.043	0.069	0.190

P: test for association (FAMHAP); CC, CT, TT: genotypes.

In contrast, association of SNP rs10761482 was observed for bipolar disorder (p = 0.015, OR 1.304, CI 1.065-1.595), exactly for the CC genotype. However, no association with schizophrenia was observed, neither with the subgroup of schizoaffective disorder nor with unipolar depression (Table 1.C4 and Table 2.C4).

Table 2.C4. Schizophrenia according to ICD10: Genotype distribution and test for association.

SNP	Cases n	Controls n	Cases CC	Controls CC	*p*	Cases CT	Controls CT	*p*	Cases TT	Controls TT	*p*
rs9804190 (C/T)	920	480	0.577	0.578	0.949	0.369	0.380	0.674	0.055	0.042	0.284
rs10994336 (C/T)	920	480	0.884	0.874	0.617	0.112	0.119	0.689	0.004	0.006	0.620
rs10761482 (C/T)	920	480	0.557	0.572	0.593	0.394	0.359	0.196	0.048	0.069	0.113

P: test for association (FAMHAP); CC, CT, TT: genotypes.

Sample was further differentiated according to Leonhard's classification. Analyzing association of SNPs with schizophrenic spectrum divided into subgroups systematic schizophrenias, unsystematic schizophrenias and cycloid psychosis provided no significances. Likewise affective disease with manic depression and monopolar depression reached no significant association.

Analyzing haplotype with FAMHAP provided no further risks haplotype concordant with observed low LD.

The study population with 1540 cases and 480 controls had a power of 55.1% to replicate the reported association with bipolar disorder and 69.1% with schizophrenia (alpha = 0.05%).

DISCUSSION

Common susceptibility genes for schizophrenia and bipolar disorder challenge traditional diagnostic categories and boundaries of these disorders. The present study attempted to replicate genetic association findings of *ANK3* as a possible common risk factor for schizophrenia and affective disorders in a case control study of > 2000 subjects of German descent.

Analysis of previously associated SNPs in different LD-Blocks, located intronic (rs9804190 and rs10761482) or 30 kb down-stream of *ANK3* (rs10994336), found a nominally significant association of SNP rs10761482 with bipolar disorder (p = 0.015, OR 1.304) but not with schizophrenia. Thus, association of this marker with schizophrenia in a GWA analysis of European samples could not be confirmed (Jenkins SM & Bennett V, 2001). The study failed to confirm an association of rs9804190 and rs10994336 with bipolar disorder reported in two previous GWA studies. Furthermore no association with either major depression or schizophrenia including subgroup of schizoaffective disorder was found (Dzhashiashvili Y et al., 2007; Kizhatil K et al., 2007). Analyzing haplotype provided no further risk haplotype concordant with observed LD between the markers. The failure to replicate previous findings could be due to an insufficient sample size. The study had a power of 55.1% to replicate reported association with bipolar disorder and 69.1% with schizophrenia (alpha = 0.05%) (Kizhatil K et al., 2007; Jenkins SM & Bennett V, 2001). However, in this study cases and controls of the same genetic background were included, minimizing a distortion regarding genetic heterogeneity.

The strength of the applied strategy is the combination of operational diagnostic criteria (ICD10) and Leonhard's categorical diagnostic approach. In search for common risk factors for schizophrenia and bipolar disorder, association was not found in the schizophrenic spectrum, neither with systematic schizophrenias nor with subgroups with a specific combination of bipolar and psychotic syndromes; Particularly the unsystematic schizophrenias and strictly defined manic depression with strong genetic background (Franzek E & Beckmann H, 1998; Pfuhlmann B et al., 2004; Stöber G et al., 1995). Other disease entities - according to Leonhard's classification - were not associated to any of the markers.

Data obtained in the study support findings from two meta-analyses of GWA-studies searching for common risk variants in *ANK3* for schizophrenia, bipolar disorder or unipolar depression: One study combining meta-anaysis and additional genotyping of a bipolar and unipolar sample from the US, the UK, Ireland, and Netherlands, found no association of variants in *ANK3* and unipolar depression. Another Meta-analysis on GWA studies based on schizophrenia and bipolar disorder cohorts with samples from UK observed no significant results for schizophrenia. Both Meta-analyses suggested a specific effect of *ANK3* for bipolar disorder (Liu Y et al., 2011; Williams HJ et al., 2011).

Since genetically associated SNPs around *ANK3* are intronic or located in downstream regions, causative variants or associated haplotype blocks are still missing. Regarding distorted gene regulation as pathophysiological causative factor, a recent study reported evidence for cis-acting regulation of *ANK3* by testing for allelic expression imbalance, but the study failed to attribute deregulation to risk-associated SNPs (Quinn EM et al., 2010).

CONCLUSIONS

From the results of this chapter of the thesis, where the principal aim had been to study the link between *ANK3* and schizophrenia and bipolar disorders, and whether *ANK3* variants could be risk factors to develop these diseases, the conclusions are:

- Ψ There is evident genetic contribution of *ANK3* to bipolar disorder, according to ICD10 classification. However, this contribution cannot be determined using Leonhard's classification.

- Ψ The rs10761482 SNP is a genetic risk factor for bipolar disorders, but not rs9804190 and rs10994336 gene variants.

- Ψ Intended replication of former association findings for schizophrenia according to ICD10 or Leonhard's classification failed for three studied SNPs (rs9804190, rs10994336, rs10761482).

- Ψ Therefore, the study cannot confirm *ANK3* as a common risk factor for schizophrenia and bipolar disorders.

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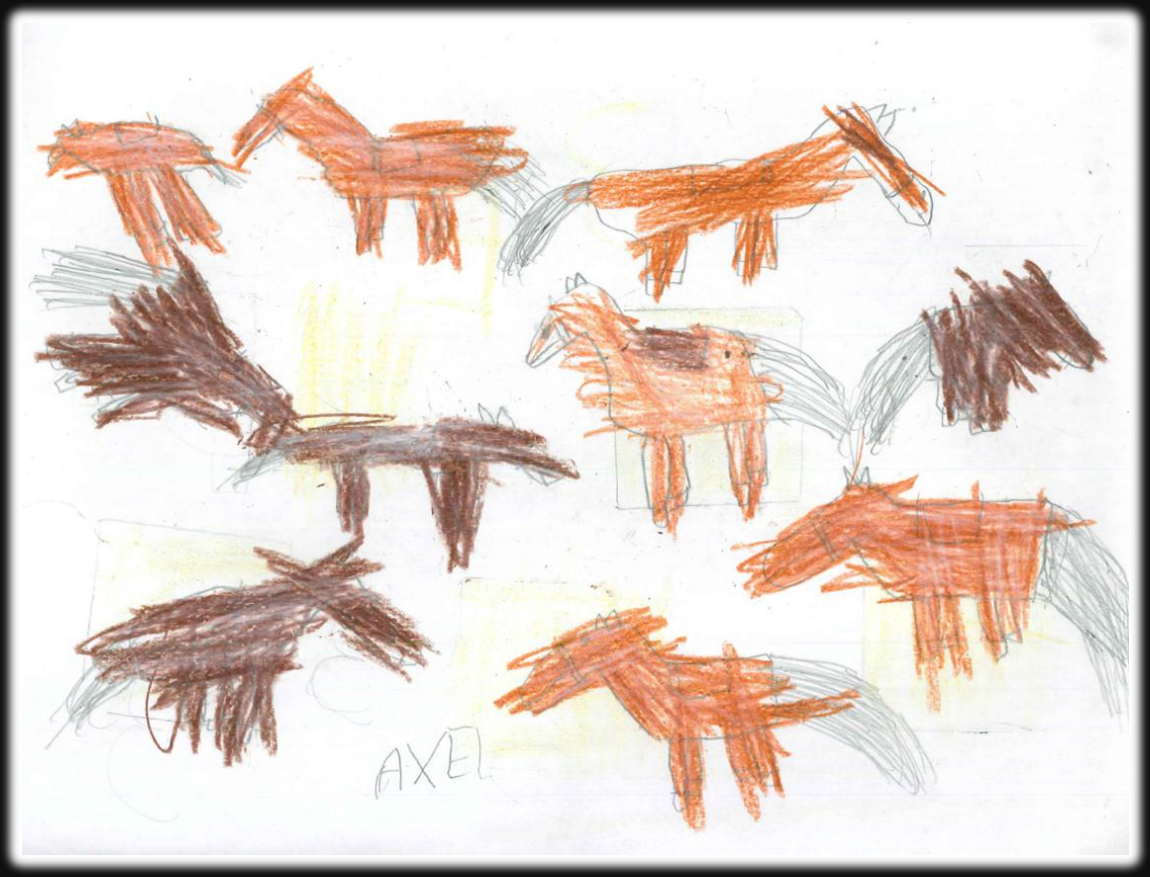

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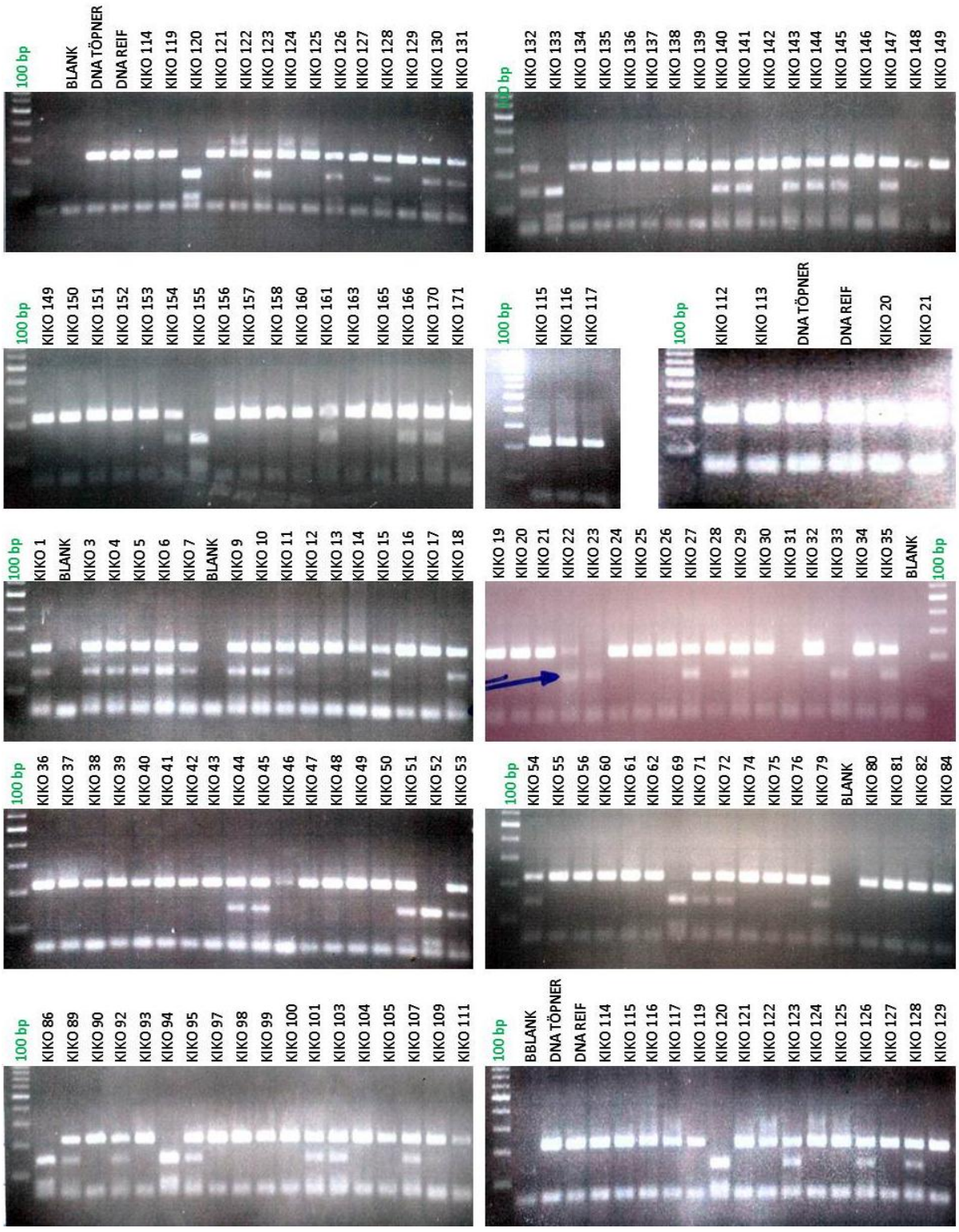


Figure 1SD. BDNF (rs6265) product digestion with NlaIII restriction enzyme of controls samples from Hospital of Child and Adolescent Psychiatry, Psychosomatic and Psychotherapy from Würzburg (Germany). Each bands profile is distinguished with the name identification sample. The loading marker used in the agarose gel resolution is equivalent at 100 bp size.

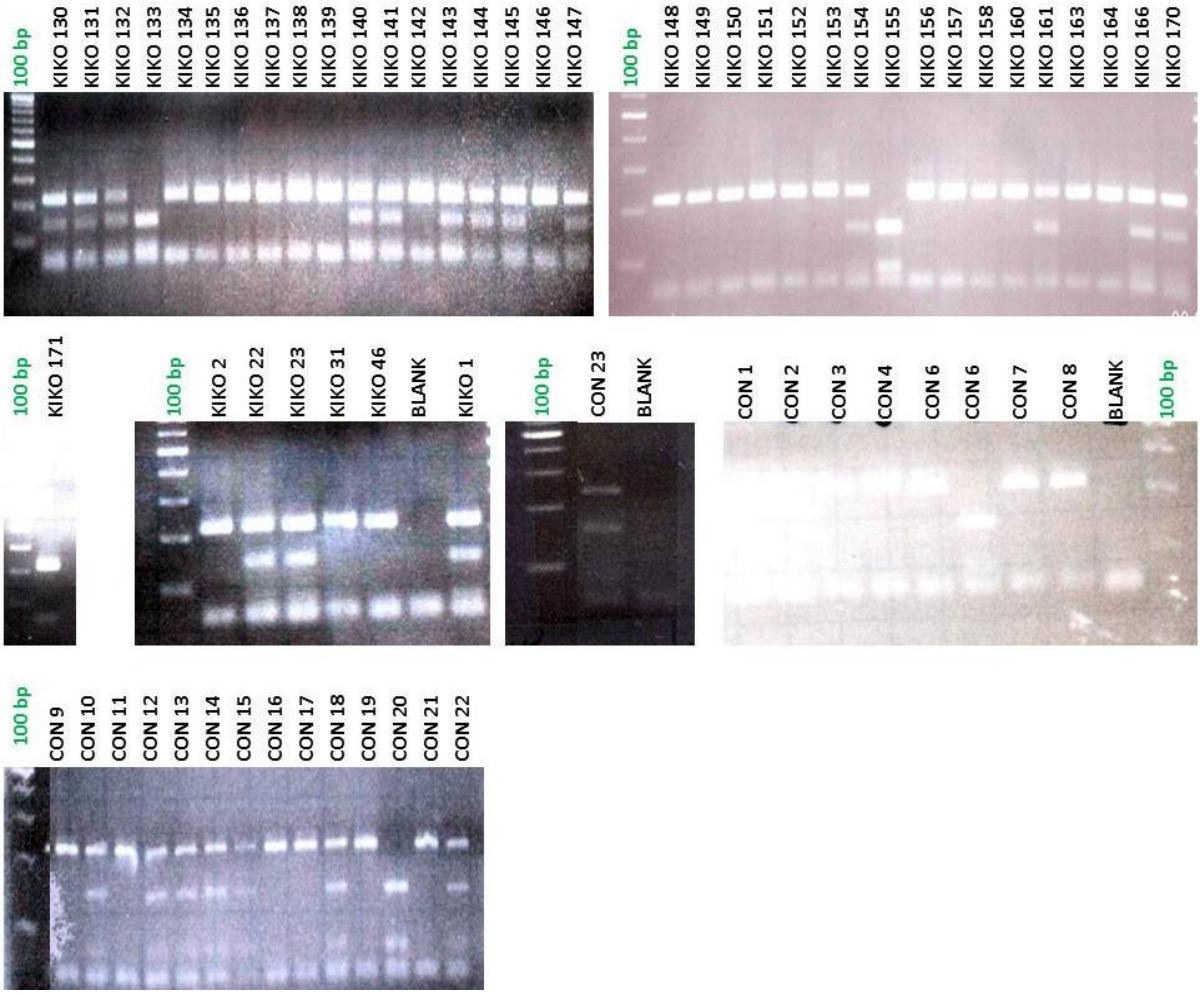


Figure 2SD. BDNF (rs6265) product digestion with NlaIII restriction enzyme of controls samples from Hospital of Child and Adolescent Psychiatry, Psychosomatic and Psychoterapy from Würzburg (Germany) and Consorci Parc Taulí from Sabadell (Spain). Each bands profile is distinguished with the name identification sample. The loading marker used in the agarose gel resolution is equivalent at 100 bp size.

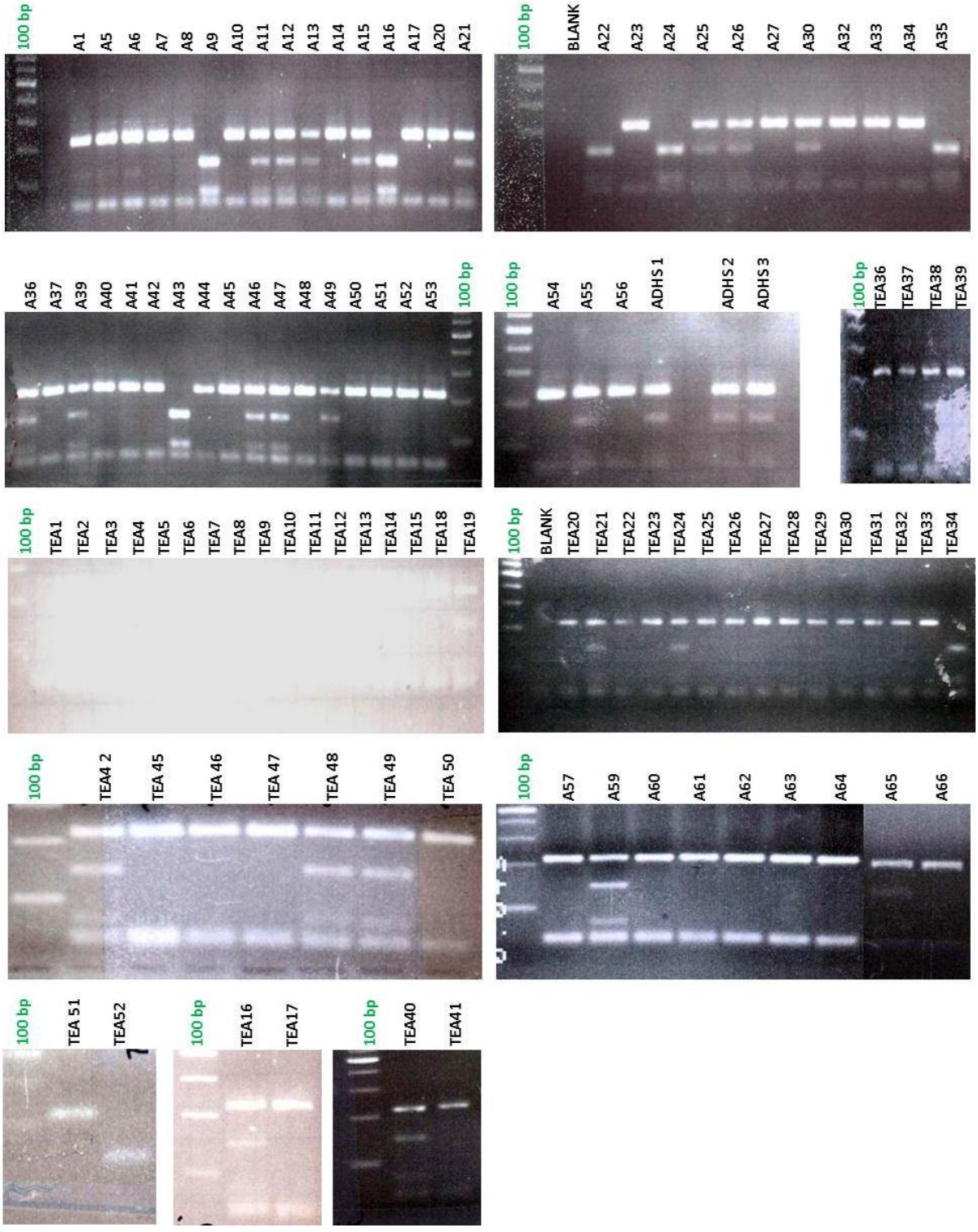
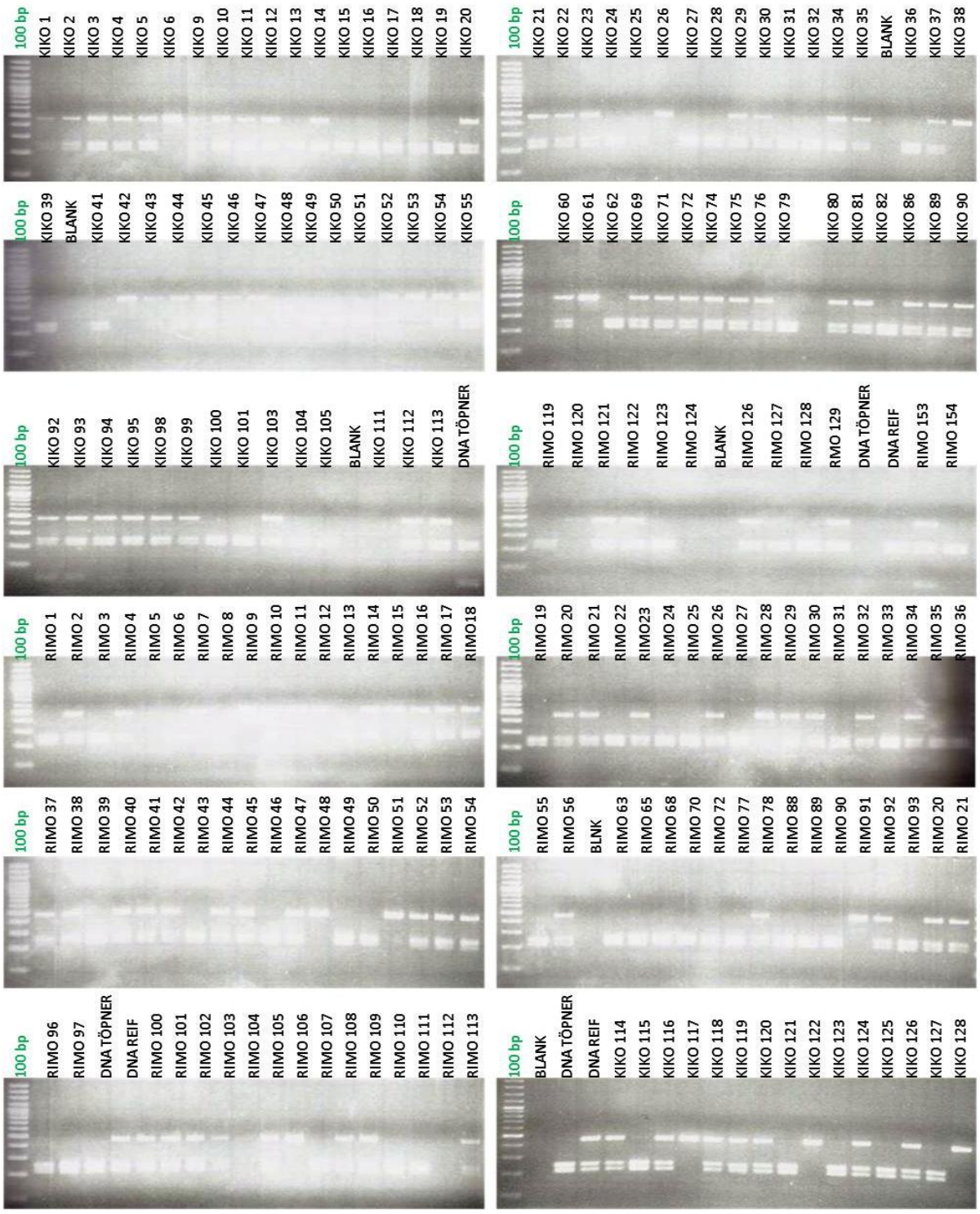


Figure 3SD. BDNF (rs6267) product digestion with NlaIII restriction enzyme of ASD samples from Hospital of Child and Adolescent Psychiatry, Psychosomatic and Psychoterapy from Würzburg (Germany), Consorci Parc Taulí from Sabadell (Spain), Fundació Althaia-CESAM from Manresa (Spain) and Escola i Centre Terapèutic Carrilet from Barcelona (Spain). Each bands profile is distinguished with the name identification sample. The loading marker used in the agarose gel resolution is equivalent at 100 bp size.



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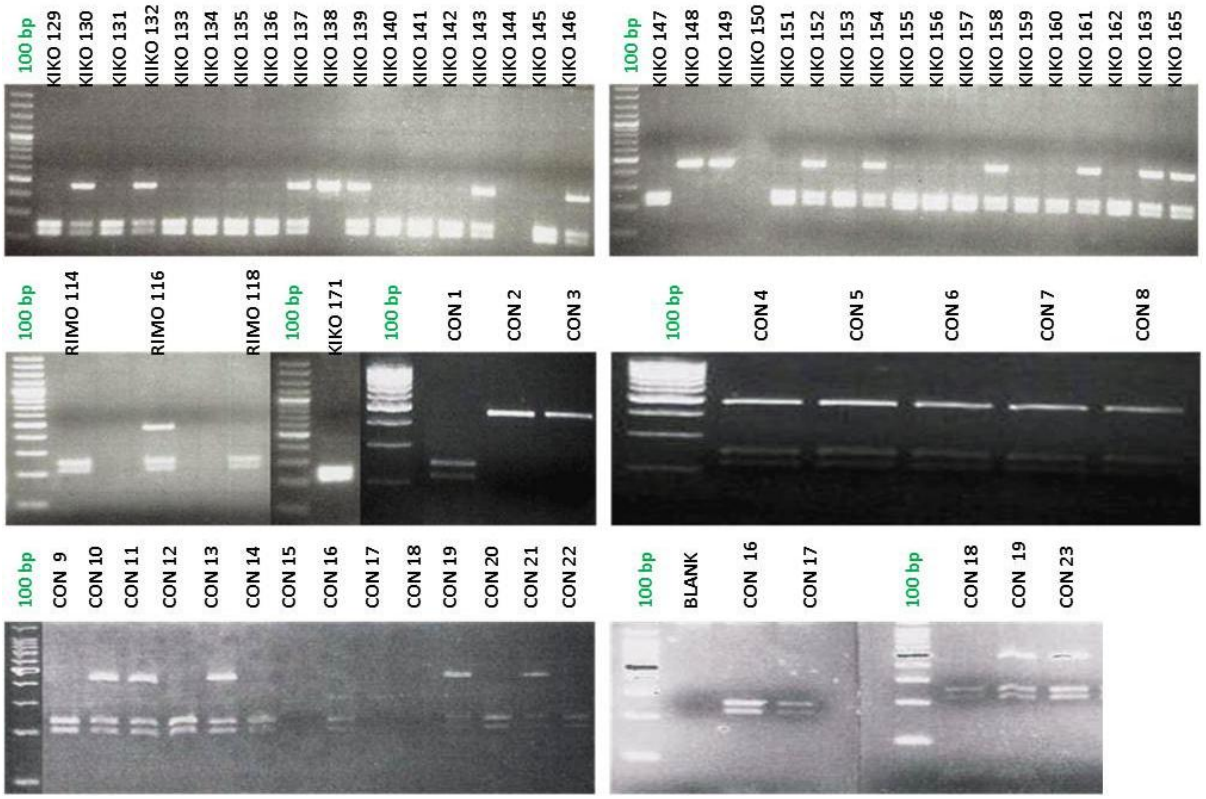

Figure 5SD. LPHN3 (rs4860079) product digestion with BsaBI restriction enzyme of control samples from Hospital of Child and Adolescent Psychiatry, Psychosomatic and Psychoterapy from Würzburg (Germany) and Consorci Parc Taulí from Sabadell (Spain). Each bands profile is distinguished with the name identification sample. The loading marker used in the agarose gel resolution is equivalent at 100 bp size.

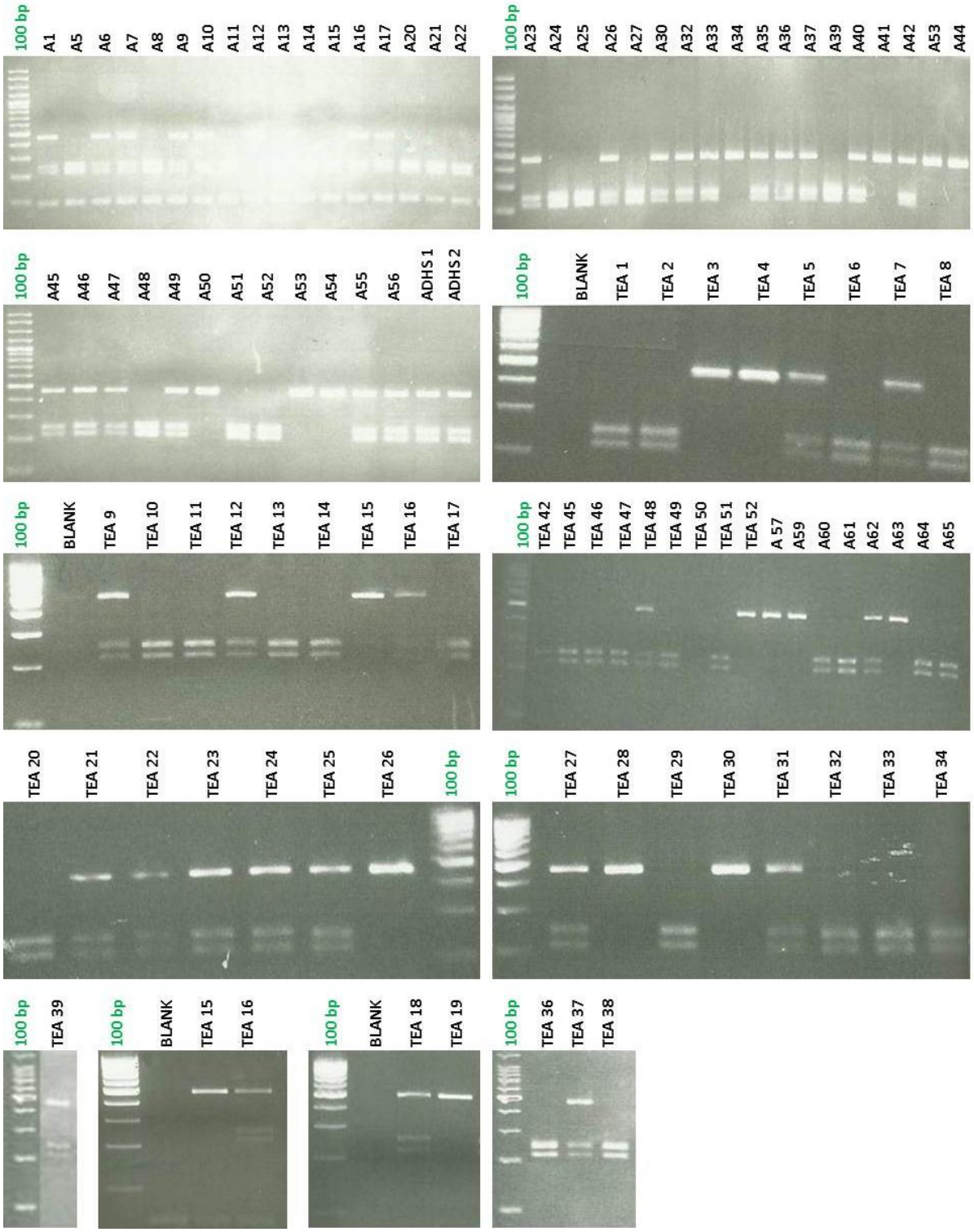

Figure 6SD. LPHN3 (rs4860079) product digestion with BsaBI restriction enzyme of ASD samples from Hospital of Child and Adolescent Psychiatry, Psychosomatic and Psychoterapy from Würzburg (Germany), Consorci Parc Taulí from Sabadell (Spain), Fundació Althaia-CESAM from Manresa (Spain) and Escola I Centre Terapèutic Carrilet from Barcelona (Spain). Each bands profile is distinguished with the name identification sample. The loading marker used in the agarose gel resolution is equivalent at 100 bp size.

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Fundació Parc Taulí
Institut Universitari UAB

INFORME DEL COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA

Doña COLOMA MORENO QUIROGA, Secretaria del Comité Ético de Investigación Clínica de la CORPORACIÓ SANITÀRIA PARC TAULÍ DE SABADELL (Barcelona)

CERTIFICA

Que este Comité ha evaluado la propuesta del promotor PROPI para que se realice el estudio titulado: "Estudio de expresión génica en el espectro autista y esquizofrénico" del protocolo y considera que:

1. Se cumplen los requisitos necesarios de idoneidad del protocolo en relación con los objetivos del estudio y están justificados los riesgos y molestias previsible para el sujeto.
2. La capacidad del investigador y los medios disponibles son apropiados para llevar a cabo el estudio.
3. Son adecuados tanto el procedimiento para obtener el consentimiento informado como la compensación prevista para los sujetos por daños que pudiera derivarse de su participación en el estudio.
4. El alcance de las compensaciones económicas previstas no interfiere con el respeto a los postulados éticos.
5. Y que el Comité acepta que dicho estudio sea realizado en el Corporació Sanitària Parc Taulí de SABADELL por PAMIAS MASSANA MONTSERRAT, como investigador principal.

Lo que firma en SABADELL a miércoles, 4 agosto 2010

Firmado:

Fundació
Parc Taulí
Institut Universitari UAB

Parc Taulí, 1
08208 Sabadell - Barcelona (Spain)

Dra. Coloma Moreno Quiroga

Ref.: 2010573

ANNEX 2

FUNDACIÓ UNIÓ

INFORME DEL COMITÉ ÉTIC D'INVESTIGACIÓ CLÍNICA

Dr. Jaume Plana, com a Secretari del Comitè Ètic d'Investigació Clínica de la FUNDACIÓ UNIO CATALANA HOSPITALS

C E R T I F I C A:

Que aquest Comitè en la seva reunió del dimarts, 25 d'octubre, ha avaluat:

La proposta d'Althaia, Xarxa Assistencial de Manresa per que es realitzi l'estudi que porta per títol "Estudi genètic dels trastorns de l'espectre autista i esquizofrènic." CEIC 11/82, i considera que:

Es compleixen els requisits necessaris d'idoneïtat del protocol en relació amb els objectius de l'estudi i que estan justificats els riscos i les molèsties previsibles per al subjecte.

La capacitat de l'investigador i els mitjans disponibles són apropiats per portar a terme l'estudi.

Són adequats tant el procediment per obtenir el consentiment informat com la compensació prevista per als subjectes per danys que es puguin derivar de la seva participació a l'estudi.

Que aquest comitè accepta que aquest estudi es digui a terme al centre Althaia, Xarxa Assistencial de Manresa amb l'Aurea Autet com investigadora principal.

I que l'investigador principal no ha estat present en les deliberacions i aprovació d'aquest estudi.

En aquesta reunió s'han complert els requisits establerts en la legislació vigent – RD 223/22004. El CEIC tant en la seva composició, com en els PNT compleix amb les normes de BPC (CPMP/ICH/135/95).

MEMBRES DEL CEIC DE LA FUNDACIÓ UNIÓ CATALANA D'HOSPITALS

Dra. Imma Guasch Jordan	President	Metge
Dr. Jaume Plana Rodríguez	Secretari	Metge
Dr. Andreu Fenellosa	Vocal	Metge
Dr. Miquel Nolla Salas	Vocal	Metge
Dra. Rosa Morros Pedrós	Vocal	Farmacòloga Clínica
Dr. Jaume Trapé Pujol	Vocal	Farmacèutic
Dra. Maria Immaculada Torre	Vocal	Farmacèutica
Dra. Concha Antolin	Vocal	Farmacèutica
Sra. Elisabet Juan Badia	Vocal	Infermera
Sra. Judith Noguera	Vocal	Infermera
Sr. Joan Pi Comellas	Vocal	Psicòleg
Sr. Josep M Bosch Vidal	Vocal	Advocat
Sra. Vanessa Massó Marigot	Vocal	C. Empresarials

Barcelona, 7 de novembre de 2011

FUNDACIÓ UNIÓ
Comitè Ètic d'Investigació Clínica
FUNDACIÓ UNIÓ CATALANA D'HOSPITALS

Dr. Jaume Plana
Secretari del CEIC

ANNEX 3

FUNDACIÓ UNIÓ

INFORME DEL COMITÉ ÉTIC D'INVESTIGACIÓ CLÍNICA

Dr. Jaume Plana, com a President del Comité Ètic d'Investigació Clínica de la FUNDACIÓ UNIO CATALANA HOSPITALS

C E R T I F I C A:

Que aquest Comitè en la seva reunió del dimarts, 27 de març, ha avaluat:

La proposta del Departament de Biologia Molecular i Cel·lular de la Facultat de Medicina i Ciències de la Salut de la Universitat Internacional de Catalunya, per que es realitzi l'estudi que porta per títol "Estudi genètic dels trastorns de l'espectre autista i esquizofrènic." CEIC 11/82, i considera que:

L'esmena consisteix és l'actualització del nom del promotor de l'estudi i la inclusió d'un nou centre.

Es compleixen els requisits necessaris d'idoneïtat del protocol en relació amb els objectius de l'estudi i que estan justificats els riscos i les molèsties previsibles per al subjecte.

La capacitat de l'investigador i els mitjans disponibles són apropiats per portar a terme l'estudi.

Són adequats tant el procediment per obtenir el consentiment informat com la compensació prevista per als subjectes per danys que es puguin derivar de la seva participació a l'estudi.

Que aquest comitè accepta que aquest estudi es digui a terme al centre Althaia, Xarxa Assistencial de Manresa amb l'Aurea Autet com investigadora principal i el centre CARRILET, amb l'Elisabet Sanchez com investigadora principal

I que l'investigador principal no ha estat present en les deliberacions i aprovació d'aquest estudi.

En aquesta reunió s'han complert els requisits establerts en la legislació vigent – RD 223/22004. El CEIC tant en la seva composició, com en els PNT compleix amb les normes de BPC (CPMP/ICH/135/95).

MEMBRES DEL CEIC DE LA FUNDACIÓ UNIÓ CATALANA D'HOSPITALS

Dr. Jaume Plana	President	Metge
Dr. Miquel Nolla	Secretari	Metge
Dr. Andreu Fenellosa	Vocal	Metge
Dra. Imma Guasch	Vocal	Metge
Dr. Jesús Montesinos	Vocal	Metge
Dra. Anna Altés	Vocal	Metge
Dra. Rosa Morros	Vocal	Farmacòloga Clínica
Dr. Jaume Trapé	Vocal	Farmacèutic
Dra. Imma Torre	Vocal	Farmacèutica
Dra. Concha Antolin	Vocal	Farmacèutica
Sra. Elisabet Juan	Vocal	Infermera
Sra. Itziar Aliri	Vocal	Advocat
Sra. Vanessa Massó	Vocal	C. Empresarials

Barcelona, 17 d'abril de 2012

FUNDACIÓ UNIÓ
Comitè Ètic d'Investigació Clínica
FUNDACIÓ UNIÓ CATALANA D'HOSPITALS

Dr. Jaume Plana
President del CEIC

ANNEX 4

INFORMACIÓ AL PARTICIPANT PER UN ESTUDI AMB MOSTRES BIOLÓGIQUES QUE ES CONSERVEN CODIFICADES

Naturalesa del projecto

El projecte d'investigació per al qual li demanem la seva participació porta per títol "Estudi genètic dels Trastorns de l'Espectre Autista i Esquizofrènic".

L'objectiu d'aquest estudi es aprofundir en el coneixement de factors genètics que puguin predisposar en el desenvolupament dels trastorns del espectre autista i esquizofrènic amb l'objectiu d'utilitzar-los com marcadors biològics de les patologies.

Els investigadors responsables d'aquest estudi pertenen a la Universitat Internacional de Catalunya i compten amb la col·laboració d'investigadors de altres centres d'invertigació i empreses privades.

Beneficis i riscos

El benefici de l'estudi es aprofundir en el coneixement dels Trastorns de l'Espectre Autista i esquizofrènic, amb l'objectiu de millorar la detecció, el diagnòstic i el tractament. Es possible que de la seva participació no obtingui un benefici directe. Per contra, la identificació de possibles factors relacionats amb aquest trastorns, podria beneficiar en un futur a altres pacients que la pateixen i això contribuir a una millora del coneixement i tractament de la malaltia.

El material genètic és un element que està present en totes les seves cèl·lules perquè l'ha rebut dels seus pares; porta un codi en forma de "gens" que determinarà les seves característiques físiques personals, com el color dels ulls, pell, etc. Així doncs, les diferencies entre unes persones i altres ens poden ajudar a explicar el per què algunes desenvolupen unes patologies i altres no.

L'estudi no suposa ningun risc que no sigui el derivat d'una extracció de sang. Així doncs, es podria notar la sensació de cremor en el punt que se li introdueix l'agulla a la pell i ocasionar un petit hematoma o una lleu infecció que desapareixerà en pocs dies. Més rarament, pot aparèixer mareig en el moment de l'extracció de sang.

Procediments

Si decideix participar en l'estudi deurà:

- Permetre que els investigadors puguin conèixer i treballar amb dades com el diagnòstic, l'edat, l'evolució de la malaltia, etc.
- Respondre a qüestionaris específics d'investigació, a més de l'entrevista i probes necessàries per poder fer el diagnòstic.
- Permetre que li practiquen una extracció de sang.

Totes les dades recollides per la investigació es guardaran informatitzades en uns fitxers on no hi apareixerà ni el seu nom ni ninguna altra dada que pugui identificar-lo.

Les mostres de sang son processades per extreure el DNA, RNA, sèrum i Limfòcits. Aquestes mostres seran congelades per al posterior anàlisis.

Lloc de la realització dels anàlisis

L'extracció de sang es realitzarà al Centre de Salut Mental de Manresa. Les mostres seran derivades al laboratori de Biologia Molecular de la Universitat Internacional de Catalunya, on seran processades segons convingui.

Ús futur de les mostres

A més, li demanem el seu consentiment per a que autoritzi als investigadors a guardar la seva mostra per realitzarà estudis genètics relacionats. Si autoritza que el material biològic sigui utilitzat en aquestes investigacions, les seves dades es mantindran codificades per tal de garantir la confidencialitat en la seva utilització, tal com preveu la legislació vigent.

Garantia de participació voluntària

Els investigadors li garanteixen que sigui quina sigui la seva decisió respecte a la participació en el projecte, la seva atenció sanitària per part del personal del Centre no es veurà afectada. A més, em cas que vostè accepti participar, ha de saber que es pot retirar en qualsevol moment, sense haver de donar explicacions i, en tal cas, la seva mostra seria retirada del banc de mostres i les seves dades dels fitxers informàtics.

Protecció de dades

D'acord amb la Lley Orgànica 15/1999 de Protecció de Dades de Caràcter Personal, les dades personals que s'obtinguin seran les necessàries per cobrir els fins de l'estudi. En cap dels informes apareixerà ni el nom ni ningun altre tipus d'identificació personal, excepte per complir amb els fins de l'estudi, y en el cas d'urgència mèdica o requeriment legal.

Qualsevol informació de caràcter personal que pugui ser identificable serà conservada per mètodes informàtics en condicions de seguretat. L'accés a dita informació quedarà restringida al personal de l'equip investigador, designat a l'efecte o a altre personal autoritzat que estarà obligat a mantenir la confidencialitat de la informació.

D'acord amb la llei vigent, te vostè dret a l'accés de les seves dades personals; així mateix, i si està justificat, te dret a la seva rectificació i cancel·lació. Si així ho desitja, deurà sol·licitar-ho al metge que l'até en l'estudi.

Implicació de la informació obtinguda en l'estudi

Si decideix participar en l'estudi, és possible que de les seves mostres s'obtingui informació rellevant per la seva salut o la de la seva família. D'acord amb la legislació vigent, te dret a ser informat de les seves dades genètiques que s'obtinguin en el curs de l'estudi.

En el cas de voler conèixer les dades genètiques obtingudes, informis a través del seu metge sobre les implicacions que aquesta informació pot tenir per la seva persona i la pròpia família. Aquesta informació se li comunicarà si així ho desitja; en cas que prefereixi no ser informat, es respectarà la seva decisió.

Preguntes

Per favor, si no ho ha fet abans, faci les preguntes que li pareguin oportunes. Li respondrem el millor que podrem.

ANNEX 5

CONSETIMENT INFORMAT PER PARE/MARE/TUTOR

ACCEPTACIÓ VOLUNTARIA DE PARTICIPACIÓ EN EL PROJECTE D'INVETSIGACIÓ "ESTUDIS GENÈTICS DELS TRASTORNS DEL ESPECTRE AUTISTA I ESQUIZOFRÈNIC"

Jo,……., lliurement, accepto que el meu fill/filla/menor tutoritzat .....participi en el projecte d'investigació "Estudis Genètics dels Trastorns del Espectre Autista i Esquizofrènic".

Sóc sabedor de que la participació consisteix en acceptar que es practiqui una extracció de 30 cc de sang, la qual serà utilitzada per extreure DNA, RNA, Sèrum i limfòcits amb l'objectiu de portar a terme estudis encaminats a identificar gens implicats en la vulnerabilitat a patir trastorns del espectre autista o esquizofrènic. A més, permeto als investigadors conèixer i treballar amb dades del diagnòstic, edat, evolució de la malaltia, etc y respondré als qüestionaris específics necessaris per a l'estudi.

He estat informat de que aquest estudi està dissenyat per incrementar els coneixements mèdics sobre aquest tiratrons i que els resultats que d'ells derivin, en cas de ser difosos, es publicaran en revistes científiques i sempre mantenint secreta la meva identitat. Així mateix, tindré dret a conèixer els resultats de l'estudi que involucrin a la meva persona o els meus descendents.

Adverteixo que puc renunciar a aquest consentiment en qualsevol moment sense donar ninguna explicació.

Dono fe que el propòsit d'aquest estudi se m'ha explicat per:……., amb qui he pogut discutir totes les qüestions que he plantejat.

Firmat,

Faig constar que he explicat a la persona voluntària la naturalesa i efecte de les probes que es realitzaran.

Firmat,

……., ….. de …… de ……..

CONSETIMENT INFORMAT PER ALS PARTICIPANTS

ACCEPTACIÓ VOLUNTARIA DE PARTICIPACIÓ EN EL PROJECTE D'INVESTIGACIÓ "ESTUDIS GENÈTICS DELS TRATRONS DEL ESPECTRE AUTISTA I ESQUIZOFRÈNIC"

Jo,……..,lliurement accepto participar en el projecte d'investigació "Genètica dels Trastorns del Espectre Autista i Esquizofrènic".

Sóc sabedor de que la participació consisteix en acceptar que se'm practiqui una extracció de 30 cc de sang, que serà utilitzada per extreure DNA, RNA, Sèrum i Limfòcits amb l'objectiu de portar a terme estudis encaminats a la identificació de gens implicats en la vulnerabilitat a patir Trastorns del Espectre Autista o Esquizofrènic. A més, permeto als investigadors conèixer i treballar amb dades del diagnòstic, edat, evolució de la malaltia, etc y respondré als qüestionaris específics necessaris per a l'estudi.

He estat informat que aquest estudi està dissenyat per incrementar els coneixements mèdics sobre aquests trastorns i que els resultats que d'ells deriven, en cas de ser difosos, es publicaran en revistes científiques i sempre mantenint secreta la meva identitat. Així mateix, tindré dret a conèixer els resultats de l'estudi que impliquin la meva persona o als meus descendents.

Adverteixo que puc renunciar a aquest consentiment en qualsevol moment sense donar cap explicació.

Dono fe que el propòsit d'aquest estudi m'ha estat explicat per: ……., amb qui he pogut discutir totes les qüestions que he plantejat.

Firmat,

Faig constar que he explicat a la persona voluntària la naturalesa i els efectes de les probes que es realitzaran.

Firmat,

……., ….. de …… de ……..

ANNEX 6

ACEPTACIÓN VOLUNTARIA DE PARTICIPACIÓN EN EL PROYECTO DE INVESTIGACIÓN "ESTUDIO DE EXPRESIÓN GÉNICA EN EL ESPECTRO AUTISTA Y ESQUIZOFRÉNICO"

Yo,……., libremente, acepto participar en el proyecto de investigación " Estudio de expresión génica en el espectro autista y esquizofrénico" .

La participación consiste en aceptar que se me practique una extracción de 30 cc de sangre, la cual será utilizada para extraer ADN y suero con el objetivo de llevar a cabo estudios encaminados a identificar los genes implicados en la vulnerabilidad a padecer trastornos del especto autista o esquizofrénico.

He sido informado de que este estudio está diseñado para incrementar los conocimientos médicos sobre este trastorno y que los resultados que de él se deriven, caso de ser difundidos, se publicarán en revistas científicas y siempre manteniendo secreta mi identidad. Así mismo, tendré derecho a conocer los resultados del estudio que conciernan a mi persona o a mis descendientes.

Advierto que puedo renunciar a este consentimiento en cualquier momento sin dar ninguna explicación.

Doy fe de que el propósito de este estudio me ha sido explicado por: ……, con quien he podido discutir todas las cuestiones que he planteado.

Firmado,

Hago constar que he explicado a la persona voluntaria la naturaleza y efectos de las pruebas que se realizarán

Firmado,

Sabadell , ….. de ……de……..

CONSENTIMIENTO INFORMADO

ACEPTACIÓN VOLUNTARIA DE PARTICIPACIÓN EN EL PROYECTO DE INVESTIGACIÓN "ESTUDIO DE EXPRESIÓN GÉNICA EN EL ESPECTRO AUTISTA Y ESQUIZOFRÉNICO"

Yo,……., libremente, acepto que mi hijo/ hija / menor tutorizado participe en el proyecto de investigación " Estudio de expresión génica en el espectro autista y esquizofrénico".

La participación consiste en aceptar que se me practique una extracción de 30 cc de sangre, la cual será utilizada para extraer ADN y suero con el objetivo de llevar a cabo estudios encaminados a identificar los genes implicados en la vulnerabilidad a padecer trastornos del especto autista o esquizofrénico.

He sido informado de que este estudio está diseñado para incrementar los conocimientos médicos sobre este trastorno y que los resultados que de él se deriven, caso de ser difundidos, se publicarán en revistas científicas y siempre manteniendo secreta mi identidad. Así mismo, tendré derecho a conocer los resultados del estudio que conciernan a mi persona o a mis descendientes.

Advierto que puedo renunciar a este consentimiento en cualquier momento sin dar ninguna explicación.

Doy fe de que el propósito de este estudio me ha sido explicado por: ……, con quien he podido discutir todas las cuestiones que he planteado.

Firmado,

Hago constar que he explicado a la persona voluntaria la naturaleza y efectos de las pruebas que se realizarán

Firmado,

Sabadell , ….. de ……..de……..

ANNEX 7

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BMC Psychiatry

RESEARCH ARTICLE Open Access

Is Ankyrin a genetic risk factor for psychiatric phenotypes?

Alejandro Gella[1], Mònica Segura[1], Núria Durany[1], Bruno Pfuhlmann[2], Gerald Stöber[2] and Micha Gawlik[2*]

Abstract

Background: Genome wide association studies reported two single nucleotide polymorphisms in *ANK3* (rs9804190 and rs10994336) as independent genetic risk factors for bipolar disorder. Another SNP in *ANK3* (rs10761482) was associated with schizophrenia in a large European sample. Within the debate on common susceptibility genes for schizophrenia and bipolar disorder, we tried to investigate common findings by analyzing association of *ANK3* with schizophrenia, bipolar disorder and unipolar depression.

Methods: We genotyped three single nucleotide polymorphisms (SNPs) in *ANK3* (rs9804190, rs10994336, and rs10761482) in a case-control sample of German descent including 920 patients with schizophrenia, 400 with bipolar affective disorder, 220 patients with unipolar depression according to ICD 10 and 480 healthy controls. Sample was further differentiated according to Leonhard's classification featuring disease entities with specific combination of bipolar and psychotic syndromes.

Results: We found no association of rs9804190 and rs10994336 with bipolar disorder, unipolar depression or schizophrenia. In contrast to previous findings rs10761482 was associated with bipolar disorder ($p = 0.015$) but not with schizophrenia or unipolar depression. We observed no association with disease entities according to Leonhard's classification.

Conclusion: Our results support a specific genetic contribution of *ANK3* to bipolar disorder though we failed to replicate findings for schizophrenia. We cannot confirm *ANK3* as a common risk factor for different diseases.

Background

Schizophrenia and bipolar disorder are genetically complex diseases with numerous proposed genetic risk factors encompassing different pathophysiological pathways of neurotransmission, brain development or synaptic plasticity with each small contribution to disease risk and inconsistent results among replication studies (Stöber et. al 2009) [1,2]. Recently genome wide association studies (GWAs) lead to identification of new susceptibility genes with genome-wide levels of significance: zinc finger gene *ZNF804A* on chromosome 2q32 or the *MHC*-locus at 6p21 on schizophrenia. For bipolar disorder the most promising results have been reported for *CACNA1C* and *ANK3* (ankyrin 3, node of Ranvier) [3-5]. Subsequently *CACNA1C* and *ZNF804A* were proposed as common risk variants for both bipolar disorder and schizophrenia and a Meta-analysis additionally added the *MHC*-locus as a common risk factor for both diseases [5].

ANK3 at 10q21.2 consists of 44 exons spanning ~700 kb on genomic DNA with multiple splicing variants. A GWA study based on pooled DNA found association with bipolar disorder and rs9804190 located intronic between exon 36 and 37 at the locus *ANK3* [6]. A Meta-analysis of GWA on bipolar patients with European ancestry reported an additional marker rs10994336, about 340 kb distal to rs9804190, at the 3-UTR of *ANK3* [7]. Further analysis suggested that each variant might contribute independently to bipolar disorder [8]. A further SNP located 3-UTR showed suggestive evidence of genome-wide association in a Han Chinese sample [9]. Subsequent studies found a genetic marker at *ANK3* to be associated with schizophrenia as well. Analysis in a GWA study of a Norwegian discovery sample with a large European replication sample reported association of rs10761482 located near 3-UTR between exon

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41 and 42 with disease at a distance of 84.5 kb to rs9804190 [10].

Ankyrin 3 is a brain expressed member of a protein-family linking the integral membrane proteins to the underlying spectrin-actin cytoskeleton. The gene product Ankyrin-G of 4377 amino acids locates on axonal initial segment and at nodes of Ranvier in the central and peripheral neurons. Ankyrin-G is proposed to play a regulatory role on sodium channel function, cell adhesion and neuronal development [11-16]. A post mortem study reported reduced immunoreactive of Ankyrin-G in pyramidal neurons in the superficial cortical layer of the dorsolateral prefrontal cortex in subjects with schizophrenia [17].

Within the debate on common susceptibility genes for schizophrenia and bipolar disorder we attempted to replicate common findings of a genetic association for different disease entities by analyzing association of *ANK3* with major psychosis in a case-control study with SNPs rs9804190, rs10994336, and rs10761482. For diagnosis we used beside ICD10 Leonhard's classification separating disease entities with specific combination of bipolar and psychotic syndromes [18].

Karl Leonhard divides psychoses into five main groups, systematic schizophrenias, unsystematic schizophrenias and cycloid psychoses. Affective psychoses are subdivided into bipolar manic depression and monopolar depression. In family and twin studies based on Leonhard's classification, a different genetic background for each diagnostic category was demonstrated [19]

Methods

Index cases were recruited from the Department of Psychiatry, Psychosomatics and Psychotherapy at of the University of Würzburg. The sample encompassed 920 cases (631 males, 68%) with psychosis according to ICD10 for schizophrenia or related diseases with an average age at onset of 26.5 years and an average age at recruitment of 41 years including 182 cases with schizoaffective disorder (ICD10 F20-F25). 400 cases (231 males, 58%) with bipolar disorder (F30-F31) with an average age at onset of 32 years and an average age at recruitment of 42.5 years and 220 cases (134 males, 61%) with unipolar depression (F32-F33). with an average age at onset of 43 years and an average age at recruitment of 51 years.

Sample was further subdivided according to Leonhard's classification systematic schizophrenias (n = 228), unsystematic schizophrenias (n = 635), cycloid psychosis (n = 309), manic depression (n = 284) and monopolar depression (n = 90) [17]. Diagnosis in differentiated psychopathology was made by repeated personal examinations of experienced psychiatrists (BP, MG, GS).

The 480 volunteer control subjects (283 males, 59%) were recruited from the blood donor centre at the University of Würzburg. The average age of recruitment was 29 years. The preponderance of males in both samples avoided gender distortion in comparison of cases and controls. All subjects were unrelated and of German Caucasian descent. The Ethics Committee of the University of Würzburg had approved the study, and written informed consent was obtained from all subjects.

PCR for allelic discrimination was performed in a final reaction volume of 20 μl containing 20 ng genomic DNA and 10 μl of 2 × TaqMan®Universal PCR Master Mix (Applied Biosystems) and 1 μl of 20 × TaqMan™ SNP genotyping assay including fluorescent tags specific for the wild type allele and the variant allele. Marker amplification was performed in microtiter plates on Biometra thermocyclers (Whatman). PCR amplification conditions were according to the manufacturer's recommendation [10 min at 95°C followed by 15 sec at 92°C and 60 sec at 60°C for 40 cycles]. Allelic discrimination with endpoint detection of fluorescence was performed at 60°C on an ABI prism 7000 sequence detection system followed by analysis with an appropriate software package (Applied Biosystems). All genotype experiments were made at least in duplicate, with quality control of automated allele calling by two independent operators blind to phenotype. The calling rate was 99%.

Software FAMHAP was used to test for association [20]. Hardy-Weinberg equilibrium (HWE) and pairwise standardized linkage disequilibrium (LD) were calculated with the program HAPLOVIEW [21]. The software "stastical power calculator" was used analyzing power for association test [22].

Results

Corresponding to HAPMAP data rs9804190 locates between LD-block 7 and 8, rs10761482 in LD-block 26 and rs10994336 in a downstream LD block of *ANK3*. Thus, linkage disequilibrium (LD) was low between the analyzed markers with LD' 0.018 between rs9804190 and rs10761482, 0.0060 between rs9804190 and rs10994336 and 0.72 between rs10761482 and rs10994336 located at 3'-UTR. All SNPs were in HWE.

Analyzing Allele and genotype frequencies in cases according to the ICD 10 classification with schizophrenia, bipolar disorder or major depression revealed no association for SNPs rs9804190 and rs10994336, (table 1 and 2). We observed no significant difference between cases and controls for subgroup with schizoaffective disorder. SNP rs10761482 was associated with bipolar disorder (p = 0.015, OR 1.304, CI 1.065 - 1.595) but not with schizophrenia, nor with subgroup schizoaffective disorder nor with unipolar depression (table 1 and 2).

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Table 1 Bipolar disorder according to ICD10: Genotype distribution and test for association

SNP	Cases n	Controls n	Cases CC	Controls CC	P	Cases CT	Controls CT	P	Cases TT	Controls TT	P
rs9804190 (C/T)	400	480	0.618	0.578	0.237	0.327	0.380	0.101	0.056	0.042	0.337
rs10994336 (C/T)	400	480	0.843	0.874	0.182	0.154	0.119	0.13	0.003	0.006	0.415
rs10761482 (C/T)	400	480	0.652	0.572	0.015	0.300	0.359	0.063	0.048	0.069	0.19

P: test for association (FAMHAP); CC, CT, TT: genotypes

Sample was further differentiated according to Leonhard's classification. Analyzing association of SNPs with schizophrenic spectrum divided into subgroups systematic schizophrenias, unsystematic schizophrenias and cycloid psychosis provided no significance. Likewise affective diseases with manic depression and monopolar depression reached no significant association.

Analyzing haplotype with FAMHAP provided no further risks haplotype concordant with observed low LD.

Our study population with 1540 cases and 480 controls had a power of 55.1% to replicate the reported association with bipolar disorder and of 69.1% with schizophrenia (alpha = 0.05%).

Discussion

Common susceptibility genes for schizophrenia and bipolar disorder challenge traditional diagnostic categories and boundaries between schizophrenia and bipolar disorder. We attempt to replicate genetic association findings of *ANK3* as a possible common risk factor for schizophrenia and affective disorders in a case control study of > 2000 subjects of German descent. Analysis of previous associated SNPs in different LD-Blocks, located intronic (rs9804190 and rs10761482) or 30 kb downstream of *ANK3* (rs10994336) found a nominally significant association of SNP rs10761482 with bipolar disorder (p = 0.015, OR 1.304) but not with schizophrenia (table 1 and 2). Thus, association of this marker with schizophrenia in a GWA analysis of European samples could not be confirmed [16].

We failed to confirm an association of rs9804190 and rs10994336 with bipolar disorder reported in two previous GWA studies. We found no association with unipolar depression or schizophrenia including subgroup of schizoaffective disorder (table 1 and 2) [13,14]. Analyzing haplotype provided no further risks haplotype concordant with observed low LD between the markers.

Our failure to replicate previous findings could be due to insufficient sample size. The study had a power of 55.1% to replicate reported association with bipolar disorder and of 69.1% with schizophrenia (alpha = 0.05%) [14,16]. However, in our study were cases and controls of the same genetic background, minimizing a distortion regarding genetic heterogeneity. The strength of our strategy is the combination of operational diagnostic criteria (ICD-10) and Leonhard's categorical diagnostic approach. In search for common risk factors for schizophrenia and bipolar disorder we found no association in the schizophrenic spectrum neither with systematic schizophrenias nor with subgroups with a specific combination of bipolar and psychotic syndromes: Particularly the unsystematic schizophrenias and strictly defined manic depression with strong genetic background [19,23,24]. Other disease entities according to Leonhard's classification were not associated to any of the markers.

Our data support findings from two meta-analyses of GWA-studies searching for common risk variants in *ANK3* for schizophrenia, bipolar disorder or unipolar depression: One study combining Meta-analysis and additional genotyping of a bipolar and unipolar sample from the US, the UK, Ireland, and Netherlands, found no association of variants in *ANK3* and unipolar depression. Another Meta-analysis on GWA studies based on schizophrenia and bipolar disorder cohorts with samples from UK observed no significant results for schizophrenia. Both Meta-analyses suggested a specific effect of *ANK3* for bipolar disorder [25,5].

Since genetically associated SNPs around *ANK3* are intronic or in downstream regions located, causative coding variants or associated haplotype blocks are still

Table 2 Schizophrenia according to ICD10: Genotype distribution and test for association

SNP	Cases n	Controls n	Cases CC	Controls CC	P	Cases CT	Controls CT	P	Cases TT	Controls TT	P
rs9804190 (C/T)	920	480	0.577	0.578	0.949	0.369	0.380	0.674	0.055	0.042	0.284
rs10994336 (C/T)	920	480	0.884	0.874	0.617	0.112	0.119	0.689	0.004	0.006	0.62
rs10761482 (C/T)	920	480	0.557	0.572	0.593	0.394	0.359	0.196	0.048	0.069	0.113

P: test for association (FAMHAP); CC, CT, TT: genotypes

missing. Regard distorted gene regulation as patho-physiological causative factor a recent study reported evidence for cis-acting regulation of *ANK3* by testing for allelic expression imbalance, but the study failed to attribute dysregulation to risk-associated SNPs [26].

Conclusions

In conclusion, our results support a genetic contribution of *ANK3* to ICD 10 bipolar disorder, though we failed to replicate findings for schizophrenia according to ICD 10 or Leonhard's classification. Our study cannot confirm *ANK3* as a common risk factor for both diseases, challenging the hypothesis that bipolar disorder and schizophrenia are just different phenotypes of the same disease.

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Authors' contributions

AG and MS performed the experiments and drafted the manuscript; ND and GS conceived the study and participated in the coordination. BP and GS carried out the diagnostic evaluation of the patients; MG carried out the statistical analyses, coordinated the study and wrote the manuscript. All authors read and approved the final manuscript.

Authors' information

To our deep regret Professor Núria Durany passed away on August 27th 2010 after an intense but short fighting an illness. We shall continue to cherish her passion for science.

Competing interests

The authors declare that they have no competing interests.

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Caminante son tus huellas
el camino y nada más;
caminante, no hay camino
se hace camino al andar.

Al andar se hace camino
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.

Caminante no hay camino
sino estelas en la mar...