UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA

PROGRAMA DE PÓS-GRADUAÇÃO EM CB: BIOQUÍMICA

DO AMBIENTE AOS GENES:

O USO DE FERRAMENTAS BIOINFORMÁTICAS NA PROCURA DE UM MÍNIMO DENOMINADOR MOLECULAR E CELULAR COMUM NO ESPECTRO AUTISTA

Fares Zeidán Chuliá

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Tese submetida ao Programa de Pós-graduação em Ciências Biológicas: Bioquímica, como requisito para a obtenção do grau de Doutor em Bioquímica

Porto Alegre, 2014

CIP - Catalogação na Publicação

Zeidán Chuliá, Fares
Do ambiente aos genes: o uso de ferramentas bioinformáticas na procura de um mínimo denominador molecular e celular comum no espectro autista / Fares Zeidán Chuliá. -- 2014.
260 f.

Orientador: José Cláudio Fonseca Moreira.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2014.

1. Autismo. 2. Ambiente. 3. Gene. 4. Proteína. 5. Rede. I. Fonseca Moreira, José Cláudio, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Dedicado

A meus pais, irmão e minhas melhores amigas, Maria José e Carmen, na Espanha

A minha querida Rosana (Rô), que me trouxe até este belo país, aquela que já considero a minha segunda terra: o Brasil

"... e quando ele falar convosco,

acreditai nele, apesar de sua voz poder esfacelar vossos sonhos

como o vento norte arruína o jardim.

Pois mesmo quando o amor vos coroa, ele vos crucifica.

O amor não dá nada além de si mesmo e não toma nada além de si mesmo.

O amor não possui nem é possuído; pois o amor é suficiente ao amor.

Quando vós amais, não deveis dizer: <<Deus está no meu coração>>,

mas sim <<Estou no coração de Deus>>.

E não pensai que podeis dirigir o curso do amor,

pois o amor, se achar que mereceis, dirige o vosso curso"

(Trechos de *"*O Profeta" de *Khalil Gibran* falando sobre o amor)

AGRADECIMENTOS

Ao Professor **José Cláudio Fonseca Moreira**, pela confiança desde o primeiro dia quando nos conhecemos e pela excelente orientação que fizeram possível a realização da presente tese.

Ao Professor **Daniel Pens Gelain**, pelo apoio constante no meu trabalho e por acreditar sempre em mim.

Aos Professores **Alexei Verkhratsky**, **Alla B. Salmina**, **Mami Noda** e **Ulvi K. Gürsoy** pela amizade e ensino fundamentais durante todos estes gratificantes anos de trabalho e **Manuel F. Casanova** e **Eija Könönen** pela parceria nos meus projetos.

Ao aluno de iniciação científica **Ben-Hur Neves de Oliveira**, pela amizade e por todas as horas e horas e mais horas de trabalho conjunto e aprendizado que fizeram deste estudo um caminho enriquecedor e também muito divertido.

Aos colegas **Guilherme Antônio Behr**, **Eduardo Antônio Kolling**, **José Luiz Rybarczyk-Filho** e **Matheus Augusto de Bittencourt Pasquali**, pela colaboração nos estudos de bioinformática e bancada.

Ao PPG Bioquímica (UFRGS) e seus funcionários, **Cleia**, **Bebel** e os meninos da secretaria, pois o seu trabalho é fundamental para todos neste departamento.

Aos organismos de fomento **CNPq**, **CAPES** e **FAPERGS** que financiaram este trabalho.

Em geral, a todas aquelas pessoas que de alguma forma contribuíram, voluntaria ou involuntariamente, ao desenvolvimento do meu trabalho, meu eterno agradecimento.

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RESUMO

O autismo pode ser definido como um transtorno associado ao desenvolvimento e caracterizado por prejuízo na interação social, na comunicação e no comportamento. Sua etiologia ainda é pouco conhecida, existindo alterações no desenvolvimento encefálico durante a embriogênese e na vida pós-natal. Sugere-se uma complexa interface entre fatores genéticos e ambientais. Existem provas que mostram uma desregulação do controle da homeostase e redes neuronais por parte de células astrogliais, ativação da microglia e respostas neuroinflamatórias no encéfalo de pacientes autistas até a idade adulta, representando uma alteração celular comum dentro do espectro autista (ASD). A grande variabilidade dos sintomas encontrados nos pacientes torna extremamente difícil a identificação de cascatas de sinalização comuns associadas com a patologia tipicamente autista, críticas para a procura de marcadores periféricos de diagnóstico e para identificar novos alvos terapêuticos. Neste trabalho, (i) caracterizamos a natureza multifatorial do autismo, funções moleculares, componentes celulares e processos biológicos associados, (ii) mostramos que *RAC1*, em particular, e a família das RHO GTPases, em geral, poderiam ter um papel crítico nos eventos neuropatológicos associados ao autismo, sendo o cálcio (Ca^{2+}) a molécula mais central na complexa interface entre fatores genéticos e ambientais e (iii) sugerimos um modelo baseado na ativação da enzima α-secretase, mediada por receptores de glutamato (NMDARs), influxo de Ca^{2+}, ativação de Erk e adaptação da mitocôndria a apoptose, como cascata de sinalização bioquímica que poderia explicar o aumento do volume encefálico e a falha da conectividade cerebral observada em crianças autistas e que, potencialmente, poderia ser tratada com derivados de magnésio e rapamicina.

ABSTRACT

Autism is a neurodevelopmental disorder characterized by specific activity patterns and aberrant social interaction and communication. Even though its etiology is not well understood, a number of neuropathological events during central nervous system development, in childhood and adolescence, have already been described. A complex interface between genetic and environmental factors is also suggested to account for the disorder. Evidence shows a deregulation of the homeostatic control of neuronal networks by astroglia, microglial activation, and neuroinflammation; changes that persist even until adulthood and may represent a common cellular disturbance in autism spectrum disorders (ASD). The great variability of symptoms found in the patients makes a difficult challenge the identification of disrupted signaling pathways associated to ASD, which is critical to identify potentially novel biomarkers for diagnoses as well as novel therapeutic targets. In the present study, (i) we characterized the multifactorial nature of autism, molecular functions, cellular components, and biological processes associated to the disorder, (ii) we showed *RAC1*, in particular, and the RHO family of GTPases, in general, could play a critical role in the neuropathological events associated with autism, with calcium (Ca^{2+}) as the most central component in interface between genetic and environmental factors, and (iii) we proposed a model of glutamate receptors (NMDARs)-mediated Erk activation of α-secretase activity and mitochondrial adaptation to apoptosis that may explain the early brain overgrowth and disruption of synaptic plasticity and connectome in autistic children, which could potentially be targeted by magnesium-based drugs and rapamycin.

LISTA DE ILUSTRAÇÕES ORIGINAIS

LISTA DE ABREVIATURAS E SIGLAS

Aβ	Peptídeo β-amiloide
APP	Proteína precursora amiloide
sAPPα	Forma secretada solúvel α da APP
AICD	Domínio intracelular da APP
ASD	Transtornos do espectro autista
EGF	Fator de crescimento epidérmico
GFAP	Proteína fibrilar acídica glial
HSP-70	Proteína de choque térmico 70
IFNγ	Interferon γ
IL1	Interleucina 1
IL6	Interleucina 6
IL8	Interleucina 8
IL10	Interleucina 10
IL12	Interleucina 12
PDD	Transtorno desintegrativo da infância
PDD-NOS	Transtorno invasivo do desenvolvimento não especificado
RELN	Relina
ROS	Espécies reativas de oxigênio
SNC	Sistema nervoso central
TGF-β1	Fator de crescimento transformante β1
TGF- β2	Fator de crescimento transformante β2
TIDs	Transtornos invasivos do desenvolvimento

*** Obs:** siglas em inglês foram mantidas quando de uso corrente ou quando estas foram associadas a nomenclaturas já normatizadas (ex.: símbolo de genes).

SUMÁRIO

APRESENTAÇÃO----------

Esta tese está estruturada em três partes. A ***Parte I*** é uma introdução geral resumindo os pontos chave dos referenciais teóricos publicados em periódicos internacionais indexados, com ênfase nas contribuições genéticas e ambientais, na vulnerabilidade genética dos indivíduos, nas características neuropatológicas do autismo, nos tratamentos e eventos moleculares típicos da doença de Alzheimer (patologia neurodegenerativa com a qual vai ser comparado este transtorno do desenvolvimento) e, finalmente, uma breve descrição das ferramentas de biologia de sistemas e propriedades de rede utilizadas na presente tese. A ***Parte II*** apresenta os referenciais teóricos a partir dos quais se fundamenta esta tese, na forma de dois artigos de revisão e capítulo de livro. A ***Parte III*** apresenta dois capítulos na forma de artigos científicos. O primeiro capítulo caracteriza as interações gene-ambiente através da revisão sistemática da literatura e análise por biologia de sistemas para determinar os processos biológicos predominantes, componentes celulares e funções moleculares intrínsecos no transtorno. Buscamos, assim, criar um modelo integrativo gene-ambiente no autismo, elucidar o(s) componente(s) mais centrais e examinar a existência de um gene nunca antes associado com uma contribuição crítica nos eventos neuropatológicos associados ao autismo. Dentro destes eventos neuropatológicos característicos está o crescimento excessivo do encéfalo e a falha na conectividade cerebral. No capítulo dois, propusemos um modelo baseado nas novas análises *in silico*, onde o mesmo pacote de genes tipicamente desregulados na doença de Alzheimer poderia ter um papel significativo no excessivo crescimento cerebral no autista e na consequente falha da conectividade encefálica. Finalmente, a ***Parte IV*** apresenta a discussão geral sobre os resultados de cada artigo científico, seguido de conclusões e perspectivas.

PARTE I----------

INTRODUÇÃO GERAL

1. Autismo: genes, ambiente e neuropatologia associada

Autismo e transtornos do espectro autista (ASD) são terminologias genéricas usadas indistintamente para representar um grupo de transtornos do desenvolvimento cerebral, os quais são detectados durante a infância e caracterizados por uma pobre habilidade social, presença de padrões repetitivos e estereotipados de comportamento, evidenciada pela insistência em determinadas rotinas ou rituais não funcionais específicos, além de estereotipias motoras e verbais (Quaak et al., 2013).

Em 1980, o autismo foi reconhecido pela primeira vez no Manual Estatístico e Diagnóstico da Associação Americana de Psiquiatria (*DSM III*). Em 1994 (*DSM IV*), novos critérios são incluídos devido à necessidade de identificação de subgrupos homogêneos de indivíduos autistas, tanto para finalidades práticas quanto de pesquisa. A versão do ano 2000 (*DSM-IV-TR*) vem acompanhada de textos atualizados sobre o autismo e outros transtornos invasivos do desenvolvimento (TIDs), mas os critérios diagnósticos permanecem os mesmos que os do DSM-IV, que nos possibilitam o diagnóstico dos TID com suas subdivisões em (Klin, 2006; Rapin & Tuchman, 2008): (i) Autismo clássico, (ii) síndrome de Rett, (iii) síndrome de Asperger, transtorno desintegrativo da infância (PDD) e transtorno invasivo do desenvolvimento não especificado (PDD-NOS). A versão mais atual, DSM-5, foi publicada recentemente, em 2013.

Nos Estados Unidos, estimou-se uma prevalência de 1 em cada 50 crianças nascidas, com uma aparente tendência a aumentar nestes últimos anos (Blumberg et al., 2013; Rutter, 2005). Porém, não está claro se estes dados epidemiológicos são o resultado de um hiper-diagnóstico ou uma maior atenção voltada ao transtorno por parte da comunidade científica (Fombonne, 2008; Wing & Potter, 2002) pela desordem. Embora a maior parte de genes responsáveis pelas diferentes formas de autismo não seja conhecida, tem sido amplamente aceito na literatura que a existência de uma predisposição genética contribui na etiologia, pois numerosos polimorfismos associados a esse transtorno já foram descritos (Mefford et al., 2012) (**Figura 1**).

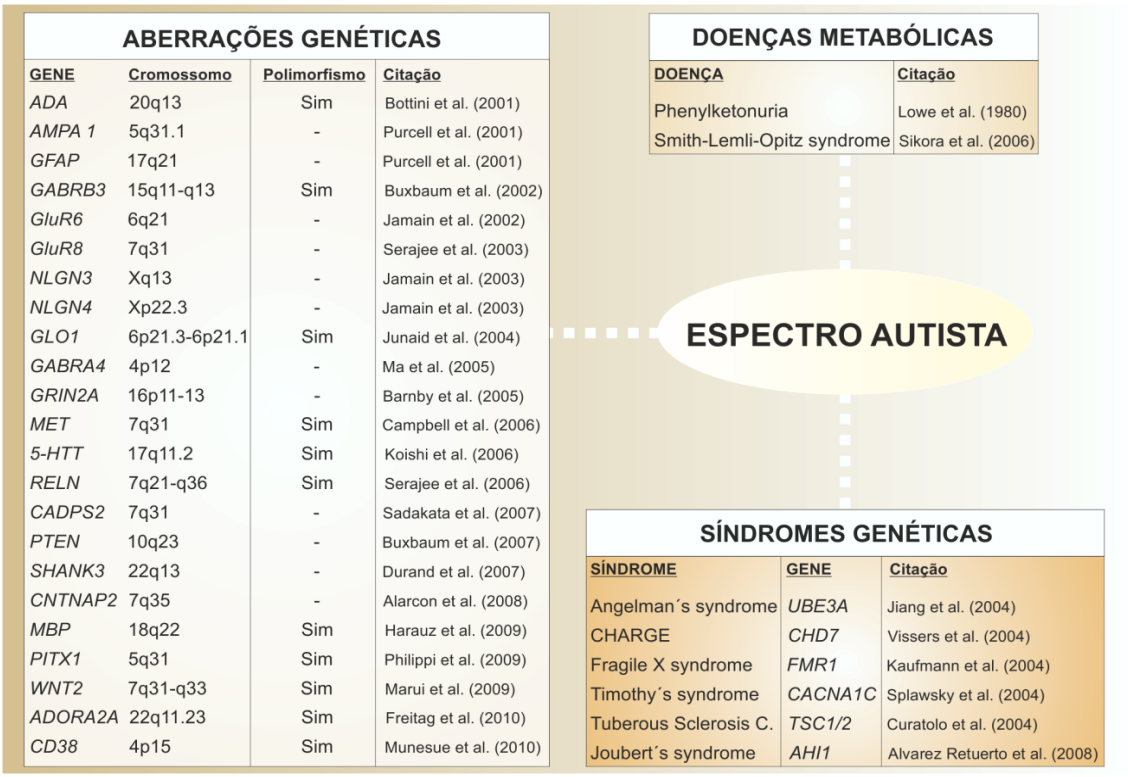

Figura 1. Genética associada ao autismo. Aberrações genéticas (com existência ou não de polimorfismos), doenças metabólicas e síndromes genéticas específicas têm sido associadas ao autismo.

Fonte: elaborado pelo autor.

Estudos em famílias mostraram uma prevalência de autismo 100 vezes maior naquelas com pelo menos um caso diagnosticado (Bailey et al., 1995; Bolton et al., 1994; Folstein & Rutter, 1977; Jorde et al., 1991). As anomalias genéticas, melhor documentadas, que estão associadas ao autismo são as síndromes genéticas e doenças metabólicas (ou transtornos diferentes de causa específica e conhecida onde o autismo é diagnosticado numa porcentagem variável de pacientes; é também chamado de "autismo sindrômico ou secundário"), e, em segundo lugar, as mutações diversas já definidas (Benvenuto et al., 2009; Kelleher and Bear, 2008). Contudo, estas contribuições genéticas não explicam por que o autismo apresenta uma grande variabilidade sintomatológica (ex.: de leve a grave retardo mental e leve a grave falha de interação social) (Landrigan, 2010; Volkmar & Pauls, 2003). Aliás, existem na literatura numerosos estudos aparentemente contraditórios onde polimorfismos em um gene (ex.: *RELN*) é associado ao autismo e outros estudos onde esta mutação não mostra qualquer correlação (Li et al., 2004; Serajee et al., 2006). É por este motivo que, na atualidade, as interações gene-ambientais são consideradas como críticas na epidemiologia associada ao ASD. Uma ampla lista de fatores ambientais tem sido descrita na etiologia autista (**Figura 2**) (Aronson et al., 1997; Christianson et al., 1994; Grabrucker, 2012; Kern et al., 2012; Windham et al., 2006; Yorbik et al., 2010; Zeidán-Chuliá et al., 2011, 2013); sendo, em algumas ocasiões, motivo de discrepância entre pesquisadores do tópico (ex.: mercúrio; ver "referencial teórico"; capítulo 1).

No nível anatômico, observa-se uma associação entre um aumento exacerbado da medida da cabeça (perímetro encefálico) durante os primeiros anos de vida da criança autista (Courchesne et al., 2003). Existem evidências sugerindo que uma característica comum a todos os pacientes autistas é a presença de estresse oxidativo, sendo este o mecanismo pelo qual os fatores ambientais afetariam o desenvolvimento

encefálico da criança (pré-, peri- ou pós-natais), e pelo qual este efeito seria exacerbado pela existência de uma suscetibilidade genética específica (Fatemi et al., 2012; Sajdel-Sulkowska et al., 2011; Zeidán-Chuliá et al., 2013a).

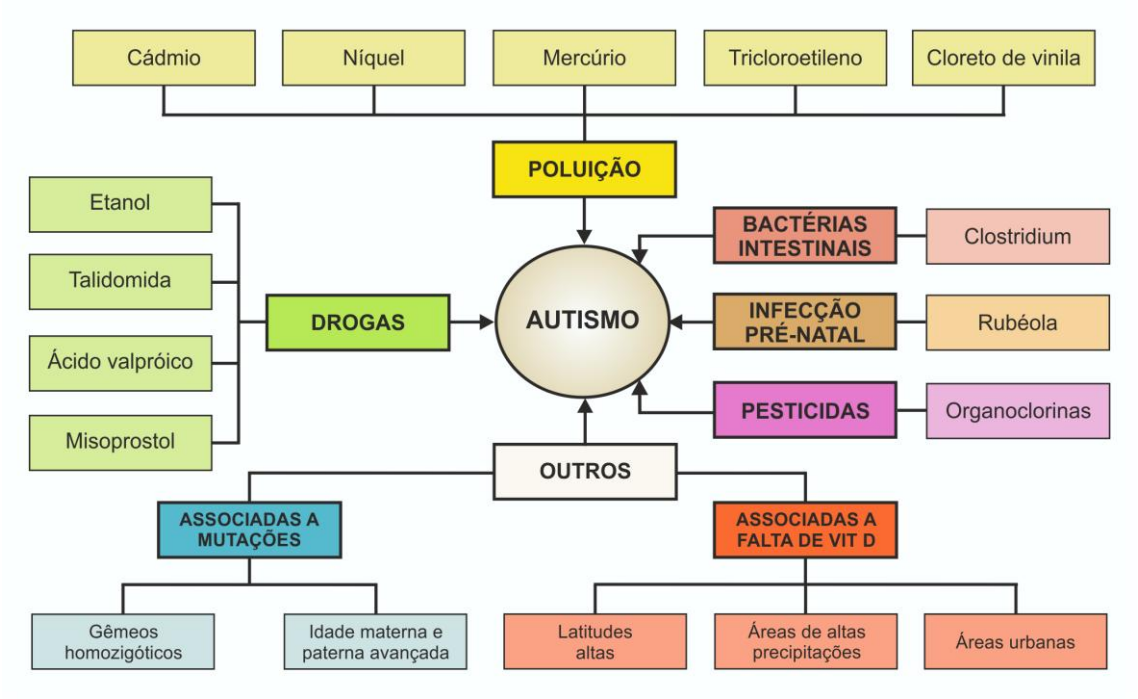

Figura 2. Fatores ambientais associados ao autismo. O diagrama representa a ampla disparidade de agentes etiológicos ambientais e, aparentemente, não relacionados, associados por um ou mais autores, na literatura, à etiologia do autismo.
Fonte: elaborado pelo autor.

Tem-se demonstrado uma diminuição de receptores GABAérgicos em córtex parietal, córtex superior frontal e cerebelo de pacientes autistas (Fatemi et al., 2009). Pelo contrário, níveis significativamente elevados de glutamato são observados em hipocampo, na região frontal e no soro de pacientes autistas em comparação com controles sadios (Shinohe et al., 2006; Brown et al., 2013). Outros estudos descreveram mudanças neuropatológicas no tecido cerebral de pacientes autistas (especialmente, no cerebelo) na forma de respostas neurogliais caracterizadas pela ativação de astroglia e microglia (ver "referencial teórico"; capítulo 2). Os resultados sugerem que pacientes

autistas com uma maior ativação microglial possuem uma maior deficiência nas suas capacidades cognitivas e estas respostas neurogliais persistem até a idade adulta (Bailey et al., 1998; Vargas et al., 2005; Suzuki et al., 2013). Em conjunto, a presença de microglia e astroglia ativada em amostras cerebrais *post mortem* de pacientes representam uma clara evidência de resposta neuroinflamatória. De fato, altos níveis de expressão de proteína fibrilar acídica glial (GFAP), por exemplo, foram observados no cerebelo acompanhado de uma perda de células de Purkinje (Bailey et al., 1998; Vargas et al., 2005), e também astrocitose no córtex frontal (Cao et al., 2012). Em geral, o processo inflamatório é caracterizado por um incremento nos níveis do fator de crescimento transformante β1 (TGF- β1), interleucinas 6 e 10 (IL6 e IL10) no encéfalo destes pacientes e também por altos níveis de citocinas inflamatórias como por exemplo, o fator de necrose tumoral α (TNF- α), interferon-γ (IFNγ), IL1, IL6, IL8 e IL12 em células mononucleares no sangue, soro, plasma e no fluido cérebro-espinhal de pacientes. No plasma, também foram encontrados níveis elevados de proteína de choque térmico 70 (HSP-70), fator de crescimento transformante β2 (TGF- β2), caspase 7 e IFNγ (El-Ansary & Al-Ayadhi, 2012).

Os espinhos dendríticos constituem as áreas principais de processamento de informação no encéfalo e, da mesma forma que para outros transtornos cognitivos, a morfologia aberrante dos espinhos parece ser uma característica associada à neuropatologia do autismo (Wei et al., 2011; Fortin et al., 2012). Casualmente, têm-se observado anormalidades dendríticas (ex.: densos, longos e finos) em pacientes e modelos animais de epilepsia (Fortin et al., 2012; Wong and Guo) e, de fato, um número significativo de pacientes autistas sofrem de crises epilépticas (Tuchman, 2013).

O aumento do volume encefálico e o desenvolvimento de espinhos dendríticos anormais representam fortes indícios de neurogênese aberrante nestes pacientes.

Alguns autores consideram o autismo como uma doença pediátrica autoimune, porque já foram encontrados anticorpos antineuronais no soro de pacientes (ex.: antigangliosídeo M1) correlacionados com a severidade da desordem (Mostafa & Al-Ayadhi, 2011 e 2012).

2. Conceito de plasticidade sináptica

As conexões neurais em nosso encéfalo não são nem fixas nem invariáveis (ainda durante a idade adulta). Estas podem mudar em resposta a estímulos intrínsecos (ex.: hormônios e diversos fatores de crescimento) e extrínsecos (ex.: fatores ambientais). Esta plasticidade e esta remodelação sináptica nos permitem compensar e adaptar a danos ou doenças que podem ocorrer no nosso sistema nervoso central (SNC) e também a novas situações como processos de aprendizado e memória (Schaefers & Teuchert-Noodt, 2013). O conceito de neuroplasticidade engloba diferentes níveis. Por exemplo, a plasticidade funcional em termos de sinapses implica mudanças na quantidade de neurotransmissor liberado ou o aumento na densidade do receptor; mudanças estruturais levam, por exemplo, a um aumento ou redução na área de contato sináptico ou até à retração ou aumento do comprimento dos espinhos, axônios e dendritos; ou, finalmente, à produção de novos neurônios mediante o processo de neurogênese.

Independentemente, as mudanças associadas à plasticidade devem sempre garantir o equilíbrio entre atividades excitatórias e inibitórias dos circuitos neurais (Wolff et al., 1989; Wolff & Missler, 1992).

A plasticidade estrutural é sempre maior nos primeiros anos de vida. Um ano depois do nascimento, o encéfalo da criança ainda tem o dobro do número de sinapses do encéfalo de um adulto. Esta superprodução de neurônios é seguida por uma fase de eliminação de sinapses durante o desenvolvimento das diferentes áreas encefálicas (Huttenlocher & Dabholkar, 1997). Assim, estes primeiros anos de vida (principalmente até os 3 anos) representam, também, um risco para a criança, porque os processos de neuroplasticidade podem ser induzidos não só por sinais ou estímulos positivos, mas também por estímulos nocivos, traumas, fármacos e/ou toxinas. Neste período da vida os insultos têm um impacto maior que em qualquer outro período da vida do indivíduo (Purves et al., 1988; Schaefers & Teuchert-noodt, 2013).

No entanto, o encéfalo imaturo adapta-se a estes insultos incorporando permanentemente esta informação fornecida pelo estímulo ambiental nas redes existentes com novos padrões de inervação neuronal (Andersen, 2003).

3. Processamento proteolítico do APP no desenvolvimento e no estado patológico

O descobrimento da existência de neurogênese no encéfalo adulto mudou significativamente nossa percepção da fisiologia e patologia do SNC. Sabe-se que a proteína precursora amilóide (APP) pode influenciar a neurogênese, especialmente,

através de sua forma secretada solúvel α (sAPPα) e seu domínio intracelular (AICD), que resulta do processamento do APP. O APP é processado por duas cascatas de sinalização opostas (**Figura 3**): (i) a via amiloidogênica mediada pela β-secretase e (ii) a via não-amiloidogênica mediada pela α-secretase. Para a formação do peptídeo β-amilóide (Aβ), o APP é, primeiramente, clivado pela β-secretase BACE1 gerando a forma β-secretada de APP (sAPPβ) e o fragmento c-terminal C99. Este é processado por uma γ-secretase gerando (Aβ) e AICD.

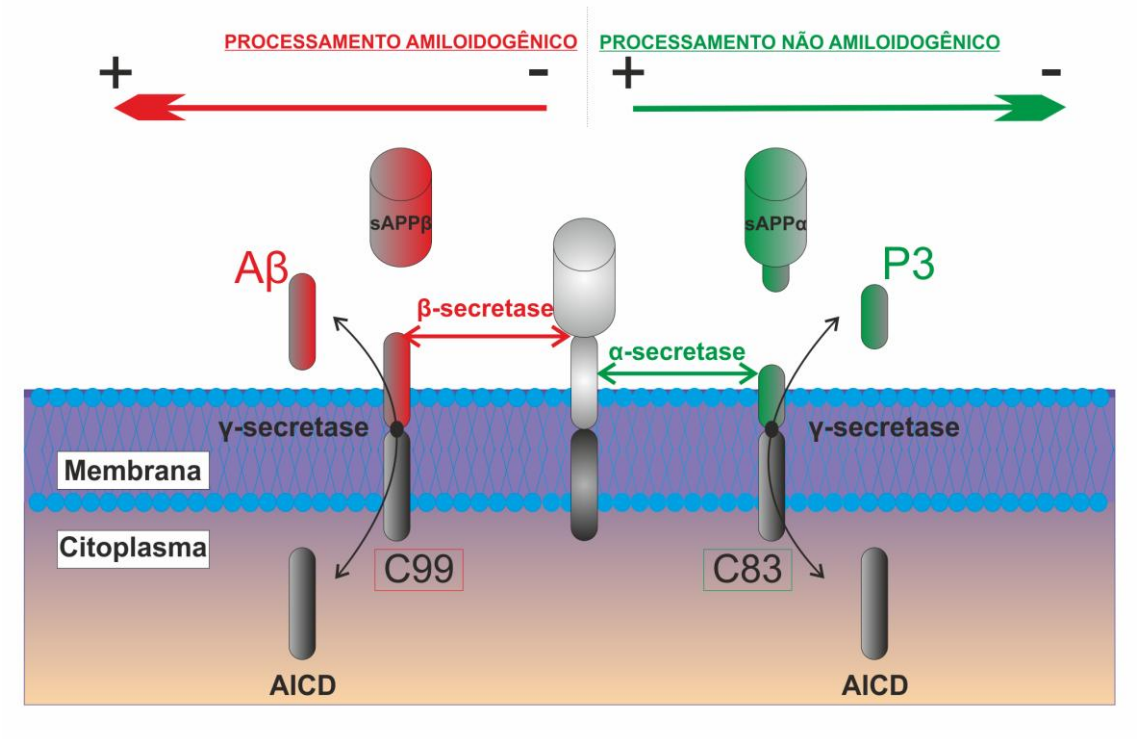

Figura 3. Processamento proteolítico do APP. O processamento amiloidogénico mediado por atividade β-secretase e, posteriormente, atividade γ-secretase gera como produtos sAPPβ, Aβ e AICD. O processamento não amiloidogénico mediado pelas atividades α- e γ-secretase gera como produtos sAPPα, peptídeo P3 e AICD.

Fonte: elaborado pelo autor.

O APP também pode ser clivado através da via não amiloidogénica, rota mediada por atividade α-secretase e, posteriormente, γ-secretase, gerando como produtos sAPPα, peptídeo P3 e AICD (Kojro & Postina, 2009). O acúmulo excessivo de

Aβ pode induzir a agregação do peptídeo em oligômeros tóxicos e induzir morte neuronal por estresse oxidativo, peroxidação lipídica, incremento do cálcio intracelular, disfunção mitocondrial e ativação da via das caspases e apoptose (Lammich et al., 1999; Ariga et al., 2008; Rothhaar et al., 2012) (ver "referencial teórico"; capítulo 3). De fato, um mecanismo patogênico amplamente aceito como relevante na doença de Alzheimer é a hipótese da cascata amilóide (Golde et al., 2006). Agregados Aβ induziriam neurotoxicidade mediada por um incremento em espécies reativas de oxigênio (ROS) e um aumento do influxo de cálcio nos neurônios (Butterfield, 2002).

Menos conhecidas são as funções fisiológicas do sAPPα, fator que tem sido relacionado com o aumento de sinaptogênese, crescimento de neuritos, sobrevivência celular e adesão celular (Mattson, 1997; Gakhar-Koppole et al., 2008). Sabe-se que o sAPPα pode ativar a proliferação de células progenitoras neurais isoladas de encéfalos embrionários, mas foi realmente em 2004 quando Caillé e colaboradores mostraram, pela primeira vez, evidências do papel do sAPPα *in vivo* na neurogênese em indivíduos adultos. Os autores mostraram evidências de sua participação na proliferação de células progenitoras neurais induzidas pelo fator de crescimento epidérmico (EGF). Os autores também observaram que a infusão do sAPPα no ventrículo lateral dos animais levava a um incremento de células progenitoras (Caillé et al., 2004).

4. Ferramentas computacionais aplicadas: redes e suas topologias

"*In silico*" é um termo referido a algo "feito no computador ou simulação computacional" e é muito utilizado na área de biologia de sistemas que modela, simula e analisa por ferramentas computacionais redes de interação biológica como uma parte integral da pesquisa moderna na Biologia (Danchin et al., 1991; Keller et al., 2013). Os

métodos *in silico* oferecem um elo entre diferentes áreas de pesquisa como Bioquímica, Biologia, Toxicologia, Farmacologia e Medicina. Estes métodos incluem bases de dados, modelos de homologia estrutural (ex.: de proteínas), análises de redes de interação protéica e outros que usam o computador como ferramenta chave (Ekins et al., 2007). Mais especificamente, a modelagem *in silico* em conjunto com as análises de expressão gênica têm guiado pesquisadores na procura de alvos terapêuticos potencialmente relevantes além de dar informação sobre interações proteína-proteína ou composto/droga-proteína no contexto de qualquer doença. Esta abordagem que tem sido aplicada com sucesso em diversos estudos (Rabelo et al., 2012; Zeidán-Chuliá et al., 2012, Zeidán-Chuliá et al., 2013b). De fato, tais abordagens podem gerar modelos de interação baseados em dados experimentais existentes na literatura para determinar a relevância de uma proteína ou grupo de proteínas dentro de uma rede de interação que representa um ou vários processos biológicos e podemos, assim, estabelecer novas hipóteses para serem testadas com experimentos na bancada (ao nível de RNA e/ou proteína) (Zeidán-Chuliá et al., 2013 a,b).

Estas redes de interação são constituídas por dois elementos principais: (i) nós que representam cada uma das partes que interagem dentro da rede e que podem ser genes, proteínas, compostos, drogas; simplesmente qualquer molécula de relevância biológica que o pesquisador quiser estudar no modelo e (ii) os conectores que representam as interações entre os nós, seja qual for a sua natureza (ex.: reação, catálise, ativação, inibição) (Rosado et al., 2011). Para entender o conceito de uma rede é necessário levar em conta o contexto em que ela está inserida. Por exemplo, é muito provável que uma rede composta por caspases (e proteínas que interagem com as mesmas) esteja caracterizando o processo de apoptose. O fluxo de informação que passa

por um nó ou grupo de nós (ex.: gene ou proteína) depende da topologia da qual fazem parte e esta se reflete na funcionalidade da rede *per se*.

Como um exemplo ilustrativo da ideia, imaginemos uma rede de interação (**Figura 4B**) formada por 7 nós: orientador, doutorando 1, doutorando 2, doutorando 3, doutorando 4, mestrando e iniciação científica. A rede está caracterizando um grupo de trabalho denominado "laboratório 32". A topologia da rede 32 vai influenciar diretamente no fluxo de ideias e resultados gerados e, portanto, na funcionalidade do laboratório (rede) como um todo (ex.: alta produção bibliográfica).

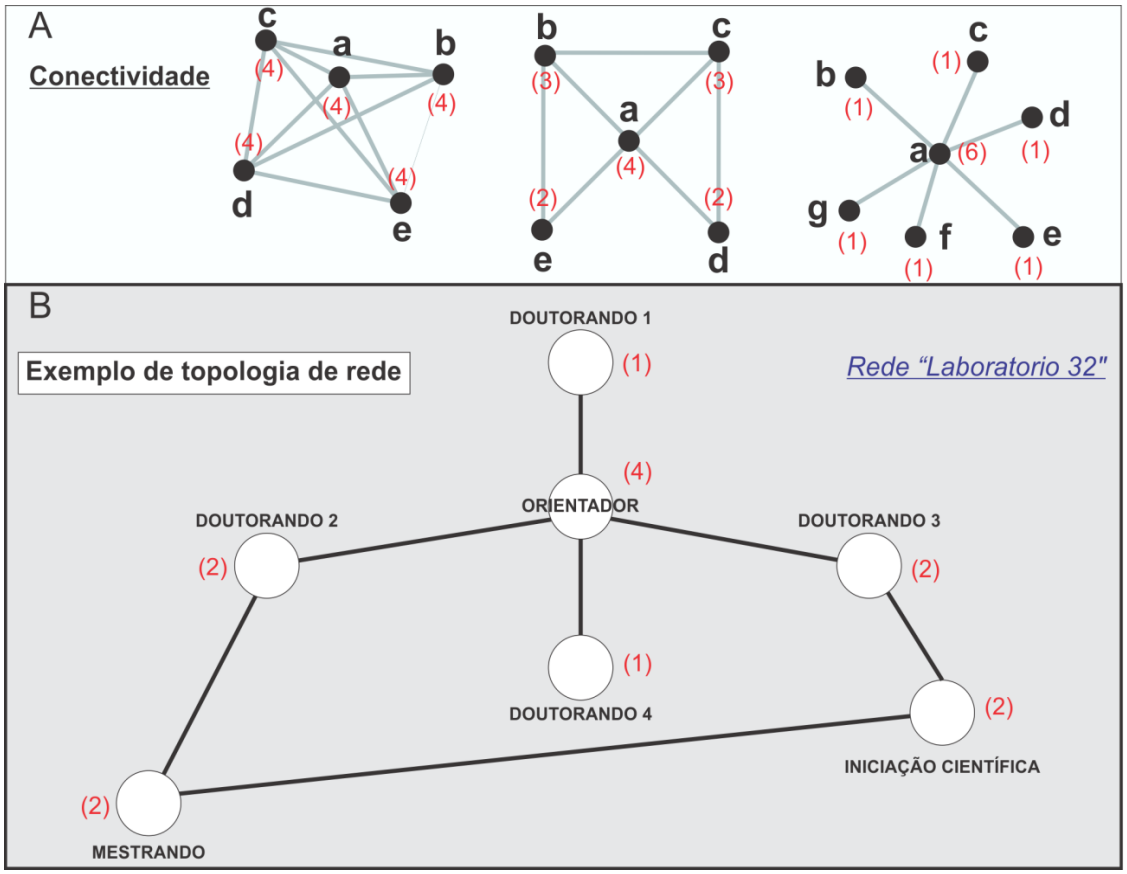

Figura 4. Exemplo de topologia de rede. A. Exemplos de medidas de conectividade de nós dentro de uma rede de interação. B. Exemplo de topologia dentro de um grupo de pesquisa ("Laboratório 32") e medidas de conectividade para cada um dos integrantes.

Fonte: elaborado pelo autor.

Assim, baseando-nos nas medidas de centralidade de rede ou medidas topológicas da rede (ex.: "*connectivity*", "*neighborhood connectivity*", "*stress*", "*betweenness*", "*closeness*" e "*clustering coefficient*") poderíamos identificar quais nós (que poderiam corresponder a genes, proteínas, compostos, contaminantes ou drogas) possuem uma posição de privilégio dentro da estrutura da rede (Wuchty & Stadler 2003; Estrada 2006; Rosado et al., 2011). Em outras palavras, aquele nó que, sendo retirado, quebraria toda a estrutura da rede de interação. Brevemente, podemos definir conectividade (do inglês, "*connectivity*") de um nó como o número de nós com os quais o referido nó interage (**Figura 4A**). Os nós com uma alta conectividade são chamados de "*hubs*" (Rosado et al., 2011). A conectividade da vizinhança de um nó "a" (do inglês, "*neighborhood connectivity"*) é definida como a conectividade média de todos os vizinhos do nó "a". Estresse (do inglês, "*stress"*) mede o número de rotas ideais (caminhos curtos) dentro de uma rede que passam através de um determinado nó "a" (Scardoni et al., 2009). O grau de intermediação (do inglês, "*Betweenness*") é muito similar ao estresse, mas a medida é calculada considerando pares de nós ("b" e "c"), contando o número de rotas ideais que conectam "b" e "c" e passam por um nó "a". Este valor é ponderado pelo número total de rotas ideais que unem "b" e "c". Todos os nós com alto grau de intermediação são chamados de gargalos ("*bottlenecks*") (Hernández et al., 2007; Yu et al., 2007). As duas topologias (estresse e grau de intermediação) dão uma ideia da relevância de um nó (ex.: gene, proteína, composto ou droga) para a disseminação da informação através de toda a rede. A proximidade (do inglês, "*closeness*") mede o grau de proximidade de um nó ao resto dos nós da rede (Hernández et al., 2007; Rosado et al., 2011; De Franceschi et al., 2012), e, portanto, fornece uma ideia de quanto tempo a informação demoraria em se dispersar de um nó ao resto da rede (del Rio et al., 2009). Finalmente, o coeficiente de agrupamento (do inglês,

"*clustering coefficient*") é uma propriedade que mede a fração de conexões entre vizinhos de um nó determinado identificando, por exemplo, genes, proteínas ou compostos em uma rede determinada que possuam vizinhos altamente conectados.

Baseados nisso, podemos definir como (i) "*hub-non-bottlenecks*" (H-NB) os nós com alta conectividade e baixo grau de intermediação; (ii) "*hub-bottlenecks*" (HB) os nós com elevada conectividade e grau de intermediação; (iii) "*non-hub-bottlenecks*" (NH-B) os nós com baixa conectividade e alto grau de intermediação, e (iv) "*non-hub-non-bottlenecks*" (NH-NB) os nós com baixa conectividade e grau de intermediação (Rosado et al., 2011). Mediante o estudo das topologias de rede em conjunto é possível determinar (e até predizer caso não tenha sido previamente descrito), genes, proteínas, compostos ou drogas de relevância num determinado contexto biológico (ex.: uma cascata de sinalização, seja qual for) ou contexto patológico (ex.: autismo ou doença de Alzheimer).

JUSTIFICATIVA

A heterogeneidade dos fatores genéticos, ambientais e sintomatologia associada aos transtornos do espectro autista fazem de seu estudo um desafio para pesquisadores e clínicos que trabalham com este transtorno, assim como constituem o que já foi denominado de "quebra-cabeças do autismo" (State, 2010). Definindo o mínimo denominador comum celular e molecular (ex.: processos biológicos, componentes celulares e funções moleculares) no ASD, teremos pistas dos mecanismos patofisiológicos envolvidos no desenvolvimento do autismo, as quais sendo

confirmadas previamente em modelos animais serão, posteriormente, aplicadas em futuras estratégias terapêuticas de pacientes.

OBJETIVO GERAL

Caracterizar a complexa interação gene-ambiente no contexto autista e determinar as rotas bioquímicas mais vulneráveis aos fatores genéticos e ambientais. Uma vez identificada esta(s) cascata(s) de sinalização, validar por ferramentas computacionais a abordagem terapêutica mais efetiva.

OBJETIVOS ESPECÍFICOS

1. Realizar uma revisão sistemática da literatura para (i) conhecer a epidemiologia associada ao autismo que constitui a sua natureza multifatorial e (ii) descobrir evidências na literatura que indiquem algum padrão patológico no nível celular no espectro autista;

2. Caracterizar *in silico* a complexa interação gene-ambiente e determinar se é possível propor um modelo de rede que possa integrar todo o conhecimento até a presente data sobre fatores epidemiológicos associados ao ASD;

3. Determinar os processos biológicos, componentes celulares e funções moleculares associados à rede associada ao ASD;

4. Fazer uma análise de propriedades topológicas da rede que caracterizam a interação gene-ambiente no contexto autista, para elucidar os genes/proteínas e/ou fatores ambientais mais relevantes já descritos nesta patologia ou até mesmo os ainda não previamente associados na literatura;

5. Elucidar se o modelo nos proporciona candidatos (genes/proteínas ou fatores ambientais) novos com relevância na patologia autista;

6. Confirmar se existe uma alteração na expressão destes genes em mostras de biópsia de pacientes autistas depositados em bancos de expressão gênica;

7. Realizar uma avaliação do arsenal terapêutico em ASD mediante uma abordagem por farmacologia de sistemas.

PARTE II----------

REFERENCIAL

TEÓRICO

Referencial Teórico 1

A DENTAL LOOK AT THE AUTISTIC PATIENT THROUGH OROFACIAL PAIN

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Acta Odontologica Scandinavica

69(4):193-200 (2011)

Article type: Review article

ISNN: 0001-6357

ISI Impact factor: 1.358 (2013)

Acta Odontologica Scandinavica, 2011; 69: 193–200

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REVIEW ARTICLE

A dental look at the autistic patient through orofacial pain

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Abstract

Autism is a neurodevelopmental disorder characterized by impaired social interaction and restricted interests, compromised communication skills, and repetitive patterns of behavior. Both social and behavioral problems, which may include hyperactivity and quick frustration, may hinder the detection of other important pathologies such as orofacial pain. This is aggravated by the invasive nature of oral exploration, which may trigger violent and self-injurious responses, such as temper tantrums and/or head banging, which make the work of professionals extremely difficult during diagnoses, follow-up examinations, and dental treatments. In addition, mercury-containing amalgams used to treat dental caries (the most common form of acute orofacial pain) have been associated with higher rates of severe autism in children. The purpose of this review is to describe the current state of the art regarding the co-occurrence of orofacial pain and autism spectrum disorder, and how these conditions may interrelate clinically and neurobiologically.

Key Words: *Amalgam fillings, autism, behavioral symptoms, dental intervention, oral health*

Introduction

Autism is a complex neurodevelopmental disorder characterized by impaired social interaction and communication, repetitive patterns of behavior, and unusual stereotyped interests [1]. Owing to the variety and grade of its characteristic features and associated symptoms among patients, it is generally termed 'Autism Spectrum Disorder' (ASD). With the exception of Asperger's syndrome, ASD is related to different ranges of mental retardation. People with ASD show diverse medical and behavioral problems, which make the management of these patients extremely complicated. Behavioral features include hyperactivity and irritability, aggression, self-injury, lack of attention, and several fits of bad temper [2,3].

Orofacial pain may be difficult to diagnose in those who suffer from developmental disabilities, aberrant social interaction, and impaired communication skills. In the case of ASD patients, this limitation may act as one potential masking factor influencing the sudden aggravation of autistic patients' behavioral core symptoms, which dramatically affects the patient's quality of life. Therefore, this article will explore the possible bi-directional influence of orofacial pain on autistic behavior (and vice versa) as well as the clinical implications of such an interrelation.

Orofacial pain in the autistic scenario

In general, pain can be classified as neuropathic (e.g. in the case of herpes zoster virus infection), nociceptive (e.g. dental pain), or mixed (e.g. in cancer scenarios). Nociceptive pain is transmitted by physiological pathways through peripheral nerves to the central nervous system (CNS) in response to potentially tissue-damaging stimuli. In contrast, neuropathic pain results from a primary lesion or dysfunction in the nervous system, either central or peripheral [4].

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(Received 2 September 2010; accepted 25 November 2010)

ISSN 0001-6357 print/ISSN 1502-3850 online
DOI: 10.3109/00016357.2010.549505

Orofacial pain is constituted by any symptom resulting from a diverse number of diseases and disorders that would lead to a sensation of discomfort or pain felt in the region of the face, mouth, nose, ears, eyes, head, and neck [5,6] (Figure 1). Its prevalence among the general population varies from 17% to 26% and it is chronic in 7–11% of these cases [7,8]. Although neuropathic orofacial pain is usually evaluated by a multidisciplinary team of neurologists, dentists, neurosurgeons, psychologists, and other healthcare professionals [4,9], it is often labeled as a 'psychopathology' whenever the etiology remains obscure [10]. It is most likely that, in any other pathological scenarios (e.g. autism) where the communication channel between healthcare professionals and patients is compromised, orofacial pain either remains undiagnosed or is tagged as 'idiopathic' in a high percentage of cases; however, this is not the only problem. Loss of teeth, a common dental event also in the autistic scenario, may be accompanied by impaired oral motor functions. Epidemiologic studies keep on revealing, among the populations of many countries, high rates of temporomandibular disorders, toothaches, headaches, and some other conditions associated with orofacial pain [11,12]. In general, the impairment of oral motor functions could potentially be aggravated by the typical seizures, stereotyped movements, and anxiety scenarios commonly present in autism [13,14], and this may synergistically influence the prevalence of orofacial pain among autistic patients and their general oral health status.

From autism to orofacial pain: the many types of orofacial motor disorders

The term 'orofacial motor disorder' (OMD) refers to a spectrum of movement aberrations, both hyper- and

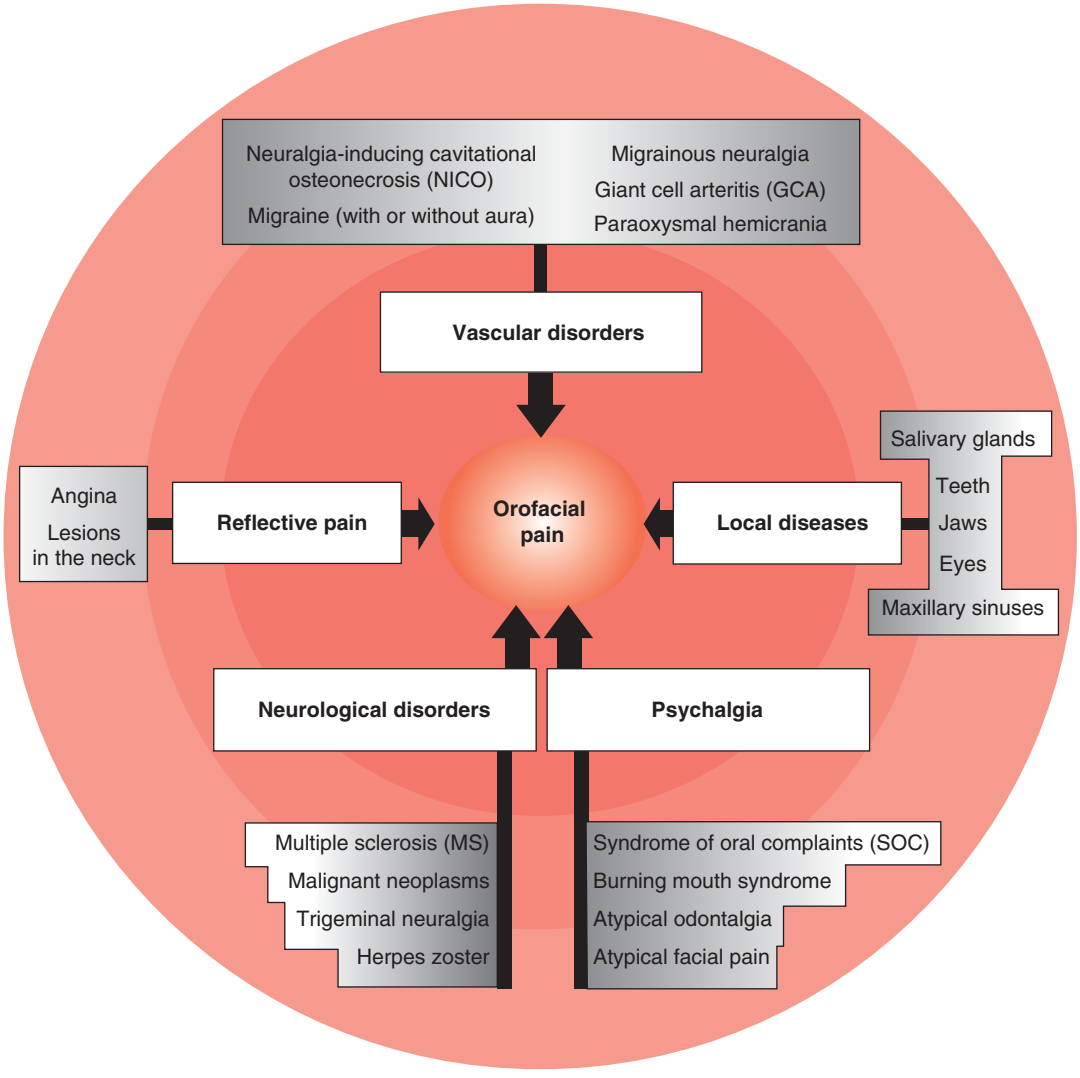

Figure 1. Revisiting the potential causes of orofacial pain. The diagram represents a number of different diseases and disorders that can potentially give rise to orofacial pain [5,6]. The multifactorial etiology of orofacial pain and the complex nature of facial structures make its diagnosis and therapeutic evaluation extremely complicated.

hypoactive, involving the muscles of the orofacial complex that are innervated by cranial nerves V, VII, and XII. OMDs generally present as localized problems that specifically affect the masticatory system, but they are actually driven by alterations in the functionality of the CNS [15]. In any case, dentists should be able to recognize and get involved in the management of such problems because OMDs can cause pain and dysfunction of the jaw, and may interfere with routine dental care of patients [15].

Bruxism

Awake bruxism is generally defined as the awareness of jaw clenching, and is mainly associated with reactions to stress and nervous tics. The pathophysiology of bruxism is still unclear. However, the most compelling hypothesis describes bruxism as a neuromotor dysregulation disorder that affects ≈10–20% of the population [16,17]. Although bruxism has widely been explained to cause pain by overloading the musculoskeletal tissue, additional experimental models may be required in order to better comprehend such a relationship [18]. The treatment options for bruxism, which include the use of splints or behavioral modification techniques, are limited in children with autism due to their poor mental skills (with the exception of Asperger's syndrome) and communication difficulties. In addition, obsessive routines, repetitive and self-injurious behaviors, and unpredictable body movements are symptoms that can potentially interfere with daily oral hygiene and professional dental care in a child with autism [16].

Dystonia

Dystonia is a syndrome of abnormal involuntary muscle movements due to sustained muscle contractions, which generates twisting, repetitive patterned movements, and/or aberrant postures [15]. Oromandibular dystonia is a form of focal dystonia that affects the orofacial region and involves the jaw openers (lateral pterygoids and anterior digastrics), tongue muscles, facial muscles (especially the orbicularis oris and buccinator), and platysma [19].

Dyskinesia

Orofacial dyskinesia is constituted by a group of repetitive, involuntary, and stereotypical movements of the tongue, lips, and, occasionally, the jaw of the patient [20,21]. This is either of spontaneous origin or induced by medication (tardive) [22]. From time to time, edentulous orodyskinesia can be confused with drug-induced oral tardive dyskinesia due to the similarities of their abnormal movements localized in the tongue, lips, and jaw. This may result in a misdiagnosis and, furthermore, inappropriate care [23].

Both dystonia and dyskinesia can lead to increased tooth wear [24] which, in the case of ASD patients, may be exaggerated by the typical seizures or anxiety scenarios associated with this neurodevelopmental syndrome [14].

Drug-induced dystonic extrapyramidal reactions

Age is a determinant of the kind of pharmacological response obtained with any psychotropic treatment, which differs between children, adolescents, and adults. Commonly used neuroleptics in children and adolescents may lead to some unwanted side effects, which include extrapyramidal symptoms, sedation, and withdrawal dyskinesias [25]. Diverse novel antipsychotics have been developed in order to decrease such side effects and, eventually, have demonstrated a relatively low risk of parallel extrapyramidal effects [26]. For instance, the atypical neuroleptic risperidone showed both efficacy and safety for the treatment of different behavioral aspects of autism, including irritability, hyperactivity, aggression, and stereotypy [27].

A neurobiological link between autism and orofacial pain?

Pain can be classified according to the mechanisms that are actually involved in it. Currently, the proposed mechanism-based classification has four main categories: nociceptive pain, functional pain, inflammatory pain (tissue injury), and neuropathic pain (nervous injury) [18,28]. The International Association for the Study of Pain® (IASP) has classified >50 fairly localized pain syndromes in the craniofacial region, including trigeminal neuralgia, post-herpetic neuralgia, odontalgia, and migraine. A complete list was published by the IASP in the mid-1980s [29].

Neuralgia (the word is derived from the Greek words *neuron*, meaning nerve, and *algos*, meaning pain) can affect people of different ages, though it rarely occurs in children. It can be triggered by a great variety of events, including tooth decay, eye strain, or shingles (an infection caused by the herpes zoster virus) [9]. The most common type of neuralgia (trigeminal neuralgia) gives rise to a brief, searing pain along the trigeminal nerve but its actual cause has not conclusively been established. Two trigeminal nerves exist: one supplying sensation to the right side of the face and the other to the left. Sensory innervations of the mouth, face, and scalp depend on the trigeminal nerve, and diseases affecting the nerve can cause orofacial pain, sensory loss, or both [7,9]. Usually, the pain only affects one side of the face, being felt either on the skin or in the mouth and teeth.

Interestingly, researchers started to associate certain cellular events concerning neuron–glial interaction with the biological basis of persistent pain [30].

For example, Okada-Ogawa et al. [31] demonstrated the involvement of astroglia (in the medullary dorsal horn) in trigeminal neuropathic pain-associated mechanisms, and Guo and colleagues [32] reported both the activation and hypertrophy of astroglia, together with elevated levels of glial fibrillary acidic protein and connexin 43, in a trigeminal model of inflammatory hyperalgesia. Moreover, *in vivo* studies have also shown that even dental extraction is sufficient to trigger neuroplastic changes as a response likely associated with the modified oral scenario [33].

Glia secrete growth factors, such as brain-derived neurotrophic factor (BDNF) and basic fibroblast growth factor, for protecting neurons that can additionally enhance pain [34]. This could be of extreme importance, since BDNF and other neurotrophins have been found to be elevated in the blood of autistic patients. Indeed, the literature strongly suggests hyperactivity of this growth factor as one of the possible etiologic factors of the autism-associated neurobiological aberrancies [35–37]. In any case, due to the different putative origins of this pain (neurological, vascular, or dental), patients can be misdiagnosed and receive dental treatment when they actually suffer from trigeminal neuralgia. This situation may be more common than it seems, since the professional consulted (i.e. a physician or dentist) is usually chosen in order to get the most plausible diagnosis [38], and orofacial pain is the main reason why patients seek a dental consultation [39].

From orofacial pain to autism: exploring the amalgam-based dental treatment

The most common form of acute orofacial pain is due to dental caries [40]. The management of dental caries includes the removal of infected hard tissues of the tooth and their replacement with filling materials, such as amalgam, composites, or glass-ionomer cements. There have been great improvements in the properties of composite materials and national regulations have limited the use of amalgam in Scandinavian countries during the last decade; however, amalgam is still the filling material of choice for posterior restorations in most countries of the world due to its handling and adequate mechanical properties [41].

Dental amalgam is a mercury-based material (50%), mixed with silver (35%), tin (9%), copper (6%), and zinc (trace amounts) [42]. Amalgam is classified as an intermetallic compound and is unstable, which means that mercury vapor leaks from amalgam over time. The amount of mercury absorbed from one dental amalgam filling ranges from 2 to 17 μg per day [43]. The amount of mercury release may increase in subjects who use chewing gum or are affected by bruxism, such as autistic patients [43]. In subjects with amalgam fillings, the mercury level in blood or urine is two to five times higher than in those without amalgam fillings, and the concentration of mercury in body tissues is two to 12 times higher in comparison with subjects without amalgam fillings [44–46]. Placental, fetal, and infant mercury burden is highly associated with the number of amalgam fillings of the mother [46–48], and mercury levels in amniotic fluid [49] and breast milk [50] correlate significantly with the number of maternal amalgam fillings.

The first report on the association between mercury and autism was published in the early 1990s; it was suggested that organic mercury is a human behavioral teratogen, being related to seizure disorders, childhood schizophrenia, early-onset emotional disturbances, and autism [51]. In 2000, the National Research Council of the US National Academy explained the mechanism behind the above-mentioned hypothesis by stating that the developing nervous system is a sensitive target organ for low-dose mercury exposure [52]. Later, other researchers reported that mercury exposure can cause immune, sensory, neurological, motor, and behavioral dysfunctions [53–55].

Evidence on the relationship between prenatal mercury exposure from maternal amalgam and the severity of autism is still limited. To the best of our knowledge, the only clinical study has been performed by Geier et al. [56], who reported that pregnant women exposed to mercury from six or more dental amalgam fillings during their pregnancy were significantly (3.2-fold) more likely to have children with severe autism rather than (mild) ASD than mothers with five or fewer amalgam restorations. The major challenge involved in performing new studies in this area is to limit the confounding variables, namely other sources of mercury exposure. In general, there are four main accepted sources of mercury coming into contact with humans, i.e. pollution, fish products, mercury-containing vaccinations, and amalgam restorations (Figure 2). Mercury can also be found in different concentrations in bleaching creams, toothpastes, lens solutions, antiseptics, and immunotherapy solutions [55–57].

With the limited information concerning amalgam hazards, it has been suggested that the mercury in dental fillings can be considered safe [58]. However, this may not be true, since the link between mercury exposure and autism also includes the subject's susceptibility. It has been proposed that when an infant or fetus has genetic or biochemical susceptibility, i.e. a decreased ability to remove mercury after exposure, can autism develop [53,55].

According to Echeverria et al. [59], coproporphyrinogen oxidase polymorphism alters the impact of mercury on cognitive mood scores. Furthermore, a quarter of the US population is polymorphic for this genotype. Another reason for the acceptance of amalgam as a safe filling material is because it does not

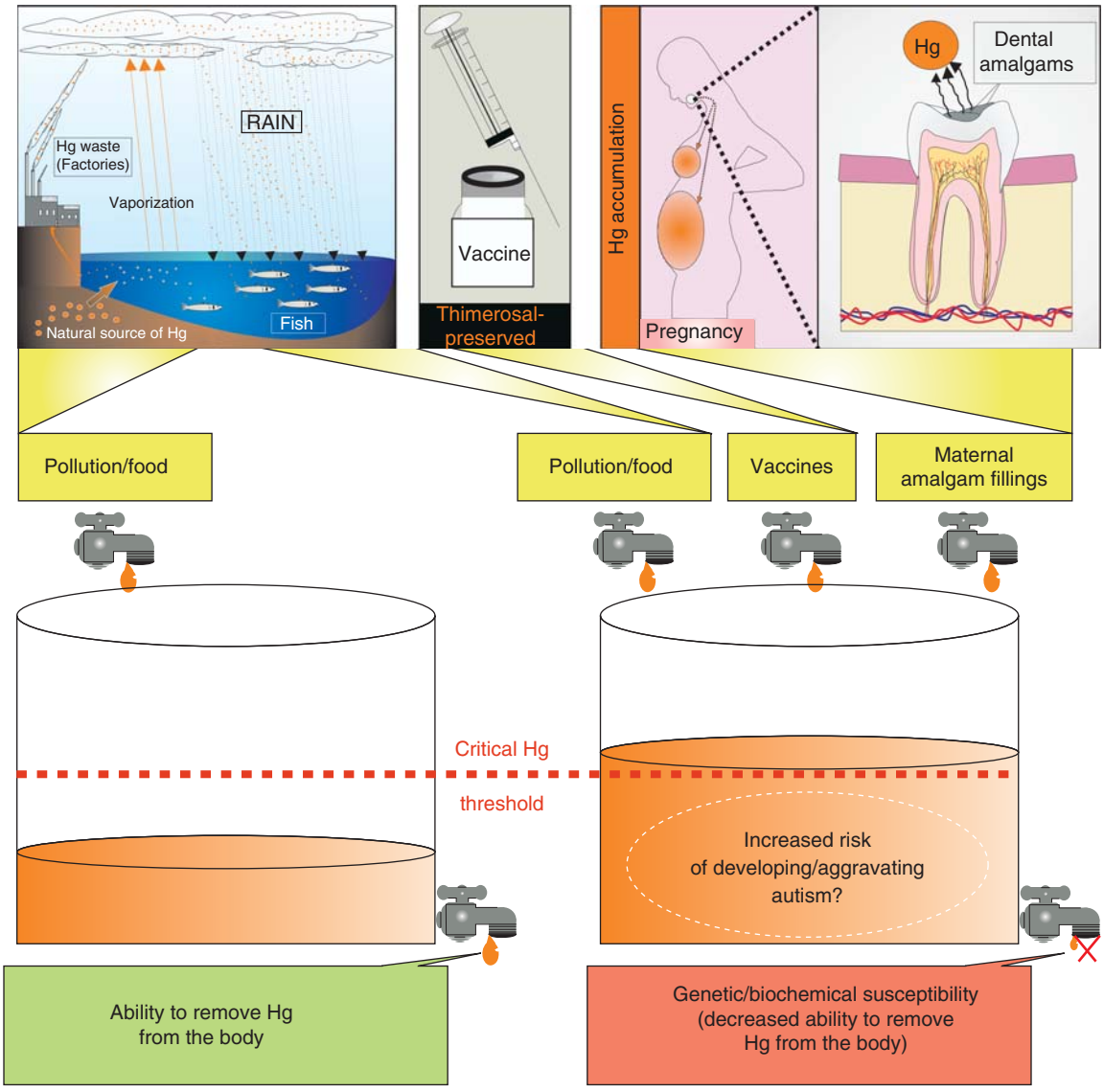

Figure 2. Cumulative mercury (Hg) exposure-based hypothesis for the development and/or aggravation of autism. There seems to be a positive correlation between the number of maternal amalgam fillings and the levels of Hg detected in both breast milk and amniotic fluid during pregnancy [47–50]. Moreover, having ≥6 amalgam fillings correlates positively with the child's probability of later being diagnosed with severe autism [56]. Therefore, it is possible that accumulated doses of Hg from different sources (e.g. pollution, maternal fish consumption, vaccinations, and dental amalgams) during infant development, together with a decreased ability to remove mercury from the body [59], could actually increase the probability of developing and/or aggravating autism among children [53,55].

raise the total blood mercury level above the safety limit (5.8 µg/l) by itself [58]. However, it must be kept in mind that accumulative doses of mercury from different sources, such as pollution, maternal fish consumption, vaccinations, and dental amalgams during infant development, may easily exceed the safety limit (Figure 2). In contrast to urine mercury levels, where the mercury falls to baseline values within weeks, mercury can stay in the brain and the CNS for up to 20 years [60]. Therefore, it is useful to consider the statement of the US Public Health Service and American Academy of Pediatrics, in which it was clearly stated that "all government agencies have to work rapidly toward reducing children's exposure to mercury from all sources" [55].

The 'challenge' of oral health interventions for autistic people

Behavioral problems, including hyperactivity and quick frustration, can definitively complicate the oral health care of patients with autism [61,62]. The invasive nature of oral care triggers violent undesired responses and self-injurious behavior, such as head banging or temper tantrums [13]. Moreover, damaging oral habits, e.g. picking at the gingiva, lip biting, and bruxism, are usually present in these patients [16]. They may exhibit abnormal responses and a higher sensitivity to various sensory stimuli, such as sound, touch, and bright colors [63]. Nevertheless, reactions vary depending on the patient

Table I. Autism-associated behavioral obstacles during oral healthcare interventions.

Management problem	Symptoms	Selected references
Behavioral	Anxiety, combative behavior, hyperactivity, quick frustration	[2]
Unusual responses to stimuli	Sound, bright colors, touch	[63]
Aversions	Oral, touch	[63]
Damaging oral habits	Bruxism, lip biting, abnormal sleep patterns	[16]
Seizures	Chip teeth or bite the tongue or cheeks	[61,67]

(Table I). Interestingly, a number of autistic patients overreact to noise and tactile stimuli, while exposure to either pain or heat may not provoke any reaction at all.

Seizures can be associated with autism but are usually controlled by the use of anticonvulsant therapy [64]. The mouth is always at risk of being damaged during a seizure because autistic patients often chip their teeth or bite their tongue or cheeks. When seizure disorders are controlled, no problems arise with these patients in the dental office. However, there are autistic patients who are extremely sensitive to changes in their environment and, therefore, need more specialized attention by healthcare professionals, who should be prepared for this particularity.

In general, knowledge among dentists and oral healthcare professionals about the characteristics of autism and the appropriate therapy will tremendously improve the clinical attention needed by these patients. Drugs commonly used for autism-associated features display diverse systemic side effects, such as depression, diarrhea, nausea, somnolence, orofacial side effects, and adverse orofacial interactions with drugs frequently used in dentistry [14,65]. For example, in the case of autistic patients with symptoms of attention-deficit hyperactivity disorder, for which the therapy often includes stimulants, such as amphetamine and dextroamphetamine, co-administration of the analgesic meperidine for the treatment of pain could result in hypotension, fever, and even respiratory collapse in the worst-case scenario. These stimulants may also interact with relatively large doses of the narcotic pain-reliever propoxyphene, producing excessive CNS stimulation and seizure activity [66]. Therefore, dental therapeutic agents should be cautiously used in these cases, taking into account possible adverse drug interactions.

Conclusions

Orofacial pain can easily elude medical diagnosis in autistic patients and remain a 'silent disease', masked by the lack of ability of affected patients to clearly express their symptoms due to deficits involving communication and reciprocal social interaction. It is also possible that symptoms remain unobserved by either the family or their physician. Better comprehension of this syndrome could alert clinicians to the need to consider orofacial pain and its relatively high prevalence within the general population as one of the possible reasons for idiopathic aggravation of autism-associated behavioral core symptoms among autistic patients.

Acknowledgements

This work was supported by the National Council for Scientific and Technological Development (CNPq) of the Federal Republic of Brazil. F. Z.-C. is grateful to the Marie Curie Early Stage Research Training (EST) program for his previous funding.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Referencial Teórico 2

THE GLIAL PERSPECTIVE OF AUTISM SPECTRUM DISORDERS

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Neuroscience & Biobehavioral Reviews

38:160-72 (2014)

Article type: Review article

ISNN: 0149-7634

ISI Impact factor: 9.440 (2013)

QUALIS A1

Neuroscience and Biobehavioral Reviews 38 (2014) 160–172

Contents lists available at ScienceDirect

Neuroscience and Biobehavioral Reviews

journal homepage: www.elsevier.com/locate/neubiorev

Review

The glial perspective of autism spectrum disorders

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ARTICLE INFO

Article history:
Received 28 August 2013
Received in revised form 3 November 2013
Accepted 21 November 2013

Keywords:
Autism
Gene
Environmental factors
Neurone
Astrocyte
Oligodendrocyte
NG2 cells
Microglia

ABSTRACT

The aetiology of autism spectrum disorders remains unclear although a growing number of associated genetic abnormalities and environmental factors have been discovered in recent decades. These advancements coincided with a remarkable increase in the comprehension of physiological functions and pathological potential of neuroglia in the central nervous system that led to a notion of fundamental contribution of glial cells into multiple neuropathologies, including neuropsychiatric and developmental disorders. Growing evidence indicates a role for deregulation of astroglial control over homeostasis and plastic potential of neural networks as well as microglial malfunction and neuroinflammatory response in the brains of autistic patients. In this review, we shall summarize the status and pathological potential of neuroglia and argue for neuroglial roots of autistic disorders.

Contents

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http://dx.doi.org/10.1016/j.neubiorev.2013.11.008

"*This very interstitial tissue of the brain and spinal marrow is one of the most frequent seats of morbid change*"

(Rudolf Virchow, discussing about neuroglia; taken from "Cellular Pathology", 1858)

1. Introduction

1.1. Neuroglia as homeostatic cells of the nervous system

Conceptually, plasticity of the brain is controlled at three different levels: subcellular compartments, single cells, and cellular networks. In the neural circuitry, intercellular connections are established by synapses operating in both chemical (mainly between neurones) and gap-junctional or electrical (mainly connecting astrocytes) varieties (Kettenmann and Ransom, 2013). Genesis, development, and functional remodelling of the highly complex neural cellular networks require precise homeostatic control executed at all these levels of organization. This homeostatic control is accomplished by neuroglia. Neuroglial cells, initially defined by Virchow as a connective tissue that "*. . .lies between the proper nervous parts, holds them together and gives the whole its form in a greater or less degree*" (Virchow, 1858), are represented by highly heterogeneous cells of neural (macroglia) and myeloid (microglia) origins (see Fig. 1). Macroglia in turn are classified into astroglia, oligodendroglia, and NG2 cells (Nishiyama et al., 2005; Verkhratsky, 2010; Verkhratsky and Butt, 2013). The common function of all these diverse cell types is to maintain homeostasis of the central nervous system (CNS), and therefore neuroglia can be defined as homeostatic cells of the CNS.

Astrocytes are arguably the most diverse neuroglial cells, being represented by protoplasmic astrocytes of the grey matter and fibrous astrocytes of the white matter, by the radial glia localized in the retina (Müller glia) and cerebellum (Bergmann glia), by the velate astrocytes of the cerebellum, by the interlaminar and polarized astrocytes of the primate cortex, by tanycytes and pituicytes, by perivascular and marginal astrocytes, etc. Astroglia also include several types of cells (ependymocytes, choroid plexus cells, and retinal pigment epithelial cells) that line the ventricles or the subretinal space (Verkhratsky and Butt, 2013). Astrocytes exert many functions, and these embrace almost every conceivable homeostatic task, from isolating the brain from the rest of the body (astrocytes control emergence and function of the blood brain barrier) to controlling neurogenesis (astrocytes in the neurogenic niches are the pluripotent stem cells), regulating ion homeostasis, supporting synaptogenesis, maintaining synaptic transmission through removing neurotransmitters and providing neurones with glutamate and GABA precursor glutamine, supplying neurones with energy substrates, and secreting scavengers of reactive oxygen species (ROS) (for comprehensive account on astroglial functions see Alvarez-Buylla and Lim, 2004; Giaume et al., 2010; Hertz et al., 1999; Iadecola and Nedergaard, 2007; Kimelberg, 2010; Kriegstein and Alvarez-Buylla, 2009; Nedergaard et al., 2003; Parpura and Verkhratsky, 2012; Verkhratsky and Butt, 2013; Wang and Bordey, 2008).

Two other classes of neuroglial cells, the oligodendrocytes and NG2 glia, are lineage related. The oligodendrocytes are critical for axon myelination, which in turn is fundamental for establishing the brain connectome and indispensable for miniaturization of the CNS (Hartline and Colman, 2007; Sporns et al., 2005; Van Essen and Ugurbil, 2012). The NG2 glia belong, from the lineage point of view, to oligodendroglial precursors, although their relatively numerous presence in the mammalian brain and their ultimate function(s) remain, to a large extend, enigmatic (Butt et al., 2005; Nishiyama et al., 2009).

Finally, microglial cells are scions of myeloid progenitors originating from the extra-embryonic yolk sac (Ginhoux et al., 2010). These myeloid progenitors enter the CNS during early embryonic development; the second wave of myeloid invasion possibly occurs in perinatal period in a form of "fountains of microglia" (Kershman, 1939), these being clearly visible around, for example, the corpus callosum. After entering the CNS, microglial cells undergo remarkable metamorphosis that converts them into surveying or "resting" microglia, which constantly scan the neighbouring neural tissue for the signs of damage (Kettenmann et al., 2011). At the same time, microglial cells have extensive array of physiological functions specifically important for the development, shaping, and fine tuning of synaptic connectivity. Of note, microglial cells are the first and only glial cells populating early embryonic brain, because astro- and oligodendrogliogenesis occur later in perinatal period. Physiological functions of microglia are many; in particular, they include (i) early synaptogenesis in which microglia can provide growth factors and thrombospondins, (ii) elimination of redundant synapses, (iii) direct modulation of synaptic transmission by secreting diverse factors such as, for example, BDNF or TNF-α; microglial cells also (iv) provide trophic support, and (v) regulate neurogenesis (for further details, see Kettenmann et al., 2013; Tremblay et al., 2010, 2011; Tyler and Boulanger, 2012).

1.2. Neuroglia in neuropathology

Neurological diseases are, by definition, failures of nervous system homeostasis in response to environmental (e.g. trauma, infection or toxic poisoning), systemic (e.g. ischaemia) or endogenous factors. Glial cells, being the central element of brain homeostasis, are ultimately involved in the pathogenesis of several neurological disorders. In addition to controlling homeostasis, neuroglia form the defensive system of the brain activated in response to every kind of lesion. Neuroglial defence defines, to a very large extend, the progression and outcome of neuropathology. The role of neuroglia in neuropathology can be primary; for instance, in Alexander disease when astroglial expression of mutated GFAP gene results in profound alterations to white matter, or in toxic assaults that render astrocytes incapable to contain glutamate load and hence trigger massive excitotoxicity; the examples of these include toxic encephalopathies such as Minamoto disease or Wernicke-Korsakoff encephalopathy. It can also be secondary; these latter being represented by variants of reactive gliosis, which is typical for virtually every type of neuropathology (Giaume et al., 2007; Verkhratsky et al., 2013).

Conceptually, gliotic reaction, which is further classified into reactive astrogliosis and activation of microglia, can be regarded as a complex, multistage, and disease specific defensive response to neuropathology (see Fig. 2). Reactive astrogliosis represents an evolutionary conserved (astrogliotic response is already in operation in arthropods) and highly versatile remodelling of astroglia aimed at neuroprotection and trophic support of stressed neurones, at isolation of the damaged area, and at reconstruction of damaged tissue after resolution of the pathology (Sofroniew, 2009; Sofroniew and Vinters, 2010; Verkhratsky and Butt, 2013). Insults of different severity and aetiology induce distinct astrogliotic programmes classified as isomorphic (i.e. preserving morphology and usually fully reversible) and anisomorphic (i.e. changing the morphology, in which astrocytes loose their domain organization and form the gliotic scar) astrogliosis. Similarly, activation of microglia is an intrinsically defensive reaction that produces multiple phenotypes, depending on severity and specificity of the pathological process, providing neuroprotection and elimination of pathogens, dead cells, or cellular debris (Hanisch and Kettenmann, 2007; Kettenmann et al., 2011; Ransohoff and Perry, 2009).

Besides reactive remodelling, numerous neurological diseases are associated with astroglial atrophy and/or functional asthenia. Atrophic and functionally weakened astrocytes are observed at the

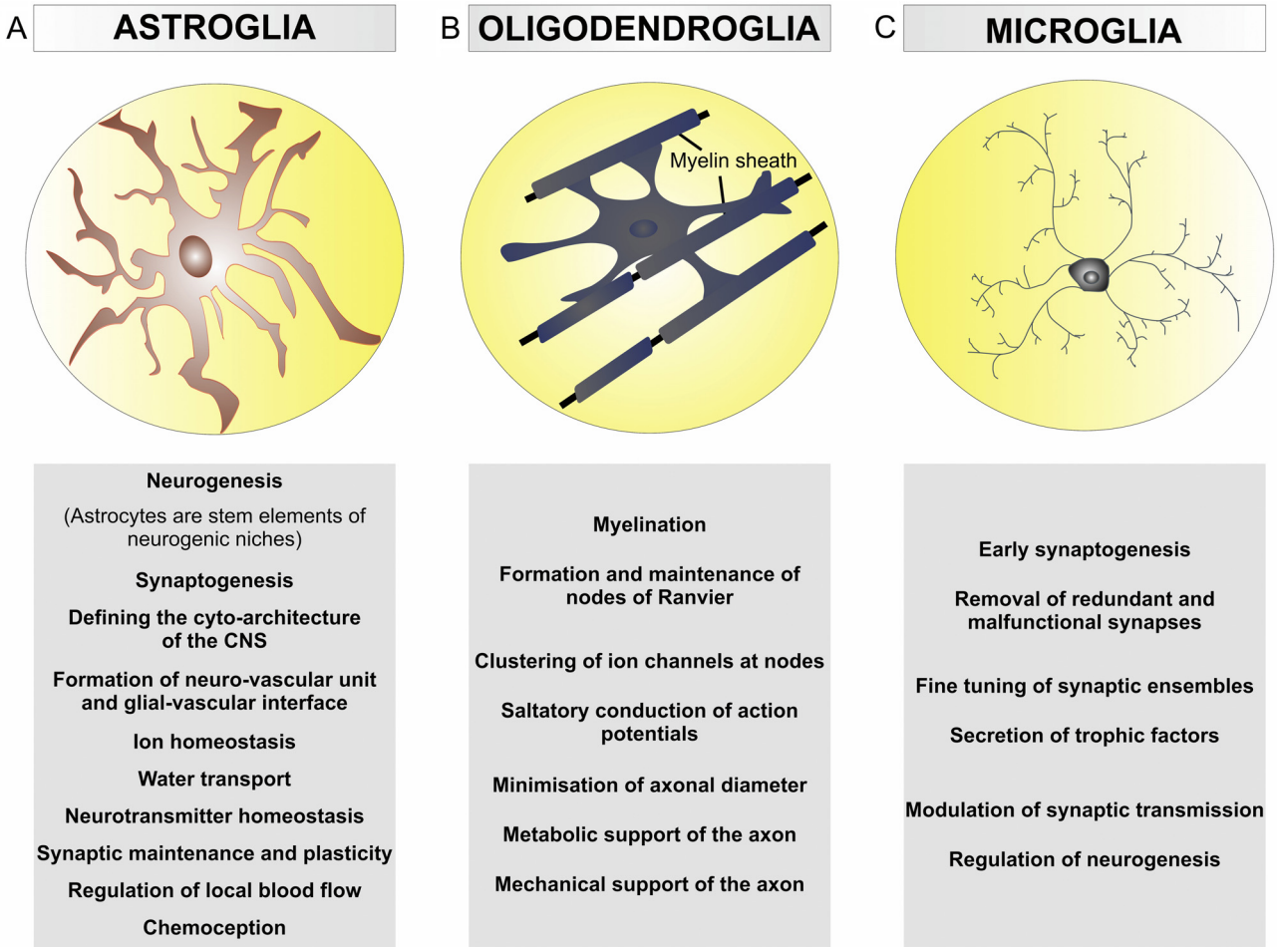

Fig. 1. The role of neuroglia in the homeostasis of the nervous system. Schematic representation of the main functions exerted by astroglia (A), oligodendroglia (B), and microglia (C) in the nervous system.

early and the late stages of neurodegenerative disorders such as amyotrophic lateral sclerosis (Rossi et al., 2008) and Alzheimer's disease (Verkhratsky et al., 2010). Astrocyte dystrophy and dysfunction also appear to be a prominent feature of psychiatric disorders such as schizophrenia and major depression (Bernstein et al., 2009; Rajkowska and Miguel-Hidalgo, 2007).

2. Autism spectrum disorders as failures of neural connectivity and CNS homeostasis: pathological potential of neuroglia

Autism spectrum disorders (ASD) is a generic term representing a group of neurodevelopmental disorders which are primarily detected during childhood and characterized by qualitative impairment in communication, aberrant social interaction, and restrictive patterns of behaviours (Quaak et al., 2013). A prevalence of 1 in every 50 children in the United States has been reported with a tendency of this prevalence to increase in recent years (Blumberg et al., 2013; Rutter, 2005). However, whether these numbers reflect a true increase or result from a hyper-diagnostic and/or greater attention paid the scientific community have been under intense discussion (Fombonne, 2008; Wing and Potter, 2002).

Genetic predisposition and environmental factors have been associated with the aetiology of ASD (Aronson et al., 1997; Christianson et al., 1994; Grabrucker, 2012; Kern et al., 2012; Windham et al., 2006; Yorbik et al., 2010; Zeidán-Chuliá et al., 2011, 2013). Abnormal formation of neuronal networks and pathological misbalance of neurotransmission lie at the very core of autistic disorders. In addition, there is evidence linking the impact of environmental conditions to oxidative stress that could be further exacerbated when combined with a genetic susceptibility (Sajdel-Sulkowska et al., 2011). Overproduction of ROS, together with reactive nitrogen intermediates, inflammatory cytokines, and proteases are responsible for the neurotoxic injury. Neuroglial cells are central for both the development of neural networks and for containment of the oxidative stress; specifically astroglia, which is the main source of ROS buffers (e.g. glutathione). Similarly, activated microglia are the main source for pro-inflammatory factors (Min et al., 2006; Shih et al., 2006). Astroglia organize the architecture of the brain, nurture synapses and perceive synaptic activity, participate in neurotransmission, neurone–astrocyte metabolic coupling, and cytokine secretion. As a result, astrocytes affect all processes associated with brain development, maturation, and ageing (Auld and Robitaille, 2003; Steinmetz et al., 2006; Verkhratsky et al., 2012). Since stress, aberrant levels of hormones, differential expression of neurotrophic factors and glial transporters, as well as changes in extracellular levels of neurotransmitters impact on glial functions, astrocyte pathophysiology can be critical for the progression of neurodegenerative or neurodevelopmental disorders (Brambilla et al., 2013; Sauvageot and Stiles, 2002). Microglial cells are, on the other hand, resident immune cells within the CNS that detect damage to the nervous system, secrete cytokines, and control neuroinflammation (Carnevale et al., 2007). Finally, dysfunction of oligodendrocytes affects the connectome and recent data highlight oligodendroglia as one of the main players in neurodegenerative and neurodevelopmental

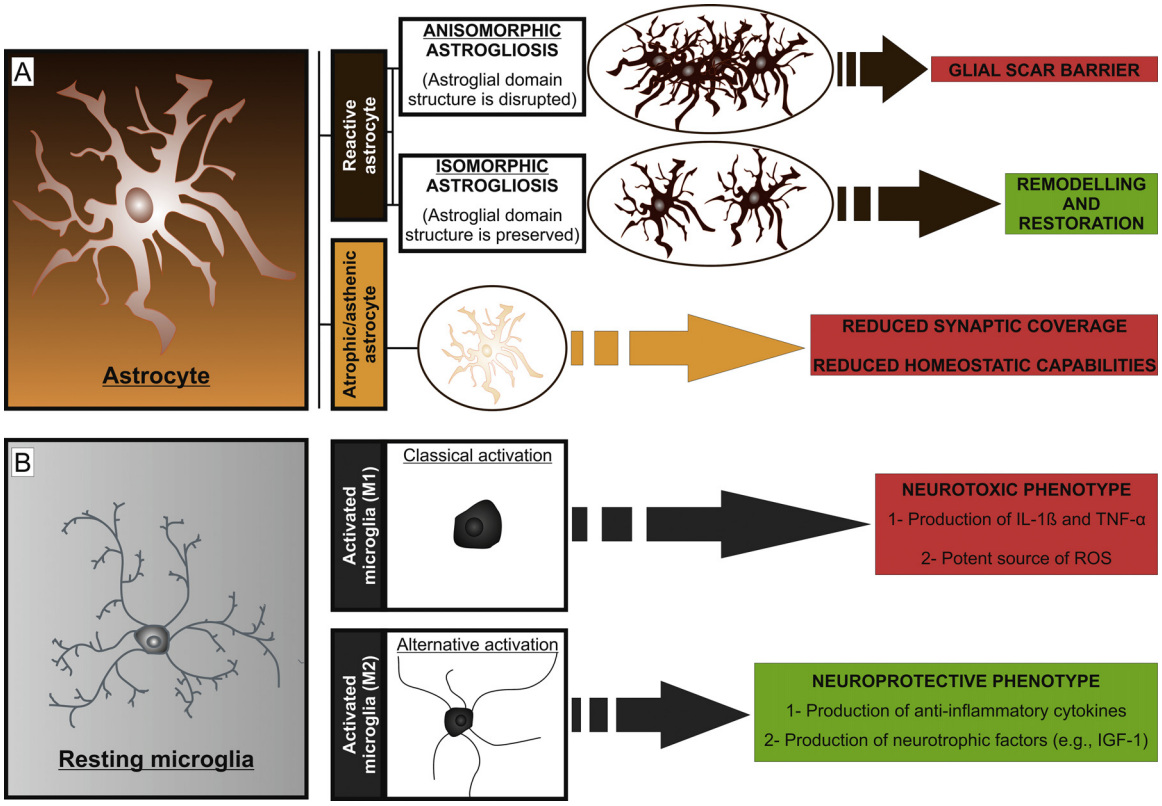

Fig. 2. The gliotic reaction. Schematic representation of the different astroglial (A) and microglial (B) defensive responses to neuropathology.

disorders (Ludolph, 2013). Taken together, these facts form a basis for the novel hypothesis that considers the disruption of homeostatic neurone–glia interactions as a primary event in the neurobiology of ASD (see Fig. 3). In the present paper, we shall introduce the idea of "autism as a glial pathology" and overview some of the most recent findings in the field of autism-associated neurobiology from this perspective.

3. From environmental and genetic factors to glial pathology in autism

Although genes responsible for the majority of forms of autism are mostly unknown, it is generally agreed that a genetic predisposition is relevant to the aetiology of ASD because numerous polymorphisms, in addition to other genetic aberrancies, have frequently been reported (Mefford et al., 2012). Family studies have revealed that the prevalence of autism can be 100 times higher in families with at least one diagnosed case. Furthermore, higher incidence is observed in monozygotic vs. dizygotic twins (Bailey et al., 1995; Bolton et al., 1994; Folstein and Rutter, 1977; Jorde et al., 1991). The best documented genetic abnormalities associated with ASD are genetic syndromes (several disorders of specific cause in which autism is diagnosed only in a variable percentage of the patients; also known as "secondary or syndromic autism", which corresponds to about 10% of autistic patients), defined mutations, and de novo copy number variation (Benvenuto et al., 2009; Kelleher and Bear, 2008). However, these aberrancies do not explain why autism displays highly variable clinical presentation and epidemiology (Landrigan, 2010). Thus, the potential contribution of environmental factors to the development of autism may account for its increasing prevalence (Altevogt et al., 2008; Calderón-Garcidueñas et al., 2008; Berg, 2009; Block and Calderón-Garcidueñas, 2009; Christianson et al., 1994; Herbert, 2010; Moore et al., 2000; Nanson, 1992; Palmer et al., 2009; Roberts et al., 2007; Satomoto et al., 2009; Waldman et al., 2008; Zeidán-Chuliá et al., 2013). Epidemiological and in vivo studies suggest the role for numerous toxic chemicals and drugs (e.g. cadmium, nickel, mercury, trichloroethylene, sevoflurane, valproic acid, and alcohol) (Aronson et al., 1997; Christianson et al., 1994; Kern et al., 2012; Satomoto et al., 2009; Windham et al., 2006; Yorbik et al., 2010). The foetal alcohol syndrome (FAS), characterized by mental and physical anomalies in children whose mothers consumed alcohol during pregnancy, shares some behavioural defects with autism. Likewise, association between prenatal exposure to ethanol and a relatively higher incidence of autistic disorder have been reported (Nanson, 1992). The most direct evidence linking environmental factors to autism derives from pharmacovigilance. Prenatal exposure to different drugs is connected with neurodevelopmental malformations and higher incidence of ASD. For instance, in utero exposure to the anticonvulsant drug valproic acid was associated with autistic phenotype with parallel development of anatomical abnormalities (Christianson et al., 1994; Moore et al., 2000).

Several studies postulated that particles from polluted air may impact on the CNS in early childhood and can damage the prefrontal cortex resulting in cognitive dysfunction in children exposed to high concentrations of air pollution (Block and Calderón-Garcidueñas, 2009; Calderón-Garcidueñas et al., 2008). Different toxic chemicals such as cadmium, nickel, mercury, and trichloroethylene, in addition to ethanol and valproic acid, exert their action through neuroglia (see Table 1) (Eskes et al., 2002; Fernandez-Lizarbe et al., 2009; Gibbons et al., 2011; Guizzetti et al., 2010; Liu et al., 2008, 2010; Sama et al., 2007; Schmidt et al., 2011; Telgkamp et al., 1996; Yang et al., 2007; Wang et al., 2011), affecting for example, glutamate uptake, which is the primary cause for excitotoxicity. This phenomenon has been extensively studied in both acute and chronic neurodegenerative diseases (Foran and Trotti,

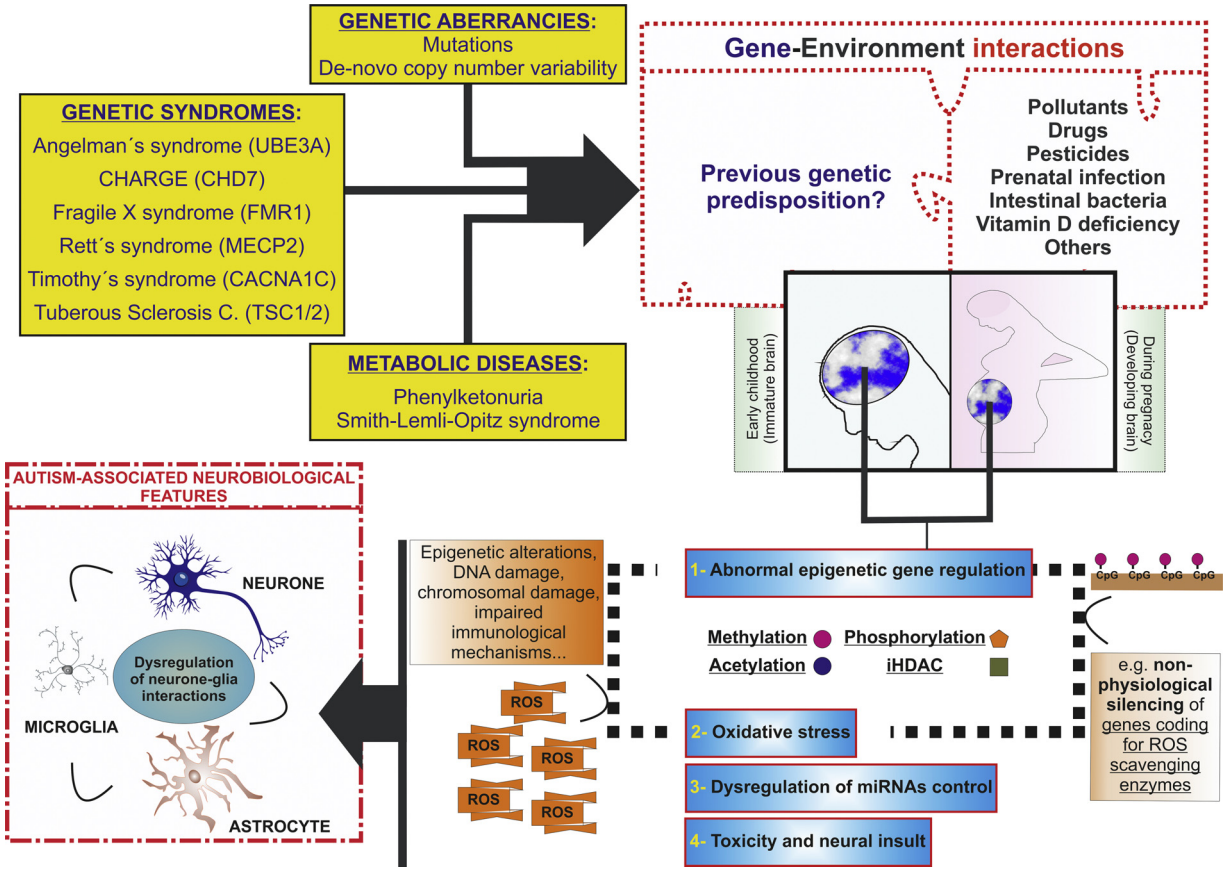

Fig. 3. From gene–environment interactions to dysregulated glial–neuronal cell physiology in autism. Numerous environmental factors, genetic predispositions, as well as genetic syndromes that have been suggested to play a role in the aetiology of autism. Multiple combinations resulting from gene–environment interactions that may occur in brain development (during pregnancy to early childhood), could explain the diverse mixture of neurobiological events described in autistic patients. Environmental factors may lead to abnormal epigenetic gene regulation as well as disruption of miRNA control. Also, it could give rise to oxidative stress scenarios, direct toxicity, and neural insults per se. Furthermore, impaired epigenetic regulation may silence genes coding for ROS scavenging enzymes causing an elevation of ROS, and excessive ROS production in turn may impair epigenetic regulation (i.e. methylation). This would constitute a positive feedback loop, making these patients even more vulnerable to oxidative stress, toxicity and neural insults.

Table 1
Examples of environmental factors that have been associated to autism with reported effects on astrocytes and/or microglia (in vitro and/or in vivo).

Factor with autism-related report	Selected reference	Factor-induced effect on astrocytes	Factor-induced effect on microglia	Selected reference(s)
Cadmium	Yorbik et al. (2010)	Down-regulation of glutamate aspartate transporter (GLAST) expression, reducing the astrocytic glutamate uptake in vitro	Increased intracellular ROS production in microglia-enriched neuronal cultures	Yang et al. (2007) and Liu et al. (2008)
Nickel	Windham et al. (2006)	Blockade of AMPA receptors in vitro	Up-regulation of the expression of inflammatory and innate immunity pathways in BV2 microglia	Telgkamp et al. (1996) and Sama et al. (2007)
Mercury	Kern et al. (2012)	Activation of microglia and its interaction with astrocytes increases the release of local IL-6		Eskes et al. (2002)
Trichloroethylene	Windham et al. (2006)	Deprivation of cellular glutathione, inactivation of glyceraldehyde-3-phosphate dehydrogenase activity, and loss of cellular viability by monochloroacetate (trichloroethylene metabolite)	Reduced mitochondrial complex I activity with elevated oxidative stress markers and activated microglia in the nigral area of adult Fisher 344 rats	Liu et al. (2010) and Schmidt et al. (2011)
Ethanol	Aronson et al. (1997)	Inhibition of neuritogenesis induced by astrocyte muscarinic receptors	Microglial activation with Toll-like receptor 4 (TLR4) response	Fernandez-Lizarbe et al. (2009) and Guizzetti et al. (2010)
Valproic acid	Christianson et al. (1994)	Induction of apoptosis in differentiating hippocampal neurons by the release of tumour necrosis factor-α (TNFα) from activated astrocytes	Decreased expression of microglial markers (PU.1 and CD45) and reduced microglial phagocytosis	Gibbons et al. (2011) and Wang et al. (2011)

2009). Moreover, several xenobiotics are widely used to produce adequate experimental models of autism (Kirsten et al., 2012; Lin et al., 2013; Roullet et al., 2013).

Despite the difficulties to link either bacterial or viral infections to neurobehavioural aberrancies, autistic hallmarks have been observed in patients prenatally affected by pathogens such as clostridia, cytomegalovirus, herpes, and rubella (Bolte, 1998; Chess, 1971; Chess et al., 1978; Finegold et al., 2012; Lipkin and Hornig, 2003; Shi et al., 2005). Incidentally, astrocytes appear to be the main cellular target for rubella virus infection in developing brain tissue (Chantler et al., 1995) suggesting that the infection may disrupt astroglial function resulting in neurological deficits.

Fundamental mechanisms in the pathogenesis of autism include: (i) alterations of brain development program and neurogenesis, (ii) neuroinflammation, (iii) synaptopathy, (iv) metabolic alterations, and (v) deregulated secretion and action of neuropeptides (e.g. oxytocin). In the following chapters we shall discuss how various aspects of neuronal–glial communication contribute to the neuropathogenesis of autism.

4. Astrocytes and metabolic dysfunction in autism

First experimental evidence for astrocytes impacting on mammalian behaviour (Halassa and Haydon, 2010) opened a new area for studying the role of astroglial cells in the pathophysiology of neurodevelopmental disorders. At the same time, there is a growing appreciation of astroglial contribution to evolution of structural and functional abnormalities specific for autistic brains (Dong and Greenough, 2004).

Reactive astrocytes are characterized by high-level expression of GFAP and by up-regulation of intermediate filaments in the cytoplasm (Wilhelmsson et al., 2006). Reactive astrogliosis in cerebral cortex plays an important role in brain repair since mature astrocytes retain the capacity to support proliferation (Buffo et al., 2008). It has been postulated that GFAP-positive astroglial cells are involved in the baseline neurogenesis in the adult mammalian CNS, positively controlling neurogenesis in the dentate gyrus of the hippocampus and in the subventricular zone during adulthood (Pekny et al., 2007; Song et al., 2002). In the autistic brain, increased expression of GFAP in the areas with disturbed neuronal architecture has been identified, therefore suggesting astrogliotic response with possible alterations in neurogenesis and neuronal migration (Laurence and Fatemi, 2005; Vargas et al., 2005). However, the role of astrocytes in ASD is not limited to regulation of neurogenesis. It is widely accepted that autistic pathology results from metabolic deregulation and toxicity (Melnyk et al., 2012; Ming et al., 2012). Chlorinated acetates, fluoxetine, ethanol, and copper are all known to disturb glucose uptake and metabolism in astrocytes (Kreft et al., 2012). Thus, we could speculate that, during early development of the brain, the action of different toxic agents might cause activation and/or dysfunction of astroglial cells, thereby affecting neuronal activity, development, and survival.

Mitochondrial metabolism in astrocytes affects neuronal activity through numerous ATP-dependent processes including secretion, ion buffering, and transport of neurotransmitters (Verkhratsky and Butt, 2013). Astrocytes utilize connexin-based gap junction channels (composed mainly from connexin 43, Cx43) for the coordination of multicellular ensembles (Nakase and Naus, 2004) and metabolic coupling within astroglial syncytium (Escartin and Rouach, 2013). Astrocytes may contribute to the genesis of mitochondrial dysfunction in neurodevelopmental disorders including autism. Astroglial mitochondrial K_{ATP}-channels regulate coupling between astrocytes in the hippocampus through the up-regulation of Cx43 expression (Wang et al., 2013). At the same time, Cx43 expression was found to be increased in astrocytes from autistic patients (Fatemi et al., 2008a), which suggests that elevated expression of the gap junctional channels may affect astroglial syncytia in ASD. Incidentally, influenza viral infection in mid pregnancy in mice affects the expression of Cx43 in astrocytes in parallel to other long-term changes in brain protein expression associated with altered brain development (Fatemi et al., 2008b).

Glutamate is the main excitatory CNS neurotransmitter and, under normal conditions, its balance in the neuropil is controlled by excitatory amino acid transporters EAAT1 and EAAT2, expressed by astrocytes, although neurones can also contribute to glutamate uptake using EAAT3, EAAT4, or EAAT5 transporters (Stobart and Anderson, 2013). When excessive extrasynaptic glutamate is taken up by astrocytes, it is partially utilized as a substrate for oxidative metabolism, partially converted to glutamine by astrocytic glutamine synthase (GS) and can be also used as a substrate for glutathione (GSH) synthesis. Glutamine is then transported to neuronal elements, converted back to glutamate by phosphate-activated glutaminase and stored into vesicles to replenish the supply of glutamate. This process is known as "glutamate–glutamine shuttle"; glutamine supply is also critical for synthesis of GABA and hence for inhibitory transmission (Dringen et al., 1999; Martinez-Hernandez et al., 1977). Astrocytes increase the release of GSH in the presence of extracellular glutamate, this release representing a powerful neuroprotective mechanism under glutamate toxicity and oxidative stress (Schulz et al., 2000). In addition to glutathione, other beneficial antioxidants produced by astrocytes include ascorbate and superoxide dismutases (SOD1/2/3) (Anderson and Swanson, 2000; Dringen, 2000; Lindenau et al., 2000; Sims et al., 2004).

Excitatory neurotransmitter signalling via glutamate receptors is critical for cognitive functions such as memory and learning, which are usually impaired in ASD (Choudhury et al., 2012; Rahn et al., 2012) (see Fig. 4). Glutamate receptors are mainly concentrated in brain regions that have been repeatedly implicated in autism such as cerebellum and hippocampus (Ozawa et al., 1998). Alterations in synaptic glutamate concentrations have been also demonstrated in a model of autism (Smith et al., 2011) and subjects with autism have specific abnormalities in glutamate transporters in the cerebellum (Purcell et al., 2001).

Another important aspect is that glutamate, in addition to fast ionotropic signalling, stimulates glycolysis (i.e. glucose utilization and lactate production) in astrocytes (Pellerin and Magistretti, 1994) and increases activity of glucose transporter-1 (GLUT1) (Loaiza et al., 2003). Both astrocytes and neurones metabolize glucose via glycolytic pentose shunt and oxidative pathways; however, only astrocytes have the ability to store glucose in the form of glycogen in an energy-requiring process. Therefore, during brain activation, glycogenolysis can also be increased in astrocytes (Hertz et al., 2007). High levels of the enzyme glycogen synthase kinase 3 (GSK-3) increase the phosphorylation of glycogen synthase, thus preventing glycogen synthesis and maintaining glucose molecules in an accessible form (Chen et al., 2007; Welsh et al., 1996). GSK-3 is present in the rough endoplasmic reticulum, in free ribosomes, and in mitochondria of neurones and astrocytes (Perez-Costas et al., 2010). Aberrant activity of GSK-3 is involved in mitochondrial dysfunction caused by neurotoxic xenobiotics (Petit-Paitel et al., 2009), in regulation of mitochondrial permeability transition (MPT) pore opening, and apoptosis. GSK-3 is also a part of β-catenin/Wnt pathway and it is affected by some drugs such as lithium and valproate, and it has been related to certain neuropsychiatric disorders (Li et al., 2002). Development of autistic-like phenotype in animals prenatally treated with valproate is associated with enhanced Wnt1 expression and activation of GSK-3β/β-catenin pathway (Go et al., 2012). Wnt/β-catenin signalling cascade is important for neurogenesis and development of astrocytes, and expression of both Wnt and β-catenin is decreased in the frontal cortex of autistic

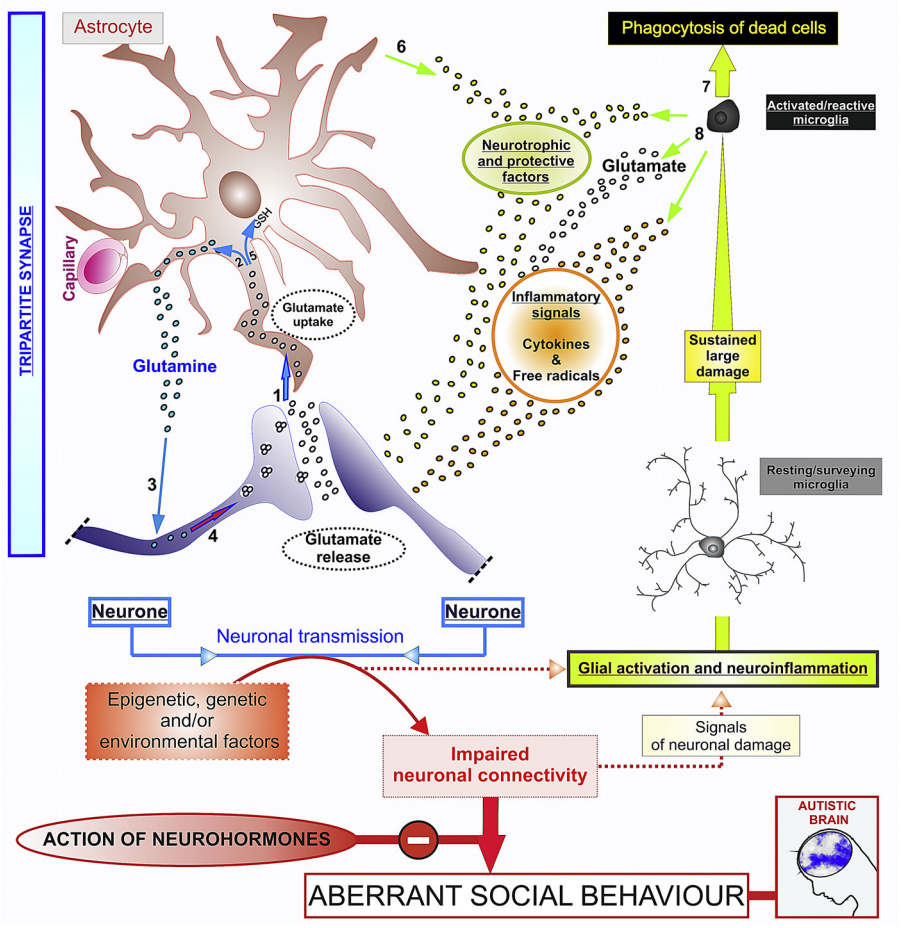

Fig. 4. Revisiting the glutamatergic signalling and neurone–glia interaction in the autistic brain. Glutamatergic signalling is strongly involved in the pathology of autism. In physiological conditions, when glutamate is released during synaptic transmission, it is taken up by astrocytes (1) and used to synthesize either glutamine (2) or glutathione (GSH) (5). Glutamine is then released and re-utilized by neurones as newly formed glutamate (3 and 4) maintaining glutamatergic transmission. However, epigenetic, genetic and/or environmental factors leading to disruption of neuronal transmission could trigger glial activation, increase astrocytic-mediated release of neurotrophic factors (6), and lead to neuroinflammatory events forcing microglia into different levels of activation. Activated microglia is expected to scavenge dead cells (7) from the CNS (e.g. apoptotic neurons), secrete neuroprotective factors in response to signals of neuronal damage. Paradoxically, same microglial activation can lead to secretion of potentially harmful molecules (8) such as free radicals, pro-inflammatory cytokines, nitric oxide (NO), and glutamate. Long-lasting disruption of neurone–glia interactions together with a dysfunctional release and action of neurohormones could have an important role in the typical aberrant social behaviour observed in autistic patients.

subjects (Cao et al., 2012). The role of GSK-3 in the development of fragile X syndrome (common genetic cause of autism) was also reported (Portis et al., 2012). Expression of DISC1, an endogenous inhibitor of GSK-3 and an important gene related to schizophrenia, is increased in the hippocampus of mice with autistic-like behavioural phenotype induced by prenatal immune activation (Tang et al., 2013). Therefore, expression of GSK-3/Wnt/β-catenin and associated proteins in astroglia may impact not only onto neurogenesis, but also on mitochondrial function and metabolic coupling between neurones and astrocytes; although contribution of these mechanisms into ASD remains to be elucidated. This is consistent with data showing that expression of mutant DISC1 downregulates endogenous DISC1 and diminishes the production of D-serine, positive regulator of NMDA receptors released by astrocytes (Ma et al., 2013). Deficient D-serine production in turn affects NMDA neurotransmission, thus linking DISC1 and NMDA to pathophysiological mechanisms of psychiatric disorders such as autism.

In general, there is a firm agreement on the association of ASD with mitochondrial dysfunction in neural cells (Lombard, 1998; Palmieri and Persico, 2010). Mitochondrial dysfunction is usually accompanied by oxidative stress, which is well-documented in ASD (Chauhan et al., 2011; Marazziti et al., 2012). Astrocytes could serve as a source of ROS (Jou, 2008), as modulators of neuronal resistance to oxidative injury, or mediators of oxidative stress-induced damage of neurones (Blanc et al., 1998a, 1998b). Several studies indicated that chronic mitochondrial stress is present in autism, reflecting metabolic dysregulation and toxicity regardless of the causal factor(s) (James et al., 2004; Pastural et al., 2009). Numerous, diverse and complex developmental, neurological, and medical phenotypes of persons with mitochondrial autism (caused by mutations in mitochondrial DNA) appear to be different from those in patients with idiopathic autism (Weissman et al., 2008). The same conclusions were drawn from the analysis of co-morbidities in autistic patients suffering from gastrointestinal dysfunction, exercise intolerance, developmental gross motor delays, and growth retardation (Bauman, 2010). Among all patients with mitochondrial autism, the electron transport chain complex I enzyme was affected most frequently, in more than 60% of the patients, followed by complex III deficiency detected in 20% of patients (Bauman, 2010). However, variability of phenotypes and significant individual variations obscure a more precise analysis of mitochondrial aetiology of ASD.

5. Astroglial control of neurosecretion in autism

Deficits in oxytocin-dependent effects are frequent in autistic patients (Tom and Assinder, 2010). Recent findings

suggest an important role for NAD^+-converting enzyme–NAD^+-glycohydrolase/CD38 in the regulation of oxytocin secretion and related changes in social behaviour in animals and humans (Higashida et al., 2010; Jin et al., 2007).

Plasticity of neurosecretory cells is controlled by several specific mechanisms and in particular, it can be regulated by morphological restructuring of neighbouring astroglia (Bonfanti and Theodosis, 2009). In different brain regions, astrocytes express oxytocin receptors (OTR), stimulation of which activates proliferation (Di Scala-Guenot and Strosser, 1992; Lucas and Salm, 1995). Pharmacological properties of OTRs are regulated by the plasmalemmal content of cholesterol that stabilizes OTRs and controls their affinity to agonists and antagonists (Gimpl, 2008; Gimpl and Fahrenholz, 2002; Reversi et al., 2006). Recent data indicate different mechanisms of cholesterol transport in neurones and astrocytes (Chen et al., 2013). Considering the essential role of cholesterol in synaptic plasticity, synaptic vesicle swelling, and synaptogenesis (that ultimately requires glia-derived cholesterol), abnormalities of cholesterol metabolism detected in ASD can then be linked to disturbed neuronal–glial interactions regulating synaptic plasticity and central action of oxytocin (Koudinov and Koudinova, 2001; Mauch et al., 2001; Tierney et al., 2006).

6. Microglial dysfunction and microglial activation in the context of autism

It is well documented that autistic patients exhibit neuroanatomical abnormalities. More specifically, the clinical onset of autism seems to be related to a reduced head size at birth and a sudden and excessive increase in the size of the head between 1 to 2 months and between 6 to 14 months (Courchesne et al., 2003; Wei et al., 2013). Incidentally, mice with higher levels of IL-6 in the brain show an increase in total brain volume and enlargement of the lateral ventricle (Wei et al., 2012). Neuroanatomical abnormalities seen in ASD patients are consistent with the reported neuroimmune alterations, where cytokines like IL-6, IL-8, TNF-α, GM-CSF, and IFN-γ were significantly increased in autistic brains when compared to control samples (Li et al., 2009; Wei et al., 2011). In general, cytokines are usually expressed/secreted by microglia under inflammatory conditions (Tambuyzer et al., 2009). Microglial cells are the primary immune cells of the CNS (Kettenmann et al., 2011) and influence numerous physiological processes including neural development, synaptic plasticity, and cognition; while microglial activation and production of immune molecules can induce stereotyped sickness behaviours as well as cognitive dysfunction (Perry and Teeling, 2013; Schwarz and Bilbo, 2012). Both microglial cells and astrocytes may, conceivably, appear as a critical link in the aetiology and pathological development of different neuropsychiatric disorders (Perry and Teeling, 2013; Schwarz and Bilbo, 2012; Verkhratsky and Butt, 2013).

Dysfunction or modification of microglial ability to perform their physiological and defensive duties (such as failure in synaptic removal or abnormal microglial activation) may certainly be fundamental for developmental brain disorders, including ASD, which is corroborated by recent findings. Firstly, deficient microglial function has been implicated in pathogenesis of pathological grooming behaviour observed in mice lacking HOXB8 gene. These mice display behavioural phenotype similar to trichotillomania, a human disease also known as a compulsive hair pulling disorder. This distinct compulsive behaviour is associated with mutant microglia as HOXB8 gene is exclusively expressed in bone marrow derived microglia. Transplantation of wild-type bone marrow into HOXB8 mutant mice eradicates pathological phenotype (Chen et al., 2010). Second, β2-adrenoceptor overstimulation with terbutaline administered to rats between 2nd and 5th postnatal days results in microglial activation associated with innate neuroinflammatory pathways and behavioural abnormalities, similar to those described in autism (Zerrate et al., 2007). Third, mutation of MECP2, an X-linked gene encoding the epigenetic factor methyl-CpG-binding protein-2 is associated with Rett syndrome (RS). Although it was thought that the primary cause resulted from a lack of functional MECP2 in neurones, it was later demonstrated that the loss of MECP2 occurs also in astroglia (Ballas et al., 2009). In addition, it was found that MECP2-null microglial cells exert a potent glutamate-mediated neurotoxic activity to hippocampal neurones. Increased levels of glutaminase and Cx32 in MECP2-null microglia were found responsible for increased glutamate production and release, respectively. Therefore, microglial activation and related glutamate toxicity may represent potential therapeutic targets for RS and hence for autism (Maezawa and Jin, 2010; Maezawa et al., 2011) (see Fig. 4). Of note, targeted expression of MECP2 into astrocytes markedly attenuates the clinical manifestations of RS (Derecki et al., 2012; Lioy et al., 2011). The correlation between microglial glutamate neurotoxicity and clinical stages of RS may seem controversial; however, many symptoms of ASD are connected with deregulation of glutamatergic neurotransmission and also display features that adhere to an immuno-excitotoxic scenario, such as enhanced activation of excitatory receptors and positive modulation by pro-inflammatory immune cytokines (Galic et al., 2012; Essa et al., 2013). As a matter of fact, inflammasome activation through Toll-like receptors (TLR3 or TLR4)-mediated pathway may contribute to well-known animal models of autism (e.g. poly(I:C) and LPS) (Lu et al., 2008; Zhou et al., 2013). Both types of TLRs are expressed in astrocytes and microglia and it seems that the response of astrocytes to TLR2 and TLR3 agonists is greatly enhanced by, and response to TLR4 agonists is completely dependent on, the presence of functional microglia (Holm et al., 2012). This latter finding emphasizes the role of microglia-derived cytokines as modulators of astroglial function in the neuroinflammatory context. TLR signalling is also responsible for microglia-mediated neurotoxicity (Lehnardt, 2010). However, it still remains to be clarified whether inflammasome signalling in glia plays a role in neurodevelopmental disorders.

The active and ongoing neuroinflammatory process in the cerebral cortex, in the white matter, and in the cerebellum of autistic persons has been well-documented (Vargas et al., 2005; Li et al., 2009; Wei et al., 2011). Cytokines released from microglial cells and astrocytes have great impact on neurogenesis, synaptogenesis, and establishment of behavioural phenotypes (Yirmiya and Goshen, 2011). Several immune-mediated mechanisms were suggested in autistic development, including abnormalities of humoral and cellular immunity, as well as autoimmune reactivity and infection-induced immune response (Pardo et al., 2005).

Microglial activation in various brain regions such as midbrain, pons, fusiform gyri, the anterior cingulate and orbitofrontal cortices, and the cerebellum (where it was most prominent) has been demonstrated by the in vivo imaging (PET) in ASD patients (Suzuki et al., 2013). Microglia appeared markedly activated in some autistic patients, with morphological alterations included soma enlargement, retraction and thickening of processes, and extension of filopodia (Morgan et al., 2010). Average volume of microglial somata was significantly increased in the white matter, with similar trend in the grey matter (Morgan et al., 2010). Furthermore, microglial cell density has been shown to be significantly increased in the grey matter, but not in the white matter (Morgan et al., 2010) in postmortem tissue; number of microglia also decreases in the cerebral cortex of autistic patients (Tetreault et al., 2012). In some regions of the autistic brain (e.g. dorsolateral prefrontal cortex), increased short-distance microglia–neurone interactions were observed. These are manifested by encirclement of neurones by microglial processes (Morgan et al., 2012).

Excessive production of ROS and proinflammatory cytokines by activated microglial cells may inhibit mitochondrial energy metabolism in the brain (Milton et al., 2008; Park et al., 1999; Rodriguez and Kern, 2011). Microglial activation could be also compromised by mitochondrial toxins (Ferger et al., 2010). Production of ROS by microglial cells and subsequent oxidative stress are implicated into the pathogenesis of neurodegenerative and neurodevelopmental diseases (Derecki et al., 2013; Lull and Block, 2010). Oxidative stress is regarded as a common feature in autism and oxidative pathways are generally regarded as relevant therapeutic targets (Villagonzalo et al., 2010).

Aberrant metabolism of fatty acids is another manifestation of inflammatory process and mitochondrial dysfunction. Persons with autism exhibit significantly increased levels of docosahexaenoic acid-phosphatidylethanolamine, docosahexaenoic acid-ethanolamine plasmalogen, saturated and poly-unsaturated very long chain fatty acids-phosphatidyl ethanolamine, thus suggesting impaired mitochondrial oxidation of fatty acids, increased cytosolic fatty acid elongation and desaturation, as well as increased peroxisomal β-oxidation and plasmalogen synthesis (Pastural et al., 2009). These abnormalities in fatty acid metabolism may be, hypothetically, induced by glutamatergic pathways expressed on neurons and astrocytes; the same pathways were further postulated to trigger microglial activation and neurodegeneration (Innis and Dyer, 2002). Plasmalogens also act as ROS scavengers in the membranes (Lessig and Fuchs, 2009), and hence alterations outlined above may be related to the compensatory activation of anti-oxidant defence.

Alterations in the activity of cyclic ADP ribose hydrolase, generally known as CD38, are implicated in neurodevelopmental disorders including autism through their role in oxytocin secretion (Jin et al., 2007). The data on expression of CD38 in microglia are controversial. Oligodendrocytes and microglia were reported to be immuno-negative for CD38 in rat brain (Yamada et al., 1997). Expression and activity of CD38 were reported to be increased by LPS/IFN-γ treatment in primary microglial cultures (Mayo et al., 2008), although these in vitro data should be always treated with a degree of healthy scepticism. Similarly, increased expression of CD38 protein in microglia was observed in the LPS-injected mouse brain, whereas CD38 was rarely detected in astrocytes (Akimoto et al., 2013). Nonetheless, dysfunction of CD38 in microglia in the context of ASD needs further scrutiny.

In summary, neuroimmune alterations do occur in the brain of autistic patients and may contribute to phenotypic diversity of autism, suggesting that further understanding of the role of neuroinflammation in the pathogenesis of the disorder may have important clinical and therapeutic implications (El-Ansary and Al-Ayadhi, 2012; Pardo et al., 2005). Interrelations between immunity and behaviour in the course of brain development, in physiological and pathophysiological environments are, arguably, one of the most promising areas for new therapeutic strategies (Careaga and Ashwood, 2012). Several antipsychotics, considered standard drugs for the treatment of both autism and schizophrenia, affect microglial activation which effects may account for their clinical efficiency in these patients (reviewed by Monji et al., 2009, 2013).

7. Oligodendrocytes in the autistic brain

Very little is known about oligodendroglial pathology in ASD, although recent findings point towards a possible role of oligodendrocytes in the neurobiology of autism:

(i) Overgrowth of frontal and temporal regions of autistic brain suggests excessive myelination (Carmody and Lewis, 2010). However, these changes seemed to be brain area specific. Myelination in both left and right medial frontal cortex was greater than expected for their age in children with ASD; but at the same time, these children showed myelination that was less than expected in left temporo-parietal junction (Carmody and Lewis, 2010).

(ii) Significant reductions in N-acetyl aspartate (NAA) in all brain regions with a specific decrease in left frontal cortex was found in autistic patients when compared to controls (Kleinhans et al., 2007). NAA is an amino acid present in the brain of vertebrates, which is synthesized and stored primarily in neurones (Baslow, 2003a; Taylor et al., 2004). NAA is transported from neurones to oligodendrocytes, where the NAA-degrading enzyme aspartoacylase (ASPA) cleaves the acetate moiety for further usage in fatty acid and steroid synthesis. The newly produced fatty acids and steroids are utilized as building blocks for myelin synthesis (Moffett et al., 2007). Thus, NAA and ASPA can be considered important for supporting myelination (Francis et al., 2012). Changes in NAA metabolism at the early stages of brain development could thus impair myelination and brain connectivity thereby leading to neurodevelopmental alterations. Incidentally, disturbances in NAA metabolism are associated with demyelinating diseases such as Canavan disease (Baslow, 2003b) and reduced concentrations of NAA have been reported in ASD (Corrigan et al., 2013; Horder et al., 2013).

(iii) The BTBR mice recapitulates the three core behavioural features that characterizes ASD, including deficits in social interactions as juveniles and adults, unusual vocalizations as infants, as well as repetitive stereotyped behaviours (McFarlane et al., 2008; Scattoni et al., 2008). An apparent increase in NG2 immunoreactivity has been described in the anterior cingulate cortex of BTBR forebrain which spanned caudally into the retrosplenial cortex (Stephenson et al., 2011). Increase in the immunostaining coincided with an increase in

Table 2
Prospects for glia-targeted pharmacotherapy of autism.

Glial cells	Key glia-controlled processes for the pharmacotherapy of ASD	Putative target molecules
Astroglia	Glucose transport and metabolism	Glycolytic enzymes, GLUTs, PI3K
	Glutamate transport and metabolism	EAATs, GDH
	NAD+ metabolism	CD38, HDAC, PARP
	Cholesterol metabolism	LXR
	Inflammation and cytokine production	Wnt/GSK-3β, HDAC, MAPKs
	Neurogenesis	NOTCH1, RAC1, WNT/GSK-3β, DISC1, DAB1
	Oxidative stress	Antioxidants, system x(c)(−) (xCT/4F2hc), HO-1, NOX
Microglia	Inflammasome activation	TLR, P2X7, PANX, NLRP3
	Cytokine production	p38 MAPK
	NAD+ metabolism	CD38, HDAC, PARP
	Metabolism of fatty acids	PPARs
	Oxidative stress	PI3K, AKT, mTOR, NOX
Oligodendroglia	Metabolism of N-acetyl aspartate	ASPA
	Myelination	mTOR, PTEN, PI3K, AKT, NPC1

size and extent of cellular processes of NG2 cells, suggesting their possible involvement in ASD pathogenesis.

8. Concluding remarks

In summary, reported signs of astroglial, oligodendroglial, and microglial dysfunction in the autistic brain indicate that ASD may be considered as a gliopathology, where progression, severity, and outcome of neurological manifestations could be controlled by neuroglia. Thus, all types of neuroglia, such as astrocytes, oligodendrocytes, NG2 cells, and microglia may represent presumptive targets for novel therapeutic strategies (see Table 2).

Conflict of interest statement

The authors declare that there is no conflict of interests.

Acknowledgements

First of all, our sincere apologies to the authors whose work have not been cited in the present review article due to space considerations. We are grateful to the Brazilian research funding agencies FAPERGS (PqG 1008860, PqG 1008857, ARD11/1893-7, PRONEX 1000274), CAPES (PROCAD 066/2007), CNPq (558289/2008-8 and 302330/2009-7), as well as PROPESQ-UFRGS for supporting this work.

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Referencial Teórico 3

TARGETING THE GSK3B/B-CATENIN SIGNALING TO TREAT ALZHEIMER´S DISEASE: PLAUSIBLE OR UTOPIC?

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Frontiers in Drug Design & Discovery

Chapter Accepted; ***In press*** **(Vol. 6)**

Article type: Book chapter

ISNN: 1574-0889

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Frontiers in Drug Design & Discovery, **2012, 6, 00-00** **1**

Targeting the GSK3β/β-catenin Signaling to Treat Alzheimer´s Disease: Plausible or Utopic?

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Abstract: Alzheimer´s disease (AD) is a neurodegenerative disorder characterized by progressive memory loss, cognitive impairment, and at the molecular level, by the presence of neurofibrillary tangles (NFTs). As opposed to degeneration, it is known that some specific regions of the brain contain neural stem cells (NSCs) able to produce neurons during adulthood. Wnt/β-catenin signaling has been described as a key pathway modulating the balance between NSC proliferation and differentiation. Wnt signaling is regulated by glycogen synthase kinase 3 (GSK3) that is constitutively active in the cells, keeps β-catenin phosphorylated on serine and threonine residues, and controls its proteosomal-mediated degradation. This raises the question whether inhibition of GSK3β activity, β-catenin stabilization, and therefore, pharmacological activation of endogenous neurogenesis would be a plausible therapeutic strategy for treating AD patients. In this chapter, we herein review the Wnt/β-catenin signaling and evaluate the strategy of inhibiting GSK3β in the disease.

Keywords: Neurofibrillary tangles, β-amyloid, drug therapy, GSK3 inhibitor, lithium, neural stem cells, diabetes, AGEs, RAGE, cognitive impairment.

1. INTRODUCTION

Alzheimer´s disease (AD) is a neurodegenerative disorder defined by progressive memory loss, cognitive impairment, and at the molecular level, by the presence of neurofibrillary tangles (NFTs) composed by hyper-phosphorylated forms of the microtubule-associated protein tau and insoluble β-amyloid (Aβ) plaques. However, it is not well understood what really triggers the development of such structures that actually appear in the patient's brain at later stages of the disease.

As opposed to degeneration, certain locations of the brain such as subventricular zone (SVZ) of telencephalic lateral ventricles [1, 2] and subgranular zone (SGZ) of

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Atta-ur-Rahman / M. Iqbal Choudhary (Eds.)

hippocampal dentate gyrus [3, 4] contain neural stem cells (NSCs) able to produce neurons during adulthood [5]. Several growth factors, ligands, and pathways have been proposed to regulate neural and glial progenitor cell behavior. Among other candidates, Wnt/β-catenin signaling has been described as a key pathway modulating the balance between NSC proliferation and differentiation, but it seems to have opposing roles in the developing and the adult brain [6-8]. For instance, Wnt/β-catenin signaling transiently induces cortical radial glia (RG) to proliferate during early neurogenesis but forces NSCs from SVZ to exit the cell cycle [9, 10].

Some studies are highlighting the existence of an altered and decreased neurogenesis in the brain of AD patients [11, 12]. Therefore, one could speculate whether both events, impairment of neurogenesis and generation of NFT and senile plaques (SP), might be directly or indirectly connected to the disease.

Activation of the canonical Wnt signaling pathway inhibits the activity of glycogen synthase kinase 3 (GSK3), a serine/threonine kinase which has a role in controlling diverse neuronal functions (e.g., neurite outgrowth, neurotransmission, synapse formation, and neurogenesis) [13]. Interestingly, its activity has already been linked to AD-associated anomalous structures [14] since inhibition of GSK3α with therapeutic concentrations of lithium is able to stop both production and accumulation of Aβ in the brains of amyloid precursor protein (APP) over-expressing mice. Besides, GSK3β was also shown to phosphorylate tau protein of the neurofibrillary tangles [15]. Since evidences are pointing towards a deficient neurogenesis in AD patients and the possible role of GSK3 activity in the generation of AD-associated aberrant structures, the question is whether pharmacological modulation of GSK3β/β-catenin signaling may represent a real therapeutic alternative for these patients. The aim of this chapter is to critically review the pros and cons of such strategy.

2. THE SCENARIO: AD HALLMARKS

Nowadays, AD is the most common irreversible neurodegenerative disorder, with more than 20 million cases worldwide [16]. It is characterized by the loss of neurons and synapses, leading to cognitive impairment, loss of memory, language, reasoning, and followed by dementia. Most cases of AD are idiopathic, and advanced age, diabetes, hypertension, hypercholesterolaemia, hyperhomocysteinaemia, and inheritance of the epsilon 4 allele of the polymorphic apolipoprotein E gene are reported major risk factors, although they are not enough to cause the disease [17]. Data from postmortem brains reveal abnormal intracellular NFTs, consisting of hyperphosphorylated microtubule-binding protein tau, and extracellular senile plaques mainly composed of Aβ oligomers deposits [18]. Even though the expression pattern of NFTs correlates with the clinical onset and progression of AD [19], the relevant role for Aβ deposits has been suggested from the association between familial AD and mutations in the genes that encode APP, presenilin-1, and presenilin-2 [20], that up-regulate the general Aβ production and more specifically, the generation of a minor 42-amino acid form (Aβ42) with increased propensity for aggregation [21]. In general, Aβ can be 40 to 42 amino acids in length and it is generated by proteolytic cleavage of the transmembrane APP [22]. This is processed by two competing cellular pathways: the amyloidogenic or β-secretase-mediated route and the non-amyloidogenic or α-secretase-mediated pathway. For the generation of Aβ, APP is first cleaved by β-secretase BACE1 (β-site amyloid precursor protein cleaving enzyme 1), a membrane-bound aspartyl-protease, generating β-secreted APP (sAPPβ),

and a C-terminal membrane-bound fragment, called C99 or β-CTF. C99 is further processed by γ-secretase, releasing the Aβ peptide. In addition to the amyloidogenic processing of APP involving β- and γ-secretase activity, APP can be cleaved in a nonamyloidogenic pathway by α-secretases [21, 23-25]. The excessive Aβ peptides may aggregate into toxic oligomers and induce neuronal loss by promotion of oxidative stress, lipid peroxidation, increase of intracellular Ca^{2+}, mitochondrial dysfunction, caspase pathway activation accompanied with increased macroautophagy and lysosomal ensuing apoptosis [26, 27].

Interestingly, the advanced glycation end products (AGEs) increase in the brain during normal aging and different studies support that AGEs are further increased in the brain in the presence of vascular or Alzheimer's dementia [28, 29]. As a matter of fact, several experimental evidence suggests that accumulation of N(epsilon)-(carboxymethyl)lysine protein (CML), the most abundant AGE *in vivo* [30], is greater in AD and diabetes than that observed in AD alone [29]. A study from Kim and colleagues (2009) proposed that type 1 and type 2 diabetes could actually contribute to AD but through different mechanisms: if hyperglycemia-mediated tau cleavage might be the key feature in type 2 diabetes, in case of type 1 diabetes, insulin deficiency would be the major contributing factor [31]. The close interrelation between AD, diabetes, and cognitive decline is under intense research within the scientific community [32, 33]. Certainly, the main pathophysiological problem in both type 1 and 2 diabetes mellitus is insulin resistance, which is characterized by the progressive reduction in the response of insulin-sensitive tissues to normal levels of insulin [34, 35]. Diverse studies have shown that reductions in both insulin receptor substrate 1 (IRS1) and/or 2 (IRS2) protein levels are associated with insulin resistance [36-38]. Furthermore, it has been shown that activation of GSK3β contributes to the induction of insulin resistance *via* phosphorylation of IRS1, triggering the ubiquitination and degradation of IRS1 [39].

GSK3 is an enzyme that, in physiological conditions, takes part in several cellular processes (e.i., regulation of body metabolism by phosphorylation of glycogen synthase (GS) and other substrates); but it has recently gained researchers' attention by incorporating the "*GSK3 hypothesis of AD*" into the field [40]. This theory proposed that over-activity of such an enzyme has a role in the elevated production of Aβ, tau phosphorylation and microglia-associated inflammatory process and, as a final consequence, in memory impairment. All these events are typical hallmarks of AD-associated pathology. Thus, the overexpression or overactivation of GSK3 could induce a series of pathological events, most of which are common hallmarks of AD and diabetes mellitus type 2 and would represent a common cross-talk between these age-dependent diseases [41].

3. THE TARGET: GSK3/B-CATENIN SIGNALING IN AD

During embryonic development, adult tissue remodeling, and even tumorigenesis, cell proliferation and differentiation are two distinct (but coupled) biological processes that require appropriate changes in the metabolic status to occur; and the Wnt signaling pathway is indeed known for integrating cellular metabolism together with tissue development and function [42]. Secreted signaling proteins belonging to the Wnt family bind to specific Frizzled (Fzd) receptor complexes on the surface of target cells, triggering an intracellular signal transduction that can occur through either β-catenin-dependent (canonical Wnt-signaling) or β-catenin-independent (non-canonical Wnt-

signaling) pathways. The less studied β-catenin-independent Wnt signaling pathways do not need the transcriptional activity of β-catenin and it is usually involved in cell migration and polarity [43, 44]. The more extensively studied canonical pathway regulates the ability of β-catenin protein to modulate the activation of specific target genes. In the absence of Wnt signal, β-catenin (transcriptional activator) is actively degraded in the cell by the action of a protein complex called the "destruction box". Within this complex, the axin and APC form a scaffold allowing β-catenin phosphorylation by CK1α (casein-kinase 1α) and GSK3β. Phosphorylation of cytosolic β-catenin marks it for subsequent ubiquitination-dependent proteasomal degradation [45].

Then, the lack of free β-catenin allows the DNA-binding T-cell factor/lymphoid enhancer factor (Tcf/Lef) proteins to interact with transcriptional co-repressors to block target expression in the nucleus. On the other hand, Wnt binding to Fzd-LRP (low-density lipoprotein receptor-related protein) receptor complexes at the membrane leads to the formation of Dishevelled (Dvl)-Fzd complexes and relocalization of Axin from the destruction complex to the cell membrane. This allows β-catenin to accumulate and enter the nucleus, where it interacts with members of the Tcf/Lef family, transforms the Tcf proteins into powerful transcriptional activators by recruiting co-activator proteins that ensures efficient activation of Wnt target genes (i.e., c-myc), and instructs the cell to actively proliferate and remain in an undifferentiated state [46-48]. Diverse studies highlight the critical role of Wnt pathway in controlling stem cell proliferation. It has been shown to participate in the regulation of stem cell expansion in a number of tissues such as the skin, intestine, hematopoietic, and nervous system [49-53]. In fact, when Wnt signaling pathway is blocked, progenitor cell proliferation is disrupted and causes severe reduction of hippocampus development [54, 55]. On the contrary, over-expression of Wnt signaling is known to lead to uncontrolled cell proliferation and tumorigenesis [56]. GSK3β is inactivated by phosphorylation of serine (Ser), and its activity is increased by phosphorylation of tyrosine (Tyr); in fact, p38MAPK directly inactivates GSK3β by phosphorylating Ser389 in the C terminus of GSK3β in the brain and thymocytes, leading to an accumulation of intracellular β-catenin. Thus, GSK3β activity is a determinant of β-catenin stabilization and its accumulation in the nucleus; a required step for normal neurogenesis [57-60]. *In vitro*, GSK3 inhibition has been shown to increase the proliferation of neural progenitors in the presence of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) with the involvement of NOTCH signaling and β-catenin stabilization. On the other hand, in the absence of growth factors, same inhibition enhances neuronal differentiation of neural progenitor cells [61]. Since the ability to generate new neurons throughout life gets compromised with age due to reduced neurogenesis and failure of newborn neurons to mature [62, 63], one could speculate whether Wnt signaling could be altered or had a role in the pathophysiology of AD. This was the aim of a study performed by He & Shen (2009), where the specific goal of their investigation was to study the neurogenic potential of glial precursor cells (GPCs) coming from AD patients, to reveal whether exogenous levels of Aβ might affect neurogenesis and the mechanism controlling such interplay [12]. To answer this question, they compared the properties of glial progenitor cells (GPCs) from the cortices of healthy controls and AD patients, showing that GPCs from patients displayed reduced renewal capability and reduced neurogenesis when compared with healthy controls. Interestingly, GPCs from patients expressed high levels of GSK3β together with an increase in phosphorylated β-catenin and a reduction in non-

phosphorylated β-catenin in relation to healthy controls. Moreover, the exposure to Aβ was able to impair the ability of GPCs from healthy controls to give rise to new neurons and leading to similar changes in β-catenin signaling proteins, all together suggesting that Aβ-induced disruption of β-catenin signaling could contribute to the impairment of neurogenesis in AD progenitor cells.

4. THE USE OF GSK3 INHIBITORS AS THERAPEUTICAL AGENTS FOR AD PATIENTS

The serine/threonine kinase GSK3 was first isolated and purified as an enzyme able to phosphorylate and inactivate the enzyme GS [57], but subsequent purification and molecular cloning revealed two isoforms as two different gene products. Even though GSK3α (51 KDa) is slightly higher in molecular weight with an extended N-terminal glycine rich domain than GSK3β (47 KDa), both isoforms have very similar biochemical characteristics [64-66]. Under physiological conditions, insulin can stimulate glycogen synthesis by dephosphorylation of GS (GS activation) at the sites targeted by GSK3 [67], thanks to simultaneous inhibition of GSK3 activity and activation of one of the glycogen-associated forms of protein phosphatase 1 [68]. Insulin-derived GSK3α and β inhibition occurs through phosphorylation of both isoforms at Ser residues in the N-terminal lobe of the protein kinase (Ser21 for GSK3α and Ser9 for GSK3β) [66]. In opposition to inhibitory serine phosphorylation, GSK3 activity can be increased by phosphorylation of a Tyr residue, Tyr216 in GSK3β and Tyr279 in GSK3α, located in the kinase domain [69]. In contrast to most protein kinases, GSK3 is constitutively active in cells and in addition to insulin, a variety of extracellular stimuli can inhibit it such as EGF, FGF, and ligands of Wnt signaling pathway [70]. GSK3 is known to participate in numerous cellular processes (e.g., glycogen metabolism, cytoskeleton regulation, intracellular vesicular transport, cell cycle progression, and apoptosis). Therefore, numerous putative substrates for this protein kinase have been identified to date and include β-catenin, axin, APC, cyclin D1, IRS1, GS, tau, and presenilin 1 (PS1) [71-78]. From those, the most studied interaction is probably the regulation of GSK3/β-catenin signaling due to the central role that GSK3 plays in the canonical Wnt signal transduction pathway, where its phosphorylation of β-catenin on key residues is required for β-catenin's cellular ubiquitination and proteasomal degradation; a signaling pathway that is well known to promote self-renewal in a number of different tissue stem cells when activated [79-82], including NSCs, and hematopoietic stem cells. Furthermore, the fact that GSK3 interacts with several neuronal proteins that have directly been related to AD raises the question whether targeting this protein kinase could be therapeutically possible. For instance, the neuron-specific microtubule-associated protein tau (main component of NFTs and known to be abnormally phosphorylated in AD) was shown to be phosphorylated by GSK3 [72]. Aβ is able to induce a significant increase in GSK3β expression *in vitro* and it is known to be elevated in AD human brains [12, 83], and it has even postulated that PS1 could act as a scaffold protein that would bring GSK3 into proximity with its substrates tau and β-catenin [70]. In that respect, the therapeutic potential of GSK3 inhibitors has become a primary focus of pharmaceutical interest. Already a decade ago, Sun and colleagues reported that lithium chloride was able to reduce in a dose-dependent manner the secreted Aβ after transient expression of APP C99 *in vitro* [84]. Lithium ions, mainly utilized as a primary mood stabilizer in the treatment of chronic patients with bipolar disorder, were demonstrated to inhibit GSK3 [85, 86]; highlighting that potential dysregulation of GSK3 may contribute to this mental

disorder [87]. Bipolar disorder has been associated with cognitive dysfunction and increased risk for dementia [88, 89]. But most interestingly, it has been reported that lithium treatment reduced the prevalence of AD in patients with bipolar disorder to levels found in the general elderly population [90]. In addition to lithium-induced GSK3 inhibition, one cannot exclude the effect of additional mechanisms since lithium induces its therapeutic effects only after chronic administration, whereas direct lithium-induced inhibition of GSK3 is rapid and mild at therapeutic concentrations of 1 mM [91]. Furthermore, lithium is known to affect neuronal inositol metabolism, to inhibit adenylate cyclase, and to activate c-Jun NH2-terminal kinases (JNKs) [68, 92-95]. Perhaps, the most peculiar characteristic of lithium is its "dual" inhibition of GSK3. GSK3 catalyzes the phosphorylation of numerous protein substrates in the presence of Mg^{2+}-ATP. Lithium can directly inhibit GSK3 by acting as competitive inhibitor of Mg^{2+}, reducing the activity of this kinase [96]. Additionally, we previously commented in this chapter that GSK3 is inactivated by phosphorylation on a serine in the N-terminal domain: Ser9 in GSK3β and Ser21 in GSK3α. Once GSK3 is inactivated by serine phosphorylation, it can be re-activated by removing the phosphate from that serine through phosphatase activity. Lithium-induced indirect inhibition of GSK3 is due to a reduced effect of this phosphatase, resulting in higher levels of phosphorylated GSK3 (inactive form) [97]. A number of studies have demonstrated that Aβ production is enhanced by GSK3 and decreased by GSK3 inhibitors [84, 98]. For instance, *in vivo*, lithium treatment abolishes GSK3β-mediated Aβ increase in the brains of GSK3β transgenics and reduces plaque burden in the brains of the PDAPP (APP_{V717F}) transgenic mice [99]. Moreover, LiCl is able to modulate GSK3β transcription *in vitro* and *in vivo* [100]. All these studies support the potential therapeutic use of lithium in AD patients.

A number of research groups and pharmaceutical companies are searching for GSK3 inhibitory activities in compounds that have already shown other biological properties [87], such as hymenialdisine, maleimides, muscarinic agonist, paullones, thiadiazolidinones, and indirubins.

Recent studies have highlighted the relevant role of cell cycle proteins in mild cognitive impairment (MCI) and AD. The levels of key cell cycle proteins (e.g., CDK2, CDK5, cyclin G1, and BRAC1) are increased in MCI brains when compared to age-matched controls [101]. Furthermore, the peptidyl-prolyl cis-trans isomerase (Pin1), a protein that plays an important role in regulating the activity of key proteins like CDK5, GSK3β, and PP2A involved in both the phosphorylation state of Tau and cell cycle, has been found to be oxidatively modified and downregulated in MCI and AD brains [102-104]. Therefore, CDK´s inhibitors may have, in theory, great potential for the treatment of AD. Indirubins, for instance, were initially identified as CDK inhibitors and they are derived from the spontaneous and non-enzymatic dimerization of isantin and indoxyl found in more than 200 species of indigo-producing plants [105]. 6-brominated indirubins [106] extracted from another natural source (Mollusk *Hexaplex trunculus*) have been shown to provide an excellent scaffold for the generation of potent kinase inhibitors selective for GSK3 (comparing to CDKs). Indirubins have been shown to be very potent inhibitors of both GSK3β and CDK5, the two major kinases involved in tau hyperphosphorylation, constituting a promising family of compounds to evaluate as therapeutic agents in AD [107].

5. CONCLUDING REMARKS

The demonstrations that autonomous Wnt signaling is a conserved feature of the neurogenic niche that preserves the delicate balance between NSC maintenance and differentiation [8], and how lithium is able to directly expand pools of adult hippocampal progenitors *in vitro*, inducing them to become neurons at therapeutically relevant concentrations [108], suggest a connection between deficient neurogenesis in AD patients and a possible role of GSK3 in this deficit. Furthermore, additional studies have linked GSK3 to long term potentiation (LTP) by showing that over-expression of GSK3β in mice is enough to prevent its induction [109]. Similarly, it has been reported that inhibition of GSK3β blocked long-term depression (LTD) [110]. These studies together indicate that GSK3β could be critical for the induction of memory formation, switching off LTD and allowing LTP to occur [40]. Thus, the development of new therapeutic approaches to inhibit GSK3β may facilitate not only the promotion of neurogenesis but also recovery of the memory loss by inducing LTP. As a matter of fact, the Wnt/β-catenin signaling pathway is considered a putative target for the treatment of different diseases [111], including neurodegenerative diseases; and the study from He & Shen (2009) reporting that interruption of β-catenin signaling reduces neurogenesis in AD patients support this possibility [12]. They proposed that Aβ elevates GSK3β, which in turn promotes the phosphorylation and degradation of β-catenin, downregulating the expression of proneural genes. However, their model may leave other open questions for further studies. For instance, does their data support previous work where calcium-selective Aβ channels were proposed as an entrance pathway associated with neuronal toxicity [112]? What is the mechanism by which Aβ might be able to increase the intracellular levels of GSK3? Could such increase be a result of an indirect Aβ-induced effect or does it happen as a consequence of a direct physical interaction between protein (Aβ) and enzyme (GSK3)? Is the Aβ-induced effect specific for GSK3 or does it affect other known β-catenin phosphorylators such as PKC (protein kinase C) [45]?

Despite preliminary data demonstrating that GSK3 inhibitors can reduce hyperglycemia, improve insulin sensitivity [113], and exert benefits in animal models of AD [114], there are two main concerns in conflict with the predicted usefulness in therapy: (I) GSK3 is a critical player belonging to the Wnt signaling pathway and its inhibition would mimic the activation of this route which has been linked to the development of several types of cancers in humans [115]; (II) *in vivo,* disruption of the murine GSK3β gene revealed an unexpected embryonic lethality due to massive hepatocyte apoptosis [116]. This is consistent with additional *in vitro* data where pharmacological inhibition of GSK3 in different cell lines has been shown to facilitate apoptosis triggered by different stimuli [117-119]. This means that GSK3 inhibitors would provide protection from intrinsic apoptosis signaling, induced by several stimuli that cause cell damage (e.g., DNA damage, oxidative stress), but could exacerbate extrinsic apoptosis signaling (receptor-mediated). An excellent example is given by an *in vivo* study, where co-infusion of the specific GSK3 inhibitor, SB216763, corrected all responses derived from Aβ infusion, excepting for the induction of gliosis and behavioral deficits. However, SB216763 alone was associated with the induction of neurodegenerative markers and behavioral deficits, supporting the role for GSK3 hyper-activation in AD pathogenesis, but also highlighting the relevance of developing novel inhibitors that do not suppress its constitutive activity [114].

In another elegant study from 2007, Tet/DN-GSK3 mice showed increased neuronal apoptosis and impaired motor coordination, effects that were reversed when DN-GSK3 expression was shut-down and GSK3 activity came back to normal levels [120]. This proves that potential neurological toxicity induced by pharmacological inhibition of GSK3, beyond physiological levels, is plausible and must be taken into consideration. Moreover, in the heart, GSK3 seems to suppress cardiac hypertrophy [121, 122]. Then, the question is whether long-term exposure to GSK3 inhibitors could also increase the incidence of severe cardiac adverse effects because these drugs are intended to treat diabetes and/or AD patients for several years. Even though long-term use of lithium ions (generally used to treat bipolar disorder) has not been associated with an increased risk of cancer so far [123], additional pharmacological tests are required to answer these questions that still remain. Nevertheless, since the activity of the majority of GSK3 inhibitors is not restricted to GSK3, to establish a direct link between pure kinase modulation and its effects could be a difficult challenge.

CONFLICT OF INTEREST

The author(s) confirm that this chapter content has no conflict of interest.

ACKNOWLEDGEMENTS

We thank the Brazilian research funding agencies FAPERGS (PqG 1008860, PqG 1008857, ARD11/1893-7, PRONEX 1000274), CAPES (PROCAD 066/2007), CNPq, PROPESQ-UFRGS, and IBN-Net #01.06.0842-00 for financial support. Fares Zeidán-Chuliá acknowledges the Marie Curie Early Stage Research Training (EST) program for his previous funding at the University of Helsinki, Finland.

ABBREVIATIONS

AD	=	Alzheimer´s disease
NFTs	=	neurofibrillary tangles
GSK3	=	glycogen synthase kinase 3
Aβ	=	β-amyloid
SVZ	=	subventricular zone
SGZ	=	subgranular zone
NSCs	=	neural stem cells
RG	=	radial glia
SP	=	senile plaques
APC	=	*adenomatous polyposis coli* protein
APP	=	amyloid precursor protein
Aβ42	=	42-amino acid form of Aβ
BACE1	=	β-site amyloid precursor protein cleaving enzyme 1
sAPPβ	=	β-secreted APP
β-CTF	=	C-terminal membrane-bound fragment

AGEs = advanced glycation end products

CML = N(epsilon)-(carboxymethyl)lysine protein

IRS1 = insulin receptor substrate 1

IRS2 = insulin receptor substrate 2

GS = glycogen synthase

Fzd = frizzled

CK1α = casein-kinase 1α

Tcf/Lef = T-cell factor/lymphoid enhancer factor proteins

LRP = low-density lipoprotein receptor-related protein

Dvl = dishevelled

bFGF = basic fibroblast growth factor

EGF = epidermal growth factor

GPCs = glial precursor cells

PS1 = presenilin 1

JNKs = c-Jun NH2-terminal kinases

MCI = mild cognitive impairment

Pin1 = peptidyl-prolyl cis-trans isomerase

LTP = long term potentiation

LTD = long-term depression

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PARTE III----------

RESULTADOS

Artigo 1

EXPLORING THE MULTIFACTORIAL NATURE OF AUTISM THROUGH COMPUTATIONAL SYSTEMS BIOLOGY: CALCIUM AND THE RHO GTPASE RAC1 UNDER THE SPOTLIGHT

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Neuromolecular Medicine

15(2):364-83 (2013)

Article type: Research article

ISNN: 1535-1084

ISI Impact factor: 4.492 (2013)

QUALIS A1

Neuromol Med (2013) 15:364–383
DOI 10.1007/s12017-013-8224-3

ORIGINAL PAPER

Exploring the Multifactorial Nature of Autism Through Computational Systems Biology: Calcium and the Rho GTPase RAC1 Under the Spotlight

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Received: 17 October 2012 / Accepted: 16 February 2013 / Published online: 2 March 2013

Abstract Autism is a neurodevelopmental disorder characterized by impaired social interaction and communication accompanied with repetitive behavioral patterns and unusual stereotyped interests. Autism is considered a highly heterogeneous disorder with diverse putative causes and associated factors giving rise to variable ranges of symptomatology. Incidence seems to be increasing with time, while the underlying pathophysiological mechanisms remain virtually uncharacterized (or unknown). By systematic review of the literature and a systems biology approach, our aims were to examine the multifactorial nature of autism with its broad range of severity, to ascertain the predominant biological processes, cellular components, and molecular functions integral to the disorder, and finally, to elucidate the most central contributions (genetic and/or environmental) in silico. With this goal, we developed an integrative network model for gene–environment interactions (GENVI model) where calcium (Ca^{2+}) was shown to be its most relevant node. Moreover, considering the present data from our systems biology approach together with the results from the differential gene expression analysis of cerebellar samples from autistic patients, we believe that RAC1, in particular, and the RHO family of GTPases, in general, could play a critical role in the neuropathological events associated with autism.

Keywords Autism spectrum disorders · Xenobiotic · Polymorphism · In silico model · Gene expression

Electronic supplementary material The online version of this article (doi:10.1007/s12017-013-8224-3) contains supplementary material, which is available to authorized users.

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Introduction

Autism, a term derived from the *Greek* word "*autos*" which means "self", is generally defined as a neurodevelopmental disorder characterized by severe impairment in social interaction, fixed and restricted patterns of behavior and interest as well as qualitative defects in communicative skills. Behavioral features include hyperactivity, irritability, aggression, self-injuring, lack of attention, and fits of bad temper, and it is considered to be highly heterogeneous (Kanner 1943). Autism and autism spectrum disorders (ASD) are indistinctively used terms to designate a group of disorders of brain development that include Rett's syndrome, Asperger's syndrome, pervasive developmental disorder not otherwise specified (PDD-NOS), and childhood disintegrative disorder (Volkmar and Rutter 1995; Volkmar et al. 1997).

Autism is one of the most frequent developmental disorders (Kogan et al. 2007; Paula et al. 2011); and data from clinical and epidemiological samples have indicated a 4:1 male to female ratio prevalence (Fombonne 2003), although the causes of such differences are not clear.

Core symptoms of autism generally represent an aberrant social development, possibly of congenital origin (Grossman et al. 1997). Evaluation of autistic hallmarks seems to be complicated since a high percentage of patients suffers from mental retardation and different levels of communicative impairment (Baron-Cohen et al. 1999; Lord and Volkmar 2002), which may vary from complete lack of functional language to diverse weakness in verbally mediated tasks, although sometimes language skills can be normal (Tager-Flusberg and Joseph 2003; Bennett et al. 2007; Tager-Flusberg and Caronna 2007). Likewise, it is rarely diagnosed before 3 years of age due to the fact that early indicators of autism, such as abnormal social interaction and unusual playing behavior, are not detectable at ages younger than 14 months (Landa et al. 2007). There seems to be an association between autism and epilepsy with a frequency varying from 5 to 49 % (Tuchman and Rapin 2002). Also, mood disorders and anxiety are commonly diagnosed in these patients (Lecavalier 2006).

Despite extensive research, numerous etiological questions remain unanswered hitherto. For example, the main neuropathological events and molecular mechanisms underlying the aberrant brain development of autistic children are virtually unknown. Therefore, the use of novel approaches is needed for studying the apparent multifactorial causes of autism as a whole, and to better understand, the challenging etiology of this disorder as well as the molecular and biological processes involved in its development. Nowadays, by using computational tools, one can develop network models from large amounts of available experimental data, and thus, to generate new hypotheses to be later confirmed by wet laboratory work (Rosado et al. 2011; Rybarczyk-Filho et al. 2011; Zeidán-Chuliá et al. 2012). Here, we systematically reviewed the literature for characterizing (in silico) the landscape of gene–environment interactions in autism and proposed a network model capable of integrating the current knowledge on this topic. Second, we elucidated the main biological processes, cellular components, and molecular functions associated with the interconnected genes/proteins within such a model and characterized the topological properties of the generated network in order to predict the potential relevance of its components (putative candidate genes, xenobiotics, or previously reported ones) for further discussion in the present study. Finally, to better illustrate our in silico results, we performed a focused microarray analysis of gene expression (RHO family GTPases and related genes) in cerebellar tissue from autistic patients versus control samples.

Methodology

Systematic Review of the Literature and Development of a Network Model for Gene–Environment Interactions (GENVI) in the Autistic Context

Our aim was to plan an approach for investigating reported risk factors (genetic and environmental) in order to provide a more comprehensive picture of the multifactorial landscape of ASD. With this aim, we performed a systematic review of both original research articles and reviews by searching for literature associated with autism or ASD in the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/). These articles were obtained by using the two terms "autism" or "ASD" and combining them with the following terms: "gene," "polymorphism," "environmental factor," "gene-environment interaction," "developmental biology," "neurobiology," "toxicology," "drug," "xenobiotic", and "metabolism," respectively. The information was collected and split into two separate lists. The first list, with autism-related genes, included gene symbol, alias and/or description, Ensembl ID, as well as chromosomal location by collecting information from KEGG GENES (http://www.genome.jp/kegg/genes.html) and GeneCards® (http://www.genecards.org/). Many of these DNA sequence variations were described as genetic polymorphisms. The second one, with factors (drugs, pollutants, and compounds in general) that had ever been linked to the etiology of autism, including the compound identifier (CID) and/or KEGG ID, target, and activity-related information that was collected by utilizing KEGG COMPOUND (http://www.genome.jp/kegg/compound/), KEGG DRUG (http://www.genome.jp/kegg/drug/), and PubChem compound (http://www.ncbi.nlm.nih.gov/pccompound) as sources.

Thereafter, we screened the possible landscape of interactions between the genes/proteins (collected during the systematic review of the literature) by using the online search tool for the retrieval of interacting genes STRING 9.0 (http://string-db.org/) (Szklarczyk et al. 2011), with "Databases" and "Experiments" as input options and a confidence score of 0.400 (medium confidence). Then, chemical–chemical and chemical–gene/protein interactions (corresponding to the drugs, pollutants, and compounds listed after review of autism-related literature) were searched by utilizing the search tool for interactions of chemicals STITCH 3.0 (http://stitch.embl.de/) (Kuhn et al. 2008), with "Databases" and "Experiments" as input options and a confidence score of 0.400 (medium confidence). Links (interaction strength) between two different nodes were saved in data files to be handled with the Medusa interface (Hooper and Bork 2005).

Identification of Cellular Components, Molecular Functions, and Biological Processes of the Genes Belonging to the Network Model in the Autistic Context and Hierarchical Classification

Next, we aimed to identify the cellular components, molecular functions, and biological processes of the genes belonging to the newly developed network model (GENVI) by using the database for annotation, visualization, and integrated discovery DAVID v6.7 (http://david.abcc.ncifcrf.gov/) (Liu et al. 2011), which provides a number of functional annotation tools to researchers for better comprehension of the biological meaning behind any large list of genes. Only those cellular components, molecular functions, and biological processes with p values $< E-07$ were selected for further hierarchical classification. Hierarchy of cellular components, molecular functions, and biological processes of the genes belonging to the network was identified by Gene Ontology (GO) (http://www.geneontology.org/) analysis. Three-dimensional representation (3D) and spatial localization of the biological processes identified in the network model (GENVI) by using ViaComplex software (http://lief.if.ufrgs.br/pub/biosoftwares/viacomplex/) (Castro et al. 2009).

In Silico Identification of the Most Central Nodes Within the Network Model of Gene–Environment Interactions in the Autistic Context and Subnetwork Construction

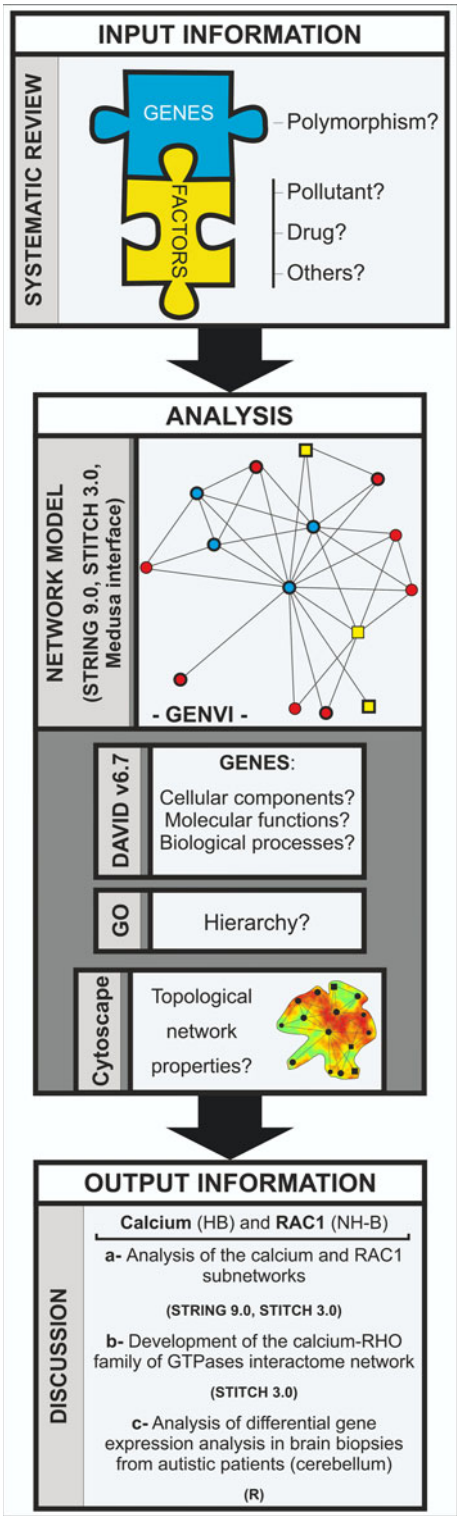
Based on network centralities (e.g., connectivity, neighborhood connectivity, stress, betweenness, closeness, and clustering coefficient), one could identify which nodes (genes, drugs, pollutants, and compounds in general) have an important position in the overall network structure (Wuchty and Stadler 2003; Estrada 2006; Rosado et al. 2011); meaning that "targeting" a node with high centrality values would considerably disrupt the whole network. Briefly, connectivity of a node is defined as the number of its interacting partners (Liu et al. 2006). Highly connected nodes in a network are named as "hubs" (Rosado et al. 2011). Neighborhood connectivity of a node "a" would be defined as the average connectivity of all neighbors of "a." Stress measures the number of shortest paths passing through a node (Scardoni et al. 2009). Betweenness, which is similar to the stress centrality, measures how frequently the shortest path connecting every pair of nodes (e.g., "a" and "b") is crossing a given node ("c"). All nodes with high betweenness values are named as "bottlenecks" (Hernández et al. 2007; Yu et al. 2007). Both stress and betweenness highlight the relevance of a gene/protein, drug or compound in general, for spreading the information through the entire network. Closeness centrality measures the grade of proximity of a node to the rest of nodes (Hernández et al. 2007; Rosado et al. 2011; De Franceschi et al. 2012), giving an idea of how long it would take an information to disperse from one network node to the rest of them. Last but not least, clustering coefficient is a centrality that measures the fraction of connections between the neighbors of a given node, identifying genes and/or factors with highly connected neighbors (del Rio et al. 2009). Therefore, hub-non-bottlenecks (H-NB) are nodes with high connectivity and low betweenness centrality, hub-bottlenecks (HB) are nodes with a value above the thresholds for both connectivity and betweenness centrality, non-hub-bottlenecks (NH-B) are nodes with low connectivity and high betweenness centrality, and non-hub-non-bottlenecks (NH-NB) are nodes with both connectivity and betweenness centrality values under the thresholds (Rosado et al. 2011).

For calculating these network centralities, Cytoscape, an open source platform for complex network analysis and visualization (http://www.cytoscape.org/), was used (Smoot et al. 2011). Numeric values concerning the properties of each node are also provided as supplementary material. For clearer visualization of the topological network properties of each node within the network model, numeric values were projected in 2D representation by using ViaComplex software (http://lief.if.ufrgs.br/pub/biosoftwares/viacomplex/) (Castro et al. 2009). Thresholds were established considering the mean value of each centrality: connectivity >40 (mean = 9.33), stress > 40,000 (mean = 30,000), betweenness centrality >0.05 (mean = 0.02), closeness centrality >0.34 (mean = 0.32), and neighborhood connectivity >60 (mean = 28.88).

Finally, for providing a more detailed analysis, interpretation, and discussion of the results, subnetworks of selected "bottlenecks" were constructed by utilizing STRING 9.0 and STITCH 3.0 (Kuhn et al. 2008; Szklarczyk et al. 2011).

Ca^{2+}-RHO Family of GTPases Interactome Network Development and Analysis of Differential Gene Expression in Cerebellar Brain Biopsies From Autistic Patients

For developing the Ca^{2+}-RHO family interactome network, STITCH 3.0 (http://stitch.embl.de/) (Kuhn et al. 2008) was utilized, with "Databases" and "Experiments" as input options, confidence score of 0.400 (medium confidence), in order to represent the interaction of calcium (Ca^{2+}) with members of the RHO family of GTPases and related gene/proteins. Links (interaction strength) between two different nodes were saved in data files to be handled with the Medusa interface (Hooper and Bork 2005). For studying the differential expression of members from the



◀ **Fig. 1** Graphical abstract summarizing the different approaches followed in the present study. A systematic review of the literature was performed for collecting information about genes and environmental factors that had been linked to the development of autism. Thereafter, a network model (GENVI) was developed for integrating the information collected during the systematic review. The present in silico model also included a number of genes/proteins and factors with no previous report on autism research. The genes were subjected to further analysis for elucidating the cellular components, molecular functions, and biological processes affected in the network, as well as their hierarchy. Then, after elucidation of a number of topological network properties, most biologically relevant nodes (genes/proteins and factors) of the in silico model were selected (e.g., Ca^{2+} and RAC1) for further discussion as well as focused subnetwork and differential gene expression analyses

RHO family of GTPases and related genes belonging to the interactome network, the microarray dataset GSE38322 from the public database of Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo/) was found by using "autism" and "brain" as keywords and downloaded for further analysis. This dataset (GSE38322) is publicly available; it was originally contributed by Ginsberg and colleagues (Ginsberg et al. 2012) and contained data from cerebellar brain tissue of autistic patients and control samples. Expression data were filtered from probes with <0.05 signal detection p values and normalized by using the lumi package from the freely available software system R (http://www.r-project.org) (Gentleman et al. 2004) and robust spline normalization (RSN). For differential gene expression analysis, normalized data of cerebellar samples from autistic patients versus controls were analyzed by utilizing the limma package from R and false discovery rate (FDR) (Pawitan et al. 2005) for statistical assessment of the microarray data (corrected p values <0.05 were considered significant).

A graphical abstract summarizing the contents and the methodological approach utilized in the present study is additionally provided (Fig. 1).

Results and Discussion

GENVI is an Integrative Network Model for Gene–Environment Interactions in Autism: Insights From Systems Biology

The analysis resulted in one single model for gene–environment interactions (GENVI) in the autistic context with 122 genes/proteins (Supplementary table S1) and 191 factors (Supplementary table S2) (313 nodes in total) connecting through 1,461 interactions (Fig. 2). *In silico*, this network model integrated not only reported gene–environment interactions in autism but also genes/proteins and factors returned by the search tools (STRING 9.0 and STITCH 3.0) for interconnecting the whole network, with no previous reported link to autistic disorder so far (listed without PMID in supplementary tables S1 and S2) and peripheral nodes interacting with genes/proteins and factors with already autism-related report in the literature. Since we believe they could represent potentially

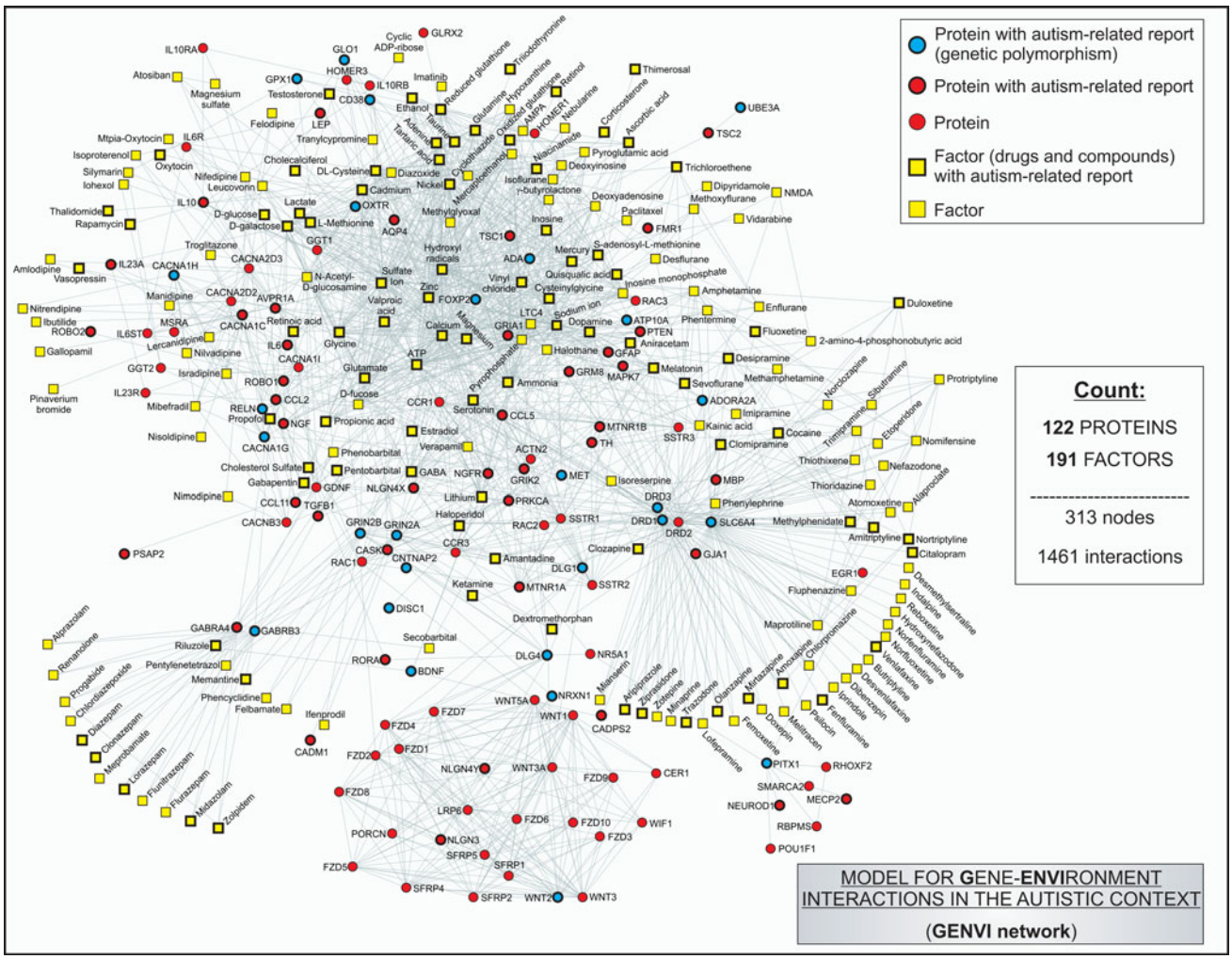


Fig. 2 *In silico* network model (GENVI) integrating proposed and novel putative gene–environment interactions in autism. The network interconnected 122 genes/proteins and 191 factors (drugs, pollutants, chemicals, and other compounds) through 1,461 interactions. The model was developed by using "Databases" and "Experiments" as input options. As explained in the inset, genetic polymorphisms and nodes with autism-related report are represented with *different shapes* and *colors* (Color figure online)

interesting candidates for future research (e.g., clinical studies, in vivo or in vitro experiments), they were all subjected to further in silico analysis. In total, 151 nodes corresponding to genes/proteins and factors with autism-related report and 162 un-reported ones were integrating this model.

Hierarchical representation of cellular components, molecular functions, and biological processes of the genes belonging to the GENVI network model is provided (Fig. 3 and supplementary table S3). In total, 16 different biological processes were shown to be affected by the genes within GENVI network model mainly corresponding to both intra- and intercellular communication, Wnt signaling, nerve impulse transmission, cell differentiation, neuronal development, neurogenesis, behavior, and locomotion. This is shown in 3D representation (Fig. 4) by utilizing the ViaComplex software (http://lief.if.ufrgs.br/pub/biosoftwares/viacomplex/) (Castro et al. 2009).

Exploration of network centralities (Supplementary table S4 and S5) revealed that RAC1, PRKCA, GABA, and CACNA1C were NH-Bs or nodes with high betweenness centrality (>0.05) but lower connectivity (<40) (Supplementary table S6, Figs. 5, 6). Additionally, Ca^{2+}, SLC6A4, sulfate, hydroxyl radicals, DRD2, sodium ion, ATP, and magnesium were HBs or nodes with high values of both betweenness centrality (>0.05) and connectivity (>40) (Supplementary table S6, Figs. 5, 6). Moreover, these bottlenecks (NH-Bs and HBs) showed high values for stress and closeness centralities above the thresholds. This demonstrates that such nodes are essential for the flow of information

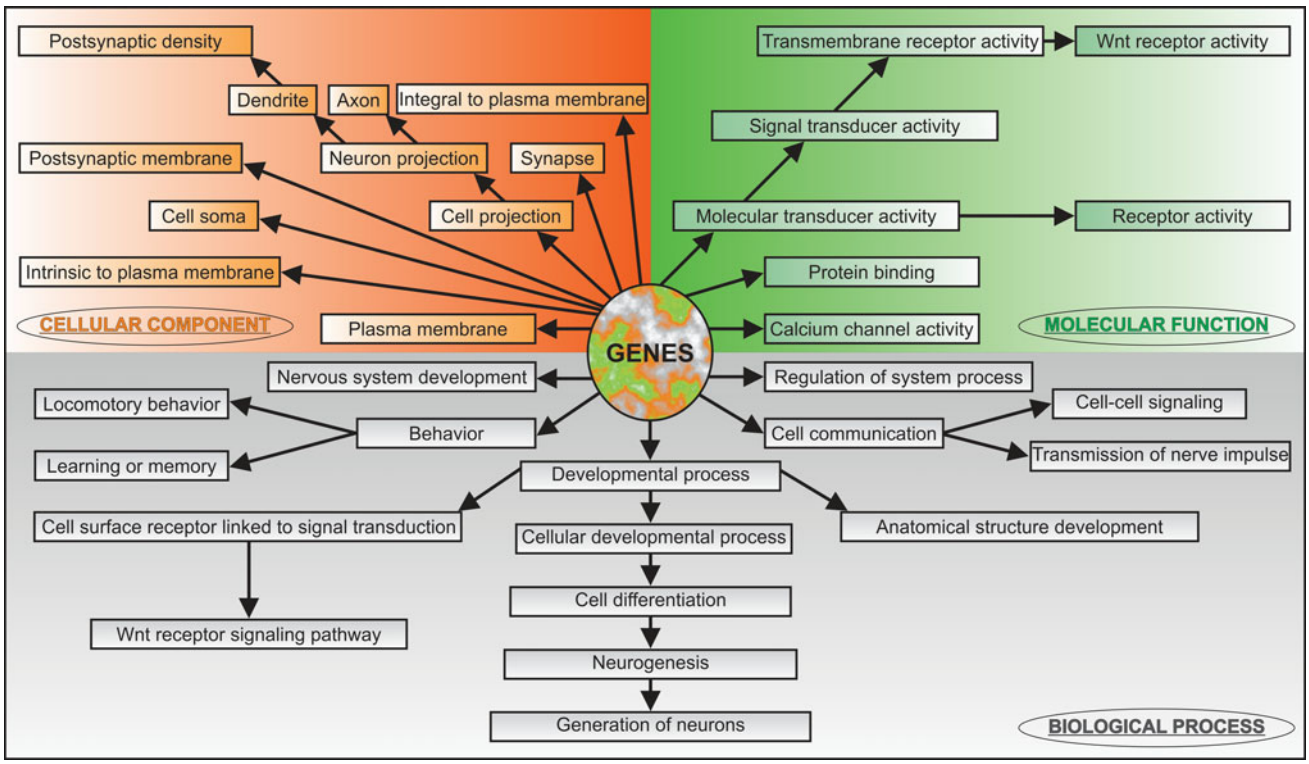


Fig. 3 Hierarchical representation of cellular components, molecular functions, and biological processes of the genes belonging to the network model (GENVI)

through the whole network (Fig. 6a, b, d). Another interesting aspect is related to tranylcypromine (an irreversible inhibitor of monoamine oxidase and a potent antidepressant) (Frieling and Bleich 2006), cholecalciferol, corticosterone, and triiodothyronine. These nodes scored the highest neighborhood connectivity values (>60) (Supplementary table S6 and Fig. 6c) and may have a strong etiological relevance since all of them are connected to Ca^{2+} and/or hydroxyl radicals (HBs of GENVI network model). Considering the clinical heterogeneity of autism, one could speculate that environmental factors (drugs, pollutants, etc.) "targeting" highly connected and stressed bottlenecks in our in silico model could easily disrupt the network and affect a higher number of biological processes. This may explain the wide symptomatology (from mild to severe) of ASD, because different type and number of genes/proteins together with its associated biological processes will be affected depending on whether the node is targeted and its position (topologically speaking) within the network of interactions (Fig. 2).

We shall herein discuss the role of Ca^{2+} (HB) in autism and the reasons why RAC1 (NH-B) may be a relevant gene deserving further investigation in this area of research.

The Neuron-Glia Communication Mediator, Ca^{+2}, is a HB of GENVI Network Model: Contextualizing its Relevance in Autism

Our data pointed to Ca^{2+} (HB) (Fig. 7) as the most relevant node of GENVI network model for gene–environment interactions in the autistic context, scoring the highest values of connectivity (>40), stress (>40,000), betweenness (>0.05), and closeness (>0.34) centralities among 313 nodes (Supplementary table S6 and Fig. 6). Thus, abnormal Ca^{2+} influx would affect numerous interactions between gene/proteins and compounds within the network and, consequently, numerous biological processes. For instance, glial cells excitability is based on variations of cytosolic Ca^{2+} concentrations rather than electrical changes localized in the membrane (Perea and Araque 2005). Astrocytes express receptors for almost all neurotransmitters at their membranes, giving the capability to detect any neurotransmitter released at the synapse, activating them through the mobilization of their intracellular Ca^{2+}. Activated astrocytes can then modulate neuronal excitability by releasing a number of neuroactive molecules or gliotransmitters such as glutamate, ATP, D-serine, nitric oxide (NO), atrial natriuretic factor (ANF), and prostaglandins,

homocysteic acid, taurine, and tumor necrosis factor-α (TNF-α) (Haydon 2001; Fellin et al. 2006; Halassa et al. 2007). This efficient interrelation allows synaptic transmission to continue. For instance, the expression of plasma-membrane glutamate transporters ensures quick clearance of the neurotransmitter from the synapse, preventing the desensitization of postsynaptic receptors that would interrupt synaptic transmission (Halassa et al. 2007). Moreover, when presynaptic neurons are activated, astrocytes release ATP which is then hydrolyzed to adenosine. If adenosine accumulates, synaptic transmission is inhibited through mediation of presynaptic adenosine A1 receptors (Zhang et al. 2003; Pascual et al. 2005). The existence of this bidirectional signaling between astrocytes and neurons gave rise to the concept of "tripartite synapse" (Perea and Araque 2005).

Neurochemical studies have focused on the involvement of neurotrophins and different neurotransmitter systems as an important part of the neurobiological bases of autism (Pardo and Eberhart 2007). The nerve growth factor family is constituted by different proteins that include nerve growth factor (NGF), neurotrophin-3, neurotrophin-4, and brain-derived neurotrophic factor (BDNF) (Lewin and Barde 1996; Huang and Reichardt 2003). BDNF production was shown to be enhanced during the neonatal period and later reduced in adult male patients of 18–26 years of age (Nelson et al. 2001; Miyazaki et al. 2004; Connolly et al. 2006; Hashimoto et al. 2006; Katoh-Semba et al. 2007). The presence of high levels of neurotrophin-4 and neurotrophin-5 in neonatal blood of children, who were later diagnosed with autism, was also reported (Nelson et al. 2006). Abnormalities in neurotransmitter systems including serotoninergic, dopaminergic, opioid, cholinergic, GABAergic, and glutamatergic may be possibly involved in the pathology of the syndrome (Lam et al. 2006). High peripheral-blood platelet concentrations of serotonin were shown to be associated with the disorder (Schain and Freedman 1961; Anderson et al. 1990; Anderson and Hoshiono 1997). Reduced serotonin transporter (SERT, also known as SLC6A4) binding capacity and low serotonin synthesis in the brain of these patients have already been described (Makkonen et al. 2008).

Additionally, D2 dopamine receptor (DRD2) gene has been implicated in schizophrenia, posttraumatic stress disorder, movement disorders, and migraine (Oades et al. 2000; Noble 2003). However, whether DRD2 may play a role in autism is not clear yet (Philippe et al. 2002).

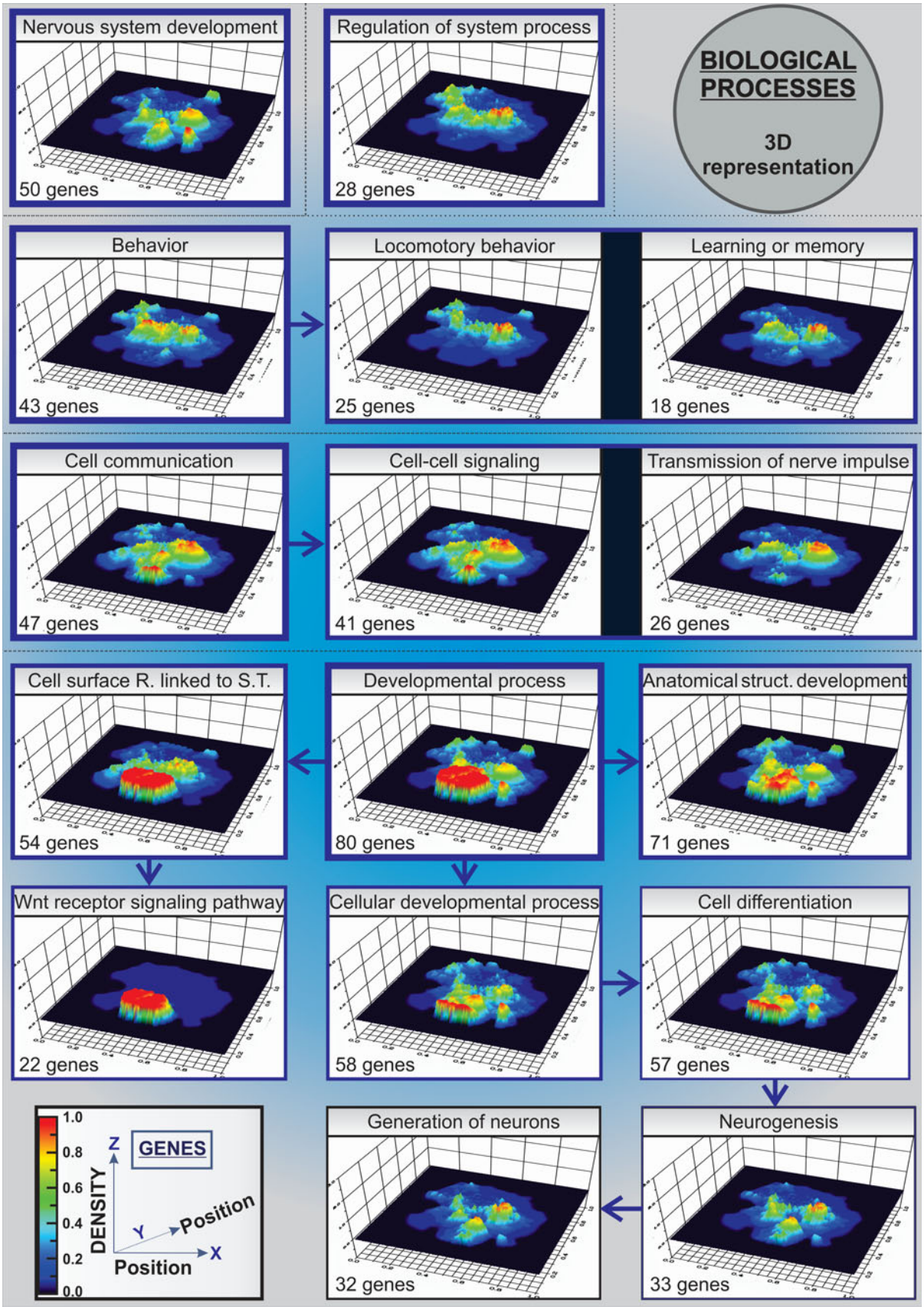
In our analysis, both SLC6A4 and DRD2 were HBs within GENVI model, displaying the highest connectivity and betweenness centrality values among 122 proteins belonging to the network (Supplementary table S6, Figs. 5, 6b), highlighting their biological relevance in silico. SLC6A4 is targeted by multiple psychoactive drugs within the network, such as cocaine and perinatal exposure to this drug has already been correlated with autism and developmental abnormalities (Davis et al. 1992). Furthermore, cocaine abuse itself has been associated with intronic polymorphisms affecting alternative splicing of human DRD2 (Moyer et al. 2011). This may represent an example of how a single environmental factor may affect different gene/proteins with potential relevance or reported association with the neuropathology of autism.

Appreciation for cholinergic transmission in autism steadily grew since it was reported that either its stimulation or disruption could affect cognitive performance (Andrews et al. 1994; Newhouse et al. 2004). Indeed, some studies have claimed that, for instance, decreased nicotinic receptor function is present in these patients (Lee et al. 2002; Martin-Ruiz et al. 2004).

Other authors have described a hyperglutaminergic state in autism and how memantine (NMDA glutamate receptor-antagonist) is able to improve the characteristic symptomatology (Shinohe et al. 2006; Chez et al. 2007). As Ghanizadeh suggested (Ghanizadeh 2010), it is interesting that higher serum levels of neurotensin (NT) had been detected in young patients with autistic disorder (Angelidou et al. 2010), considering the imbalance in glutamate-to-GABA ratios (Harada et al. 2011) and the lower expression of GABA(A) receptors found in these patients (Buxbaum et al. 2002; Ma et al. 2005; Fatemi et al. 2009); since it is known that NT is also able to promote endogenous glutamate signaling in certain brain regions (Antonelli et al. 2007). Moreover, single-nucleotide polymorphisms in both GRIN2A and GRIN2B genes (NMDA receptor 2A and 2B, respectively) were associated with the disorder (Barnby et al. 2005; Myers et al. 2011; O'Roak et al. 2011). This all together may reflect in a glutamate-induced brain injury as well as an inflammatory scenario in autism.

Then, which are the direct implications that could be expected from an abnormal Ca^{2+} influx in the context of autism?

(1) In general, synaptic control of glial Ca^{2+} has been shown in different brain areas such as cerebellum, cortex, and hippocampus and the responsiveness of astrocytes to the release of some neurotransmitters by synaptic terminals such as noradrenaline, acetylcholine, GABA, glutamate, or NO is well described in the literature (Perea and Araque 2005). This necessarily means that disturbances excessively elevating intracellular Ca^{2+} would directly affect synaptic function, neuron-astrocyte metabolic coupling, and may have an impact in other glial cells with involvement of Ca^{2+} signaling like oligodendrocytes and microglia (Agrawal et al. 2000; Schipke et al. 2002; Perea and


Nervous system development
50 genes
Regulation of system process
28 genes
BIOLOGICAL PROCESSES
3D representation
Behavior
43 genes
Locomotory behavior
25 genes
Learning or memory
18 genes
Cell communication
47 genes
Cell-cell signaling
41 genes
Transmission of nerve impulse
26 genes
Cell surface R. linked to S.T.
54 genes
Developmental process
80 genes
Anatomical struct. development
71 genes
Wnt receptor signaling pathway
22 genes
Cellular developmental process
58 genes
Cell differentiation
57 genes
1.0
0.8
0.6
0.4
0.2
0.0
DENSITY
Z
GENES
Y
Position
X
Position
Generation of neurons
32 genes
Neurogenesis
33 genes

◀ **Fig. 4** Three-dimensional representation (3D) and spatial localization of the biological processes identified in the in silico model (GENVI). In the present figure, hierarchy of the biological processes is additionally represented with *arrows*. The number of affected genes corresponding to each biological process is shown with *color grading* in the network (from *dark blue* for the *lowest to dark red* for the *highest* density of genes) (Color figure online)

Araque 2005). Moreover, low sulphation or sulphoconjugation capacity has been noticed in "low-functioning" autistic children, which is the mechanism that effectively metabolize phenolic amines, including catecholamines functioning as neurotransmitters, and this deficiency would result in aberrant inactivation of neurotransmitters (Alberti et al. 1999).

(2) Exposure to environmental stressors (e.g., pollutants or drugs) may lead to overproduction of reactive oxygen species (ROS) (e.g., hydroxyl radicals) with ensuing neurotoxicity. In fact, xenobiotics and heavy metals are able to inhibit metabolic pathways that synthesize GSH and keep physiological levels of its reduced form (Carvalho et al. 2008; Herbert 2010). In autism, the endogenous antioxidant and detoxifier, glutathione (GSH), may be specifically important for the pathogenesis of the disorder since polymorphisms of genes encoding gluthatione-dependent enzymes were reported (e.g., glyoxalase 1 or GLO1 as well as glutathione peroxidase 1 or GPX1) (Junaid et al. 2004; Ming et al. 2010). Individuals with compromised

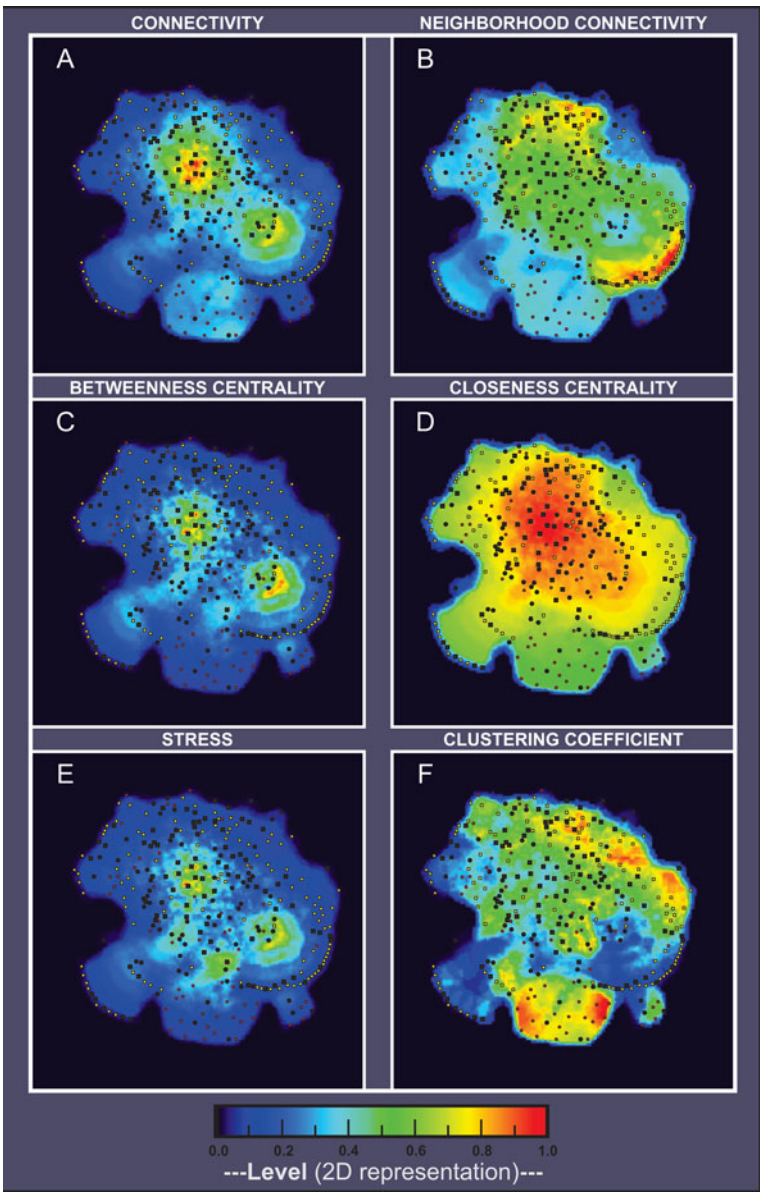


Fig. 5 Network centrality measures and topological analysis of the network model (GENVI). Numeric values corresponding to the properties of each node (stress, connectivity, neighborhood connectivity, betweenness, and closeness centralities) are plotted in a bi-dimensional (2D) *color grading* representation (from *dark blue* for the *lowest to dark red* for the *highest* values) (Color figure online)

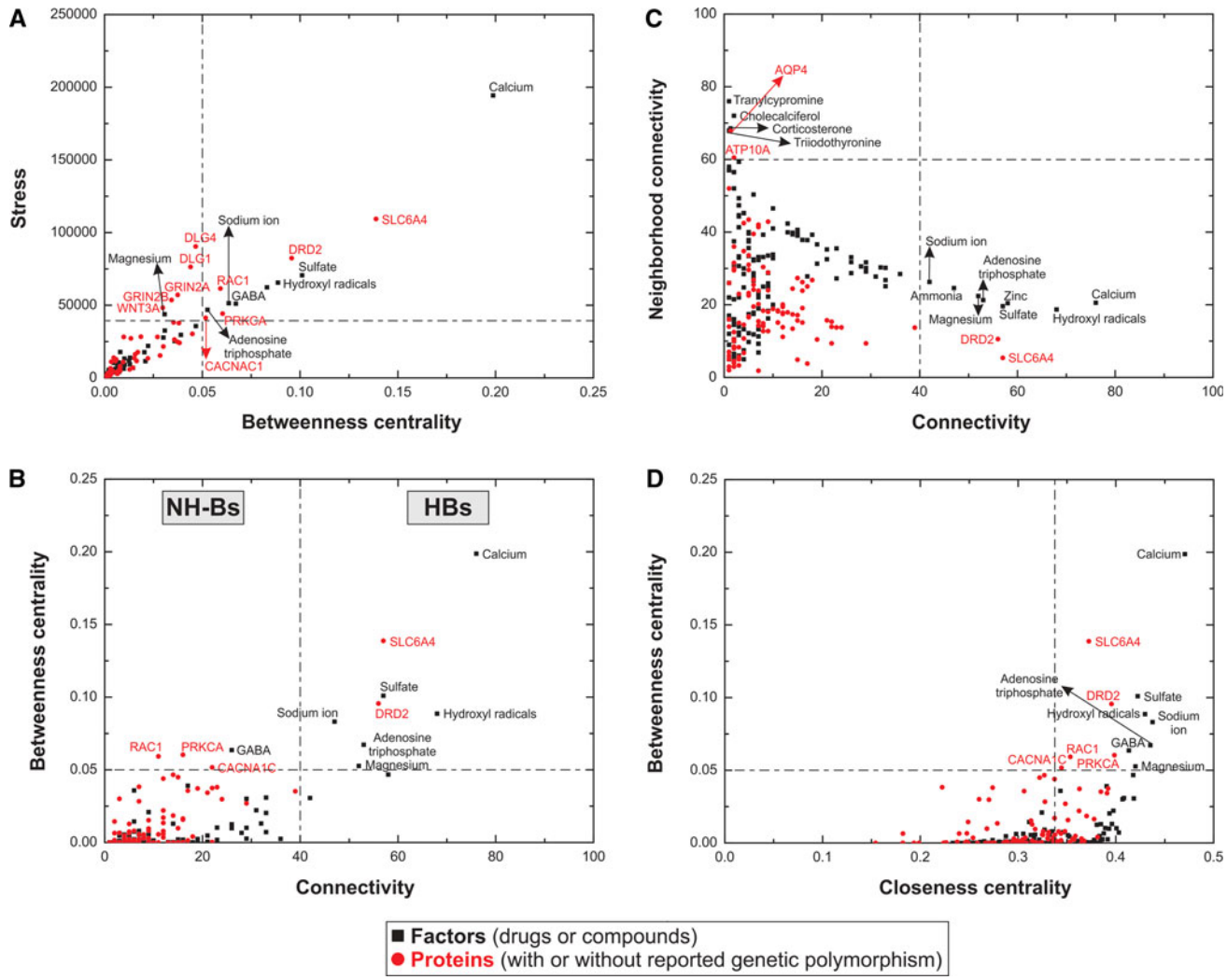


Fig. 6 Analysis of the topological properties of the nodes belonging to the network model (GENVI). *Dashed lines* are indicating the threshold value for each property. In the graphs, genes/proteins are represented by *red circles* and factors (drugs, pollutants, chemicals, and other compounds) are plotted as *black squares*. Note that NH-Bs and HBs distinguish non-hub-bottlenecks from hub-bottlenecks, respectively (Color figure online)

antioxidant defenses would be under oxidative stress and may easily reach toxic thresholds when compared to healthy people (Herbert 2010). It is well known that oxidative stress levels can trigger cell death by increasing the cytoplasmic Ca^{2+} concentration, giving rise to a Ca^{2+} influx into mitochondria where it may accelerate and disrupt the normal cellular metabolism of these patients (Ermak and Davies 2002). Furthermore, excessive ROS production would also affect the epigenetic regulation of gene expression due to lower methionine synthase activity. In oxidative stress scenarios, decreased methylation capability (in addition to compromised antioxidant/detoxification capacity) would be then expected in autism (Deth et al. 2008; James et al. 2008). In other words, oxidative stress-induced decreased DNA methylation could up-regulate the expression of genes that are usually controlled by methylation gene silencing, representing one of the possible cross-talks between environmental influences and genetic alterations in ASD.

The Rho GTPase RAC1 is a NH-B of GENVI Network Model: A New Genetic Link to the Neurobiology of Autism?

It is generally agreed that the characteristic fixed patterns of behavior and lack of social interaction in ASD indicate a disruption of critical neurodevelopmental routes and, thus, aberrant pre- and postnatal development of important brain structures (Muhle et al. 2004). These cerebral areas were previously linked to social development in patients (Adolphs 2001) and implicate parts of the frontal lobe, superior temporal cortex, parietal cortex, and amygdala

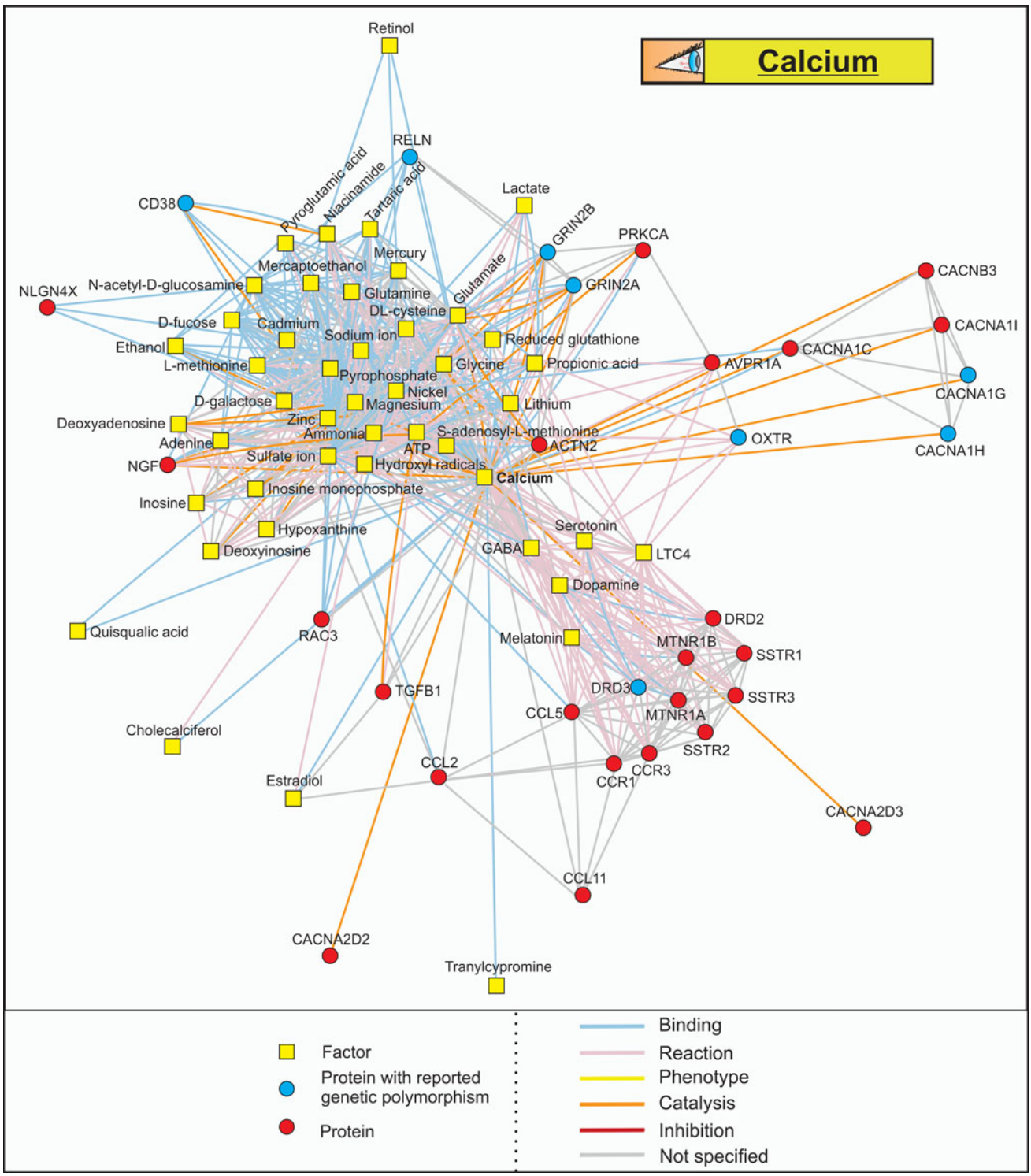


Fig. 7 Ca^{2+} subnetwork analysis. Landscape of interactions of Ca^{2+}-gene/protein and Ca^{2+}-factor (drugs, pollutants, chemicals, and other compounds) through either "binding," "reaction," "phenotype," "catalysis," "inhibition," or "not specified" type of interconnection. Reported genetic polymorphisms are marked with *blue circles*, as indicated in the *inset* (Color figure online)

(Amaral et al. 2008). Postmortem studies revealed a specific cytoarchitecture in both cortical and subcortical structures of autistic patients. Several disturbances were demonstrated, including the decreased number of neurons and reduced dendritic arborisation in different parts of the limbic system (amygdala, hippocampus, septum, and anterior cingulated cortex) (Baron-Cohen et al. 2000; Kemper and Bauman 2002; Sweeten et al. 2002; Volkmar

and Pauls 2003; Schumann and Amaral 2006). Similar aberrations were reported in the cerebellum where a decreased number of Purkinje and granule cells were observed (Ritvo et al. 1986). Furthermore, both number and structure of neocortical "minicolumns" (or "microcolumns") seem to be altered. In the brains of ASD patients, Casanova and colleagues found significantly higher numbers of cortical minicolumns when compared to the healthy controls (Casanova et al. 2002, 2006; Casanova and Trippe 2009). They additionally observed a decreased intercolumnar width (smaller space between cell body-defined minicolumnar structures) and reduced neuronal size.

In physiological conditions, during development and adulthood, cellular migration is a crucial biological process for neurons and glial cells to occupy characteristic positions in the central (CNS) and peripheral nervous system (PNS) (Torrence 1991). Cytoskeleton reorganization is required for axonal outgrowth and neuronal migration (Dent and Gertler 2003). Interestingly, several studies have implicated Rho GTPases in the development of axons and dendrites (Gualdoni et al. 2007). In particular, normal levels of RAC1 seem to be essential for early dendritic development of mouse hippocampal neurons (Gualdoni et al. 2007). Moreover, migration of differentiated hippocampal neurons in a RAC and PI3-kinases-dependent manner has been observed (Leemhuis et al. 2004).

Our in silico analysis showed RAC1 to be a gene with high betweenness centrality (>0.05) in our network model (GENVI) (Supplementary table S6 and Fig. 6). It is also remarkable that RAC1 was identified in nine of the sixteen different biological processes representing the whole network model for gene–environment interactions in the autistic context (GENVI), such as behavior, developmental process, nervous system development, anatomical structure development, cell differentiation, cellular developmental process, locomotory behavior, neurogenesis, and generation of neurons (Supplementary table S3; Figs. 3, 4).

Despite extensive research, there are no studies directly linking RAC1 to ASD. It is an opened question whether mutations in RAC1 gene or impaired functionality of the protein could have a role in the neuropathology of this disorder. On the other hand, there are some circumstantial facts suggesting RAC1 as an interesting candidate gene/protein to pay attention to:

(1) RAC1 is an ubiquitously expressed member of the RHO monomeric GTPase family that participates in the regulation of gene expression, cytoskeletal rearrangement, cell activation, and cell motility (Chatterjee et al. 2010). RAC1 has been suggested to have an important role in toll-like receptors (TLR) functioning in immune cells (Ruse and Knaus 2006). Additionally, it has been reported the existence of differential monocyte responses to TLR ligands in children with ASD (Enstrom et al. 2010). Therefore, alterations of innate immunity due to TLR-mediated neuroimmune interactions, sustained inflammatory response, or even infection- or vaccine-induced imbalance between innate and adaptive immunity in autistic patients may have an interpretation through the altered RAC1-associated signaling pathways (Jyonouchi et al. 2005, 2008; Hagberg et al. 2012).

(2) It has been noted that a significant percentage of autistic patients were undergoing extensive antimicrobial treatment and suffering from chronic diarrhea before subsequent gradual evolution of their autistic symptoms (Bolte 1998; Sandler et al. 2000). Some reports suggest that disruption of protective intestinal microbiota after several rounds of treatment with broad-spectrum antibiotics may create a favorable environment for opportunistic neurotoxin-producing pathogens such as *Clostridium tetani*, which might become a pathological element to consider in the context of the idiopathic increase of autism rates as well as when multiple cases were reported in the same family (Sandler et al. 2000; Finegold 2008). As a matter of fact, both qualitative and quantitative differences between gastrointestinal microflora of autistic children and healthy controls have been reported as well, especially associated with *Clostridium spp.* (Finegold et al. 2002; Song et al. 2004; Parracho et al. 2005). Furthermore, autistic children have showed some improvement after oral vancomycin treatment (which is not absorbed in the gut) (Sandler et al. 2000). Even though the connection between intestinal anaerobic bacteria and autism is still under debate (Martirosian et al. 2009), it is noteworthy that a number of different *Clostridium* species can produce large molecular mass cytotoxins, able to trigger effects on the actin cytoskeleton and to disrupt actin stress fibers (Popoff et al. 1996). RAC, RAP, and RAS small GTP-binding proteins are indeed targets for *Clostridium* toxins (Just et al. 1996; Popoff et al. 1996; Leemhuis et al. 2004; Geny et al. 2010).

(3) An additional circumstantial fact is that biological activities of RHO family of GTPases (like CDC42, RHOA, and RAC1) are controlled by their guanine nucleotide binding states in cells. RhoGAPs (regulatory molecules of RHO GTPases) use magnesium (Fig. 8) as a cofactor to reach catalytic efficiency and specificity in GTP hydrolysis (Zhang et al. 2000). A significant decrease in the concentration of magnesium and selenium in the hair and nail samples of autistic patients, correlating with their

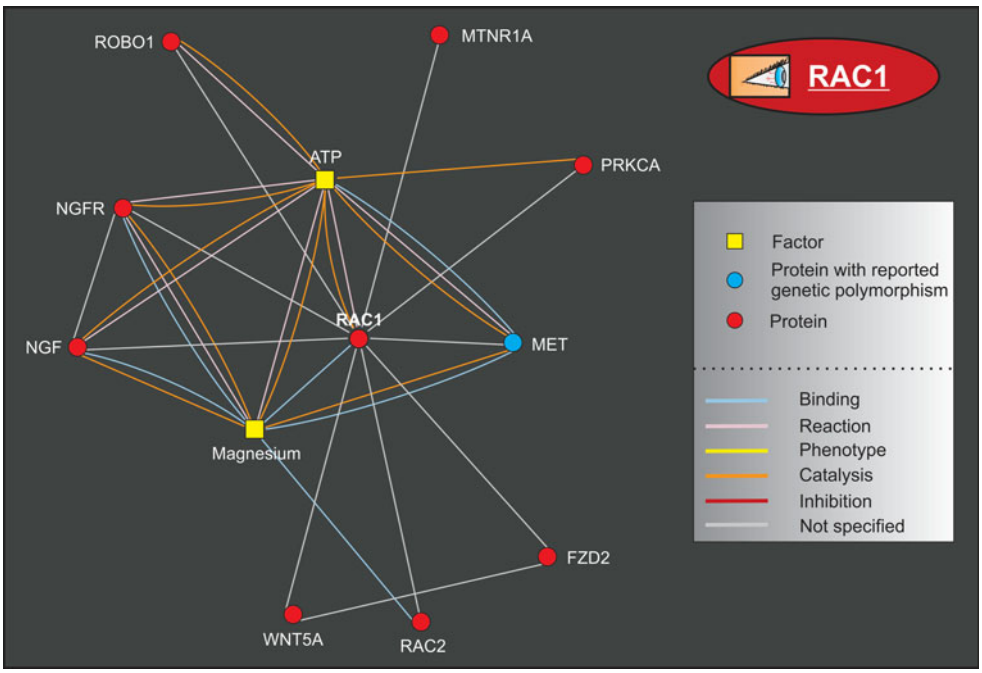


Fig. 8 RAC1 subnetwork analysis. Landscape of interactions of RAC1-gene/protein and RAC1-factor (drugs, pollutants, chemicals, and other compounds) through either "binding," "reaction," "phenotype," "catalysis," "inhibition," or "not specified" type of interconnection. Reported genetic polymorphisms are marked with *blue circles*, as indicated in the *inset* (Color figure online)

degrees of severity, has already been reported (Lakshmi Priya and Geetha 2011). Thus, if physiological levels of magnesium are reduced in autistic patients, one could predict that both catalytic efficiency and specificity of RhoGAPs would be also compromised.

(4) An elegant study by Adams et al. (2011) reporting significantly low levels of ATP in children with autism may also support RAC1 as an interesting candidate gene/protein in the autistic scenario since RAC1 is known to be sensitive to ATP (Fig. 8) and GTP levels in vitro, displaying a short-term moderate decrease in activity after nucleotide triphosphate depletion (Hallett et al. 2003).

In other words, based on the in silico evidences presented in our analysis together with the facts mentioned above, we believe that RAC1, in particular, and the RHO family of GTPases, in general, may have a role in the neuropathological events associated with autism, presumably, due to altered neuroimmune interactions.

Analysis of the Genes Belonging to the Ca^{2+}-RHO Family of GTPases Interactome Network Reveals a Differential Gene Expression in the Cerebellum of Autistic Brains

It is well described that several small GTPases collaborate with Ca^{2+} signaling in regulating different cellular processes, such as cell adhesion, cell migration, and exocytosis (Aspenström 2004). Our in silico results demonstrated a topological relevance of Ca^{2+} and RAC1 in the proposed model for gene–environment interactions in autism (GENVI) (Figs. 2, 6). Consequently, a constant information flow in the interaction network is being trafficked through these nodes. Thus, to further characterized the interplay between Ca^{2+}, RAC1, and other RHO family GTPases and related proteins, we elaborated a list genes/proteins (Supplementary table S7), and by utilizing STITCH 3.0, the Ca^{2+}-RHO family of GTPases interactome network was developed (Fig. 9). All the genes/proteins from the original list (43 nodes) were interconnected, and 5 of them (CHP, ITPR1, PLCG1, PRKCA, and RAC3) were directly linked to Ca^{2+} in this newly developed network (Supplementary table S7 and Fig. 9).

Synaptic control of glial Ca^{2+} is well described in different brain areas such as cerebellum, cortex, and hippocampus (Perea and Araque 2005). Diverse lines of evidence suggest the involvement of apoptosis in the cerebellum of autism subjects, including loss and atrophy of Purkinje cells (Kern 2003; Whitney et al. 2008). Furthermore, it has been reported brain region-specific deficits in expression levels of mitochondrial electron transport chain complexes in the cerebellum and the frontal and temporal cortices of children with autism (Chauhan et al. 2011). Therefore, we checked whether the genes belonging to the Ca^{2+}-RHO family of GTPases interactome network could be differentially expressed in the cerebellum of these patients. Very interestingly, we found that 15 from the 43 genes belonging to the Ca^{2+}-RHO family of GTPases interactome network (Table 1) were differentially

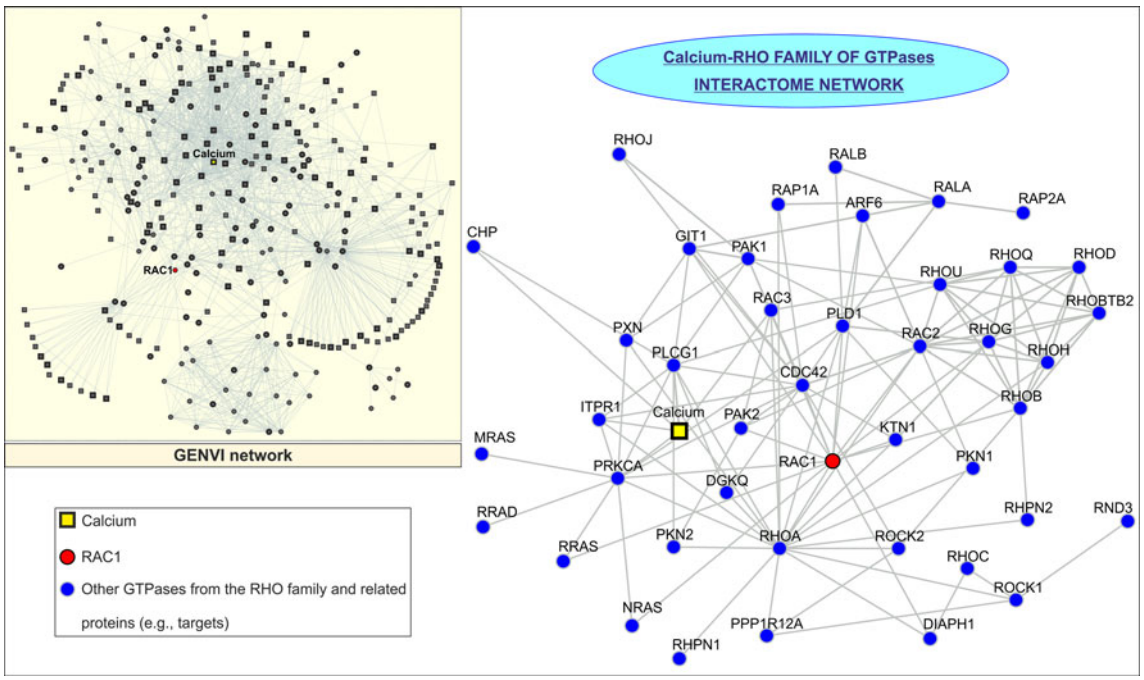


Fig. 9 Ca^{2+}-RHO family of GTPases interactome network. The network interconnected Ca^{2+} with 43 GTPases from the RHO family and related genes/proteins (e.g., targets). The model was developed by using "Databases" and "Experiments" as input options

expressed (corrected p value <0.05) in the cerebellum of autistic patients when compared to control samples. Three from the 5 genes that interconnected to Ca^{2+} in the interactome network were in this group of either sub- or overexpressed genes (PRKCA, RAC3, and CHP). In addition, RAC1 directly connected with a number of differentially expressed genes (e.g., CDC42, PRKCA, and RHOB) (Table 1; Fig. 9), which is consistent with our in silico results where RAC1 was shown to be a bottleneck of the GENVI network model, or an essential key connector node critically relevant for the flow of information through the entire network (Yu et al. 2007).

In our array analysis, CDC42 was strongly subexpressed (corrected p value = 0.00200014), while RHOB displayed a significant overexpression (corrected p value = 0.01534273) in the cerebellum of autistic patients (Table 1). Even though RAC1, CDC42, and RHOA/RHOB GTPases are known to be all expressed by migrating precerebellar neurons (PCN), they seem to have opposed roles on neuritogenesis. For instance, inhibition of RAC and CDC42 subfamilies impairs neurite outgrowth of PCN without affecting migration, whereas pharmacological inhibition of RHO enhances axon outgrowth of PCN and prevents nuclei migration (Causeret et al. 2004). Likely, our results may reflect a compromised neurite outgrowth in cerebellar neurons of autistic patients. Besides, RAC1 and RAC3 are important for the development of the nervous system, wherein they play complementary roles during late stages of brain development (Corbetta et al. 2009). Both RAC1 and RAC3 GTPases synergistically control the development of cortical and hippocampal GABAergic interneurons (Vaghi et al. 2012), areas of the CNS where decreased number of neurons and reduced dendritic arborisation have been described in autism (Baron-Cohen et al. 2000; Kemper and Bauman 2002; Sweeten et al. 2002; Volkmar and Pauls 2003; Schumann and Amaral 2006). In an elegant study by Bolis et al. (2003), RAC3 was implicated in Purkinje cell development, at times of neuronal differentiation and synaptogenesis. Our own results showed subexpression of RAC3 in the cerebellum of autistic brains (corrected p value = 0.01470489) (Table 1). Considering that RAC3-knockout mice are viable and recent studies started to indicate a relevant role of RAC3 in cognitive development (Corbetta et al. 2008), the specific role of these GTPases in the context of autism may deserve further research.

Concluding Remarks

A general tendency is emerging toward the combination of different etiologies to explain the heterogeneity of autism. Casanova (2007) proposed a "Triple Hit Hypothesis of Autism," with three different degrees of etiological factors such as precise time window of brain development, genetic susceptibility, and different environmental stressors. Williams and Casanova (2010) further suggested that ultrasound

Table 1 Differentialy expressed genes from the Ca^{2+}-RHO family of GTPases interactome network in the cerebellum of autistic brains versus control samples (corrected p values <0.05 were considered significant)

Gene symbol	Alias and/or description	Ensembl ID (ENSP)	Differential gene expression	Corrected p value	Interact. with calcium
CDC42	Cell division cycle 42 (GTP-binding protein, 25 kDa)	ENSP00000314458	Down	0.00200014	NO
PRKCA	PKC-α; protein kinase C, α	ENSP00000284384	Up	0.00353275	YES
RALA	v-ral simian leukemia viral oncogene homolog A (ras related)	ENSP00000005257	Up	0.00628922	NO
RALB	v-ral simian leukemia viral oncogene homolog B (ras related)	ENSP00000272519	Down	0.00803662	NO
RHOQ	Ras homolog gene family member Q; Plasma membrane-associated small GTPase	ENSP00000238738	Up	0.01193934	NO
RAC3	Ras-related C3 botulinum toxin substrate 3 (rho family, small GTP-binding protein Rac3)	ENSP00000304283	Down	0.01470489	YES
RHOB	Ras homolog gene family member B	ENSP00000272233	Up	0.01534273	NO
RHOU	Ras homolog gene family member U	ENSP00000355652	Up	0.02181148	NO
ROCK1	Rho-associated coiled-coil containing protein kinase 1	ENSP00000382697	Up	0.02843342	NO
PLD1	Phospholipase D1	ENSP00000342793	Up	0.03381597	NO
PKN2	Protein kinase N2	ENSP00000359552	Up	0.034955	NO
GIT1	G protein-coupled receptor kinase interacting ArfGAP 1	ENSP00000378338	Up	0.03796387	NO
RHOG	Ras homolog gene family member G (rho G)	ENSP00000339467	Up	0.03837408	NO
PXN	Paxillin	ENSP00000228307	Up	0.04056128	NO
CHP	Calcium-binding protein p22	ENSP00000335632	Down	0.04455404	YES

examination, commonly used in obstetrics, could be one of these environmental stressors by exerting teratogenic/toxic effects on the CNS. Another hypothesis proposed that accumulative doses of mercury from different sources (e.g., pollution, maternal fish consumption, dental amalgams, and vaccinations) during infant development, together with a decreased ability to remove mercury from the body, could increase the probability of developing and/or aggravating autism (Zeidán-Chuliá et al. 2011).

In this study, after systematic review of the literature, we aimed to develop a model for gene–environment interactions in ASD able to integrate the current knowledge and findings in the topic. Thereafter, we characterized it in detail by using systems biology tools (Fig. 1), and finally, we further discussed the most relevant genes and/or factors according to the output information from this approach and the results from the microarray analysis of samples from autistic patients. Two key conclusions can be summarized according to our data:

(1) Ca^{2+}- signaling molecule was the most central node of our model of gene–environment interactions in the autistic context (GENVI). Communication of Ca^{2+} signals between brain cells is a typical feature of glial cells. In fact, astroglia-regulated neurogenesis, neuronal structural plasticity, and synaptic rearrangement may have an important role in the dynamics of social functioning (Mercadante et al. 2008). Normal development of nervous system would require well-organized neuronal migration, axon guidance, target selection, dendritic growth, synapse formation, and elimination (Bolton and Eroglu 2009). But under stressful conditions (e.g., sensory deprivation, separation stress, toxic action of xenobiotics), reactive structural plasticity can occur in an immature developing brain which is accompanied by alterations of glial cell activation (Musholt et al. 2009). Neuroanatomical abnormalities observed in autism, such as reduced brain size at birth and a sudden and excessive brain overgrowth in the areas of cerebellum, frontal lobe, and limbic structures, with increased cerebral white matter and decrease in cerebral cortex and hippocampal/amygdala volumes, are changes associated with delays in neuronal maturation and increased astrogliosis (Pardo et al. 2005). These evidences together with studies reporting that astroglial responses in autism, manifested by increased astrocyte/neuron ratio in brain cortex, alterations in intercellular communication within the astroglial syncytium, and reactive astrogliosis (Laurence and Fatemi 2005; Pardo et al. 2005; Fatemi et al. 2008) point toward a dysregulation of neuron–glia interactions and Ca^{2+}-mediated signaling in the developing brain of autistic children.

(2) RAC1, in particular, and the RHO family of GTPases, in general, are highlighted as potentially relevant genes/proteins the context of autism. Certainly, the

maintenance of dendritic arbor complexity during development and into adulthood is critical for the preservation of functional circuitry and connectivity critical for learning and complex behaviors (Srivastava et al. 2012). The Rho family of small GTPases (especially, RAC, CDC42, and RHOA) is indeed a key regulator of the actin cytoskeleton in response to extracellular cues, and disturbances in their activities often give rise to dramatic effects in dendritic morphogenesis (Scott et al. 2003).

Finally, we believe that our approach (combination of systematic review of the literature with systems biology and microarray analyses) offers an attractive option to aid researchers in the field of PDD for finding potentially relevant genes, factors, or gene–environment interactions, which could be later confirmed by clinical and/or basic science studies.

Acknowledgments First of all, we apologize to all our colleagues whose studies were not cited due to lack of space. We thank the Brazilian research funding agencies FAPERGS (PqG 1008860, PqG 1008857, ARD11/1893-7, PRONEX 1000274), CAPES (PROCAD 066/2007), CNPq, PROPESQ-UFRGS, and IBN-Net #01.06.0842-00 for supporting this work. A.B.S is supported by the grant from the Federal Program of the Russian Federation (N 8061, 2012–2013). We are very grateful to Prof. Alexei Verkhratsky (University of Manchester, Manchester, UK) and Dr. Marcio L. Acencio (Universidade Estadual Paulista, São Paulo, Brasil) for reading the manuscript.

Conflict of interest The authors declare that there are no conflicts of interest.

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SUPPLEMENTARY MATERIAL

Supplementary table S1. Reported and putative genetic variations interconnecting through the network model for gene-environment interactions in the autistic context ("GENVI" network). Underlined PMIDs distinguish reports where no significant correlation with autism of such a gene/protein was found.

Gene symbol	**Alias and/or description**	**Ensembl ID** (ENSP)	**Chromosome** (Cytogenetic band)	**Polymorphism** (Yes/---)	**Selected autism-related report** (PMID)
ACTN2	Actinin, α 2	ENSP00000355537	1q42-q43	---	---
ADA	Adenosine deaminase	ENSP00000361965	20q13	Yes	11354825
ADORA2A	Adenosine A2a receptor	ENSP00000336630	22q11.23	Yes	19565319
AQP4	Aquaporin 4	ENSP00000372654	18q11.2-q12.1	---	18435417
ATP10A	ATPase, class V, type 10A	ENSP00000349325	15q11.2	Yes	20609483
AVPR1A	Arginine vasopressin receptor 1A	ENSP00000299178	12q14-q15	---	12082568
BDNF	Brain-derived neurotrophic factor	ENSP00000414303	11p13	Yes	21547716 11857571
CACNA1C	Calcium channel, voltage-dependent, L type, α 1C subunit	ENSP00000266376	12p13.3	---	15454078
CACNA1G	Calcium channel, voltage-dependent, T type, α 1G subunit	ENSP00000352011	17q22	Yes	19455149
CACNA1H	Calcium channel, voltage-dependent, T type, α 1H subunit	ENSP00000334198	16p13.3	Yes	16754686
CACNA1I	Calcium channel, voltage-dependent, T type, α 1I subunit	ENSP00000385019	22q13.1	---	---
CACNA2D2	Calcium channel, voltage-dependent, α 2/ δ subunit 2	ENSP00000390329	3p21.3	---	---
CACNA2D3	Calcium channel, voltage-dependent, α 2/ δ subunit 3	ENSP00000288197	3p21.1	---	---
CACNB3	Calcium channel, voltage-dependent, β 3 subunit	ENSP00000301050	12q13	---	---
CADM1	Cell adhesion molecule 1	ENSP00000329797	11q23.2	---	18957284
CADPS2	Ca++-dependent secretion activator 2	ENSP00000400401	7q31.3	---	17380209
CASK	Calcium/calmodulin-dependent serine protein kinase (MAGUK family)	ENSP00000367408	Xp11.4	---	18054859 20574149

CCL2	MCP-1; chemokine (C-C motif) ligand 2	ENSP00000225831	17q11.2-q12	---	21095018
CCL5	RANTES; chemokine (C-C motif) ligand 5	ENSP00000293272	17q11.2-q12	---	21095018
CCL11	Eotaxin; chemokine (C-C motif) ligand 11	ENSP00000302234	17q21.1-q21.2	---	21095018
CCR1	RANTES receptor; chemokine (C-C motif) receptor 1	ENSP00000296140	3p21	---	---
CCR3	Eosinophil eotaxin receptor; chemokine (C-C motif) receptor 3	ENSP00000350003	3p21.3	---	---
CD38	CD38 molecule	ENSP00000226279	4p15	Yes	20435366
CER1	Cerberus 1, cysteine knot superfamily, homolog (Xenopus laevis)	ENSP00000370297	9p23-p22	---	---
CNTNAP2	AUTS15, NRXN4; contactin associated protein-like 2	ENSP00000354778	7q35	Yes	18179894
DISC1	Disrupted in schizophrenia 1	ENSP00000355596	1q42.1	Yes	17579608
DLG1	Discs, large homolog 1 (Drosophila)	ENSP00000345731	3q29	Yes	20830797
DLG4	Discs, large homolog 4 (Drosophila)	ENSP00000293813	17p13.1	Yes	20952458
DRD1	Dopamine receptor D1	ENSP00000327652	5q35.1	Yes	18205172
DRD2	Dopamine receptor D2	ENSP00000354859	11q23	---	11857571
DRD3	Dopamine receptor D3	ENSP00000373169	3q13.3	Yes	21691864
EGR1	Early growth response 1	ENSP00000239938	5q31.1	---	---
FMR1	Fragile X mental retardation 1	ENSP00000359506	Xq27.3	---	2031184
FOXP2	Forkhead box P2	ENSP00000386200	7q31	Yes	15108192 11894222
FZD1	Frizzled homolog 1 (Drosophila)	ENSP00000287934	7q21	---	---
FZD2	Frizzled homolog 2 (Drosophila)	ENSP00000323901	17q21.1	---	---
FZD3	Frizzled homolog 3 (Drosophila)	ENSP00000240093	8p21	---	---
FZD4	Frizzled homolog 4 (Drosophila)	ENSP00000311581	11q14.2	---	---
FZD5	Frizzled homolog 5 (Drosophila)	ENSP00000354607	2q33.3	---	---
FZD6	Frizzled homolog 6 (Drosophila)	ENSP00000351605	8q22.3-q23.1	---	---
FZD7	Frizzled homolog 7 (Drosophila)	ENSP00000286201	2q33	---	---
FZD8	Frizzled homolog 8 (Drosophila)	ENSP00000363826	10p11.21	---	---
FZD9	Frizzled homolog 9 (Drosophila)	ENSP00000345785	7q11.23	---	---
FZD10	Frizzled homolog 10 (Drosophila)	ENSP00000229030	12q24.33	---	---
GABRA4	γ-aminobutyric acid (GABA) A receptor, α 4	ENSP00000264318	4p12	---	16080114
GABRB3	γ-aminobutyric acid (GABA) A receptor, β 3	ENSP00000299267	15q11.2-q12	Yes	11920158
GDNF	Glial cell derived neurotrophic factor	ENSP00000317145	5p13.1-p12	---	---

GFAP	Glial fibrillary acidic protein	ENSP00000253408	17q21	---	11706102
GGT1	γ-glutamyltransferase 1	ENSP00000248923	22q11.23	---	---
GGT2	γ-glutamyltransferase 8 pseudogene	ENSP00000385721	22q11.21	---	---
GJA1	CX43, connexin-43; gap junction protein, α 1, 43kDa	ENSP00000282561	6q21-q23.2	---	18435417
GLO1	Glyoxalase I	ENSP00000362463	6p21.3-6p21.1	Yes	15386471
GLRX2	Glutaredoxin 2	ENSP00000356410	1q31.2-q31.3	---	---
GPX1	Glutathione peroxidase 1	ENSP00000407375	3p21.3	Yes	19195803
GRIA1	GLUR1; glutamate receptor, ionotropic, AMPA 1	ENSP00000285900	5q31.1	---	11706102
GRIK2	GLUR6; glutamate receptor, ionotropic, kainate 2	ENSP00000397026	6q16.3-q21	---	11920157
GRIN2A	NMDAR2A; glutamate receptor, ionotropic, N-methyl D-aspartate 2A	ENSP00000332549	16p13.2	Yes	15830322
GRIN2B	NMDAR2B; glutamate receptor, ionotropic, N-methyl D-aspartate 2B	ENSP00000279593	12p12	Yes	21383861 21572417
GRM8	GLUR8; glutamate receptor, metabotropic 8	ENSP00000344173	7q31.3-q32.1	---	12676915
HOMER1	Homer homolog 1 (Drosophila)	ENSP00000334382	5q14.2	---	---
HOMER3	Homer homolog 3 (Drosophila)	ENSP00000348150	19p13.11	---	---
IL6	Interleukin 6 (interferon, β 2)	ENSP00000258743	7p21	---	15546155
IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	ENSP00000338799	5q11.2	---	---
IL6R	Interleukin 6 receptor	ENSP00000357470	1q21	---	---
IL10	Interleukin 10	ENSP00000412237	1q31-q32	---	15546155
IL10RA	Interleukin 10 receptor, α	ENSP00000227752	11q23	---	---
IL10RB	Interleukin 10 receptor, β	ENSP00000290200	21q22.11	---	---
IL23A	Interleukin 23, alpha subunit p19	ENSP00000228534	12q13.3	---	19800697
IL23R	Interleukin 23 receptor	ENSP00000321345	1p31.3	---	---
LEP	Leptin	ENSP00000312652	7q31.3	---	20478355
LRP6	Low density lipoprotein receptor-related protein 6	ENSP00000261349	12p13.2	---	---
MAPK7	Mitogen-activated protein kinase 7	ENSP00000311005	17p11.2	---	21848643
MBP	Myelin basic protein	ENSP00000348273	18q23	---	7682457
MECP2	Methyl CpG binding protein 2 (Rett syndrome)	ENSP00000395535	Xq28	---	11106359
MET	Hepatocyte growth factor receptor; met proto-oncogene	ENSP00000317272	7q31	Yes	17053076
MSRA	Methionine sulfoxide reductase A	ENSP00000313921	8p23.1	---	---
MTNR1A	Melatonin receptor 1A	ENSP00000302811	4q35.1	---	20377855

MTNR1B	Melatonin receptor 1B	ENSP00000257068	11q21-q22	---	20377855
NEUROD1	Neurogenic differentiation 1	ENSP00000295108	2q32	---	14593429 15542242
NGF	Nerve growth factor (β polypeptide)	ENSP00000358525	1p13.1	---	---
NGFR	Nerve growth factor receptor (TNFR superfamily, member 16)	ENSP00000172229	17q21-q22	---	---
NLGN3	Neuroligin 3	ENSP00000363163	Xq13.1	---	12669065 15389766 18189281
NLGN4X	Neuroligin 4, X-linked	ENSP00000275857	Xp22.33	---	12669065 18189281
NLGN4Y	Neuroligin 4, Y-linked	ENSP00000342535	Yq11.221	---	18628683
NR5A1	Nuclear receptor subfamily 5, group A, member 1	ENSP00000362690	9q33	---	---
NRXN1	Neurexin 1	ENSP00000385142	2p16.3	Yes	18179900
OXTR	Oxytocin receptor	ENSP00000324270	3p25	Yes	20094064
PITX1	Paired-like homeodomain 1	ENSP00000265340	5q31.1	Yes	18628683
PORCN	Porcupine homolog (Drosophila)	ENSP00000322304	Xp11.23	---	---
POU1F1	POU class 1 homeobox 1	ENSP00000342931	3p11	---	---
PRKCA	PKC-α; protein kinase C, α	ENSP00000284384	17q22-q23.2	---	---
PSAP2	SHANK3; SH3 and multiple ankyrin repeat domains 3	ENSP00000262795	22q13.3	---	17173049
PTEN	Phosphatase and tensin homolog	ENSP00000361021	10q23.3	---	17427195
RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	ENSP00000348461	7p22	---	---
RAC2	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	ENSP00000249071	22q13.1	---	---
RAC3	Ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	ENSP00000304283	17q25.3	---	---
RBPMS	HERMES; RNA binding protein with multiple splicing	ENSP00000340176	8p12	---	---
RELN	Reelin	ENSP00000392423	7q22	Yes	16311013 15048648
RHOXF2	Rhox homeobox family, member 2	ENSP00000360441	Xq24	---	---
ROBO1	Roundabout, axon guidance receptor, homolog 1 (Drosophila)	ENSP00000381450	3p12	---	18270976
ROBO2	Roundabout, axon guidance receptor, homolog 2 (Drosophila)	ENSP00000417164	3p12.3	---	18270976

					21859478
RORA	RAR-related orphan receptor A	ENSP00000261523	15q22.2	---	20375269
SFRP1	Secreted frizzled-related protein 1	ENSP00000220772	8p11.21	---	---
SFRP2	Secreted frizzled-related protein 2	ENSP00000274063	4q31.3	---	---
SFRP4	Secreted frizzled-related protein 4	ENSP00000410715	7p14.1	---	---
SFRP5	Secreted frizzled-related protein 5	ENSP00000266066	10q24.1	---	---
SLC6A4	SERT, 5-HTT; solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	ENSP00000261707	17q11.2	Yes	18804097
SMARCA2	SWI2; SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	ENSP00000265773	9p22.3	---	---
SSTR1	Somatostatin receptor 1	ENSP00000267377	14q13	---	---
SSTR2	Somatostatin receptor 2	ENSP00000350198	17q24	---	---
SSTR3	Somatostatin receptor 3	ENSP00000330138	22q13.1	---	---
TGFB1	Transforming growth factor, β 1	ENSP00000221930	19q13.2	---	17030376
TH	Tyrosine hydroxylase	ENSP00000370571	11p15.5	---	7786965 11857571
TSC1	Tuberous sclerosis 1	ENSP00000298552	9q34	---	9813776 12023313 16901420
TSC2	Tuberous sclerosis 2	ENSP00000219476	16p13.3	---	9813776 12023313 16901420
UBE3A	Ubiquitin protein ligase E3A	ENSP00000381045	15q11.2	Yes	20609483
WIF1	WNT inhibitory factor 1	ENSP00000286574	12q14.3	---	---
WNT1	Wingless-type MMTV integration site family, member 1	ENSP00000293549	12q13	---	---
WNT2	Wingless-type MMTV integration site family, member 2	ENSP00000265441	7q31.2	Yes	19895723 15048648
WNT3	Wingless-type MMTV integration site family, member 3	ENSP00000225512	17q21	---	---
WNT3A	Wingless-type MMTV integration site family, member 3A	ENSP00000284523	1q42	---	---
WNT5A	Wingless-type MMTV integration site family, member 5A	ENSP00000264634	3p21-p14	---	---

Supplementary table S2. Reported and putative drugs, pollutants, chemicals and other compounds interconnecting through the network model for gene-environment interactions in the autistic context ("GENVI" network). Underlined PMIDs distinguish reports where no significant correlation with autism of such a factor was found.

FACTOR	**Compound identifier** (CID)	**KEGG** (ID)	**Target/Comment**	**Activity**	**Selected autism-related report** (PMID)
2-amino-4-phosphonobutyric acid	CID000002207	---	---	---	---
Adenine	CID000000190	D00034	---	---	10699370
Adenosine triphosphate (ATP)	CID000005957	C00002	---	---	21651783
Alaproclate	CID000002081	D02787	Serotonin reuptake inhibitor	Antidepressant	---
Alprazolam	CID000002118	D00225	GABAA-receptor (benzodiazepine binding site) agonist	Sedative-hypnotic	---
Amantadine	CID000002130	D07441	Influenza M2 proton channel blocker; N-methyl-D-aspartate receptor antagonist	Antiviral; antiparkinsonian	11392343
Amitriptyline	CID000002160	D07448	Serotonin transporter inhibitor; noradrenalin transporter inhibitor	Antidepressant (tricyclic)	---
Amlodipine	CID000002162	D07450	Calcium channel L type blocker	Antihypertensive (calcium antagonist)	---
Ammonia	CID000000222	C00014	---	---	16613869
Amoxapine	CID000002170	D00228	Serotonin transporter inhibitor; noradrenalin transporter inhibitor	Antidepressant (tricyclic)	12707553
AMPA	CID000001221	C13672	AMPA receptor agonist	---	---
Amphetamine	CID000003007	D07445	Noradrenalin transporter inhibitor; dopamine transporter	Psychostimulant	---

			inhibitor; serotonin transporter inhibitor		
Aniracetam	CID000002196	D01883	---	Mental performance enhancer	12070527
Aripiprazole	CID000060795	D01164	Dopamine D2-receptor agonist/antagonist; 5-HT2A-receptor antagonist; 5-HT1A-receptor agonist/antagonist	Antipsychotic; antischizophrenic	17389666
Ascorbic acid	CID000005785	D00018	---	Vitamin (antiscorbutic); acidifier (urinary)	8255984
Atomoxetine	CID000054840	D07473	Noradrenalin transporter inhibitor	Antidepressant	17003665
Atosiban	CID000068613	D03008	Oxytocin receptor antagonist	Antagonist (oxytocin)	---
Butriptyline	CID000021771	D07601	---	Antidepressant (tricyclic)	---
Cadmium	CID000002514	C01413	---	---	19688188
Calcium	CID000000271	C00076	---	---	20956852
Chlordiazepoxide	CID000002712	D00267	GABAA-receptor	Tranquilizer (minor)	---
Chlorpromazine	CID000002726	D00270	5-HT2-receptor antagonist; H1 receptor antagonist; α2-adrenergic receptor antagonist; dopamine D2-receptor antagonist; muscarinic cholinergic receptor antagonist	Anti-emetic; antipsychotic	---
Cholecalciferol	CID000002735	C05443	---	---	17920208
Cholesterol sulfate	CID000065076	C18043	---	---	22098722
Citalopram	CID000002771	D07704	Serotonin transporter inhibitor	Antidepressant	21667200
Clomipramine	CID000002801	D07727	Serotonin transporter inhibitor; noradrenalin transporter inhibitor	Antidepressant (tricyclic)	11476129
Clonazepam	CID000002802	D00280	GABAA-receptor (benzodiazepine binding site) agonist	Anticonvulsant	11494958
Clozapine	CID000002818	D00283	(5-HT1A, 5-HT2A, 5-HT2C, and 5-HT7)-receptor antagonist; α1-adrenergic receptor antagonist; dopamine (D2 and D4)-receptor	Antipsychotic	11590976

			antagonist		
Cocaine	CID000446220	D00110	Serotonin transporter inhibitor; dopamine transporter inhibitor; noradrenalin transporter inhibitor; voltage-gated potassium channel (KCNH2) blocker; voltage-gated sodium channel (SCN1A, SCN2A, SCN3A, SCN4A, SCN5A, SCN8A, SCN9A, SCN10A) blocker	Anesthetic	1380564
Corticosterone	CID000005753	C02140	Glucocorticoid	---	20298706
Cyclic ADP-ribose	CID000123847	C13050	---	---	---
Cyclothiazide	CID000002910	D01256	---	Diuretic; antihypertensive	---
Cysteinylglycine	CID000065270	C01419	---	---	19056591
Deoxyadenosine	CID000013730	C00559	---	---	---
Deoxyinosine	CID000065058	C05512	---	---	---
Desflurane	CID000042113	D00546	---	Anesthetic	---
Desipramine	CID000002995	D07791	Serotonin transporter inhibitor; noradrenalin transporter inhibitor	Antidepressant (tricyclic)	1536276
Desmethylsertraline	CID000114743	---	---	---	---
Desvenlafaxine	CID000125017	D07793		Antidepressant; neurotransmitter antagonist; serotonin antagonist	---
Dextromethorphan	CID005360696	D03742	N-methyl-D-aspartate receptor antagonist	Antitussive	16134036
Diazepam	CID000003016	D00293	GABAA-receptor (benzodiazepine binding site) agonist	Sedative-hypnotic	16173223
Diazoxide	CID000003019	D00294	ATP-sensitive potassium channel (SUR1/Kir6.2) opener	Antihypertensive	---
Dibenzepin	CID000009418	D07812	---	Antidepressant (tricyclic)	---
Dipyridamole	CID000003108	D00302	Phosphodiesterase (PDE) V inhibitor; phosphodiesterase (PDE) III inhibitor	Vasodilator (coronary)	---
Dopamine	CID000000681	D07870	Dopamine (D1, D2, D3, D4,	Antihypotensive; cardiotonic	21763301

			and D5) receptor agonist		
Doxepin	CID000003158	D07875	---	Antidepressant (tricyclic)	---
Duloxetine	CID000060834	D07880	Serotonin transporter inhibitor; noradrenalin transporter inhibitor (serotonin norepinephrine reuptake inhibitor)	Antidepressant	21731837
D-fucose	CID000000840	C01018	---	---	---
D-galactose	CID000000206	C00124	---	---	---
D-glucose	CID000005793	C00031	---	---	9247387
DL-cysteine	CID000000594	C00736	---	---	15585776
Enflurane	CID000003226	D00543	---	Anesthetic (inhalation)	---
Estradiol	CID000005757	C00951	---	---	21359227
Ethanol	CID000000702	C00469	---	---	9344050
Etoperidone	CID000040589	D04105	---	Antidepressant	---
Felbamate	CID000003331	D00536	---	Antiepileptic	---
Felodipine	CID000003333	D00319	Calcium channel L type blocker	Vasodilator	---
Femoxetine	CID000043103	---	Serotonin reuptake inhibitor	Antidepressant	---
Fenfluramine	CID000003337	D07945	---	Anorexic	8369641
Flunitrazepam	CID000003380	D01230	GABAA-receptor (benzodiazepine binding site) agonist	Sedative-hypnotic	---
Fluoxetine	CID000003386	D00326	Serotonin transporter inhibitor	Antidepressant	22015434
Fluphenazine	CID000003372	D07977	Dopamine D2-receptor antagonist	Neuroleptic; antipsychotic	---
Flurazepam	CID000003393	D00329	GABAA-receptor (benzodiazepine binding site) agonist	Anxiolytic	---
GABA	CID000000119	D00058	GABAA-receptor (benzodiazepine binding site) agonist; GABAB-receptor agonist	---	21763301
Gabapentin	CID000003446	D00332	GABA analogue	Anticonvulsant	15007160
Gallopamil	CID000001234	D08009	Calcium channel L type blocker	Calcium antagonist; coronary vasodilator	---

Glutamate	CID000000611	C00025	---	---	21998651
Glutamine	CID000000738	C00303	---	---	21998651 16863675
Glycine	CID000000750	C00037	---	---	16863675
γ-butyrolactone	CID000007302	C01770	---	---	---
Haloperidol	CID000003559	D00136	Dopamine D2-receptor antagonist	Antidyskinetic (in Gilles de la Tourette's disease); antipsychotic	11476129
Halothane	CID000003562	D00542	---	Anesthetic (inhalation)	---
Hydroxyl radicals	CID000000961	C01328	---	---	16766163
Hydroxynefazodone	CID000127096	---	---	---	---
Hypoxanthine	CID000000790	C00262	---	---	---
Ibutilide	CID000060753	D08060	Voltage-gated potassium channel (KCNH2) blocker	Antiarrhythmic	---
Ifenprodil	CID000003689	D08064	N-methyl-D-aspartate receptor antagonist; α1-adrenergic receptor antagonist	Vasodilator	---
Imatinib	CID000005291	D08066	Bcr-Abl tyrosine kinase inhibitor; PDGFRA tyrosine kinase inhibitor; PDGFRB tyrosine kinase inhibitor; c-KIT tyrosine kinase inhibitor	Antineoplastic	---
Imipramine	CID000003696	D08070	Serotonin transporter inhibitor; noradrenalin transporter inhibitor	Antidepressant (tricyclic)	---
Indalpine	CID000044668	---	---	---	---
Inosine	CID000000804	C00294	---	---	21651783
Inosine monophosphate	CID000008582	C00130	---	---	---
Iohexol	CID000003730	D01817	---	Diagnostic aid	---
Iprindole	CID000021722	D04605	---	Antidepressant	---
Isoflurane	CID000003763	D00545	---	Anesthetic (inhalation)	---
Isoproterenol	CID000003779	D01390	β1, β2, and β3-adrenergic receptor agonist	Bronchodilator	---
Isoreserpine	CID000005052	---	---	---	---
Isradipine	CID000003784	D00349	Calcium channel L type blocker	Antagonist (calcium channel)	---
Kainic acid	CID000010255	C12819	Kainate receptor agonist	---	---

Ketamine	CID000003821	D08098	N-methyl-D-aspartate receptor antagonist	Anesthetic (intravenous)	19234837
Lactate	CID000061503	C00186	---	---	21119085
Lercanidipine	CID000065866	D08111	Calcium channel blocker	Calcium antagonist	---
Leucovorin	CID000000143	D07986	---	Antidote against folic acid antagonists	---
Lithium	CID000028486	D08133	---	Antidepressant	3429701
Lofepramine	CID000003947	D08140	Serotonin transporter inhibitor; noradrenalin transporter inhibitor	Antidepressant (tricyclic)	---
Lorazepam	CID000003958	D00365	GABAA-receptor (benzodiazepine binding site) agonist	Tranquilizer (minor)	20888413
LTC4 (Leukotriene C4)	CID005280493	C02166	---	---	---
L-Methionine	CID000006137	D00019	---	Amino acid	15585776
Magnesium	CID000000888	C00305	---	---	20625937
Magnesium sulfate	CID000024843	D01108	---	Anticonvulsant; laxative; replenisher (electrolyte)	---
Manidipine	CID000004008	D08155	Calcium channel L type blocker	Calcium antagonist	---
Maprotiline	CID000004011	D02566	Noradrenalin transporter inhibitor (norepinephrine reuptake inhibitor)	Antidepresant (tetracyclic)	---
Melatonin	CID000000896	D08170	---	Free oxygen radical scavenger; hypnotic sedative; anticonvulsant	11186124
Melitracen	CID000025381	D08171	---	Antidepressant (tricyclic)	---
Memantine	CID000004054	D08174	N-methyl-D-aspartate receptor antagonist	Anti-Alzheimer's	16848669
Meprobamate	CID000004064	D00376	GABAA-receptor agonist	Sedative-hypnotic	---
Mercaptoethanol	CID000001567	C00928	---	---	---
Mercury	CID000023931	C00703	---	---	21231817
Methamphetamine	CID000001206	D08187	Dopamine transporter inhibitor; noradrenalin transporter inhibitor; serotonin transporter inhibitor	Antihypotensive; sympathomimetic; psychostimulant	---
Methoxyflurane	CID000004116	D00544	---	Anesthetic (inhalation)	---

Methylglyoxal	CID000000880	C00546	---	---	---
Methylphenidate	CID000004158	D04999	Piperidine derivatives	Stimulant (central)	18752063
Mianserin	CID000004184	D08216	(5-HT1 and 5-HT2)-receptor antagonist; (α1 and α2)-adrenergic receptor antagonist	Antidepressant (tetracyclic)	---
Mibefradil	CID000060662	D08217	Calcium channel T type blocker	Calcium antagonist; vasodilator	---
Midazolam	CID000004192	D00550	GABAA-receptor (benzodiazepine binding site) agonist	Anesthetic	19234837
Minaprine	CID000004199	D05039	---	Psychotropic	---
Mirtazapine	CID000004205	D00563	(5-HT2 and 5-HT3)-receptor antagonist; α2-adrenergic receptor antagonist	Antidepressant	19364298
Mtpia-Oxytocin	CID003083145	---	---	---	---
Nebularine	CID000068368	C01736	---	---	---
Nefazodone	CID000004449	D08257	(5-HT2A and 5-HT2C) - receptor antagonist	Antidepressant	---
Niacinamide	CID000000936	D00036	---	Vitamin (enzyme co-factor)	21651783
Nickel	CID000000934	C00291	---	---	16966102
Nifedipine	CID000004485	D00437	Calcium channel L type blocker	Vasodilator (coronary)	---
Nilvadipine	CID000004494	D01908	Calcium channel L type blocker	Antagonist (calcium channnel)	---
Nimodipine	CID000004497	D00438	Calcium channel L type blocker	Vasodilator	---
Nisoldipine	CID000004499	D00618	Calcium channel L type blocker	Vasodilator (coronary)	---
Nitrendipine	CID000004507	D00629	Calcium channel L type blocker	Antihypertensive	---
NMDA	CID000022880	C12269	N-methyl-D-aspartate receptor agonist	---	---
Nomifensine	CID000004528	D05200	Noradrenalin transporter inhibitor	Antidepressant	---
Norclozapine	CID000002820	---	---	---	---
Norfenfluramine	CID000015897	---	---	---	---
Norfluoxetine	CID000004541	---	---	---	---
Nortriptyline	CID000004543	D08288	Serotonin transporter inhibitor; noradrenalin transporter inhibitor	Antidepressant (tricyclic)	5338335
N-acetyl-D-	CID000000899	C00140	---	---	---

glucosamine					
Olanzapine	CID000004585	D00454	(5-HT2A and 5-HT2C)-receptor antagonist; H1-receptor antagonist; α1-adrenergic receptor antagonist; dopamine (D2, D3 and D4)-receptor antagonist	Antipsychotic	17389666
Oxidized glutathione	CID000065359	C00127	---	---	15585776
Oxytocin	CID000439302	D00089	Posterior pituitary hormone	Oxytocic	9513736
Paclitaxel	CID000036314	D00491	β-tubulin depolymerization inhibitor	Antineoplastic	---
Pentobarbital	CID000004737	D00499	GABAA-receptor (picrotoxin binding site) agonist; barbiturates	Sedative-hypnotic	15902433
Pentylenetetrazol	CID000005917	D07409	GABAA-receptor (benzodiazepine binding site) antagonist	Analeptic	---
Phencyclidine	CID000006468	D05453	N-methyl-D-aspartate receptor antagonist	Anesthetic	---
Phenobarbital	CID000004763	D00506	---	---	---
Phentermine	CID000004771	D05458	---	Anorexic	---
Phenylephrine	CID000004782	D08635	---	α-sympathomimetic	---
Pinaverium bromide	CID000040703	D07094	---	---	---
Progabide	CID006314395	D05621	---	Anticonvulsant; relaxant (muscle)	---
Propionic acid	CID000001032	C00163	---	---	20937326
Propofol	CID000004943	D00549	GABAA-receptor agonist	Anesthetic (intravenous)	19157349
Protriptyline	CID000004976	D08447	---	Antidepressant (tricyclic)	---
Psilocin	CID000004980	C08312	---	---	---
Pyroglutamic acid	CID000007405	C01879	---	---	---
Pyrophosphate	CID000001023	C00013	---	---	---
Quisqualic acid	CID000040539	C08296	---	---	---
Rapamycin	CID005284616	D00753	Mammalian target of rapamycin (mTOR) inhibitor	Immunosuppressant	21115397
Reboxetine	CID000065856	D08472	Noradrenalin transporter inhibitor	Antidepressant	---

Reduced glutathione	CID000124886	C00051	---	---	19307255
Renanolone	CID000068930	---	---	---	---
Retinoic acid	CID000444795	C00777	---	---	21528155
Retinol	CID000445354	C00473	---	---	10867750
Riluzole	CID000005070	D00775	Glutamate release inhibitor	Amyotrophic lateral sclerosis treatment	21823915
Secobarbital	CID000031143	D00430	GABAA-receptor (picrotoxin binding site) agonist	Sedative-hypnotic	---
Serotonin	CID000005202	C00780	---	---	2252319
Sevoflurane	CID000005206	D00547	---	Anesthetic (inhalation)	19212262
Sibutramine	CID000005210	D08513	Serotonin norepinephrine reuptake inhibitor (SNRI)	Anorexic; Antidepressant	---
Silymarin	CID000031553	D08515	---	Hepatic protectant	---
Sodium ion	CID000000923	C01330	---	---	19781542
Sulfate ion	CID000001117	C00059	---	---	10435209
S-adenosyl-L-methionine	CID009865603	C00019	---	---	15585776
Tartaric acid	CID000000875	C00898	---	---	18510798
Taurine	CID000001123	C00245	---	---	21925797
Testosterone	CID000005408	D00075	Androgen receptor agonist (component of Metharmon-F (TN))	Androgen	21359227
Thalidomide	CID000005426	D00754	---	Sedative-hypnotic	8157157
Thimerosal	CID000067361	D00864	---	Anti-infective, topical; pharmaceutic aid (preservative)	16818529
Thioridazine	CID000005452	D00373	Dopamine D2-receptor antagonist	Antipsychotic; sedative-hypnotic	---
Thiothixene	CID000941651	D00374	Dopamine D2-receptor antagonist; dopamine D1 receptor antagonist	Antipsychotic	---
Tranylcypromine	CID000005530	D08625	---	Antidepressant; MAO-inhibitor	---
Trazodone	CID000005533	D08626	Serotonin transporter inhibitor; 5-HT2A-receptor antagonist	Tranquilizer; antidepressant	1918430
Trichloroethene	CID000006575	C06790	---	---	16966102
Triiodothyronine	CID000005920	C02465	---	---	6927746

Trimipramine	CID000005584	D00394	Noradrenalin transporter inhibitor; serotonin transporter inhibitor	Antidepressant (tricyclic)	---
Troglitazone	CID000005591	D00395	---	Antidiabetic	---
Valproic acid	CID000003121	D00399	GABA transaminase inhibitor; calcium channel T type blocker; histone acetyltransferase inhibitor	Anticonvulsant	7512516
Vasopressin	CID011979316	D00101	---	Hormone (antidiuretic)	16504417
Venlafaxine	CID000005656	D08670	Serotonin transporter inhibitor; noradrenalin transporter inhibitor	Antidepressant	16307837
Verapamil	CID000002520	D02356	Calcium channel L type blocker; potassium inwardly-rectifying channel (KCNA3, KCNA7, KCNA10, KCNC2, and KCNJ6) blocker	Vasodilator (coronary)	---
Vidarabine	CID000021704	D00406	DNA polymerase inhibitor	Antiviral	---
Vinyl chloride	CID000006338	C06793	---	---	16966102
Zinc	CID000023994	C00038	---	---	20625937
Ziprasidone	CID000060853	D08687	5-HT2-receptor antagonist; dopamine (D2)-receptor antagonist	Neuroleptic	17389666
Zolpidem	CID000005732	D08690	GABAA-receptor (benzodiazepine binding site) agonist	Hypnotic	10619284
Zotepine	CID000005736	D01321	5-HT2A-receptor antagonist; dopamine (D2)-receptor antagonist	Neuroleptic	---

Supplementary table S3. Biological processes, cellular components, and molecular functions (*p*-value <E-07) of genes integrating the network model for gene-environment interactions in the autistic context ("GENVI" network).

GENE ONTOLOGY identifier (GO ID)	**NAME**	**Count**	**P-value**	**Pop hits**	**Bonferroni**	**Benjamini**	**FDR**	**GENES**
	Biological process							
GO:0007610	Behavior	43	1,56E-31	469	3,42E-28	3,42E-28	2,71E-28	DRD1, CCL2, DRD3, ADORA2A, GRIK2, DRD2, CCR1, TH, OXTR, CCL5, GDNF, PTEN, IL10, ADA, BDNF, CHD7, RAC2, GRIN2B, ROBO1, RAC1, DLG4, ROBO2, ROBO3, FZD9, EGR1, PRKCA, IL6, MET, GRIN2A, NLGN3, IL6R, FOXP2, LEP, CCL11, GRIA1, CCR3, NLGN4X, TSC2, AVPR1A, RELN, CACNA1C, MTNR1A, NGF
GO:0007154	Cell communication	47	2,15E-26	795	4,73E-23	2,36E-23	3,74E-23	WNT5A, DRD1, DRD3, GABRB3, ADORA2A, DRD2, GRIK2, WNT3A,

								CCR1, TH, SLC6A4, OXTR, GJA1, CCL5, GDNF, TGFB1, IL10, MBP, GLRX2, WNT2, BDNF, WNT3, GRIN2B, DLG4, CNTNAP2, AGRN, PRKCA, IL6, GRIN2A, FZD1, NLGN3, NRXN1, FZD2, HOMER1, LEP, SSTR2, SSTR3, TSC1, SSTR1, GRIA1, GRM8, SFRP2, AVPR1A, MTNR1B, NEUROD1, CACNA1C, NR5A1
GO:0007267	Cell-cell signaling	41	4,93E-25	600	1,08E-21	3,61E-22	8,57E-22	WNT5A, DRD1, GABRB3, DRD3, ADORA2A, DRD2, GRIK2, WNT3A, CCR1, TH, SLC6A4, GJA1, OXTR, CCL5, GDNF, IL10, MBP, WNT2, BDNF, WNT3, GRIN2B, DLG4, AGRN, PRKCA, IL6, GRIN2A, FZD1, NLGN3, FZD2, NRXN1, HOMER1, LEP, SSTR2, SSTR3, SSTR1, GRIA1, GRM8, MTNR1B, NEUROD1, CACNA1C, NR5A1
GO:0032502	Developmental process	80	1,48E-23	3148	3,24E-20	8,11E-21	2,57E-20	CADM1, WNT3A, SLC6A4, AQP4, GJA1, RORA, GDNF, PTEN, IL10, TGFB1, MBP, WNT2, WNT1, GPX1, BDNF, WNT3, GRIN2B, ROBO1, DLG4, CNTNAP2, ROBO2, ROBO3, DISC1, PITX1, DLG1, EGR1, PRKCA,

								FMR1, GRIN2A, IL6R, NRXN1, RELN, NGFR, MAPK7, NGF, WNT5A, CER1, DRD1, CCL2, DRD3, ADORA2A, UBE3A, DRD2, TH, OXTR, POU1F1, CCL5, ADA, GLRX2, CHD7, RAC3, RAC1, AGRN, FZD9, FZD8, IL6, MET, FZD1, FZD3, FZD2, FZD5, HOMER1, FZD4, FZD7, FOXP2, FZD6, SFRP5, LEP, FZD10, SFRP1, TSC1, SFRP2, SFRP4, TSC2, LRP6, AVPR1A, CACNA1H, NEUROD1, WIF1, NR5A1
GO:0007399	Nervous system development	50	2,12-E23	1088	4,66E-20	9,32E-21	3,69E-20	CADM1, WNT3A, SLC6A4, AQP4, GJA1, RORA, PTEN, GDNF, TGFB1, MBP, WNT2, WNT1, BDNF, ROBO1, DLG4, CNTNAP2, ROBO2, ROBO3, PITX1, DISC1, PRKCA, FMR1, GRIN2A, NRXN1, RELN, NGFR, NGF, DRD1, DRD3, ADORA2A, DRD2, UBE3A, TH, OXTR, POU1F1, CHD7, RAC3, RAC1, AGRN, FZD9, IL6, MET, FZD3, FZD6, FOXP2, LEP, TSC1, TSC2, AVPR1A, NEUROD1
GO:0048856	Anatomical structure	71	1,99E22	2527	4,36E-19	6,23E-20	3,45E-19	CADM1, WNT3A, SLC6A4, AQP4, GJA1, RORA, GDNF, PTEN, IL10, TGFB1, MBP, WNT2, WNT1, GPX1,

	development							BDNF, WNT3, GRIN2B, ROBO1, DLG4, CNTNAP2, ROBO2, ROBO3, DISC1, PITX1, DLG1, EGR1, PRKCA, FMR1, GRIN2A, IL6R, NRXN1, RELN, NGFR, NGF, WNT5A, CER1, DRD1, CCL2, DRD3, ADORA2A, UBE3A, DRD2, TH, OXTR, POU1F1, ADA, CHD7, RAC3, RAC1, AGRN, FZD9, IL6, MET, FZD1, FZD3, FZD2, FZD5, HOMER1, FOXP2, FZD6, SFRP5, LEP, SFRP1, TSC1, SFRP2, TSC2, LRP6, AVPR1A, CACNA1H, NEUROD1, NR5A1
GO:0030154	Cell differentiation	57	2,68E-21	1637	5,88E-18	7,35E-19	4,66E-18	CADM1, WNT3A, GJA1, RORA, PTEN, GDNF, IL10, TGFB1, MBP, WNT2, WNT1, GPX1, BDNF, WNT3, ROBO1, CNTNAP2, ROBO2, ROBO3, PITX1, PRKCA, EGR1, GRIN2A, NRXN1, RELN, NGFR, MAPK7, NGF, WNT5A, DRD1, DRD3, ADORA2A, DRD2, TH, POU1F1, ADA, GLRX2, CHD7, RAC3, RAC1, AGRN, FZD9, IL6, MET, FZD1, FZD2, FZD5, HOMER1, LEP, SFRP5, TSC1, SFRP1, SFRP2, SFRP4, AVPR1A, CACNA1H, NEUROD1, NR5A1

GO:0048869	Cellular developmental process	58	3,04E-21	1706	6,68E-18	7,42E-19	5,29E-18	CADM1, WNT3A, GJA1, RORA, GDNF, PTEN, IL10, TGFB1, MBP, WNT2, WNT1, GPX1, BDNF, WNT3, ROBO1, CNTNAP2, ROBO2, ROBO3, PITX1, DLG1, PRKCA, EGR1, GRIN2A, NRXN1, RELN, NGFR, MAPK7, NGF, WNT5A, DRD1, DRD3, ADORA2A, DRD2, TH, POU1F1, ADA, GLRX2, CHD7, RAC3, RAC1, AGRN, FZD9, IL6, MET, FZD1, FZD2, FZD5, HOMER1, LEP, SFRP5, TSC1, SFRP1, SFRP2, SFRP4, AVPR1A, CACNA1H, NEUROD1, NR5A1
GO:0016055	Wnt receptor signaling pathway	22	6,00E-21	133	1,32E-17	1,20E-18	1,04E-17	FZD9, WNT5A, FZD8, WNT3A, FZD1, FZD3, FZD2, FZD5, FZD4, FZD7, PORCN, FZD6, WNT2, SFRP5, WNT1, FZD10, WNT3, SFRP1, SFRP2, SFRP4, LRP6, WIF1
GO:0044057	Regulation of system process	28	7,85E-20	309	1,72E-16	1,32E-17	1,36E-16	DRD1, CCL2, DRD3, ADORA2A, GRIK2, DRD2, IL6ST, TH, OXTR, GDNF, ADA, IL10, BDNF, GRIN2B, DLG4, EGR1, PRKCA, GRIN2A, NLGN3, LEP, CD38, SSTR2, GRM8, CACNA1G, AVPR1A, CACNA1H,

								CACNA1C, NGF
GO:0007626	Locomotory behavior	25	1,05E-17	274	2,30E-14	1,53E-15	1,82E-14	DRD1, CCL2, DRD3, DRD2, ADORA2A, CCR1, TH, CCL5, GDNF, IL10, CHD7, RAC2, ROBO1, RAC1, ROBO2, ROBO3, PRKCA, IL6, IL6R, CCL11, CCR3, TSC2, RELN, CACNA1C, NGF
GO:0022008	Neurogenesis	33	4,81E-17	601	1,05E-13	6,59E-15	8,35E-14	DRD1, DRD3, ADORA2A, DRD2, WNT3A, TH, GJA1, RORA, PTEN, GDNF, TGFB1, MBP, WNT2, WNT1, BDNF, ROBO1, RAC3, RAC1, CNTNAP2, ROBO2, AGRN, ROBO3, FZD9, PRKCA, IL6, MET, GRIN2A, NRXN1, LEP, NEUROD1, RELN, NGFR, NGF
GO:0048699	Generation of neurons	32	5,22E-17	559	1,15E-13	6,75E-15	9,08E-14	DRD1, DRD3, ADORA2A, DRD2, WNT3A, TH, GJA1, RORA, PTEN, GDNF, TGFB1, MBP, WNT2, WNT1, BDNF, ROBO1, RAC3, RAC1, CNTNAP2, ROBO2, AGRN, ROBO3, FZD9, PRKCA, IL6, MET, NRXN1, LEP, NEUROD1, RELN, NGFR, NGF
GO:0007611	Learning or	18	6,82E-17	111	2,44E-13	1,35E-14	1,89E-13	PRKCA, FZD9, EGR1, DRD1, DRD3, DRD2, TH, GRIN2A, OXTR, NLGN3,

	memory							PTEN, FOXP2, BDNF, GRIN2B, GRIA1, DLG4, CACNA1C, NGF
GO:0007166	Cell surface receptor linked to signal transduction	54	1,62E-16	1856	2,44E-13	1,29E-14	1,89E-13	GRIK2, IL6ST, WNT3A, PTEN, TGFB1, WNT2, WNT1, WNT3, GRIN2B, HOMER3, GRIN2A, SSTR2, SSTR3, GRM8, SSTR1, CCR3, NGF, CER1, WNT5A, DRD1, CCL2, DRD3, ADORA2A, DRD2, CCR1, OXTR, CACNB3, CCL5, AGRN, FZD9, FZD8, GABRA4, MET, FZD1, FZD3, FZD2, HOMER1, FZD5, HOMER2, FZD4, FZD7, PORCN, FZD6, SFRP5, FZD10, SFRP1, SFRP2, TSC2, SFRP4, AVPR1A, LRP6, MTNR1B, WIF1, MTNR1A
GO:0019226	Transmission of nerve impulse	26	2,57E-16	350	4,87E-13	2,43E-14	3,89E-13	DRD1, GABRB3, DRD3, ADORA2A, GRIK2, DRD2, SLC6A4, TH, OXTR, TGFB1, MBP, WNT2, GRIN2B, DLG4, CNTNAP2, AGRN, PRKCA, GRIN2A, NLGN3, NRXN1, HOMER1, TSC1, GRM8, GRIA1, MTNR1B, CACNA1C
	Cellular component							
GO:0005887	Integral to plasma	39	1,06E-14	1188	2,33E-12	1,17E-12	1,34E-11	DRD1, GABRB3, DRD3, ADORA2A, IL6ST, DRD2, GRIK2, CCR1, SLC6A4,

	membrane							GJA1, OXTR, AQP4, GRIN2B, ROBO1, IL10RB, DLG4, IL6, GABRA4, MET, GRIN2A, FZD3, FZD2, IL6R, NRXN1, FZD5, HOMER1, FZD6, SSTR2, FZD10, SSTR3, SSTR1, GRM8, GRIA1, CCR3, NLGN4X, AVPR1A, MTNR1B, NGFR, MTNR1A
GO:0043005	Neuron projection	23	1,40E-14	342	3,09E-12	1,03E-12	1,78E-11	DRD1, CADM1, GABRB3, ADORA2A, DRD2, GRIK2, TH, GRIN2A, ACTN2, ADA, TGFB1, MBP, TSC1, GRIN2B, ROBO1, GRM8, RAC3, TSC2, CACNA1G, CACNA1H, ROBO2, RELN, CACNA1C
GO:0031226	Intrinsic to plasma membrane	39	2,17E-14	1215	4,81E-12	1,20E-12	2,77E-11	DRD1, GABRB3, DRD3, ADORA2A, IL6ST, DRD2, GRIK2, CCR1, SLC6A4, GJA1, OXTR, AQP4, GRIN2B, ROBO1, IL10RB, DLG4, IL6, GABRA4, MET, GRIN2A, FZD3, FZD2, IL6R, NRXN1, FZD5, HOMER1, FZD6, SSTR2, FZD10, SSTR3, SSTR1, GRM8, GRIA1, CCR3, NLGN4X, AVPR1A, MTNR1B, NGFR, MTNR1A
GO:0042995	Cell projection	30	4,66E-14	697	1,03E-11	2,06E-12	5,94E-11	DRD1, CADM1, GABRB3, DRD3, ADORA2A, GRIK2, DRD2, TH, OXTR,

								ADA, TGFB1, MBP, WNT2, GRIN2B, RAC3, ROBO1, RAC1, ROBO2, FZD9, MET, GRIN2A, ACTN2, SSTR3, TSC1, GRM8, TSC2, CACNA1G, CACNA1H, RELN, CACNA1C
GO:0045202	Synapse	21	2,93E-12	355	6,48E-10	1,08E-10	3,73E-09	CADM1, GABRA4, GABRB3, ADORA2A, GRIK2, TH, GRIN2A, CASK, NLGN3, NRXN1, HOMER1, HOMER2, CADPS2, GRIN2B, GRM8, GRIA1, HOMER3, DLG4, AGRN, CACNA1C, DLG1
GO:0005886	Plasma membrane	65	5,93E-12	3777	1,31E-09	1,87E-10	7,56E-09	CADM1, GABRB3, IL6ST, GRIK2, SLC6A4, AQP4, GJA1, CASK, MBP, WNT2, GRIN2B, ROBO1, HOMER3, DLG4, ROBO2, DLG1, PRKCA, GRIN2A, ACTN2, NRXN1, IL6R, CD38, SSTR2, SSTR3, SSTR1, GRM8, CCR3, NGFR, DRD1, DRD3, ADORA2A, DRD2, CCR1, TH, OXTR, CACNB3, ADA, IL10RB, IL10RA, RAC1, FZD9, IL6, IL23R, GABRA4, CACNA1I, MET, FZD3, FZD2, FZD5, HOMER1, HOMER2, FZD7, FZD6, FZD10, CADPS2, GRIA1, NLGN4X, TSC2, MTNR1B, LRP6, AVPR1A, CACNA1G,

								CACNA1H, CACNA1C, MTNR1A
GO:0043025	Cell soma	14	5,17E-10	168	1,14E-07	1,27E-08	6,58E-07	DRD1, CCL2, GRIK2, ADORA2A, TH, TGFB1, ADA, MBP, RAC3, GRM8, TSC2, CACNA1G, CACNA1H, CACNA1C
GO:0014069	Postsynaptic density	10	3,40E-09	71	7,52E-07	7,52E-08	4,33E-06	GRIN2B, ADORA2A, HOMER3, GRIA1, DLG4, GRIN2A, CACNA1C, HOMER1, HOMER2, DLG1
GO:0030425	Dendrite	13	4,38E-09	163	9,67E-07	8,79E-08	5,57E-06	DRD1, CADM1, DRD2, GRIK2, ADORA2A, ACTN2, ADA, GRIN2B, TSC2, CACNA1G, CACNA1H, RELN, CACNA1C
GO:0045211	Postsynaptic membrane	12	6,87E-09	135	1,52E-06	1,26E-07	8,75E-06	GABRB3, GRIN2B, GABRA4, ADORA2A, GRIK2, HOMER3, GRIA1, DLG4, GRIN2A, HOMER1, HOMER2, DLG1
GO:0030424	Axon	11	3,95E-07	159	8,74E-05	5,14E-06	5,04E-04	DRD1, CADM1, GABRB3, ADORA2A, GRIK2, ROBO1, DRD2, TH, ROBO2, TGFB1, MBP
	Molecular function							

GO:0004871	Signal transducer activity	57	2,03E-15	2270	7,37E-13	7,37E-13	2,75E-12	GABRB3, IL6ST, GRIK2, WNT3A, GJA1, RORA, WNT2, WNT1, WNT3, GRIN2B, ROBO1, ROBO2, ROBO3, GRIN2A, ACTN2, NRXN1, IL6R, CD38, SSTR2, SSTR3, GRM8, SSTR1, CCR3, MAPK7, NGFR, NGF, WNT5A, DRD1, CCL2, DRD3, ADORA2A, DRD2, CCR1, OXTR, CCL5, IL10RB, IL10RA, AGRN, FZD9, FZD8, IL23R, GABRA4, MET, FZD1, FZD3, FZD2, FZD5, FZD4, FZD7, FZD6, FZD10, GRIA1, AVPR1A, LRP6, MTNR1B, MTNR1A, NR5A1
GO:0060089	Molecular transducer activity	57	2,03E-15	2270	7,37E-13	7,37E-13	2,75E-12	GABRB3, IL6ST, GRIK2, WNT3A, GJA1, RORA, WNT2, WNT1, WNT3, GRIN2B, ROBO1, ROBO2, ROBO3, GRIN2A, ACTN2, NRXN1, IL6R, CD38, SSTR2, SSTR3, GRM8, SSTR1, CCR3, MAPK7, NGFR, NGF, WNT5A, DRD1, CCL2, DRD3, ADORA2A, DRD2, CCR1, OXTR, CCL5, IL10RB, IL10RA, AGRN, FZD9, FZD8, IL23R, GABRA4, MET, FZD1, FZD3, FZD2, FZD5, FZD4, FZD7, FZD6, FZD10, GRIA1, AVPR1A, LRP6, MTNR1B, MTNR1A, NR5A1

GO:0042813	Wnt receptor activity	8	8,28E-14	9	3,06E-11	1,53E-11	1,14E-10	FZD9, FZD8, FZD10, FZD3, FZD2, FZD4, FZD7, FZD6
GO:0004888	Transmembrane receptor activity	39	2,88E-12	1305	1,06E-09	3,54E-10	3,97E-09	DRD1, GABRB3, DRD3, ADORA2A, IL6ST, GRIK2, DRD2, CCR1, OXTR, GRIN2B, ROBO1, IL10RB, IL10RA, ROBO2, FZD9, FZD8, GABRA4, MET, GRIN2A, FZD1, FZD3, FZD2, IL6R, FZD5, FZD4, FZD7, FZD6, SSTR2, FZD10, SSTR3, SSTR1, GRM8, GRIA1, CCR3, AVPR1A, LRP6, MTNR1B, NGFR, MTNR1A
GO:0004872	Receptor activity	45	2,41E-11	1838	8,88E-09	2,22E-09	3,32E-08	DRD1, DRD3, GABRB3, ADORA2A, IL6ST, DRD2, GRIK2, CCR1, OXTR, RORA, GRIN2B, ROBO1, IL10RB, IL10RA, ROBO2, ROBO3, FZD9, FZD8, IL23R, GABRA4, MET, GRIN2A, FZD1, FZD3, FZD2, IL6R, NRXN1, FZD5, FZD4, FZD7, FZD6, CD38, SSTR2, FZD10, SSTR3, SSTR1, GRIA1, GRM8, CCR3, AVPR1A, MTNR1B, LRP6, NGFR, MTNR1A, NR5A1
GO:0005515	Protein binding	97	1,64E-07	8154	6,06E-05	1,21E-05	2,26E-04	DRD3, ADORA2A, DRD2, CACNB3, CCL5, ADA, IL23A, RAC2, RAC3, RAC1, AGRN, RHOXF2, CACNA1I,

								MET, HOMER1, HOMER2, PORCN, FOXP2, SFRP5, SFRP1, TSC1, NLGN4Y, SFRP2, SFRP4, NLGN4X, TSC2, AVPR1A, CACNA1C, GFAP, GJA1, PTEN, GPX1, DLG4, CNTNAP2, DLG1, PRKCA, NRXN1, IL6R, NGFR, CER1, UBE3A, CCR1, TH, OXTR, POU1F1, IL10RB, IL10RA, FZD8, IL6, GGTLC3, FZD1, NLGN3, FZD3, FZD2, FZD5, FZD4, LEP, CCL11, FZD10, GRIA1, LRP6, NEUROD1, NR5A1
GO:0005262	Calcium channel activity	9	2,92E-07	79	1,08E-04	1,80E-05	4,03E-04	GRIN2B, CACNA1I, CACNA1G, GRIN2A, CACNA1H, CACNB3, CACNA2D3, CACNA1C, CACNA2D2

Supplementary table S4. Genes/proteins from "GENVI" network model with their values for betweenness, closeness, clustering coefficient, connectivity, neighborhood connectivity, and stress centralities.

Node	Betweenness centrality	Closeness centrality	Clustering coefficient	Connectivity	Neighborhood connectivity	Stress
ACTN2	0,0186	0,37011	0,32967	14	24,42857	28376
ADA	0,01568	0,35096	0,35	16	23,875	16250
ADORA2A	0,00276	0,35576	0,42857	8	30,375	3194
AQP4	0	0,30087	0	1	68	0
ATP10A	0	0,32198	1	2	60,5	0
AVPR1A	0,0221	0,36237	0,30303	12	19,41667	13480
BDNF	9,52E-04	0,29462	0	4	6,75	686
CACNA1C	0,05175	0,34437	0,12987	22	9,40909	41242
CACNA1G	0,00556	0,33766	0,50909	11	15,09091	11626
CACNA1H	0,0059	0,33086	0,40909	12	13,83333	12772
CACNA1I	0,00556	0,33766	0,50909	11	15,09091	11626
CACNA2D2	0,00553	0,32534	0	8	14,125	12504
CACNA2D3	0,00569	0,32466	0	7	15,42857	11908
CACNB3	0,00375	0,33584	0,64286	8	18,375	5430
CADM1	0	0,24684	0	1	7	0
CADPS2	7,96E-04	0,28729	0	2	30	320
CASK	0,02841	0,32739	0,14286	7	14,28571	15426
CCL2	0,00794	0,34136	0,28788	12	20,08333	7990
CCL5	0,00412	0,38142	0,75163	18	26,77778	4684
CCL11	4,87E-04	0,32399	0,73333	6	24,16667	692
CCR1	0,00141	0,36749	0,82353	17	25,05882	1538
CCR3	0,00141	0,36749	0,82353	17	25,05882	1538
CD38	0,00641	0,34361	0,6	5	43,4	4188
CER1	0	0,22382	1	5	22,6	0
CNTNAP2	0	0,24684	0	1	7	0

DISC1	0	0,27036	0	1	14	0
DLG1	0,04396	0,3373	0,27273	12	17,58333	76416
DLG4	0,04659	0,3267	0,20879	14	13,42857	90492
DRD1	0,02701	0,34821	0,04187	29	9,37931	28184
DRD2	0,09565	0,39544	0,10195	56	10,57143	82438
DRD3	0,03527	0,38471	0,18758	39	13,69231	38178
EGR1	0,0145	0,2488	0	2	8	6964
FMR1	0	0,29602	0	1	52	0
FOXP2	0	0,29602	0	1	52	0
FZD1	0,00347	0,25386	0,63636	12	17,5	11028
FZD2	0,01343	0,27882	0,56061	12	17,83333	27282
FZD3	1,37E-05	0,22462	0,77778	10	18,9	20
FZD4	0,00985	0,26804	0,69524	15	17,13333	28296
FZD5	1,94E-05	0,22527	0,85714	14	17,35714	26
FZD6	1,37E-05	0,22478	0,81818	11	18,45455	20
FZD7	0,00985	0,26804	0,69524	15	17,13333	28296
FZD8	1,37E-05	0,22511	0,87179	13	17,92308	20
FZD9	1,37E-05	0,22462	0,77778	10	18,9	20
FZD10	1,37E-05	0,22462	0,77778	10	18,9	20
GABRA4	0,03572	0,30618	0,00735	17	3,82353	26430
GABRB3	0,04497	0,32198	0,00952	15	4,86667	30358
GDNF	0,00804	0,32568	0,06667	6	19,5	4860
GFAP	5,11E-04	0,30291	0	2	12,5	358
GGT1	0,00954	0,3174	0,16667	9	12,77778	10984
GGT2	0,00291	0,29573	0	4	10,75	4982
GJA1	0	0,28969	1	2	10	0
GLO1	4,55E-04	0,31772	0,63889	9	28,88889	282
GLRX2	0	0,27586	0	1	26	0
GPX1	0,00244	0,30439	0,5	5	23,2	2030
GRIA1	0,03726	0,36279	0,14035	19	10,47368	24896
GRIK2	0,01707	0,3373	0,15152	12	11,16667	17630
GRIN2A	0,03744	0,39196	0,17316	22	15,36364	57158

GRIN2B	0,03422	0,39098	0,19048	21	15,71429	53670
GRM8	0,0016	0,3142	0,16667	4	14,5	866
HOMER1	3,20E-04	0,27441	0	2	10,5	174
HOMER3	0,00147	0,29942	0	2	29,5	790
IL6	0,00365	0,30739	0,17857	8	12,75	3342
IL6ST	6,50E-05	0,30558	0,73333	6	25,5	72
IL6R	6,62E-05	0,30648	0,73333	6	26,83333	82
IL10	0,01525	0,31325	0,16667	9	18,11111	13562
IL10RA	7,11E-05	0,28545	0,33333	3	15	48
IL10RB	1,88E-04	0,29914	0,66667	3	23	190
IL23A	7,12E-04	0,28916	0,4	5	16,8	1238
IL23R	2,24E-05	0,25849	0,66667	3	8,33333	20
LEP	8,57E-04	0,30203	0	3	22	454
LRP6	1,08E-04	0,2256	0,60833	16	15,125	94
MAPK7	0,00146	0,33621	0,5	4	34,75	712
MBP	0	0,28519	0	1	16	0
MECP2	0	0,15423	0	1	2	0
MET	0,00112	0,33987	0,6	5	33,6	1736
MSRA	1,88E-05	0,29799	0	2	36	36
MTNR1A	0,01639	0,37772	0,875	16	26,25	11636
MTNR1B	0	0,36491	1	15	27,33333	0
NEUROD1	0	0,18203	0	1	7	0
NGF	0,00298	0,36491	0,72222	9	42,88889	6618
NGFR	0,00259	0,33086	0,42857	7	23,85714	1528
NLGN3	8,57E-04	0,24723	0	2	8,5	500
NLGN4X	0,00473	0,35862	0,5	4	42,5	13894
NLGN4Y	0	0,1986	0	1	3	0
NR5A1	0,03005	0,26022	0	3	5	21570
NRXN1	0,00667	0,24762	0	3	3,33333	2972
OXTR	0,00798	0,34783	0,5	9	24	7230
PITX1	0,03832	0,22238	0,04762	7	1,85714	24156
PORCN	0	0,22382	1	5	22,6	0

POU1F1	0	0,18203	0	1	7	0
PRKCA	0,06034	0,39847	0,125	16	19,125	44290
PSAP2	0	0,24586	0	1	6	0
PTEN	0,00184	0,34399	0,6	6	39,5	2880
RAC1	0,05928	0,35334	0,21818	11	18,18182	61578
RAC2	0	0,30891	1	2	31,5	0
RAC3	0,00218	0,35698	0,61905	7	42	1562
RBPMS	0	0,18214	1	2	4,5	0
RELN	5,27E-04	0,35294	0,71429	7	41,42857	1208
RHOXF2	0	0,18214	1	2	4,5	0
ROBO1	0,00739	0,33121	0,4	5	29,6	5078
ROBO2	0	0,249	0	1	5	0
RORA	7,53E-04	0,2378	0	2	3	438
SFRP1	7,68E-05	0,22543	0,6381	15	15,66667	76
SFRP2	7,68E-05	0,22543	0,6381	15	15,66667	76
SFRP4	7,68E-05	0,22543	0,6381	15	15,66667	76
SFRP5	7,68E-05	0,22543	0,6381	15	15,66667	76
SLC6A4	0,13882	0,37232	0,02694	57	5,42105	109430
SMARCA2	0,00641	0,18224	0	2	4	4020
SSTR1	0	0,36491	1	15	27,33333	0
SSTR2	0	0,36491	1	15	27,33333	0
SSTR3	0	0,36491	1	15	27,33333	0
TGFB1	0,00169	0,34361	0,4	5	31	3282
TH	0,00324	0,33016	0,07143	8	14,875	2334
TSC1	0,01283	0,3142	0,3	5	16,6	5946
TSC2	0,00641	0,23963	0	2	3	2960
UBE3A	0	0,19343	0	1	2	0
WIF1	0	0,22414	1	7	20,14286	0
WNT1	2,71E-04	0,22658	0,59307	22	13,90909	188
WNT2	2,71E-04	0,22658	0,59307	22	13,90909	188
WNT3	2,71E-04	0,22658	0,59307	22	13,90909	188
WNT3A	0,02978	0,27013	0,52174	24	13,75	48308

WNT5A	0,03802	0,27392	0,54545	23	13,73913	37842

Supplementary table S5. Environmental factors (drugs, pollutants, chemicals, and other compounds) from "GENVI" network model with their values for betweenness, closeness, clustering coefficient, connectivity, neighborhood connectivity, and stress centralities.

Node	Betweenness centrality	Closeness centrality	Clustering coefficient	Connectivity	Neighborhood connectivity	Stress
2-amino-4-phosphonobutyric acid	1,04E-04	0,27036	0,5	4	5	44
Adenine	1,82E-04	0,3791	0,78363	19	36,63158	362
Adenosine triphosphate (ATP)	0,06721	0,43515	0,25399	53	21,28302	51158
Alaproclate	0	0,27154	0	1	57	0
Alprazolam	1,73E-05	0,24606	0	2	16	28
Amantadine	0,00222	0,31643	0,2	5	33,4	5548
Amitriptyline	4,53E-04	0,30558	0,63889	9	24,88889	1540
Amlodipine	3,14E-05	0,257	0	2	14,5	26
Ammonia	0,03068	0,41823	0,40418	42	26,28571	43768
Amoxapine	2,39E-04	0,30409	0,3	5	37,6	978
AMPA	3,07E-04	0,32332	0,33333	3	44,66667	388
Amphetamine	0,03907	0,39049	0,27206	17	27,82353	29624
Aniracetam	0,00438	0,35254	0,4	5	35,6	4038
Aripiprazole	1,96E-04	0,3038	0,16667	4	45,25	932
Ascorbic acid	1,17E-04	0,34437	0,71111	10	32,8	192
Atomoxetine	6,48E-05	0,29686	0	2	56,5	312
Atosiban	0,00194	0,27392	0,33333	4	7,5	2156
Butriptyline	0	0,27154	0	1	57	0
Cadmium	0,02223	0,39745	0,44086	31	27,77419	22020
Calcium	0,19867	0,47059	0,19439	76	20,53947	194318
Chlordiazepoxide	1,73E-05	0,24606	0	2	16	28
Chlorpromazine	1,96E-04	0,30409	0,5	5	37,4	932
Cholecalciferol	0	0,33227	1	2	72	0
Cholesterol sulfate	0,00629	0,30528	0,33333	3	30,66667	4456

Citalopram	0	0,27154	0	1	57	0
Clomipramine	0,00212	0,33657	0,41667	9	28,77778	4062
Clonazepam	1,73E-05	0,24606	0	2	16	28
Clozapine	3,76E-04	0,30769	0,1	5	37,8	1202
Cocaine	0,02094	0,32432	0,47222	9	22,22222	11512
Corticosterone	0	0,30087	0	1	68	0
Cyclic ADP-ribose	0	0,25595	0	1	5	0
Cyclothiazide	3,36E-04	0,31772	0,5	4	28,5	364
Cysteinylglycine	4,54E-04	0,33405	0,67857	8	33,125	740
Deoxyadenosine	4,36E-04	0,37545	0,86667	15	40	1054
Deoxyinosine	3,26E-04	0,36491	0,88462	13	39,92308	786
Desflurane	2,92E-04	0,28261	0,90476	7	9,28571	528
Desipramine	0,00199	0,33693	0,47222	9	25,88889	3780
Desmethylsertraline	0	0,27154	0	1	57	0
Desvenlafaxine	0	0,27154	0	1	57	0
Dextromethorphan	0,00484	0,31643	0,33333	3	33,33333	10448
Diazepam	1,73E-05	0,24606	0	2	16	28
Diazoxide	0,00107	0,28703	0	3	12	902
Dibenzepin	0	0,27154	0	1	57	0
Dipyridamole	0	0,26	0	1	16	0
Dopamine	0,01011	0,40051	0,45074	29	29,17241	13356
Doxepin	6,48E-05	0,29686	0	2	56,5	312
Duloxetine	4,44E-05	0,30499	0,93333	6	17,83333	36
D-fucose	0,00474	0,38329	0,70952	21	35,2381	5800
D-galactose	0,00657	0,38758	0,54497	28	30,57143	9058
D-glucose	1,34E-04	0,32534	0,33333	3	44,33333	246
DL-cysteine	0,00261	0,38614	0,60345	29	31,68966	3878
Enflurane	2,92E-04	0,28261	0,90476	7	9,28571	528
Estradiol	0,03579	0,34323	0,2	6	28,66667	25442
Ethanol	3,07E-05	0,36836	0,93333	10	46,5	58
Etoperidone	6,48E-05	0,29686	0	2	56,5	312
Felbamate	0	0,2821	1	2	21,5	0

Felodipine	0,00195	0,30922	0,16667	4	24,75	1706
Femoxetine	6,48E-05	0,29686	0	2	56,5	312
Fenfluramine	0	0,27154	0	1	57	0
Flunitrazepam	1,73E-05	0,24606	0	2	16	28
Fluoxetine	0	0,32332	1	2	52	0
Fluphenazine	1,45E-05	0,2905	0,33333	3	41,33333	76
Flurazepam	1,73E-05	0,24606	0	2	16	28
GABA	0,06356	0,41325	0,49538	26	31,76923	51360
Gabapentin	0,00228	0,31611	0,13333	6	17,5	2644
Gallopamil	0	0,25637	0	1	22	0
Glutamate	0,03087	0,40838	0,37311	33	26,78788	32600
Glutamine	1,51E-04	0,38049	0,84314	18	39,27778	320
Glycine	0,00714	0,4031	0,51136	33	30,15152	10736
γ-butyrolactone	1,72E-04	0,33227	0,7	5	40	130
Haloperidol	0,00801	0,33476	0,09524	7	33,14286	17878
Halothane	0,00912	0,32704	0,47222	9	18,33333	8792
Hydroxyl radicals	0,08867	0,42975	0,20764	68	18,69118	65544
Hydroxynefazodone	0	0,27154	0	1	57	0
Hypoxanthine	4,51E-04	0,37636	0,84559	17	37,82353	1074
Ibutilide	0	0,25637	0	1	22	0
Ifenprodil	0	0,2821	1	2	21,5	0
Imatinib	0	0,2949	0	1	58	0
Imipramine	0,00137	0,33476	0,52381	7	32,28571	2976
Indalpine	0	0,27154	0	1	57	0
Inosine	3,21E-04	0,37321	0,88462	13	42,38462	794
Inosine monophosphate	2,26E-05	0,36406	0,87619	15	39,26667	26
Iohexol	1,00E-05	0,24149	0	2	6,5	8
Iprindole	0	0,27154	0	1	57	0
Isoflurane	0,00579	0,3174	0,58333	9	16,22222	6280
Isoproterenol	8,45E-04	0,28058	0	3	12,33333	238
Isoreserpine	3,76E-04	0,30769	0,1	5	37,8	1202
Isradipine	1,06E-04	0,25806	0,4	6	11,83333	106

Kainic acid	0,00568	0,33227	0,28571	7	19	3926
Ketamine	0,00101	0,312	0,33333	3	33	2376
Lactate	2,47E-04	0,35135	0,75	8	39,25	730
Lercanidipine	0	0,25637	0	1	22	0
Leucovorin	4,16E-06	0,34361	0,93333	6	50,33333	14
Lithium	0,01297	0,38471	0,50549	14	39,35714	9408
Lofepramine	6,48E-05	0,29686	0	2	56,5	312
Lorazepam	1,73E-05	0,24606	0	2	16	28
LTC4 (Leukotriene C4)	0,0102	0,38903	0,5415	23	27,26087	9174
L-Methionine	0,0016	0,3662	0,82857	15	39,33333	1216
Magnesium	0,0527	0,41992	0,29412	52	22,42308	46950
Magnesium sulfate	0	0,23318	1	2	5,5	0
Manidipine	4,77E-05	0,2549	0,3	5	9,8	56
Maprotiline	4,83E-04	0,30558	0,61111	9	24,88889	1578
Melatonin	0,00233	0,38614	0,73684	19	31,31579	4396
Melitracen	0	0,27154	0	1	57	0
Memantine	0	0,2821	1	2	21,5	0
Meprobamate	0	0,23459	0	1	17	0
Mercaptoethanol	0,00177	0,38424	0,72727	23	35,52174	3100
Mercury	0,00981	0,39295	0,64	26	33,11538	6274
Methamphetamine	0,00223	0,32165	0,47222	9	22,22222	1650
Methoxyflurane	2,92E-04	0,28261	0,90476	7	9,28571	528
Methylglyoxal	6,86E-05	0,32298	0,80556	9	33,55556	134
Methylphenidate	2,89E-04	0,32066	0,78571	8	25	792
Mianserin	1,96E-04	0,3038	0,16667	4	45,25	932
Mibefradil	1,33E-04	0,25828	0,47619	7	11,28571	118
Midazolam	1,73E-05	0,24606	0	2	16	28
Minaprine	1,51E-04	0,3035	0	3	47,33333	704
Mirtazapine	1,45E-05	0,2905	0,33333	3	41,33333	76
Mtpia-Oxytocin	0	0,26644	1	2	10,5	0
Nebularine	0	0,3142	1	3	47,33333	0
Nefazodone	6,48E-05	0,29686	0	2	56,5	312

Niacinamide	4,94E-04	0,37681	0,82857	15	40,6	654
Nickel	0,01309	0,39594	0,53427	32	30,28125	13926
Nifedipine	0,00621	0,29799	0,33333	7	13,28571	5284
Nilvadipine	1,06E-04	0,25806	0,4	6	11,83333	106
Nimodipine	0	0,25658	1	2	15	0
Nisoldipine	0	0,25637	0	1	22	0
Nitrendipine	3,13E-05	0,25721	0,33333	3	14	30
NMDA	0	0,26667	1	3	5,66667	0
Nomifensine	1,84E-04	0,32033	0,85714	7	23,14286	474
Norclozapine	1,96E-04	0,3038	0,16667	4	45,25	932
Norfenfluramine	0	0,27154	0	1	57	0
Norfluoxetine	0	0,27154	0	1	57	0
Nortriptyline	1,79E-04	0,29856	0,66667	7	22	560
N-acetyl-D-glucosamine	0,02038	0,39344	0,39015	33	25,12121	17702
Olanzapine	1,96E-04	0,3038	0,16667	4	45,25	932
Oxidized glutathione	5,24E-04	0,34024	0,75556	10	33,8	612
Oxytocin	0	0,26644	1	2	10,5	0
Paclitaxel	0	0,32099	1	3	59,33333	0
Pentobarbital	0,00108	0,26531	0	3	14,66667	1190
Pentylenetetrazol	0	0,2821	1	2	21,5	0
Phencyclidine	0	0,2821	1	2	21,5	0
Phenobarbital	0,00467	0,28756	0,1	5	12,2	4120
Phentermine	0,00538	0,35096	0,4	5	39,6	5384
Phenylephrine	0	0,28519	0	1	16	0
Pinaverium bromide	0	0,25637	0	1	22	0
Progabide	1,73E-05	0,24625	0,66667	3	11,66667	28
Propionic acid	0,00204	0,37772	0,64762	15	39,06667	2248
Propofol	0,00544	0,31294	0,16667	4	30	5644
Protriptyline	3,62E-05	0,2766	0,67857	8	14,625	24
Psilocin	0	0,27154	0	1	57	0
Pyroglutamic acid	2,77E-04	0,36879	0,79121	14	39,85714	456
Pyrophosphate	0,00258	0,39147	0,5254	36	28,47222	4100

Quisqualic acid	0,00746	0,34628	0,33333	6	26,66667	4278
Rapamycin	0	0,23871	0	1	9	0
Reboxetine	0	0,27154	0	1	57	0
Reduced glutathione	0,01253	0,38049	0,46154	26	28,69231	11924
Renanolone	1,73E-05	0,24606	0	2	16	28
Retinoic acid	0,00184	0,33051	0,26667	6	32,16667	1504
Retinol	0	0,32704	1	3	44,33333	0
Riluzole	9,35E-04	0,29213	0,16667	4	13,25	548
Secobarbital	6,68E-04	0,26131	0	2	14,5	796
Serotonin	0,03007	0,40678	0,43842	29	30,13793	27834
Sevoflurane	2,92E-04	0,28261	0,90476	7	9,28571	528
Sibutramine	0	0,28571	1	2	31	0
Silymarin	3,64E-05	0,25121	0,33333	3	6	16
Sodium ion	0,08308	0,43759	0,3173	47	24,61702	62402
Sulfate ion	0,10103	0,42219	0,22368	57	19,63158	70684
S-adenosyl-L-methionine	5,96E-04	0,36967	0,63333	21	32,52381	950
Tartaric acid	0,00193	0,37818	0,74167	16	37,875	2164
Taurine	0,0014	0,36069	0,66667	10	41	1958
Testosterone	0	0,312	1	3	49,33333	0
Thalidomide	0	0,23871	0	1	9	0
Thimerosal	0	0,28235	0	1	26	0
Thioridazine	2,00E-04	0,30439	0,6	6	32,5	934
Thiothixene	1,45E-05	0,2905	0,33333	3	41,33333	76
Tranylcypromine	0	0,32033	0	1	76	0
Trazodone	1,51E-04	0,3035	0	3	47,33333	704
Trichloroethene	1,76E-04	0,25263	0,71429	7	6,85714	80
Triiodothyronine	0	0,30087	0	1	68	0
Trimipramine	3,88E-04	0,30588	0,64444	10	20	1152
Troglitazone	6,24E-04	0,30439	0,2	5	17,4	302
Valproic acid	0	0,28312	1	2	10	0
Vasopressin	0	0,26621	0	1	12	0
Venlafaxine	0	0,27154	0	1	57	0

Verapamil	0,01453	0,31138	0,4	6	27,5	6836
Vidarabine	0	0,26	0	1	16	0
Vinyl chloride	0,00116	0,30291	0	2	37,5	1954
Zinc	0,04672	0,41767	0,25711	58	20,46552	35654
Ziprasidone	1,96E-04	0,3038	0,16667	4	45,25	932
Zolpidem	1,73E-05	0,24625	0,66667	3	11,66667	28
Zotepine	1,96E-04	0,3038	0,16667	4	45,25	932

Supplementary table S6. Selected nodes from "GENVI" network model based on their respective values (above the thresholds) for betweenness, closeness, clustering coefficient, connectivity, neighborhood connectivity, and stress centralities.

Node	**Betweenness centrality**	**Closeness centrality**	**Clustering coefficient**	**Connectivity**	**Neighborhood connectivity**	**Stress**
Adenosine triphosphate (ATP)	0,06721	0,43515	0,25399	53	21,28302	51158
Ammonia	0,03068	0,41823	0,40418	42	26,28571	43768
AQP4	0	0,30087	0	1	68	0
ATP10A	0	0,32198	1	2	60,5	0
CACNA1C	0,05175	0,34437	0,12987	22	9,40909	41242
Calcium	0,19867	0,47059	0,19439	76	20,53947	194318
Cholecalciferol	0	0,33227	1	2	72	0
Corticosterone	0	0,30087	0	1	68	0
DLG1	0,04396	0,3373	0,27273	12	17,58333	76416
DLG4	0,04659	0,3267	0,20879	14	13,42857	90492
DRD2	0,09565	0,39544	0,10195	56	10,57143	82438

GABA	0,06356	0,41325	0,49538	26	31,76923	51360
GRIN2A	0,03744	0,39196	0,17316	22	15,36364	57158
GRIN2B	0,03422	0,39098	0,19048	21	15,71429	53670
Hydroxyl radicals	0,08867	0,42975	0,20764	68	18,69118	65544
Magnesium	0,0527	0,41992	0,29412	52	22,42308	46950
PRKCA	0,06034	0,39847	0,125	16	19,125	44290
RAC1	0,05928	0,35334	0,21818	11	18,18182	61578
SLC6A4	0,13882	0,37232	0,02694	57	5,42105	109430
Sodium ion	0,08308	0,43759	0,3173	47	24,61702	62402
Sulfate	0,10103	0,42219	0,22368	57	19,63158	70684
Tranylcypromine	0	0,32033	0	1	76	0
Triiodothyronine	0	0,30087	0	1	68	0
WNT3A	0,02978	0,27013	0,52174	24	13,75	48308
Zinc	0,04672	0,41767	0,25711	58	20,46552	35654

Supplementary table S7. Ensembl protein identifiers of the genes belonging to the Ca^{2+}-RHO family of GTPases interactome network.

Gene symbol	**Alias and/or description**	**Ensembl ID** (ENSP)	**Interaction with calcium**
ARF6	ADP-ribosylation factor 6	ENSP00000298316	NO
CDC42	Cell division cycle 42 (GTP binding protein, 25kDa)	ENSP00000314458	NO
CHP	Calcium-binding protein p22	ENSP00000335632	YES
DGKQ	Diacylglycerol kinase θ (110kDa)	ENSP00000273814	NO
DIAPH1	Diaphanous homolog 1 (Drosophila)	ENSP00000381565	NO
GIT1	G protein-coupled receptor kinase interacting ArfGAP 1	ENSP00000378338	NO

ITPR1	Inositol 1,4,5-triphosphate receptor type 1	ENSP00000405934	YES
KTN1	Kinectin 1 (kinesin receptor)	ENSP00000348562	NO
MRAS	Muscle RAS oncogene homolog	ENSP00000289104	NO
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	ENSP00000358548	NO
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1	ENSP00000278568	NO
PAK2	p21 protein (Cdc42/Rac)-activated kinase 2	ENSP00000314067	NO
PKN1	Protein kinase N1	ENSP00000343325	NO
PKN2	Protein kinase N2	ENSP00000359552	NO
PLCG1	Phospholipase C γ1	ENSP00000244007	YES
PLD1	Phospholipase D1	ENSP00000342793	NO

PPP1R12A	Protein phosphatase 1, regulatory (inhibitor) subunit 12A	ENSP00000261207	NO
PRKCA	PKC-α; protein kinase C, α	ENSP00000284384	YES
PXN	Paxillin	ENSP00000228307	NO
RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	ENSP00000348461	NO
RAC2	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	ENSP00000249071	NO
RAC3	Ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	ENSP00000304283	YES

RALA	v-ral simian leukemia viral oncogene homolog A (ras related)	ENSP00000005257	NO
RALB	v-ral simian leukemia viral oncogene homolog B (ras related)	ENSP00000272519	NO
RAP1A	RAP1A member of RAS oncogene family	ENSP00000348786	NO
RAP2A	RAP2A, member of RAS oncogene family	ENSP00000245304	NO
RHOA	Ras homolog gene family member A	ENSP00000400175	NO
RHOB	Ras homolog gene family member B	ENSP00000272233	NO
RHOBTB2	Rho-related BTB domain containing 2	ENSP00000251822	NO
RHOC	Ras homolog gene	ENSP00000285735	NO

	family member C		
RHOD	Ras homolog gene family member D	ENSP00000308576	NO
RHOG	Ras homolog gene family member G (rho G)	ENSP00000339467	NO
RHOH	Ras homolog gene family member H	ENSP00000371219	NO
RHOJ	Ras homolog gene family member J	ENSP00000316729	NO
RHOQ	Ras homolog gene family member Q; Plasma membrane-associated small GTPase	ENSP00000238738	NO
RHOU	Ras homolog gene family member U	ENSP00000355652	NO
RHPN1	Rhophilin, Rho GTPase binding protein 1	ENSP00000289013	NO

RHPN2	Rhophilin, Rho GTPase binding protein 2	ENSP00000254260	NO
RND3	Rho family GTPase 3	ENSP00000263895	NO
ROCK1	Rho-associated coiled-coil containing protein kinase 1	ENSP00000382697	NO
ROCK2	Rho-associated coiled-coil containing protein kinase 2	ENSP00000317985	NO
RRAD	Ras-related associated with diabetes	ENSP00000299759	NO
RRAS	Related RAS viral (r-ras) oncogene homolog	ENSP00000246792	NO

Artigo 2

ALTERED EXPRESSION OF ALZHEIMER´S DISEASE-RELATED GENES IN THE CEREBELLUM OF AUTISTIC PATIENTS: A MODEL FOR DISRUPTED BRAIN CONNECTOME AND THERAPY

Fares Zeidán-Chuliá, Ben-Hur Neves de Oliveira, Alla B. Salmina, Manuel F. Casanova, Daniel Pens Gelain, Mami Noda, Alexei Verkhratsky & José Cláudio Fonseca Moreira

Cell Death & Disease (npg; nature publishing group)

Manuscript Accepted (CDDIS-14-0230R); ***In press***

Article type: Research article

ISSN (online): 2041-4889

ISI Impact factor: 6.044 (2013)

QUALIS A1

CDDIS-14-0230R (Manuscript Accepted)

Altered expression of Alzheimer´s disease-related genes in the cerebellum of autistic patients: a model for disrupted brain connectome and therapy

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Running title: Alzheimer´s disease-related genes in autism

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Abbreviations: Aβ, amyloid-β; AD, Alzheimer´s disease; APP, amyloid-β precursor protein; ASD, autism spectrum disorders; BDNF, brain-derived neurotrophic factor; ER, endoplasmic reticulum; FDR, false discovery rate; GFAP, glial fibrillary acidic protein; GM-CSF granulocyte-macrophage colony-stimulating factor; H-NBs, hub-non-bottlenecks; HBs, hub-bottlenecks; IGF-1, insulin growth factor 1; IGF-2, insulin growth factor 2; IGFBP-3, insulin-like growth factor-binding protein 3; IFN-γ, interferon-γ; IL-6, interleukin 6; IL-8, interleukin 8; NFT, neurofibrillary tangles; NH-Bs, non-hub-bottlenecks; NH-NBs, non-hub-non-bottlenecks; RSN, robust spline normalization; sAPPα, secreted amyloid-β precursor protein α; sAPPβ, secreted amyloid-β precursor protein β; TNF-α, tumor necrosis factor-α.

Keywords: proliferation; mitochondria; APP; magnesium; rapamycin.

ABSTRACT

Autism and Alzheimer´s disease (AD) are, respectively, neurodevelopmental and degenerative diseases with an increasing epidemiological burden. The AD-associated amyloid-β precursor protein α (sAPPα) has been shown to be elevated in severe autism, leading to the "anabolic hypothesis" of its etiology. Here, we performed a focused microarray analysis of genes belonging to NOTCH and WNT signaling cascades as well as genes related to AD and apoptosis pathways in cerebellar samples from autistic individuals, to provide further evidence for pathological relevance of these cascades for autism. By using the limma package from R and false discovery rate (FDR), we demonstrated that 31% (116 out of 374) of the genes belonging to these pathways displayed significant changes in expression (corrected *p*-values <0.05), with mitochondria-related genes being the most downregulated. We also found upregulation of *GRIN1*, the channel forming subunit of NMDA glutamate receptors, and *MAP3K1*, known activator of JNK and ERK pathways with anti-apoptotic effect. Expression of *PSEN2* (presinilin 2) and *APBB1* (or *F65*) were significantly lower when compared to control samples. Based on these results, we propose a model of NMDA glutamate receptors-mediated ERK activation of α-secretase activity and mitochondrial adaptation to apoptosis that may explain the early brain overgrowth and disruption of synaptic plasticity and connectome in autism. Finally, systems pharmacology analyses of the model that integrates all these genes together (NOWADA), highlighted Mg^{2+} and rapamycin as most efficient drugs to target this network model *in silico*. Their potential therapeutic application, in the context of autism, is therefore discussed.

Introduction

Autism is a neurodevelopmental disorder characterized by specific activity patterns and aberrant social interaction and communication.[1] Genetic and environmental factors have been proposed to account for apparently increasing rates of autism diagnostics.[2,3] Its neurobiological basis is, however, poorly understood and the behavioral phenotypes display a high grade of heterogeneity, leading to the definition of "autism spectrum disorders" (ASD). It has been reported that clinical onset of ASD seems to be preceded by a phase of brain overgrowth in the first years of life followed by decreased growth rates between 5 and 12 years of age, when compared to controls.[4] These abnormalities are paralleled by higher levels of brain-derived neurotrophic factor (BDNF), insulin growth factor 1 and 2 (IGF-1 and 2), insulin-like growth factor-binding protein 3 (IGFBP-3), growth hormone binding protein (GHBP), neurotrophin-4 (NT-4), and a neuroinflammatory response in the ASD brains.[5-8] Changes in trophic support may account for abnormal brain growth and an altered connectome, thus defining multiple ASD symptoms. Recent pieces of evidence also indicate the role for deregulated processing of the Alzheimer's disease (AD) associated amyloid-β precursor protein (APP) in ASD-associated pathology. Amyloid-β (Aβ) is considered to be central for the pathophysiology of AD, which in its genetic and sporadic forms appears to be the most common cause of age-associated dementia. AD is characterized by specific pathological hallmarks represented by extracellular deposits of fibrillar Aβ, intraneuronal accumulation of neurofibrillary tangles (NFT) due to hyperphosphorylation of cytoskeletal Tau filaments, as well as impairment of neurogenesis. The end result is massive neuronal death and consequent decline in cognitive functions.[9-11] In ASD, the AD-associated APP, in particular its neuroprotective processing product or secreted APPα (sAPPα), was found to be elevated.[12] Current research indicates that

abnormalities of the cerebellum, now believed to play a role in cognitive function, are associated with autism; being one of the brain regions where cellular and growth abnormalities are most pronounced in the disorder.[13-15]

Here, we address the question of whether changes of expression in the same set of genes observed in AD neuropathology could also be found in autism; and if this is the case, whether drugs tested not only in the context of autism but also in AD may be considered for therapeutics in these patients. Our study shows altered expression of genes belonging to NOTCH, WNT, AD, and apoptosis pathways in cerebellar samples from autistic individulas. In addition, the systems pharmacology analyses of such a pathway that integrates NOTCH, WNT, AD, and apoptosis-related genes suggest magnesium (Mg^{2+}) and rapamycin for further therapeutic exploration in the context of autism; two compounds/drugs which therapeutic use have already been discussed for different AD models.[16-17]

Results

Subnetwork analyses of AD-related pathways reveal transcriptional changes in the cerebellum of autistic individuals. To perform a systems level analysis of potential transcriptional changes in genes belonging to pathways typically associated to AD pathology in autism, we constructed four subnetworks models of NOTCH (Supplementary figure S1A), WNT (Supplementary figure S2A), AD (Figure 1A), and apoptosis (Supplementary figure S3A) pathways. These subnetworks contained 41, 136, 171, and 70 respective genes/proteins (Supplementary tables S1-S4). Next, we performed a focused microarray analysis of the genes belonging to each subnetwork by using samples from the cerebellum of autistic patients vs. controls. In 31% (116 out of

374) of the genes belonging to these *in silico* models were differentially expressed in ASD samples (corrected *p*-value <0.05) (Table 1). More specifically, ~ 30% for NOTCH (12 genes), ~ 23% for WNT (31 genes), more than 40% for AD (69 genes), and ~ 26% for apoptosis (18 genes). *UQCRC1*, *NDUFS3*, *NDUFA9*, *JUN*, *CIDEB*, *CDC42*, *NDUFA6*, *NDUFB7*, *NDUFA1*, and *COX4I1* (Table 1) were the top ten genes that displayed most significant changes in their expression. The downregulated genes were cytochrome c reductase and NADH dehydrogenase-related. Among the AD-related genes, upregulation of *GRIN1*, the channel forming subunit of NMDA glutamate receptors, and *MAP3K1*, known activator of JNK and ERK pathways with anti-apoptotic effect, were also found, together with significantly lower expression of *PSEN2* and *APBB1* (Table 1).

The *in silico* models were subsequently subjected to further analysis with the ViaComplex software, which plots the expression mean values derived from each diseased sample (autism) over the expression of healthy control samples (Z-axis) in the network model (NOTCH, WNT, AD, and apoptosis), presenting them as two dimensional ViaComplex-generated landscapes (Supplementary figures S1B-S3B; Figure 1B). The generated plot visualizes the general landscape of gene expression of diseased samples (autism) vs. healthy controls for each subnetwork. Expression levels are color coded with warm colors (yellow to red) being indicative of increased expression, whereas cold (green to blue) colors indicate decreased gene expression, when compared to control samples. The 2D ViaComplex plots revealed a general upregulation of NOTCH pathway (Supplementary figure S1B) and specific decrease in the expression of mitochondria-related genes (network cluster on the upper left; Figure 1B).

NOWADA is an integrative network model for NOTCH, WNT, AD, and apoptosis subnetworks. Since results presented above showed significant changes in expression of genes belonging to NOTCH, WNT, AD, and apoptosis pathways in cerebellar brain biopsies from ASD patients, our next aim was to test the feasibility of an *in silico* model able to integrate these four subnetworks into one single network and therefore to characterize the relevant molecular route. The Venn diagram, which visualizes the level of subnetwork information integration (Figure 2), shows that that the four subnetworks contain common nodes (genes/proteins) allowing communication with one another. Then, by using STRING 9.05 ("Experiments" and "Databases"; confidence score of 0.600) and plotting with Cytoscape software, we developed a **NO**TCH-**W**NT-**A**lzheimer´s **D**isease-Apoptosis (NOWADA) gene/protein interaction network model able to characterize *in silico* these molecular interactions (Figure 3). The model is composed of 374 genes/proteins interconnecting through 3665 interactions, with only 5 nodes (*PPP3CA*, *PPP3CB*, *PPP3CC*, *PPP3R1*, and *PSEN1*) contributing to maximum three groups (NOTCH, WNT, AD, or apoptosis subnetworks) at the same time.

Identification of hey hub genes/proteins within NOWADA network in the context of autism. In order to describe the global characteristics of the network, we measured centralities[18] or topological network properties: (i) "stress", representing how much a node (gene/protein) is traversed by a high number of ideal routes or short paths in a network. Then, a gene/protein traversed by higher number of short paths will be, by definition, more stressed; (ii) "connectivity or degree", which quantifies the local topology of each node by summing up the number of its adjacent nodes; (iii) "betweenness", which is similar to stress as a topological network property, provides a more informative and elaborated centrality index since it measures how frequently the

shortest path, connecting every pair of nodes, is going through a third given node. In other words, both "stress" and "betweenness" give information about the influence of a node over the spread of information throughout the network. Finally, (iv) "closeness", defined by the inverse of the average length of the shortest paths to access all other proteins in the network, gives an idea about the level of proximity of a node to other nodes or how long it will take the information to spread from a given node to other nodes in the network. Logically, the larger the value the faster the information spreads. Our focus in this part of the study is to target vulnerable (central) components in NOWADA network with 31% of its genes displaying significant changes in expression in the cerebellum of autistic patients (corrected *p*-values <0.05). Upon calculation of each centrality value for each gene/protein (Supplementary table S5), we identified the hub-bottlenecks (HBs) of the network that also displayed higher values of "stress" and closeness centralities. These network nodes were (in alphabetical order) *APP*, *CALM1*, *CASP3*, *CTNNB1*, *DVL2*, *DVL3*, *EP300*, *FZD5*, *GSK3B*, *MYC*, *NOTCH1*, *PLCB2*, *PRKCA*, and *TP53* (Supplementary table S6; Figure 4). As central members, will control the flow of biological information within the network and its disruption (e.g., by drug interaction) could destroy the entire network into small components. Remarkably, *CTNNB1*, gene encoding for β-catenin protein (Supplementary table S2), was the node with top values for all the measured centralities (Supplementary table S6; Figure 4).

Systems pharmacology analysis of NOWADA network suggests Mg^{2+} and rapamycin as most efficient drugs to target the model *in silico*. Previous analysis allowed us to identify central nodes (bottlenecks) with high topological network values. At this stage, since NOWADA network can be considered as a common pathway model for AD and autism, these fourteen nodes (genes/proteins) could represent potential

targets for both pathologies *in silico*. With the aim of elucidating the most suitable and efficient drugs targeting the network in the context of autism, differential gene expression values were taken into account. Our selection criteria was then as follows: (i) to select drugs targeting higher number of genes/proteins within the network, (ii) from those, to choose the drugs affecting/associated to higher numbers of bottlenecks (preferably, HBs with high values of "stress" and "closeness" centralities), and (iii) to select drugs which targets, within the network (NOWADA), include higher number of genes with significant changes in expression. Upon systematic review of the literature (see Material and Methods section), 47 used/proposed drugs for autism therapy were divided in 15 groups and tested for potential interaction/s with genes/proteins of the network model. The groups are acetylcholinesterase inhibitors, allosteric modulators of metabotropic glutamate receptors, antidepressants (SSRI and tricyclic), antiepileptics/anticonvulsants, antiopioids, antipsychotics, complementary and alternative medicines, inhibitors of mTOR, neurohormones, NMDAR (agonists and antagonists), mood stabilizers, psycoestimulants, and sympatholytic medications. Incidentally, 36 out of the 47 autism-related drugs have already been studied in different models of AD (in humans or *in vivo*) for potential therapeutic use[19-22] (Supplementary table S7). Upon using STITCH 3.1, we confirmed that 16 out of the 47 tested drugs had positive hits with at least one gene/protein of NOWADA network (with "Experiments" and "Databases" as input options; confidence score of 0.600). These therapeutic agents were (in alphabetical order): chlorpromazine, clozapine, D-cycloserine, fluoxetine, galantamine, haloperidol, imipramine, lithium, luteolin, Mg^{2+}, melatonin, memantine, olanzapine, rapamycin, valproic acid, and ziprasidone. Most targeted nodes were *GSK3B* (6 hits), *AKT1* (5 hits), *CALM1* (3 hits), *GRIN1* (3 hits), *GRIN2B* (3 hits), *CHRNA7* (2 hits), *GRIN2A* (2 hits), and *JUN* (2 hits) (Supplementary table S9). Except

for *CHRNA7*, our systematic review of the literature could confirmed the expression of the indicated gene products in neuronal and/or glial cells (astroglia, microglia, and oligodendroglia) (Supplementary table S9), thus representing both neuronal and glial targets. When looking into the characteristics of the tested compounds/drugs-genes/proteins interactions, Mg^{2+} and rapamycin are highlighted as the most efficient drugs *in silico* to target this disrupted pathway, which is here represented *in silico* by NOWADA network (Supplementary table S10). More specifically, Mg^{2+} interacts with 32 genes/proteins; among those ones, 8 differentially expressed genes in the cerebellum of autistic patients: the upregulated *EIF2AK2* (corrected *p*-value= 0.002850857) and *CAMK2D* (corrected *p*-value= 0.034576294) as well as the downregulated *CDC42* (corrected *p*-value= 0.002000135), *COX5A* (corrected *p*-value= 0.003530677), *NDUFV1* (corrected *p*-value= 0.011017543), *NDUFS8* (corrected *p*-value= 0.014197263), *RAC3* (corrected *p*-value= 0.014704894), and *CALM2* (corrected *p*-value= 0.032263251) (Figure 5). In order to elucidate the biological processes that Mg^{2+} could be influencing in this context, a functional enrichment analysis of the genes belonging to the magnesium subnetwork was performed. The top ten affected processes are as follows: phosphorylation, phosphorus metabolic process or phosphate-containing compound metabolic process, neuromuscular process, regulation of neurological system process, protein aminoacid autophosphorylation, small GTPase mediated signal transduction, mitochondrial electron transport (NADH to ubiquinone), positive regulation of cell projection organization, and intracellular signaling cascade (corrected *p*-value <0.05) (Supplementary table S11).

Discussion

Here, we studied the possible neurobiological mechanism involved in the excessive rates of early brain overgrowth observed in autism,[4] based on the recent evidence indicating a possible role for the AD-associated APP processing pathway in ASD.[23] Proliferation in the brain results from balancing proliferative rates and developmental apoptosis (e.i., more proliferation and less death will result in a larger brain).[24] APP is proteolytically processed by two competing pathways: the amyloidogenic (β-secretase-mediated) and the non-amyloidogenic (α-secretase-mediated) pathways.[25] sAPPβ (secreted amyloid-β precursor protein β) and the neurotoxic Aβ peptide are generated from APP and released to the extracellular site by proteolytic cleavage of both β- and γ-secretases.[26] Aβ has been suggested to play a critical role in neurotoxicity and apoptotic events seen in AD.[27] In contrast, the non-amyloidogenic product sAPPα and the p3 peptide are formed by proteolytic cleavage of both α- and γ-secretases[26] sAPPα exerts proliferative effects on neural progenitor cells isolated from embryonic brains and is able to enhance synaptogenesis, neurite outgrowth, cell survival, and cell adhesion;[28-31] and APP seems to be able to modulate the neuronal precursors migration.[32] Since γ-secretase cleaves several proteins that include APP and Notch,[33] the potential role of NOTCH signaling pathway in the genesis of AD is also considered.[34] Focused microarray analysis employed in this study demonstrated that expression of 40% of the AD-related genes was altered in the cerebellum of autistic patients (corrected *p*-value <0.05), these changes mainly represented by downregulation of NADH dehydrogenases and cytochrome c oxidases (Table 1). These results reflect deficient mitochondrial respiratory chain, which is consistent with recent reports describing mitochondrial dysfunction in ASD;[35] or may indirectly confirm tight Notch-mitochondria interconnections.[36] An upregulation of *GRIN1* (corrected *p*-value = 0.03916101), the channel forming subunit NR1 of NMDA glutamate receptors and *MAP3K1* (corrected

p-value = 0.038406592), also known as *MEKK1* or *MAPK/ERK Kinase Kinase 1*, which activates JNK and ERK pathways with anti-apoptotic effect,[37] were also found among the AD-related genes in ASD samples (Table 1). *In vivo* studies have shown that stressful events during gestation can increase the expression of NMDA receptors in the brain.[38] The altered expression of NMDA receptors may be related to epigenetic regulation (e.g., DNA hypomethylation) because histone lysine methylation at gene promoters is involved in developmental regulation and maintenance of region-specific expression patterns of ionotropic and metabotropic glutamate receptors in the brain.[39] Furthermore, glutamate receptors expression and/or their activity can modulate the APP processing mechanisms.[40] High glutamate receptors activation and consequent increase in intracellular calcium activate the ERK signaling cascade, enhancing α-secretase cleavage of APP, and thus reducing APP processing into Aβ. An increase in α-secretase activity may lead to higher production of sAPPα.[26] In this study, we hypothesize that deregulated glutamatergic synaptic transmission/plasticity, caused by increased expression of *GRIN1* and hence increased density of NMDA receptors may affect neuronal growth in autism by shifting APP processing to the production of sAPPα through ERK-mediated α-secretase activity. $_{S}$APPα, in turn, may facilitate proliferation by activating the PI3K/Akt/mTOR pathway.[41,42] Based on the present data and existing literature, we proposed this model (Figure 6) as a mechanism that could explain the focal modification of neurogenesis, migration, and alterations of the cytoarchitecture of brain cortex, subcortical structures and cerebellum observed in individuals with autism.[43] Consequently, the decrease in expression of mitochondrial enzymes observed in our array (Table 1) may represent a compensatory mechanism to contain Ca^{2+} overload-induced apoptosis in these cells. It has already been reported that compromised mitochondria accumulate (observed by increased mitochondrial mass) in

pyramidal neurons in temporal cortex of younger ASD subjects.[44] Moreover, presinilin 1 and 2 (PSEN1 and PSEN2) are believed to have almost overlapping cellular functions and contribute to diverse physiological processes, such as regulation of Ca^{2+} homeostasis, protein transport and turnover, autophagy, cell adhesion, neurotransmitter release and axon guidance.[45] PSEN2, however, seems to be responsible for endoplasmic reticulum (ER) and mitochondria interactions.[46] Closer ER-mitochondria juxtaposition (regulated by PSEN2) may expose mitochondria to excessive Ca^{2+} stimulation, triggering the apoptotic cascade by mitochondria Ca^{2+} overload. In our array analysis, we found that *PSEN2* is downregulated (corrected *p*-value= 0.005968353) in the cerebellum of autistic patients (Table 1) which could represent another compensatory mechanism to reduce Ca^{2+} overload-induced apoptosis. Our hypothesis is further supported by the following observations: (i) mutant mice in which glutamate receptors are overstimulated by knocking out glutamate transporters GLAST and GLT1 and thus leading to excessive glutamatergic signaling in the prenatal stage, compromises early brain development via overstimulation of NMDARs. NR1 deletion in double knockout mice almost completely rescued multiple brain defects including cortical, hippocampal, and olfactory bulb disorganization and defective corticothalamic and thalamocortical axonal projections;[47] (ii) opposite to what it is seen in AD patients, higher levels of sAPPα have been described in plasma from autistic children when compared to controls, favoring an increased α-secretase pathway;[12,48] (iii) evidence of autoimmunity and persistent systemic immune activation have been reported in ASD[49] and mice overexpressing human sAPPα indeed exhibit higher T-cell cytokine secretion together with reduced $CD4^+$ and higher $CD8^+$ T-cell populations in splenocytes when compared to wild-type animals. These animals also display hypoactivity, impaired social interaction, elevated levels of brain glial fibrillary acidic protein (GFAP) expression,

and altered Notch1 and IL-6 levels.[50] In the brain of autistic individuals, increased expression of GFAP in the areas with disturbed neuronal architecture has been detected together with interleukin 6 (IL-6), IL-8, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF), suggesting astrogliotic response and potential alterations in neurogenesis and neuronal migration.[51-54] Finally (iv), we observed a significant decrease (corrected *p*-value= 0.024237411) in expression of *APBB1* (amyloid β A4 precursor protein-binding, family B, member 1; or *F65*) (Table 1). *In vitro* F65 overexpression induces a dramatic increase in Aβ secretion, whereas Aβ secretion is decreased in Fe65 knockdown cells and also in hippocampal neurons of Fe65/Fe65L1 knockout (KO) mice.[55-57]

Systems pharmacology analyses of NOWADA network revealed that Mg^{2+} and rapamycin could efficiently target this pathway *in silico*, when compared to other drugs (Supplementary table S10). RhoGAPs, regulatory molecules of RHO GTPases (e.g., *CDC42*, *RHOA*, and *RAC1*), which altered expression were shown in autism,[3] use Mg^{2+} as a cofactor to reach catalytic efficiency and specificity in GTP hydrolysis (Supplementary table S11).[58] Furthermore, glycogen synthase kinase 3β (*GSK3B*), a HB in our network model (Supplementary table S6), is a ubiquitous serine/threonine kinase that catalyzes the transfer of γ-phosphate of ATP to the hydroxyl oxygen of the Ser and Thr residues of a kinase-specific protein substrate is a Mg^{2+}-dependent kinase.[59] Its aberrant function has been linked to diverse neurological disorders such as AD.[60] Lower concentrations of Mg^{2+} were described in children with autism.[61] Similarly, in AD patients, Mg^{2+} concentrations in the brain seem to be significantly lower when compared with age-matched normal individuals.[62] Recently, it was suggested that elevation of brain Mg^{2+} concentration in the brain exerts substantial synaptoprotective effects in an *in vivo* model of AD and hence Mg^{2+} may have therapeutic potential for

treating AD in humans.[17] In autism, positive behavioral effects of combined Mg^{2+} and pyridoxine treatment were described;[63] combination that was also proposed to be beneficial in ASD patients that experience seizures.[64] The therapeutic usage of rapamycin (mTOR inhibitor) in ASD is nowadays discussed. TSC1 and TSC2 are upstream inhibitory regulators of mTOR activity and studies performed in $TSC2^{+/-}$ mice showed that treatment with rapamycin can rescue synaptic plasticity and reverse the abnormal late-phase of long-term potentiation.[20] These results encourage future clinical trials with TOR inhibitors as pharmacological treatment of ASD.

We believe that the computational analyses performed in this study gave valuable clues to i) develop a model that may explain the aberrant brain overgrowth and impaired neuronal connectivity observed in these children and ii) refer to the need of further therapeutic exploration of Mg^{2+}- and rapamycin-based treatments in animal models and clinical trials with autistic patients.

Materials and Methods

Development of gene/protein network models. NOTCH, WNT, AD, and apoptosis subnetwork models were developed through (i) extracting the information provided by the KEGG PATHWAY database (http://www.genome.jp/kegg/pathway.html; NOTCH pathway: map04330, WNT pathway: map 04310; AD pathway: map05010, and apoptosis pathway: map04210) and (ii) by establishing the interactions between genes/proteins with the database resource Search Tool for the Retrieval of Interacting Genes/Proteins STRING version 9.05 (http://string-db.org/); with "Experiments" and "Databases" as input options and a confidence score of 0.600. STRING is a well-known public database with information about direct and indirect functional protein-protein

interactions. The genes/proteins belonging to NOTCH, WNT, AD, and apoptosis subnetwork models were identified by the HUGO Gene Symbol[65] and Ensemble protein ID (Suplementary tables S1-S4). Once they were selected, the links between two different nodes (genes/proteins), provided by the STRING database, are saved in data files to be processed in the Medusa interface[66] and visualized as a subnetwork (NOTCH, WNT, AD, or apoptosis). The Venn diagram was constructed by using the freely available software system R (http://www.r-project.org)[67] in order to visualize the grade of molecular relation (common genes/proteins) between the generated subnetworks. The *in silico* network model integrating NOTCH, WNT, AD, and apoptosis subnetworks (NOWADA) was developed by using again STRING version 9.05 with "Experiments" and "Databases" as input options and a confidence score of 0.600 (0.400 is considered "medium confidence") and plotted by utilizing Cytoscape, an open source platform for complex network analysis and visualization (http://www.cytoscape.org/).[68]

Data acquisition and processing, determination of variably expressed genes, statistics, and gene expression network visualization. Microarray data (raw data; .cel files) were obtained from the Geo DataSets database (http://www.ncbi.nlm.nih.gov/geo/). The dataset (GSE38322), originally contributed by Ginsberg and colleagues,[69] is publicly available; it contains data from cerebellar brain tissue of autistic patients and control subjects. Experimental assays are described in full in the original publication and details include the selection criteria of patients (both inclusion and exclusion criteria) and diagnostic profile.[69] For elucidating the differential expression of members from the NOTCH, WNT, AD, and apoptosis subnetworks, expression data were filtered from probes with <0.05 signal detection *p* values and

normalized by using the lumi package from R and robust spline normalization (RSN). For differential gene expression analysis, normalized data of cerebellar samples from patients versus controls were analyzed by utilizing the limma package from R[67] and false discovery rate (FDR) control for statistical assessment of the microarray data (corrected *p*-values <0.05 were considered significant). Specific corrected *p*-values for each differentially expressed gene are provided (Table 1). In order to visualize the general landscape of gene expression *in silico*, gene expression in samples from autism were plotted vs. the expression found in healthy controls to be visualized with the ViaComplex software, a tool that was previously developed and validated in our laboratory.[70] This software plots gene expression over the Medusa network (in a color scale) by overlapping functional input data (microarray expression data) with interaction information (NOTCH, WNT, AD, and apoptosis subnetworks) and distributes the microarray signal according to the coordinates of the nodes and its interactions in the network of interest.

Elucidation of network centralities to predict the relevance of genes in the overall network architecture. Topological network properties of the genes/proteins integrating the network (NOWADA) were calculated by using Cytoscape.[68] Numeric values corresponding to the properties of each node are additionally provided (Supplementary table S5). Thresholds for selection criteria of the most relevant nodes according to their centrality values, were established considering one standard deviation of the mean for each topological network properties: “stress”: 48919.44 (mean= 15251.9); “connectivity or degree”: 35.84815 (mean= 19.59893); “betweenness”: 0.022270086 (mean= 0.005765439); “closeness”: 0.372488254 (mean= 0.325097663). Centrality values were plotted in graphs by using OriginLab

(http://www.originlab.com/index.aspx?go=Products/OriginPro). Nodes (genes/proteins) with high connectivity (above the threshold) and low betweenness centrality (below the threshold) are considered hub-non-bottlenecks (H-NBs) and those ones with a value above the thresholds for both connectivity and betweenness centrality are referred to as HBs. Similarly, non-hub-bottlenecks (NH-Bs) are nodes with low connectivity and high betweenness centrality, and non-hub-non-bottlenecks (NH-NBs) are nodes with both connectivity and betweenness centrality values below the thresholds.[18]

In silico **development of compound (drug)-gene/protein network model.** To construct the compound (drug)-gene/protein network models we firstly performed a systematic review of relevant literature associated with autism or AD therapy in the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/). These articles were obtained by using the two terms ‘‘autism’’ and ‘‘ASD’’ and combining them with the following terms: “Alzheimer´s disease”, ‘‘therapy’’, ‘‘therapeutics’’, ‘‘treatment’’, and/or ‘‘drug’’, respectively. Moreover, compound identifier (CID), formula, description, and selected autism- and AD-related reports (with PMID) for these drugs (Supplementary table S6) are additionally provided with KEGG DRUG (http://www.genome.jp/kegg/drug/), the Search Tool for Interactions of Chemicals STITCH version 3.1 (http://stitch.embl.de/), and PubMed database as sources of information. Second, these drugs were submitted to *in silico* analysis by searching the number of genes/proteins within NOWADA network that are targeted by the selected drugs. To achieve this goal, STITCH version 3.1 (http://stitch.embl.de/) was utilized with “Experiments” and “Databases” as input options and a confidence score of 0.600 (0.400 is considered “medium confidence”). A list with the most drug-targeted network nodes (genes/proteins) is also provided (Supplementary table S8). Again, the links

between two different nodes (drug-genes/proteins), provided by the STITCH database, is saved in data files to be handled in the Medusa interface and visualized as a subnetwork. For screening potential cellular targets of the most drug-targeted network nodes (NOWADA), another systematic review of both original research articles and reviews was performed in PubMed database. These articles were obtained by using the genes/proteins name or symbol and combining them with the following terms: ‘‘Neuronal’’, “Neuron”, ‘‘Astroglia’’, “Astrocyte”, “Microglia”, “Oligodendroglia”, and/or ‘‘Oligodendrocyte’’ (Supplementary table S9).The selected references confirm the expression of the indicated gene products in brain cells. Note that the selected references do not cover therapeutic modalities, age- or brain region-specificity.

Functional enrichment analysis (biological processes). The biological processes of the genes products belonging to the subnetwork model of interactions was determined by using the database for annotation, visualization, and integrated discovery DAVID v6.7 (http://david.abcc.ncifcrf.gov/), giving a number of functional annotation tools to researchers for better comprehension of the biological meaning behind any large list of genes. Only those biological processes with corrected *p*-value <0.05 (FDR) were selected. Specific corrected *p*-values for each biological process are provided (Supplementary table S9).

A graphical abstract, summarizing the contents and the methodological approaches used for the present study, is also provided (Figure 7).

Conflict of Interest

The authors declare that no competing interests exist.

Acknowledgements. First of all, our sincere apologies to the authors whose work have not been cited in the present review article due to space considerations. We thank Brazilian research funding agencies FAPERGS (PqG 1008860, PqG 1008857, ARD11/1893-7, PRONEX 1000274), CAPES (PROCAD 066/2007), CNPq (558289/2008-8 and 302330/2009-7), as well as PROPESQ-UFRGS for supporting this work.

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Figure Legends

Figure 1 Alzheimer´s disease subnetwork analysis. **(A)** General landscape of interactions between genes/proteins belonging to the Alzheimer´s disease pathway (map05010; KEGG Pathway; http://www.genome.jp/kegg/pathway.html). The *in silico* network model was developed by using the STRING 9.05 database resource search tool, under a confidence score of 0.600 and using "Databases" and "Experiments" as input options. **(B)** Focused microarray analyses of Alzheimer´s disease-related genes in the cerebellum of autistic patients vs. healthy controls over the *in silico* model. The Z-axis for representing the relative gene expression was constructed by using the ViaComplex software.

Figure 2 Venn diagram showing information integration from the studied subnetworks. NOTCH, WNT, Alzheimer´s disease, and apoptosis models contain common nodes (genes/proteins) with, at least, one subnetwork.

Figure 3 Network model of interactions between genes/protein belonging to NOTCH, WNT, Alzheimer´s disease, and apoptosis subnetworks (NOWADA network). The present model was developed by using the STRING 9.05 database resource search tool, under a confidence score of 0.600 and using "Databases" and "Experiments" as input options and visualized by plotting it with Cytoscape software. The number of subnetwork contributions of each gene/protein within the network is represented in the figure as indicated in the inset.

Figure 4 Analysis of the topological properties of nodes (genes/proteins) belonging to the network (NOWADA). Dashed lines are indicating the threshold value for each property. Upregulated and downregulated genes in the subnetwork are marked with red and blue triangles, respectively, as indicated in the inset. Note that NH-Bs and HBs distinguish non-hub-bottlenecks from hub-bottlenecks, respectively.

Figure 5 Magnesium subnetwork analysis. **(A)** *In silico* network model of the interactions between magnesium and genes/proteins belonging to the network (NOWADA), developed by using STITCH 3.1 with "Experiments" and "Databases" as input options and a confidence score of 0.600. **(B)** Actions view representation between the network nodes by "catalysis" (yellow). **(C)** Actions view representation between the network nodes by "binding" (white color). **(D)** Actions view representation between the network nodes by "inhibition" (red color). Upregulated and downregulated genes in the subnetwork are marked with red and blue triangles, respectively, as indicated in the inset.

Figure 6 Hypothetical model of the synaptic mechanism regulating Aβ production and the link to aberrant brain growth and connectivity in autism. The development of living organisms parallels to a so-called physiological "intrinsic developmental stress", which is associated to massive internal changes during morphogenesis and thus contributes to the time window of vulnerability to the environment. Development and severity of ASD are likely to arise from complex interactions between pre-existing genetic vulnerability/ies in these children, the exposure to noxious environmental factors, and the timing of the stressful event(s); given that prenatal life, infancy, childhood, and adolescence are critical periods characterized by increased vulnerability to stressors.[2,3] Glial activation and neuroinflammation seem to persist during adulthood in autism, and early brain overgrowth in autism could be a consequence of an over-activation of neural proliferation in a trial to compensate the cellular loss induced by environmental stress/ors, triggering precipitous/uncontrolled migration and misdistribution of neurons among different brain areas. Our model proposed that aberrant epigenetic regulation may lead to increased density of NMDA receptors and therefore, to increased Ca^{+2} entry and

stimulation of ERK-dependent α-secretase activity. The decrease in mitochondrial enzymatic activity together with the downregulation of PSEN2 at the endoplasmic reticulum (ER) may represent a compensatory mechanism to reduce the Ca^{2+} overload-induced apoptosis. PSEN2 downregulation may diminish the interaction between ER and mitochondria, reducing its Ca^{+2} uptake. In turn, mitochondrial mass would increase most likely to maintain the cellular ATP levels. Higher levels of sAPPα would activate the PI3K/Akt/mTOR pathway resulting in proliferation, aberrant brain growth and disruption of synaptic plasticity and connectome.

Figure 7 Abstract workflow summarizing the different approaches utilized for the present study. The information from the different pathways (NOTCH, WNT, Alzheimer´s disease, and apoptosis) was gathered from KEGG Pathway (http://www.genome.jp/kegg/pathway.html) to develop the subnetworks. The differential expression of the genes belonging to each subnetwork was analyzed in the cerebellum of autistic patients by using R. A Venn diagram showed common genes/proteins between NOTCH, WNT, Alzheimer´s disease, and apoptosis pathways. Thereafter, a network model was developed for integrating these subnetworks into one single gene/protein interaction network model (NOWADA network) by using STRING 9.05 and Cytoscape tools. Further elucidation of the topological network properties revealed a number of potential molecular targets or "vulnerable" points of the *in silico* model and therapeutic agents used in autism targeting central nodes of the network were studied (STITCH 3.1). Finally, a subnetwork representing the interaction between magnesium (Mg^{2+}) and genes/proteins from NOWADA network was constructed and a functional enrichment analysis was performed (DAVID v6.7) to elucidate the biological processes potentially affected by this compound.

Supplementary Figure Legends

Supplementary figure S1 NOTCH subnetwork analysis. **(A)** General landscape of interactions between genes/proteins belonging to the NOTCH pathway (map04330; KEGG Pathway; http://www.genome.jp/kegg/pathway.html). The *in silico* network model was developed by using the STRING 9.05 database resource search tool, under a confidence score of 0.600 and using "Databases" and "Experiments" as input options. **(B)** Focused microarray analyses of NOTCH-related genes in the cerebellum of autistic patients vs. healthy controls over the *in silico* model. The Z-axis for representing the relative gene expression was constructed by using the ViaComplex software.

Supplementary figure S2 WNT subnetwork analysis. **(A)** General landscape of interactions between genes/proteins belonging to the WNT pathway (map04310; KEGG Pathway; http://www.genome.jp/kegg/pathway.html). The *in silico* network model was developed by using the STRING 9.05 database resource search tool, under a confidence score of 0.600 and using "Databases" and "Experiments" as input options. **(B)** Focused microarray analyses of WNT-related genes in the cerebellum of autistic patients vs. healthy controls over the *in silico* model. The Z-axis for representing the relative gene expression was constructed by using the ViaComplex software.

Supplementary figure S3 Apoptosis subnetwork analysis. **(A)** General landscape of interactions between genes/proteins belonging to the apoptosis pathway (map04210; KEGG Pathway; http://www.genome.jp/kegg/pathway.html). The *in silico* network model was developed by using the STRING 9.05 database resource search tool, under a confidence score of 0.600 and using "Databases" and "Experiments" as input

options. **(B)** Focused microarray analyses of apoptosis-related genes in the cerebellum of autistic patients vs. healthy controls over the *in silico*.

Supplementary Table Legends

Supplementary table S1 Genes/proteins belonging to the NOTCH subnetwork.

Supplementary table S2 Genes/proteins belonging to the WNT subnetwork.

Supplementary table S3 Genes/proteins belonging to the Alzheimer´s disease (AD) subnetwork.

Supplementary table S4 Genes/proteins belonging to the apoptosis subnetwork.

Supplementary table S5 Genes/proteins belonging to the interaction network model (NOWADA network) with their respective values for stress, connectivity (degree), betweenness, and closeness centralities, together with the subnetwork contribution/s of each node (gene/protein). "NOT", "WNT", "AD", and "APO" represent NOTCH, WNT, Alzheimer´s disease, and apoptosis subnetworks, respectively.

Supplementary table S6 Selected genes/proteins from the interaction network (NOWADA network) considering their respective values for stress, connectivity (degree), betweenness, and/or closeness centralities over the thresholds (value/s falling within one standard deviation of the mean). "NOT", "WNT", "AD", and "APO" represent NOTCH, WNT, Alzheimer´s disease, and apoptosis subnetworks, respectively.

Supplementary table S7 Drugs utilized for the *in silico* screening and potential target identification within NOWADA network. Sources of information: KEGG DRUG (http://www.genome.jp/kegg/drug/), STITCH 3.1 (http://stitch.embl.de/), and PubMed (http://www.ncbi.nlm.nih.gov/pubmed/).

Supplementary table S8 Drug-targeted network nodes of NOWADA network.

Supplementary table S9 Most drug-targeted network nodes of the network (NOWADA) and potential cellular targets (Yes or Not determined/ND) and selected reference/s (PMID).

Supplementary table S10 Characteristics of compound (drug)-gene/protein interactions in NOWADA network.

Supplementary table S11 Functional enrichment analysis (biological processes) of the genes belonging to the magnesium (Mg^{2+}) subnetwork (corrected *p*-value <0.05).

Figures

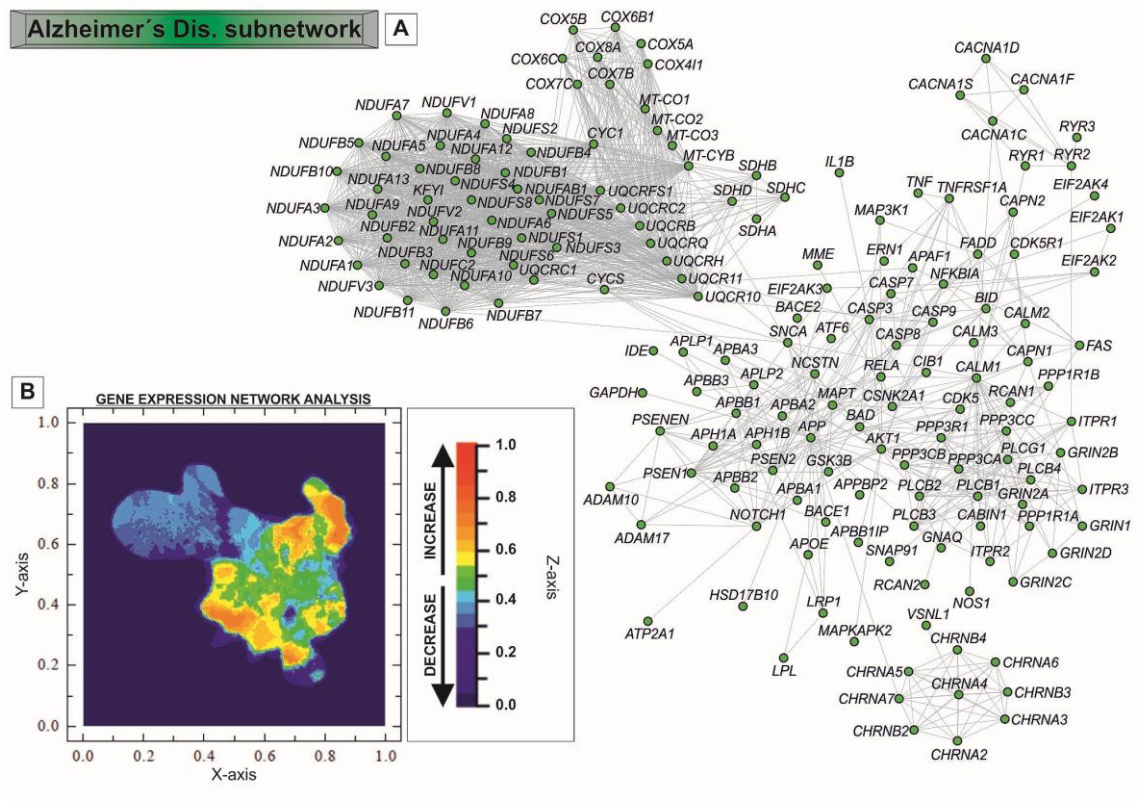


Figure 1

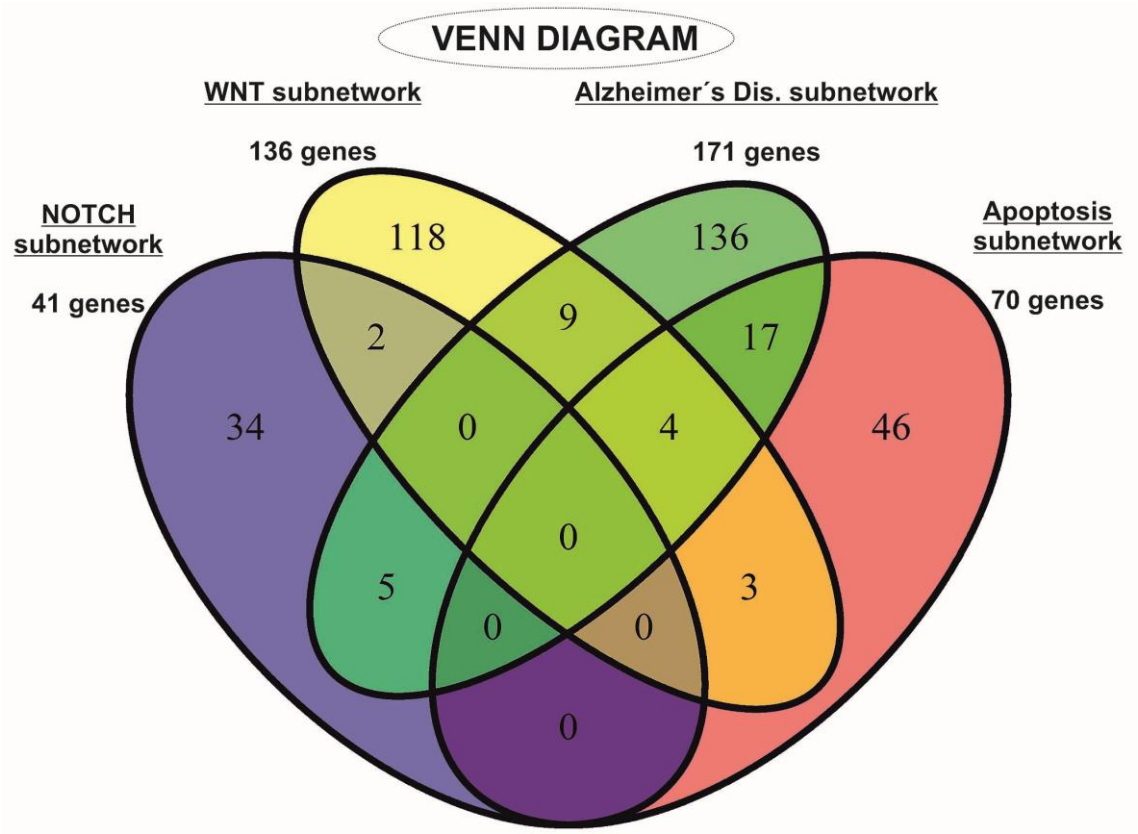

VENN DIAGRAM
WNT subnetwork
136 genes
Alzheimer´s Dis. subnetwork
171 genes
NOTCH subnetwork
41 genes
Apoptosis subnetwork
70 genes
118
136
2
9
17
34
0
4
46
0
5
0
0
3
0

Figure 2

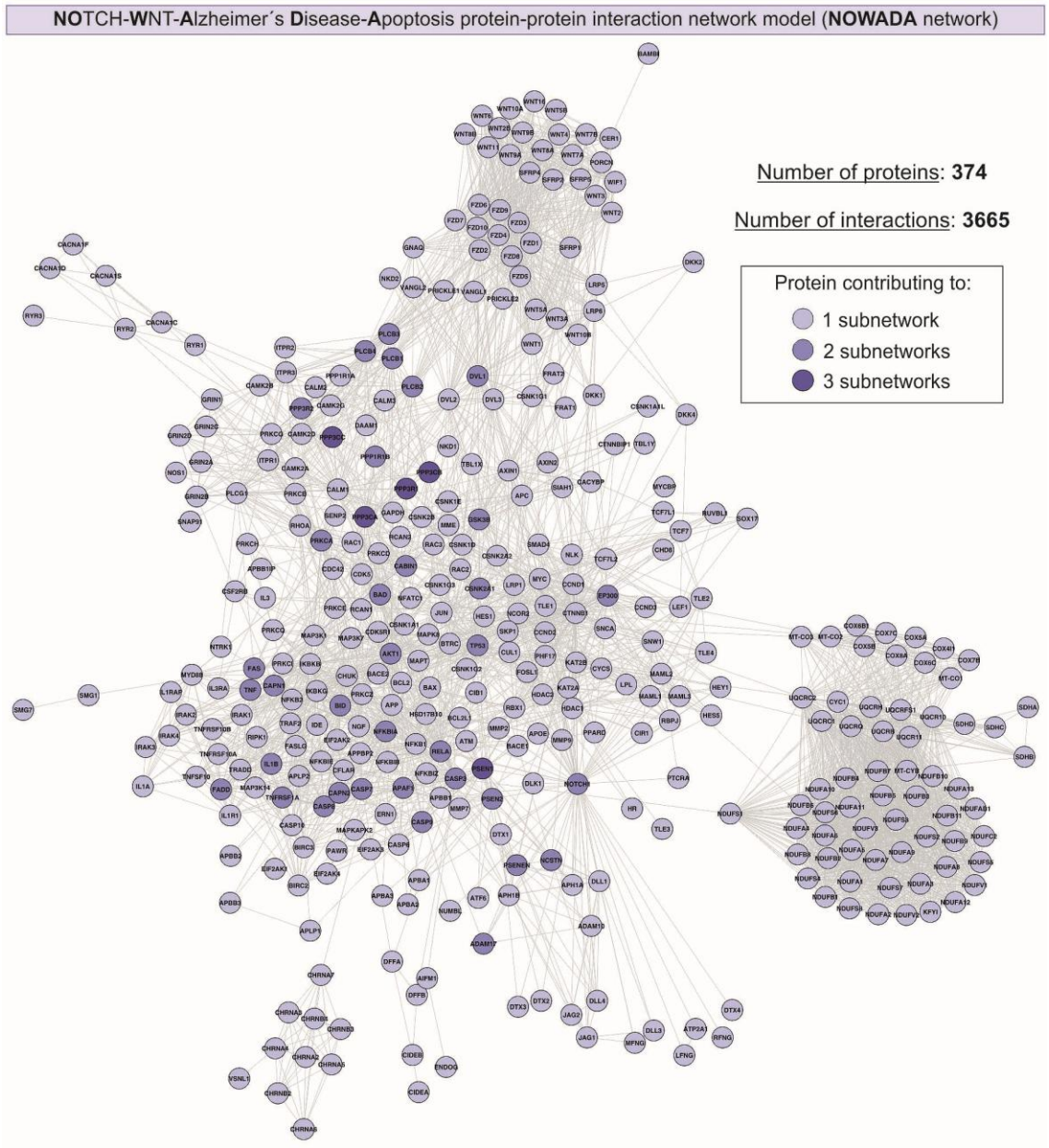

NOTCH-WNT-Alzheimer´s Disease-Apoptosis protein-protein interaction network model (NOWADA network)
Number of proteins: 374
Number of interactions: 3665
Protein contributing to:
1 subnetwork
2 subnetworks
3 subnetworks

Figure 3

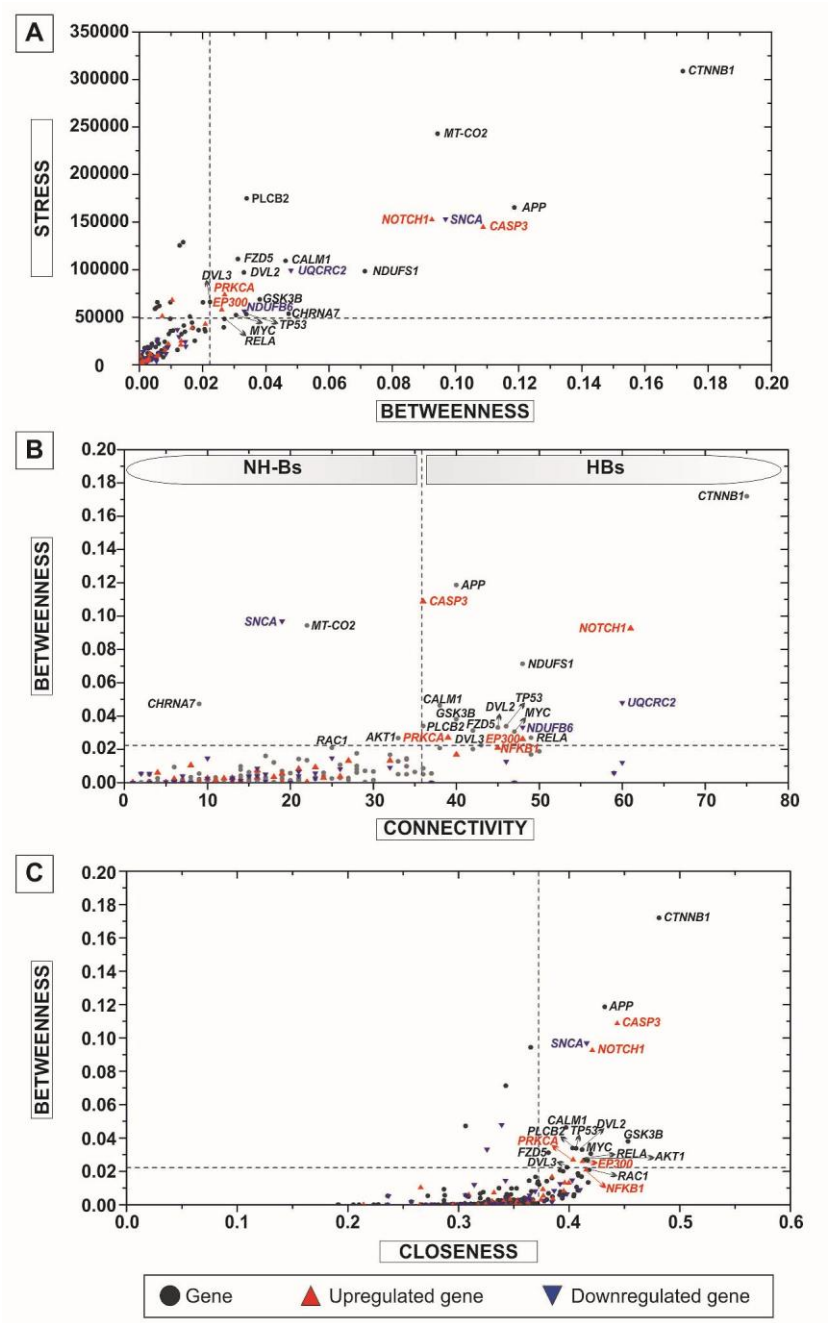

A
STRESS
BETWEENNESS
CTNNB1
MT-CO2
PLCB2
APP
NOTCH1
SNCA
CASP3
FZD5
CALM1
DVL3
DVL2
UQCRC2
NDUFS1
PRKCA
EP300
GSK3B
NDUFB6
CHRNA7
TP53
MYC
RELA
B
NH-Bs
HBs
BETWEENNESS
CONNECTIVITY
C
CLOSENESS
Gene
Upregulated gene
Downregulated gene

Figure 4

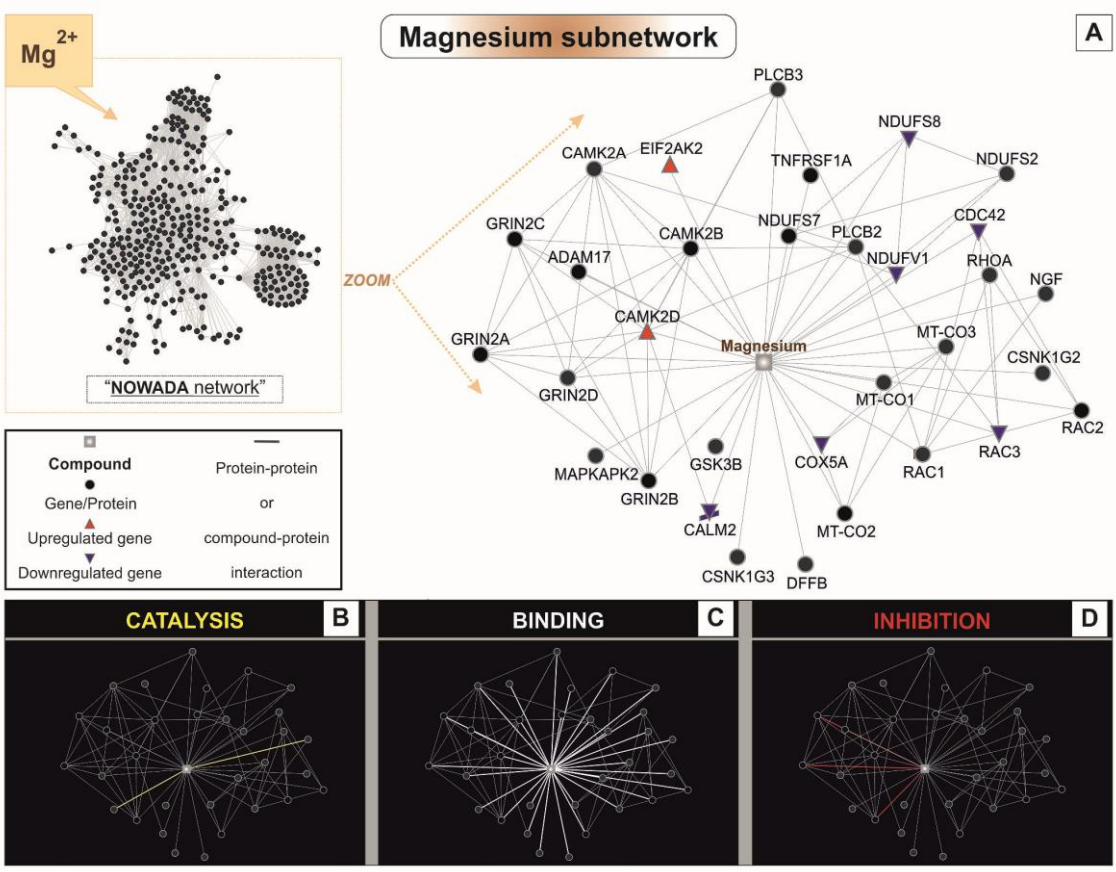

Mg 2+
"NOWADA network"
ZOOM
Magnesium subnetwork
A
PLCB3
NDUFS8
CAMK2A
EIF2AK2
TNFRSF1A
NDUFS2
GRIN2C
CAMK2B
NDUFS7
PLCB2
CDC42
ADAM17
NDUFV1
RHOA
NGF
CAMK2D
GRIN2A
Magnesium
MT-CO3
CSNK1G2
GRIN2D
MT-CO1
RAC2
RAC3
MAPKAPK2
GSK3B
COX5A
RAC1
GRIN2B
CALM2
MT-CO2
CSNK1G3
DFFB
Compound
Gene/Protein
Upregulated gene
Downregulated gene
Protein-protein
or
compound-protein
interaction
CATALYSIS
B
BINDING
C
INHIBITION
D

Figure 5

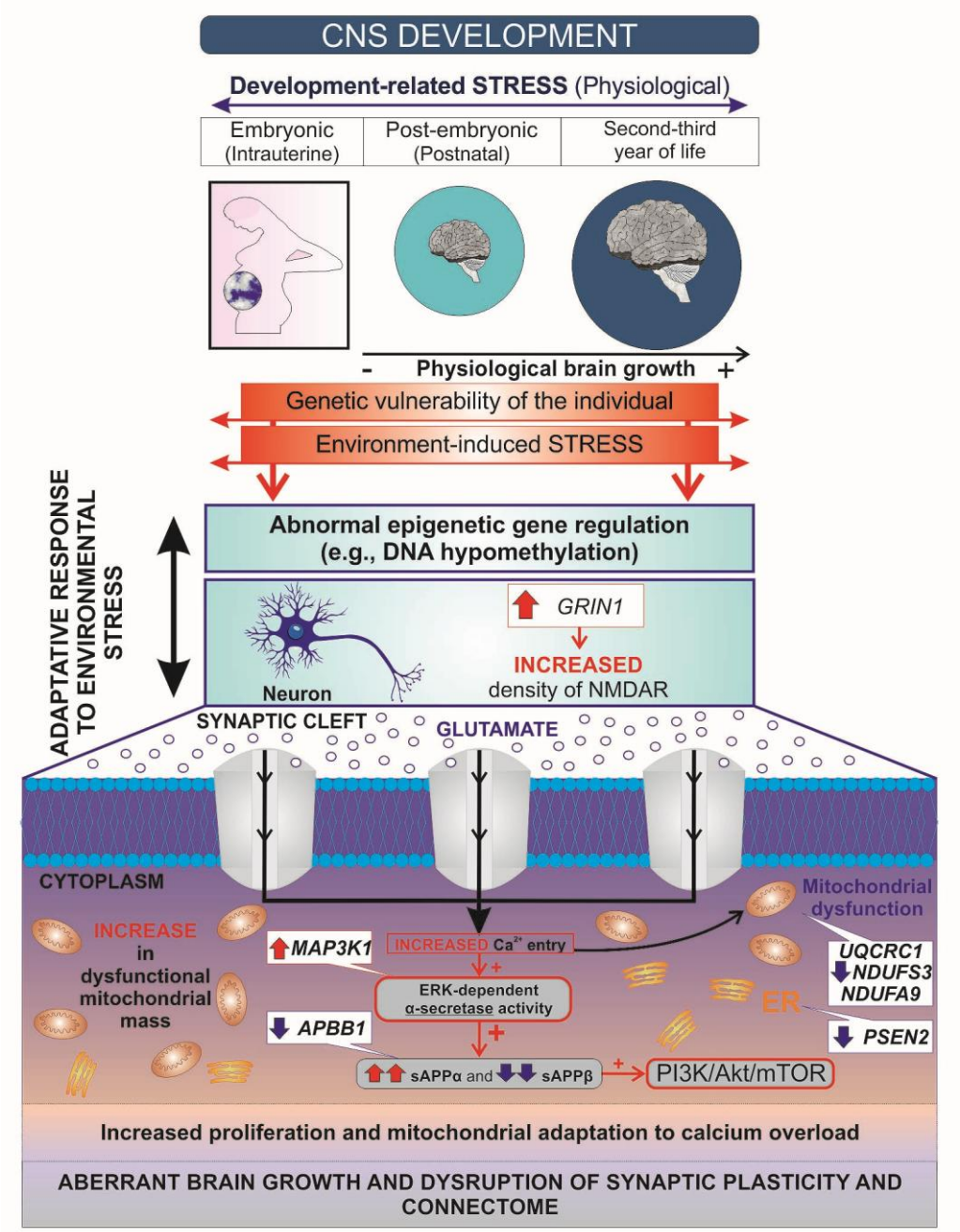

CNS DEVELOPMENT
Development-related STRESS (Physiological)
Embryonic (Intrauterine)
Post-embryonic (Postnatal)
Second-third year of life
- Physiological brain growth +
Genetic vulnerability of the individual
Environment-induced STRESS
ADAPTATIVE RESPONSE TO ENVIRONMENTAL STRESS
Abnormal epigenetic gene regulation (e.g., DNA hypomethylation)
GRIN1
INCREASED density of NMDAR
Neuron
SYNAPTIC CLEFT
GLUTAMATE
CYTOPLASM
Mitochondrial dysfunction
INCREASE in dysfunctional mitochondrial mass
MAP3K1
INCREASED Ca2+ entry
ERK-dependent α-secretase activity
APBB1
UQCRC1
NDUFS3
NDUFA9
ER
PSEN2
sAPPα and sAPPβ
PI3K/Akt/mTOR
Increased proliferation and mitochondrial adaptation to calcium overload
ABERRANT BRAIN GROWTH AND DYSRUPTION OF SYNAPTIC PLASTICITY AND CONNECTOME

Figure 6

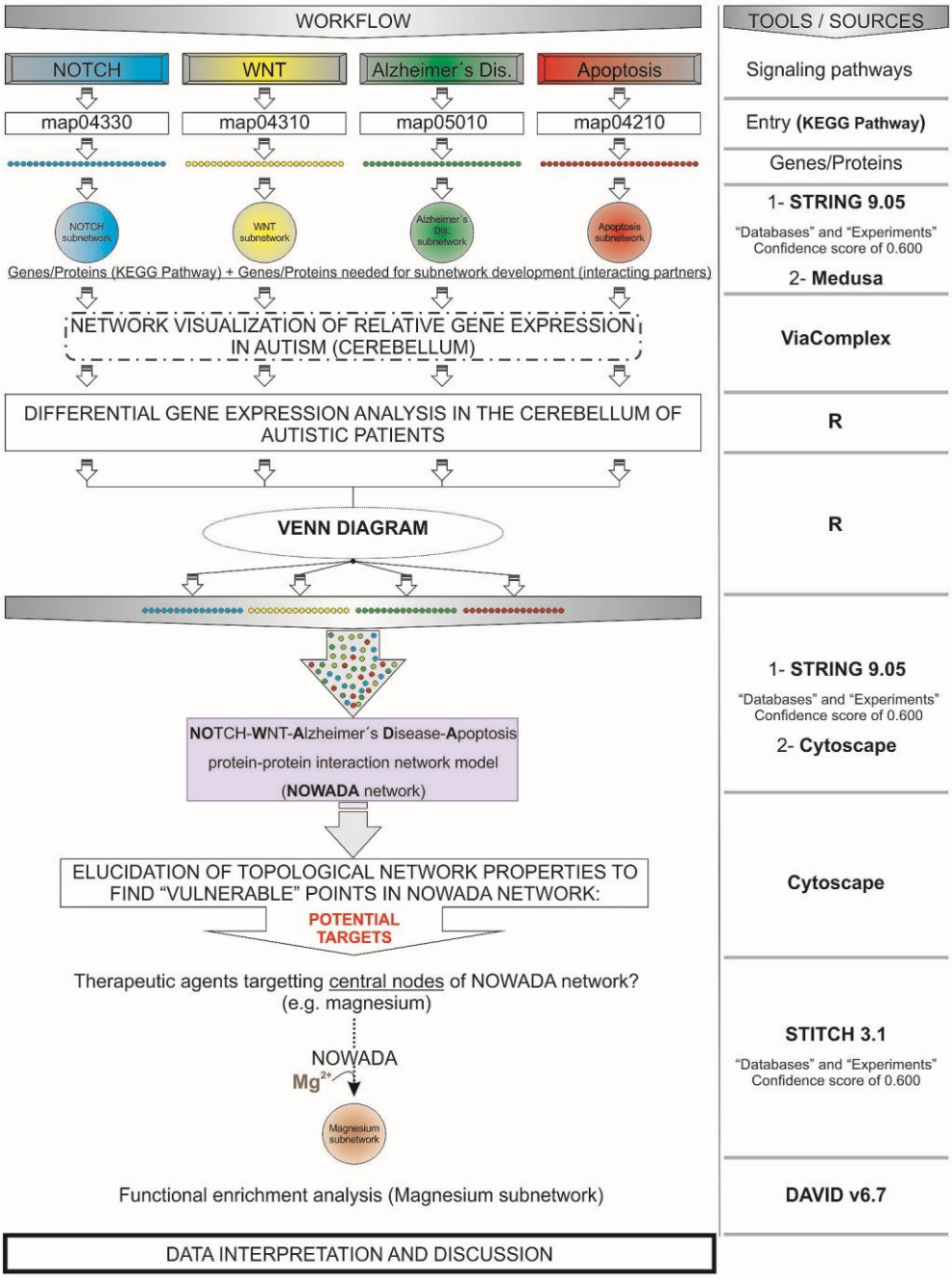
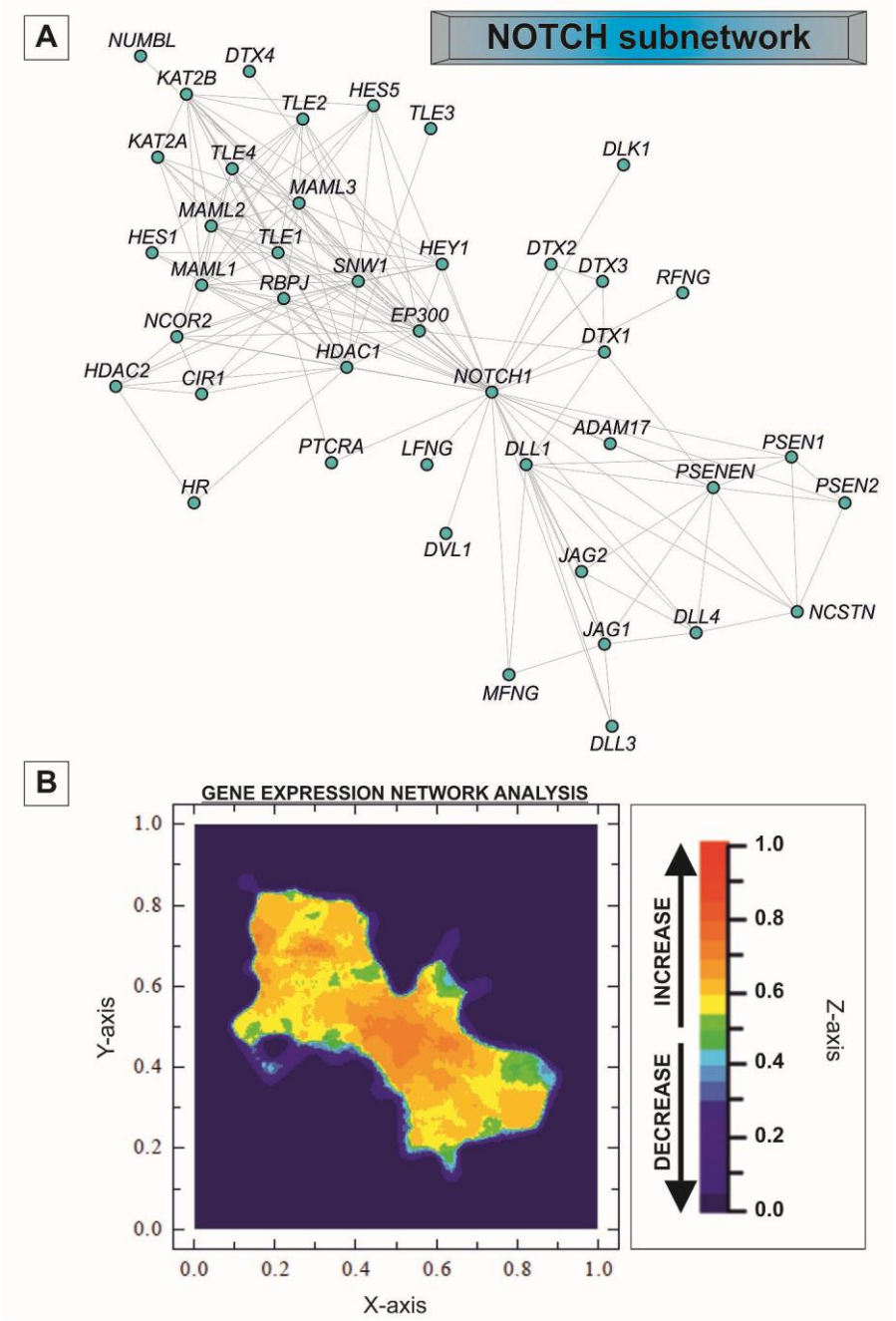
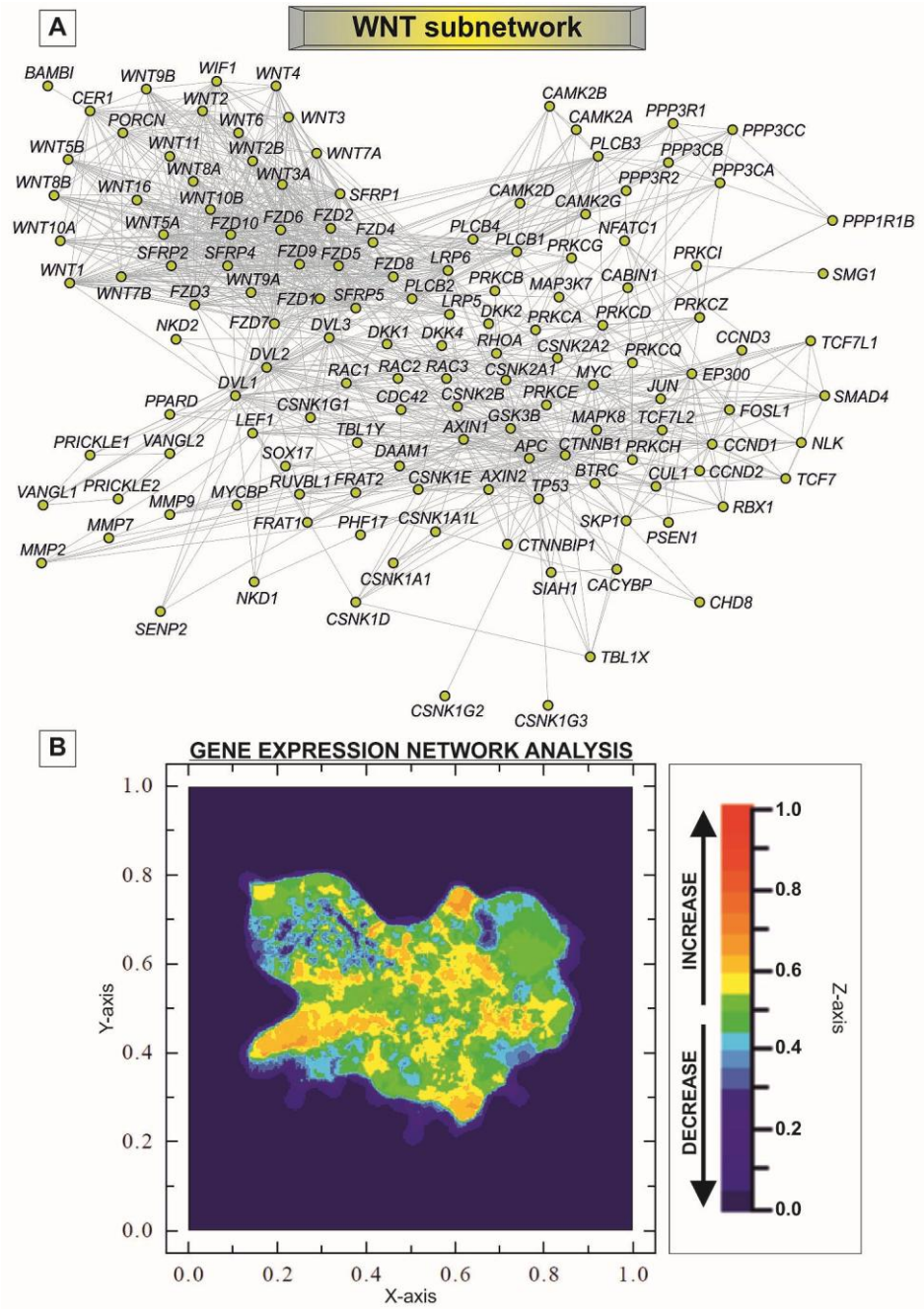

WORKFLOW
TOOLS / SOURCES
NOTCH
WNT
Alzheimer´s Dis.
Apoptosis
Signaling pathways
map04330
map04310
map05010
map04210
Entry (KEGG Pathway)
Genes/Proteins
NOTCH subnetwork
WNT subnetwork
Alzheimer´s Dis. subnetwork
Apoptosis subnetwork
1- STRING 9.05
"Databases" and "Experiments"
Confidence score of 0.600
Genes/Proteins (KEGG Pathway) + Genes/Proteins needed for subnetwork development (interacting partners)
2- Medusa
NETWORK VISUALIZATION OF RELATIVE GENE EXPRESSION IN AUTISM (CEREBELLUM)
ViaComplex
DIFFERENTIAL GENE EXPRESSION ANALYSIS IN THE CEREBELLUM OF AUTISTIC PATIENTS
R
VENN DIAGRAM
R
1- STRING 9.05
"Databases" and "Experiments"
Confidence score of 0.600
NOTCH-WNT-Alzheimer´s Disease-Apoptosis protein-protein interaction network model (NOWADA network)
2- Cytoscape
ELUCIDATION OF TOPOLOGICAL NETWORK PROPERTIES TO FIND "VULNERABLE" POINTS IN NOWADA NETWORK:
POTENTIAL TARGETS
Cytoscape
Therapeutic agents targetting central nodes of NOWADA network?
(e.g. magnesium)
NOWADA
Mg2+
Magnesium subnetwork
STITCH 3.1
"Databases" and "Experiments"
Confidence score of 0.600
Functional enrichment analysis (Magnesium subnetwork)
DAVID v6.7
DATA INTERPRETATION AND DISCUSSION

Figure 7

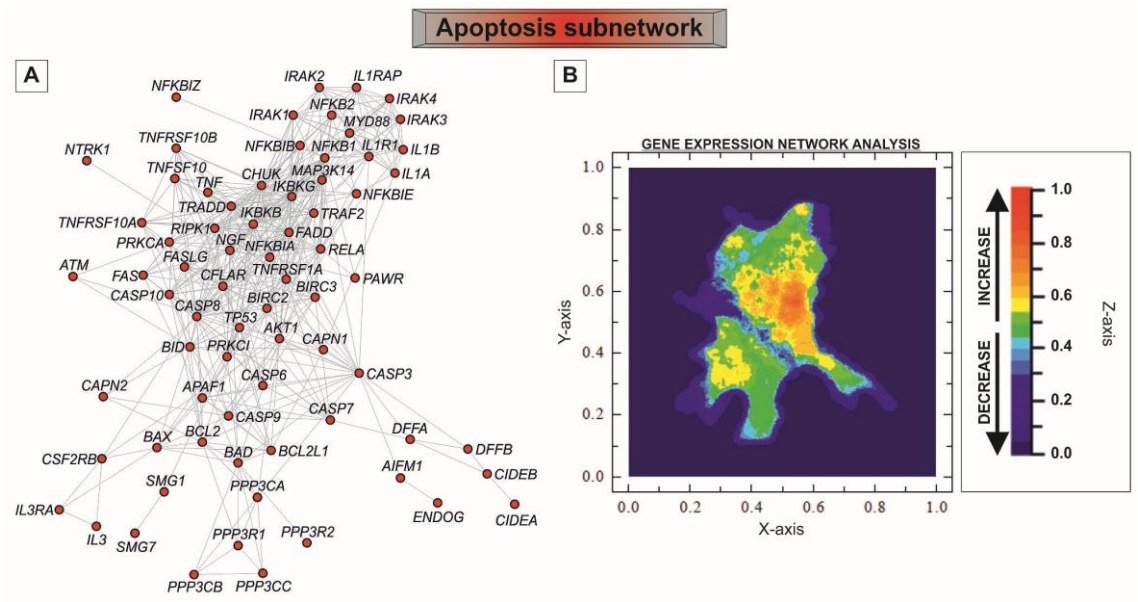
Supplementary Figures



Supplementary figure 1



Supplementary figure 2



Supplementary figure 3

Tables

Table 1. Differentially expressed genes from the NOTCH, WNT, Alzheimer´s disease, and apoptosis subnetworks in the cerebellum of autistic patients. Corrected *p*-values <0.05 were considered significant. "NOT", "WNT", "AD", and "APO" represent NOTCH, WNT, Alzheimer´s disease, and apoptosis subnetworks, respectively.

Gene symbol	**Alias and/or description**	**Ensembl ID** (ENSP)	**Differential gene expression** (UP/DOWN)	**Corrected *p*-value** (FDR)	**Subnetwork contribution/s**
UQCRC1	Ubiquinol-cytochrome c reductase core protein I	ENSP00000203407	**DOWN**	0.001062657	**AD**
NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)	ENSP00000263774	**DOWN**	0.001722868	**AD**
NDUFA9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9, 39kDa	ENSP00000266544	**DOWN**	0.00179604	**AD**
JUN	Jun oncogene	ENSP00000360266	**UP**	0.001921098	**WNT**
CIDEB	Cell death-inducing DFFA-like effector b	ENSP00000258807	**DOWN**	0.001949191	**APO**
CDC42	Cell division cycle 42 (GTP binding protein, 25kDa)	ENSP00000314458	**DOWN**	0.002000135	**WNT**
NDUFA6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14kDa	ENSP00000330937	**DOWN**	0.002033295	**AD**
NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa	ENSP00000215565	**DOWN**	0.002205663	**AD**
NDUFA1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5kDa	ENSP00000360492	**DOWN**	0.002205663	**AD**

COX4I1	Cytochrome c oxidase subunit IV isoform 1	ENSP00000253452	**DOWN**	0.002246413	**AD**
NCSTN	Nicastrin	ENSP00000294785	**UP**	0.002354256	**AD NOT**
UQCRC2	Ubiquinol-cytochrome c reductase core protein II	ENSP00000268379	**DOWN**	0.002354256	**AD**
NDUFS4	NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase)	ENSP00000296684	**DOWN**	0.002459308	**AD**
CYC1	Cytochrome c-1	ENSP00000317159	**DOWN**	0.002656014	**AD**
COX8A	Cytochrome c oxidase subunit 8A (ubiquitous)	ENSP00000321260	**DOWN**	0.002656014	**AD**
NDUFB4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4, 15kDa	ENSP00000184266	**DOWN**	0.002688877	**AD**
EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	ENSP00000233057	**UP**	0.002850857	**AD**
BID	BH3 interacting domain death agonist	ENSP00000318822	**DOWN**	0.002892499	**AD APO**
NDUFA4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4, 9kDa	ENSP00000339720	**DOWN**	0.003092192	**AD**
WNT3	Wingless-type MMTV integration site family, member 3	ENSP00000225512	**UP**	0.003122302	**WNT**
NDUFAB1	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, 8kDa	ENSP00000007516	**DOWN**	0.003262718	**AD**
NDUFA11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 11, 14.7kDa	ENSP00000311740	**DOWN**	0.003262718	**AD**
COX5A	Cytochrome c oxidase subunit Va	ENSP00000317780	**DOWN**	0.003530677	**AD**
PRKCA	Protein kinase C, alpha	ENSP00000284384	**UP**	0.003532751	**APO WNT**
NDUFB9	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9, 22kDa	ENSP00000276689	**DOWN**	0.003532751	**AD**
NDUFA2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8kDa	ENSP00000252102	**DOWN**	0.003622328	**AD**
COX5B	Cytochrome c oxidase subunit Vb	ENSP00000258424	**DOWN**	0.003627494	**AD**
BCL2	B-cell CLL/lymphoma 2	ENSP00000329623	**UP**	0.003686548	**APO**
PRICKLE2	Prickle homolog 2 (Drosophila)	ENSP00000295902	**UP**	0.003858642	**WNT**
SDHB	Succinate dehydrogenase complex, subunit B, iron	ENSP00000364649	**DOWN**	0.003949261	**AD**

	sulfur (Ip)				
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa	ENSP00000252711	**DOWN**	0.004023111	**AD**
NDUFB2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2, 8kDa	ENSP00000247866	**DOWN**	0.00417954	**AD**
NDUFB6	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6, 17kDa	ENSP00000369176	**DOWN**	0.00420016	**AD**
NDUFA7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7, 14.5kDa	ENSP00000301457	**DOWN**	0.004449939	**AD**
NDUFB3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa	ENSP00000237889	**DOWN**	0.004449939	**AD**
UQCRB	Ubiquinol-cytochrome c reductase binding protein	ENSP00000287022	**DOWN**	0.004918915	**AD**
NCOR2	Nuclear receptor co-repressor 2	ENSP00000348551	**UP**	0.005223194	**NOT**
LRP5	Low density lipoprotein receptor-related protein 5	ENSP00000294304	**UP**	0.005717875	**WNT**
BAD	BCL2-associated agonist of cell death	ENSP00000309103	**DOWN**	0.005763042	**AD APO**
UQCRH	Ubiquinol-cytochrome c reductase hinge protein	ENSP00000309565	**DOWN**	0.005906155	**AD**
PSEN2	Presenilin 2 (Alzheimer disease 4)	ENSP00000355747	**DOWN**	0.005968353	**AD NOT**
NDUFA13	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13	ENSP00000380364	**DOWN**	0.00609761	**AD**
NDUFB10	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa	ENSP00000268668	**DOWN**	0.006262982	**AD**
COX6C	Cytochrome c oxidase subunit VIc	ENSP00000297564	**DOWN**	0.006681564	**AD**
UQCRQ	Ubiquinol-cytochrome c reductase, complex III subunit VII, 9.5kDa	ENSP00000367934	**DOWN**	0.00745614	**AD**
NDUFA3	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3, 9kDa	ENSP00000398290	**DOWN**	0.007600014	**AD**
HEY1	Hairy/enhancer-of-split related with YRPW motif 1	ENSP00000338272	**DOWN**	0.00784934	**NOT**
DAAM1	Dishevelled associated activator of morphogenesis 1	ENSP00000247170	**UP**	0.007909188	**WNT**
PPP3CC	Protein phosphatase 3 (formerly 2B), catalytic	ENSP00000240139	**DOWN**	0.008118636	**AD APO WNT**

	subunit, gamma isoform				
COX7B	Cytochrome c oxidase subunit VIIb	ENSP00000417656	**DOWN**	0.008265168	**AD**
NDUFB11	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11, 17.3kDa	ENSP00000276062	**DOWN**	0.008503556	**AD**
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	ENSP00000226574	**UP**	0.008570685	**APO**
KAT2B	K(lysine) acetyltransferase 2B	ENSP00000263754	**UP**	0.009111103	**NOT**
NDUFA12	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12	ENSP00000330737	**DOWN**	0.009479959	**AD**
UQCRFS1	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	ENSP00000306397	**DOWN**	0.009524703	**AD**
NDUFS5	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa (NADH-coenzyme Q reductase)	ENSP00000362058	**DOWN**	0.009998423	**AD**
BAX	BCL2-associated X protein	ENSP00000293288	**DOWN**	0.010349538	**APO**
CYCS	Cytochrome c, somatic	ENSP00000307786	**DOWN**	0.010843806	**AD**
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	ENSP00000322450	**DOWN**	0.011017543	**AD**
RBX1	Ring-box 1	ENSP00000216225	**DOWN**	0.01185897	**WNT**
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	ENSP00000216797	**UP**	0.012804007	**AD APO**
AXIN2	Axin 2	ENSP00000302625	**UP**	0.01310984	**WNT**
CSNK1G1	Casein kinase 1, gamma 1	ENSP00000305777	**UP**	0.013381084	**WNT**
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)	ENSP00000315774	**DOWN**	0.014197263	**AD**
HSD17B10	Hydroxysteroid (17-beta) dehydrogenase 10	ENSP00000168216	**DOWN**	0.014254764	**AD**
RAC3	Ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	ENSP00000304283	**DOWN**	0.014704894	**WNT**
DTX3	Deltex homolog 3 (Drosophila)	ENSP00000338050	**DOWN**	0.015144277	**NOT**
CDK5	Cyclin-dependent kinase 5	ENSP00000297518	**DOWN**	0.015567567	**AD**
CABIN1	Calcineurin binding protein 1	ENSP00000263119	**UP**	0.015953582	**AD WNT**

COX7C	Cytochrome c oxidase subunit VIIc	ENSP00000247655	**DOWN**	0.016686515	**AD**
RUVBL1	RuvB-like 1 (E. coli)	ENSP00000318297	**DOWN**	0.016765972	**WNT**
DFFA	DNA fragmentation factor, 45kDa, alpha polypeptide	ENSP00000366237	**UP**	0.017000844	**APO**
NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa	ENSP00000299166	**DOWN**	0.01782766	**AD**
COX6B1	Cytochrome c oxidase subunit VIb polypeptide 1 (ubiquitous)	ENSP00000246554	**DOWN**	0.017845204	**AD**
TLE1	Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	ENSP00000365682	**UP**	0.018030294	**NOT**
NOTCH1	Notch homolog 1	ENSP00000277541	**UP**	0.018222797	**AD NOT**
RYR2	Ryanodine receptor 2 (cardiac)	ENSP00000355533	**UP**	0.019085894	**AD**
CACYBP	Calcyclin binding protein	ENSP00000356652	**DOWN**	0.02050623	**WNT**
CSNK2B	Casein kinase 2, beta polypeptide	ENSP00000415615	**DOWN**	0.02050623	**WNT**
SENP2	SUMO1/sentrin/SMT3 specific peptidase 2	ENSP00000296257	**UP**	0.021603075	**WNT**
CHD8	Chromodomain helicase DNA binding protein 8	ENSP00000406288	**UP**	0.021898019	**WNT**
DTX2	Deltex homolog 2 (Drosophila)	ENSP00000322885	**UP**	0.022277856	**NOT**
SDHC	succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa	ENSP00000356953	**DOWN**	0.023024554	**AD**
LRP1	Prolow-density lipoprotein receptor-related protein 1 Precursor (LRP)	ENSP00000243077	**UP**	0.023159082	**AD**
DLL1	Delta-like 1 (Drosophila)	ENSP00000355718	**UP**	0.023184387	**NOT**
APBB1	Amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)	ENSP00000299402	**DOWN**	0.024237411	**AD**
MAP3K7	mitogen-activated protein kinase kinase kinase 7	ENSP00000358335	**DOWN**	0.02453243	**WNT**
NDUFC2	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2, 14.5kDa	ENSP00000281031	**DOWN**	0.025489098	**AD**
APLP1	Amyloid beta (A4) precursor-like protein 1	ENSP00000221891	**UP**	0.026382742	**AD**
BIRC2	Baculoviral IAP repeat-containing 2	ENSP00000227758	**DOWN**	0.026521867	**APO**

NDUFB5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16kDa	ENSP00000259037	**DOWN**	0.027921676	**AD**
PPARD	Peroxisome proliferator-activated receptor delta	ENSP00000310928	**UP**	0.029747209	**WNT**
SKP1	S-phase kinase-associated protein 1	ENSP00000231487	**DOWN**	0.03098415	**WNT**
PRKCB	Protein kinase C, beta	ENSP00000305355	**DOWN**	0.031488508	**WNT**
CTNNBIP1	Catenin, beta interacting protein 1	ENSP00000366466	**DOWN**	0.031553064	**WNT**
RCAN1	Regulator of calcineurin 1	ENSP00000320768	**DOWN**	0.031896183	**AD**
AIFM1	Apoptosis-inducing factor, mitochondrion-associated, 1	ENSP00000287295	**DOWN**	0.031972517	**APO**
CALM2	Calmodulin 2 (phosphorylase kinase, delta)	ENSP00000272298	**DOWN**	0.032263251	**AD**
CAMK2D	Calcium/calmodulin-dependent protein kinase II delta	ENSP00000339740	**UP**	0.034576294	**WNT**
SMG7	Smg-7 homolog, nonsense mediated mRNA decay factor (C. elegans)	ENSP00000340766	**UP**	0.034586995	**APO**
TBL1X	Transducin (beta)-like 1X-linked	ENSP00000217964	**UP**	0.036511455	**WNT**
CASP3	Caspase 3, apoptosis-related cysteine peptidase	ENSP00000311032	**UP**	0.037829198	**AD APO**
FRAT2	Frequently rearranged in advanced T-cell lymphomas 2	ENSP00000360058	**UP**	0.037835094	**WNT**
TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)	ENSP00000358404	**UP**	0.03801246	**WNT**
PRICKLE1	Prickle homolog 1 (Drosophila)	ENSP00000345064	**UP**	0.038401928	**WNT**
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	ENSP00000382423	**UP**	0.038406592	**AD**
MAML1	Mastermind-like 1 (Drosophila)	ENSP00000292599	**UP**	0.038709571	**NOT**
GRIN1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	ENSP00000360616	**UP**	0.03916101	**AD**
SNCA	Synuclein, alpha (non A4 component of amyloid precursor)	ENSP00000338345	**DOWN**	0.040205801	**AD**
IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	ENSP00000358622	**DOWN**	0.04087009	**APO**
PRKCZ	Protein kinase C, zeta	ENSP00000367830	**DOWN**	0.041055303	**WNT**

EP300	MicroRNA 1281	ENSP00000263253	**UP**	0.041373019	**NOT WNT**
ENDOG	Endonuclease G	ENSP00000361725	**DOWN**	0.042737537	**APO**
PPP3CB	Protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	ENSP00000378306	**DOWN**	0.046120071	**AD APO WNT**
BCL2L1	BCL2-like 1	ENSP00000302564	**UP**	0.046646273	**APO**
NDUFV2	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa	ENSP00000327268	**DOWN**	0.046908433	**AD**

Supplementary tables

Supplementary table S1.

Gene symbol	**Alias and/or description**	**Ensembl ID** (ENSP)
ADAM17	ADAM metallopeptidase domain 17	ENSP00000309968
CIR1	Corepressor interacting with RBPJ, 1	ENSP00000339723
DLK1	Delta-like 1 homolog (Drosophila)	ENSP00000340292
DLL1	Delta-like 1 (Drosophila)	ENSP00000355718
DLL3	Delta-like 3 (Drosophila)	ENSP00000205143
DLL4	Delta-like 4 (Drosophila)	ENSP00000249749
DTX1	Deltex homolog 1 (Drosophila)	ENSP00000257600
DTX2	Deltex homolog 2 (Drosophila)	ENSP00000322885
DTX3	Deltex homolog 3 (Drosophila)	ENSP00000338050
DTX4	Deltex homolog 4 (Drosophila)	ENSP00000227451
DVL1	Dishevelled, dsh homolog 1 (Drosophila)	ENSP00000368169
EP300	MicroRNA 1281	ENSP00000263253
HDAC1	Histone deacetylase 1	ENSP00000362649
HDAC2	Histone deacetylase 2	ENSP00000381331
HES1	Hairy and enhancer of split 1, (Drosophila)	ENSP00000232424
HES5	Hairy and enhancer of split 5 (Drosophila)	ENSP00000367714
HEY1	Hairy/enhancer-of-split related with YRPW motif 1	ENSP00000338272
HR	Hairless homolog (mouse)	ENSP00000370826
JAG1	Jagged 1 (Alagille syndrome)	ENSP00000254958
JAG2	Jagged 2	ENSP00000328169
KAT2A	K(lysine) acetyltransferase 2A	ENSP00000225916
KAT2B	K(lysine) acetyltransferase 2B	ENSP00000263754
LFNG	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	ENSP00000222725
MAML1	Mastermind-like 1 (Drosophila)	ENSP00000292599
MAML2	Mastermind-like 2 (Drosophila)	ENSP00000412394
MAML3	Mastermind-like 3 (Drosophila)	ENSP00000408478
MFNG	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	ENSP00000349490
NCOR2	Nuclear receptor co-repressor 2	ENSP00000348551
NCSTN	Nicastrin	ENSP00000294785
NOTCH1	Notch homolog 1, translocation-associated (Drosophila)	ENSP00000277541
NUMBL	Numb homolog (Drosophila)-like	ENSP00000252891
PSEN2	Presenilin 2 (Alzheimer disease 4)	ENSP00000355747
PSENEN	Presenilin enhancer 2 homolog (C. elegans)	ENSP00000222266
PTCRA	Pre T-cell antigen receptor alpha	ENSP00000304447
RBPJ	Recombination signal binding protein for immunoglobulin kappa J region	ENSP00000345206
RFNG	RFNG O-fucosylpeptide 3-beta-N-	ENSP00000307971

	acetylglucosaminyltransferase	
SNW1	SNW domain containing 1	ENSP00000261531
TLE1	Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	ENSP00000365682
TLE2	Transducin-like enhancer of split 2 (E(sp1) homolog, Drosophila)	ENSP00000262953
TLE3	Transducin-like enhancer of split 3 (E(sp1) homolog, Drosophila)	ENSP00000319233
TLE4	Transducin-like enhancer of split 4 (E(sp1) homolog, Drosophila)	ENSP00000365735

Supplementary table S2.

Gene symbol	Alias and/or description	Ensembl ID (ENSP)
APC	Adenomatous polyposis coli	ENSP00000257430
AXIN1	Axin 1	ENSP00000262320
AXIN2	Axin 2	ENSP00000302625
BAMBI	BMP and activin membrane-bound inhibitor homolog (Xenopus laevis)	ENSP00000364683
BTRC	Beta-transducin repeat containing	ENSP00000359206
CABIN1	Calcineurin binding protein 1	ENSP00000263119
CACYBP	Calcyclin binding protein	ENSP00000356652
CAMK2A	Calcium/calmodulin-dependent protein kinase II alpha	ENSP00000381412
CAMK2B	Calcium/calmodulin-dependent protein kinase II beta	ENSP00000258682
CAMK2D	Calcium/calmodulin-dependent protein kinase II delta	ENSP00000339740
CAMK2G	Calcium/calmodulin-dependent protein kinase II gamma	ENSP00000319060
CCND1	Cyclin D1	ENSP00000227507
CCND2	Cyclin D2	ENSP00000261254
CCND3	Cyclin D3	ENSP00000362082
CDC42	Cell division cycle 42 (GTP binding protein, 25kDa)	ENSP00000314458
CER1	Cerberus 1, cysteine knot superfamily, homolog (Xenopus laevis)	ENSP00000370297
CHD8	Chromodomain helicase DNA binding protein 8	ENSP00000406288
CSNK1A1	Casein kinase 1, alpha 1	ENSP00000261798
CSNK1A1L	Casein kinase 1, alpha 1-like	ENSP00000369126
CSNK1D	Casein kinase 1, delta	ENSP00000324464
CSNK1E	Casein kinase 1, epsilon	ENSP00000352929
CSNK1G1	Casein kinase 1, gamma 1	ENSP00000305777
CSNK1G2	Casein kinase 1, gamma 2	ENSP00000255641
CSNK1G3	Casein kinase 1, gamma 3	ENSP00000353904
CSNK2A1	Casein kinase 2, alpha 1 polypeptide pseudogene	ENSP00000217244
CSNK2A2	Casein kinase 2, alpha prime polypeptide	ENSP00000262506
CSNK2B	Casein kinase 2, beta polypeptide	ENSP00000415615
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	ENSP00000344456
CTNNBIP1	Catenin, beta interacting protein 1	ENSP00000366466
CUL1	Cullin 1	ENSP00000326804
DAAM1	Dishevelled associated activator of morphogenesis 1	ENSP00000247170
DKK1	Dickkopf homolog 1 (Xenopus laevis)	ENSP00000363081
DKK2	Dickkopf homolog 2 (Xenopus laevis)	ENSP00000285311
DKK4	Dickkopf homolog 4 (Xenopus laevis)	ENSP00000220812

DVL1	Dishevelled, dsh homolog 1 (Drosophila)	ENSP00000368169
DVL2	Dishevelled, dsh homolog 2 (Drosophila)	ENSP00000005340
DVL3	Dishevelled, dsh homolog 3 (Drosophila)	ENSP00000316054
EP300	MicroRNA 1281	ENSP00000263253
FOSL1	FOS-like antigen 1	ENSP00000310170
FRAT1	Frequently rearranged in advanced T-cell lymphomas 1	ENSP00000360060
FRAT2	Frequently rearranged in advanced T-cell lymphomas 2	ENSP00000360058
FZD1	Frizzled homolog 1 (Drosophila)	ENSP00000287934
FZD10	Frizzled homolog 10 (Drosophila)	ENSP00000229030
FZD2	Frizzled homolog 2 (Drosophila)	ENSP00000323901
FZD3	Frizzled homolog 3 (Drosophila)	ENSP00000240093
FZD4	Frizzled homolog 4 (Drosophila)	ENSP00000311581
FZD5	Frizzled homolog 5 (Drosophila)	ENSP00000354607
FZD6	Frizzled homolog 6 (Drosophila)	ENSP00000351605
FZD7	Frizzled homolog 7 (Drosophila)	ENSP00000286201
FZD8	Frizzled homolog 8 (Drosophila)	ENSP00000363826
FZD9	Frizzled homolog 9 (Drosophila)	ENSP00000345785
GSK3B	Glycogen synthase kinase 3 beta	ENSP00000324806
JUN	Jun oncogene	ENSP00000360266
LEF1	Lymphoid enhancer-binding factor 1	ENSP00000265165
LRP5	Low density lipoprotein receptor-related protein 5	ENSP00000294304
LRP6	Low density lipoprotein receptor-related protein 6	ENSP00000261349
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	ENSP00000358335
MAPK8	Mitogen-activated protein kinase 8	ENSP00000353483
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	ENSP00000219070
MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	ENSP00000260227
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	ENSP00000361405
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	ENSP00000367207
MYCBP	c-myc binding protein	ENSP00000380702
NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	ENSP00000327850
NKD1	Naked cuticle homolog 1 (Drosophila)	ENSP00000268459
NKD2	Naked cuticle homolog 2 (Drosophila)	ENSP00000296849
NLK	Nemo-like kinase	ENSP00000384625
PHF17	PHD finger protein 17	ENSP00000226319
PLCB1	Phospholipase C, beta 1 (phosphoinositide-specific)	ENSP00000338185
PLCB2	Phospholipase C, beta 2	ENSP00000260402
PLCB3	Phospholipase C, beta 3 (phosphatidylinositol-specific)	ENSP00000279230
PLCB4	Phospholipase C, beta 4	ENSP00000334105
PORCN	Porcupine homolog (Drosophila)	ENSP00000322304
PPARD	Peroxisome proliferator-activated receptor delta	ENSP00000310928
PPP1R1B	Protein phosphatase 1, regulatory (inhibitor)	ENSP00000254079

	subunit 1B	
PPP3CA	Protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	ENSP00000378323
PPP3CB	Protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	ENSP00000378306
PPP3CC	Protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform	ENSP00000240139
PPP3R1	Protein phosphatase 3 (formerly 2B), regulatory subunit B, alpha isoform	ENSP00000234310
PPP3R2	Protein phosphatase 3 (formerly 2B), regulatory subunit B, beta isoform	ENSP00000363939
PRICKLE1	Prickle homolog 1 (Drosophila)	ENSP00000345064
PRICKLE2	Prickle homolog 2 (Drosophila)	ENSP00000295902
PRKCA	Protein kinase C, alpha	ENSP00000284384
PRKCB	Protein kinase C, beta	ENSP00000305355
PRKCD	Protein kinase C, delta	ENSP00000331602
PRKCE	Protein kinase C, epsilon	ENSP00000306124
PRKCG	Protein kinase C, gamma	ENSP00000263431
PRKCH	Protein kinase C, eta	ENSP00000329127
PRKCI	Protein kinase C, iota	ENSP00000295797
PRKCQ	Protein kinase C, theta	ENSP00000263125
PRKCZ	Protein kinase C, zeta	ENSP00000367830
PSEN1	Presenilin 1	ENSP00000326366
RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	ENSP00000348461
RAC2	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	ENSP00000249071
RAC3	Ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	ENSP00000304283
RBX1	Ring-box 1	ENSP00000216225
RHOA	Ras homolog gene family, member A	ENSP00000400175
RUVBL1	RuvB-like 1 (E. coli)	ENSP00000318297
SENP2	SUMO1/sentrin/SMT3 specific peptidase 2	ENSP00000296257
SFRP1	Secreted frizzled-related protein 1	ENSP00000220772
SFRP2	Secreted frizzled-related protein 2	ENSP00000274063
SFRP4	Secreted frizzled-related protein 4	ENSP00000410715
SFRP5	Secreted frizzled-related protein 5	ENSP00000266066
SIAH1	Seven in absentia homolog 1 (Drosophila)	ENSP00000349156
SKP1	S-phase kinase-associated protein 1	ENSP00000231487
SMAD4	SMAD family member 4	ENSP00000341551
SMG1	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase (C. elegans)	ENSP00000402515
SOX17	SRY (sex determining region Y)-box 17	ENSP00000297316
TBL1X	Transducin (beta)-like 1X-linked	ENSP00000217964
TBL1Y	Transducin (beta)-like 1, Y-linked	ENSP00000328879
TCF7	Transcription factor 7 (T-cell specific, HMG-box)	ENSP00000340347
TCF7L1	Transcription factor 7-like 1 (T-cell specific, HMG-box)	ENSP00000282111
TCF7L2	Transcription factor 7-like 2 (T-cell specific,	ENSP00000358404

	HMG-box)	
TP53	Tumor protein p53	ENSP00000269305
VANGL1	Vang-like 1 (van gogh, Drosophila)	ENSP00000310800
VANGL2	Vang-like 2 (van gogh, Drosophila)	ENSP00000357040
WIF1	WNT inhibitory factor 1	ENSP00000286574
WNT1	Wingless-type MMTV integration site family, member 1	ENSP00000293549
WNT10A	Wingless-type MMTV integration site family, member 10A	ENSP00000258411
WNT10B	Wingless-type MMTV integration site family, member 10B	ENSP00000301061
WNT11	Wingless-type MMTV integration site family, member 11	ENSP00000325526
WNT16	Wingless-type MMTV integration site family, member 16	ENSP00000222462
WNT2	Wingless-type MMTV integration site family member 2	ENSP00000265441
WNT2B	Wingless-type MMTV integration site family, member 2B	ENSP00000358698
WNT3	Wingless-type MMTV integration site family, member 3	ENSP00000225512
WNT3A	Wingless-type MMTV integration site family, member 3A	ENSP00000284523
WNT4	Wingless-type MMTV integration site family, member 4	ENSP00000290167
WNT5A	Wingless-type MMTV integration site family, member 5A	ENSP00000264634
WNT5B	Wingless-type MMTV integration site family, member 5B	ENSP00000308887
WNT6	Wingless-type MMTV integration site family, member 6	ENSP00000233948
WNT7A	Wingless-type MMTV integration site family, member 7A	ENSP00000285018
WNT7B	Wingless-type MMTV integration site family, member 7B	ENSP00000341032
WNT8A	Wingless-type MMTV integration site family, member 8A	ENSP00000381739
WNT8B	Wingless-type MMTV integration site family, member 8B	ENSP00000340677
WNT9A	Wingless-type MMTV integration site family, member 9A	ENSP00000272164
WNT9B	Wingless-type MMTV integration site family, member 9B	ENSP00000290015

Supplementary table S3.

Gene symbol	Alias and/or description	Ensembl ID (ENSP)
ADAM10	ADAM metallopeptidase domain 10	ENSP00000260408
ADAM17	ADAM metallopeptidase domain 17	ENSP00000309968
AKT1	v-akt murine thymoma viral oncogene homolog 1	ENSP00000270202
APAF1	Apoptotic peptidase activating factor 1	ENSP00000353059
APBA1	Amyloid beta (A4) precursor protein-binding, family A, member 1	ENSP00000265381
APBA2	Amyloid beta (A4) precursor protein-binding, family A, member 2	ENSP00000219865
APBA3	Amyloid beta (A4) precursor protein-binding, family A, member 3	ENSP00000315136
APBB1	Amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)	ENSP00000299402
APBB1IP	Amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein	ENSP00000365411
APBB2	Amyloid beta (A4) precursor protein-binding, family B, member 2	ENSP00000295974
APBB3	Amyloid beta (A4) precursor protein-binding, family B, member 3	ENSP00000346378
APH1A	Anterior pharynx defective 1 homolog A (C. elegans)	ENSP00000358105
APH1B	Anterior pharynx defective 1 homolog B (C. elegans)	ENSP00000261879
APLP1	Amyloid beta (A4) precursor-like protein 1	ENSP00000221891
APLP2	Amyloid beta (A4) precursor-like protein 2	ENSP00000263574
APOE	Apolipoprotein E	ENSP00000252486
APP	Amyloid beta (A4) precursor protein	ENSP00000284981
APPBP2	Amyloid beta precursor protein (cytoplasmic tail) binding protein 2	ENSP00000083182
ATF6	Activating transcription factor 6	ENSP00000356919
ATP2A1	ATPase, Ca^{++} transporting, cardiac muscle, fast twitch 1	ENSP00000349595
BACE1	Beta-site APP-cleaving enzyme 1	ENSP00000318585
BACE2	Beta-site APP-cleaving enzyme 2	ENSP00000332979
BAD	BCL2-associated agonist of cell death	ENSP00000309103
BID	BH3 interacting domain death agonist	ENSP00000318822
CABIN1	Calcineurin binding protein 1	ENSP00000263119
CACNA1C	Calcium channel, voltage-dependent, L type, alpha 1C subunit	ENSP00000266376
CACNA1D	Calcium channel, voltage-dependent, L type, alpha 1D subunit	ENSP00000288139
CACNA1F	Calcium channel, voltage-dependent, L type, alpha 1F subunit	ENSP00000365441
CACNA1S	Calcium channel, voltage-dependent, L type, alpha 1S subunit	ENSP00000355192

CALM1	Calmodulin 1	ENSP00000349467
CALM2	Calmodulin 2	ENSP00000272298
CALM3	Calmodulin 3	ENSP00000291295
CAPN1	Calpain 1, (mu/I) large subunit	ENSP00000279247
CAPN2	Calpain 2, (m/II) large subunit	ENSP00000295006
CASP3	Caspase 3, apoptosis-related cysteine peptidase	ENSP00000311032
CASP7	Caspase 7, apoptosis-related cysteine peptidase	ENSP00000298700
CASP8	Caspase 8, apoptosis-related cysteine peptidase	ENSP00000351273
CASP9	Caspase 9, apoptosis-related cysteine peptidase	ENSP00000330237
CDK5	Cyclin-dependent kinase 5	ENSP00000297518
CDK5R1	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	ENSP00000318486
CHRNA2	Cholinergic receptor, nicotinic, alpha 2 (neuronal)	ENSP00000240132
CHRNA3	Cholinergic receptor, nicotinic, alpha 3	ENSP00000315602
CHRNA4	Cholinergic receptor, nicotinic, alpha 4	ENSP00000359285
CHRNA5	Cholinergic receptor, nicotinic, alpha 5	ENSP00000299565
CHRNA6	Cholinergic receptor, nicotinic, alpha 6	ENSP00000276410
CHRNA7	Cholinergic receptor, nicotinic, alpha 7	ENSP00000303727
CHRNB2	Cholinergic receptor, nicotinic, beta 2 (neuronal)	ENSP00000357461
CHRNB3	Cholinergic receptor, nicotinic, beta 3	ENSP00000289957
CHRNB4	Cholinergic receptor, nicotinic, beta 4	ENSP00000261751
CIB1	Calcium and integrin binding 1 (calmyrin)	ENSP00000333873
COX4I1	Cytochrome c oxidase subunit IV isoform 1	ENSP00000253452
COX5A	Cytochrome c oxidase subunit Va	ENSP00000317780
COX5B	Cytochrome c oxidase subunit Vb	ENSP00000258424
COX6B1	Cytochrome c oxidase subunit VIb polypeptide 1 (ubiquitous)	ENSP00000246554
COX6C	Cytochrome c oxidase subunit VIc	ENSP00000297564
COX7B	Cytochrome c oxidase subunit VIIb	ENSP00000417656
COX7C	Cytochrome c oxidase subunit VIIc	ENSP00000247655
COX8A	Cytochrome c oxidase subunit 8A (ubiquitous)	ENSP00000321260
CSNK2A1	Casein kinase 2, alpha 1 polypeptide pseudogene	ENSP00000217244
CYC1	Cytochrome c-1	ENSP00000317159
CYCS	Cytochrome c, somatic	ENSP00000307786
EIF2AK1	Eukaryotic translation initiation factor 2-alpha kinase 1	ENSP00000199389
EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	ENSP00000233057
EIF2AK3	Eukaryotic translation initiation factor 2-alpha kinase 3	ENSP00000307235
EIF2AK4	Eukaryotic translation initiation factor 2 alpha kinase 4	ENSP00000263791
ERN1	Endoplasmic reticulum to nucleus signaling 1	ENSP00000401445
FADD	Fas (TNFRSF6)-associated via death domain	ENSP00000301838
FAS	Fas (TNF receptor superfamily, member 6)	ENSP00000347979
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	ENSP00000229239
GNAQ	Guanine nucleotide binding protein (G protein), q polypeptide	ENSP00000286548

GRIN1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	ENSP00000360616
GRIN2A	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	ENSP00000332549
GRIN2B	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	ENSP00000279593
GRIN2C	Glutamate receptor, ionotropic, N-methyl D-aspartate 2C	ENSP00000293190
GRIN2D	Glutamate receptor, ionotropic, N-methyl D-aspartate 2D	ENSP00000263269
GSK3B	Glycogen synthase kinase 3 beta	ENSP00000324806
HSD17B10	Hydroxysteroid (17-beta) dehydrogenase 10	ENSP00000168216
IDE	Insulin-degrading enzyme	ENSP00000265986
IL1B	Interleukin 1, beta	ENSP00000263341
ITPR1	Inositol 1,4,5-triphosphate receptor, type 1	ENSP00000405934
ITPR2	Inositol 1,4,5-triphosphate receptor, type 2	ENSP00000370744
ITPR3	Inositol 1,4,5-triphosphate receptor, type 3	ENSP00000363435
KFYI	NADH dehydrogenase [ubiquinone] 1 subunit C1, mitochondrial Precursor (NADH-ubiquinone oxidoreductase KFYI subunit)(Complex I-KFYI)(CI-KFYI)	ENSP00000377770
LPL	Lipoprotein lipase	ENSP00000309757
LRP1	Prolow-density lipoprotein receptor-related protein 1 Precursor (LRP)	ENSP00000243077
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	ENSP00000382423
MAPKAPK2	Mitogen-activated protein kinase-activated protein kinase 2	ENSP00000356070
MAPT	Microtubule-associated protein tau	ENSP00000340820
MME	Membrane metallo-endopeptidase	ENSP00000353679
MT-CO1	Cytochrome c oxidase subunit 1 (EC 1.9.3.1)(Cytochrome c oxidase polypeptide I)	ENSP00000354499
MT-CO2	Mitochondrially encoded cytochrome c oxidase II	ENSP00000354876
MT-CO3	Cytochrome c oxidase subunit 3 (Cytochrome c oxidase polypeptide III)	ENSP00000354982
MT-CYB	Mitochondrially encoded cytochrome b	ENSP00000354554
NCSTN	Nicastrin	ENSP00000294785
NDUFA1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1	ENSP00000360492
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10	ENSP00000252711
NDUFA11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 11	ENSP00000311740
NDUFA12	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12	ENSP00000330737
NDUFA13	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13	ENSP00000380364
NDUFA2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2	ENSP00000252102
NDUFA3	NADH dehydrogenase (ubiquinone) 1 alpha	ENSP00000398290

	subcomplex, 3	
NDUFA4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4	ENSP00000339720
NDUFA5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5	ENSP00000347988
NDUFA6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6	ENSP00000330937
NDUFA7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7	ENSP00000301457
NDUFA8	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8	ENSP00000362873
NDUFA9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9	ENSP00000266544
NDUFAB1	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1	ENSP00000007516
NDUFB1	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 1	ENSP00000330787
NDUFB10	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10	ENSP00000268668
NDUFB11	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11	ENSP00000276062
NDUFB2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2	ENSP00000247866
NDUFB3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3	ENSP00000237889
NDUFB4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4	ENSP00000184266
NDUFB5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5	ENSP00000259037
NDUFB6	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6	ENSP00000369176
NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7	ENSP00000215565
NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8	ENSP00000299166
NDUFB9	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9	ENSP00000276689
NDUFC2	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2	ENSP00000281031
NDUFS1	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa (NADH-coenzyme Q reductase)	ENSP00000233190
NDUFS2	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase)	ENSP00000356972
NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)	ENSP00000263774
NDUFS4	NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase)	ENSP00000296684
NDUFS5	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa (NADH-coenzyme Q reductase)	ENSP00000362058

NDUFS6	NADH dehydrogenase (ubiquinone) Fe-S protein 6, 13kDa (NADH-coenzyme Q reductase)	ENSP00000274137
NDUFS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20kDa (NADH-coenzyme Q reductase)	ENSP00000233627
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)	ENSP00000315774
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1	ENSP00000322450
NDUFV2	NADH dehydrogenase (ubiquinone) flavoprotein 2	ENSP00000327268
NDUFV3	NADH dehydrogenase (ubiquinone) flavoprotein 3	ENSP00000346196
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	ENSP00000216797
NOS1	Nitric oxide synthase 1 (neuronal)	ENSP00000320758
NOTCH1	Notch homolog 1, translocation-associated (Drosophila)	ENSP00000277541
PLCB1	Phospholipase C, beta 1 (phosphoinositide-specific)	ENSP00000338185
PLCB2	Phospholipase C, beta 2	ENSP00000260402
PLCB3	Phospholipase C, beta 3 (phosphatidylinositol-specific)	ENSP00000279230
PLCB4	Phospholipase C, beta 4	ENSP00000334105
PLCG1	Phospholipase C, gamma 1	ENSP00000244007
PPP1R1A	Protein phosphatase 1, regulatory (inhibitor) subunit 1A	ENSP00000257905
PPP1R1B	Protein phosphatase 1, regulatory (inhibitor) subunit 1B	ENSP00000254079
PPP3CA	Protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	ENSP00000378323
PPP3CB	Protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	ENSP00000378306
PPP3CC	Protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform	ENSP00000240139
PPP3R1	Protein phosphatase 3 (formerly 2B), regulatory subunit B, alpha isoform	ENSP00000234310
PSEN1	Presenilin 1	ENSP00000326366
PSEN2	Presenilin 2 (Alzheimer disease 4)	ENSP00000355747
PSENEN	Presenilin enhancer 2 homolog (C. elegans)	ENSP00000222266
RCAN1	Regulator of calcineurin 1	ENSP00000320768
RCAN2	Regulator of calcineurin 2	ENSP00000329454
RELA	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	ENSP00000384273
RYR1	Ryanodine receptor 1	ENSP00000352608
RYR2	Ryanodine receptor 2	ENSP00000355533
RYR3	Ryanodine receptor 3	ENSP00000373884
SDHA	Succinate dehydrogenase complex, subunit A	ENSP00000264932
SDHB	Succinate dehydrogenase complex, subunit B	ENSP00000364649
SDHC	Succinate dehydrogenase complex, subunit C	ENSP00000356953

SDHD	Succinate dehydrogenase complex, subunit D	ENSP00000364699
SNAP91	Synaptosomal-associated protein, 91kDa homolog	ENSP00000195649
SNCA	Synuclein, alpha (non A4 component of amyloid precursor)	ENSP00000338345
TNF	Tumor necrosis factor (TNF superfamily, member 2)	ENSP00000392858
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	ENSP00000162749
UQCR10	Cytochrome b-c1 complex subunit 9 (Ubiquinol-cytochrome c reductase complex 7.2 kDa protein)(Cytochrome c1 non-heme 7 kDa protein)(Complex III subunit X)(Complex III subunit 9)	ENSP00000332887
UQCR11	Ubiquinol-cytochrome c reductase, 6.4kDa subunit	ENSP00000262946
UQCRB	Ubiquinol-cytochrome c reductase binding protein	ENSP00000287022
UQCRC1	Ubiquinol-cytochrome c reductase core protein I	ENSP00000203407
UQCRC2	Ubiquinol-cytochrome c reductase core protein II	ENSP00000268379
UQCRFS1	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	ENSP00000306397
UQCRH	Ubiquinol-cytochrome c reductase hinge protein	ENSP00000309565
UQCRQ	Ubiquinol-cytochrome c reductase, complex III subunit VII, 9.5kDa	ENSP00000367934
VSNL1	Visinin-like 1	ENSP00000295156

Supplementary table S4.

Gene symbol	Alias and/or description	Ensembl ID (ENSP)
AIFM1	Apoptosis-inducing factor, mitochondrion-associated, 1	ENSP00000287295
AKT1	v-akt murine thymoma viral oncogene homolog 1	ENSP00000270202
APAF1	Apoptotic peptidase activating factor 1	ENSP00000353059
ATM	Ataxia telangiectasia mutated	ENSP00000278616
BAD	BCL2-associated agonist of cell death	ENSP00000309103
BAX	BCL2-associated X protein	ENSP00000293288
BCL2	B-cell CLL/lymphoma 2	ENSP00000329623
BCL2L1	BCL2-like 1	ENSP00000302564
BID	BH3 interacting domain death agonist	ENSP00000318822
BIRC2	Baculoviral IAP repeat-containing 2	ENSP00000227758
BIRC3	Baculoviral IAP repeat-containing 3	ENSP00000263464
CAPN1	Calpain 1, (mu/I) large subunit	ENSP00000279247
CAPN2	Calpain 2, (m/II) large subunit	ENSP00000295006
CASP10	Caspase 10, apoptosis-related cysteine peptidase	ENSP00000286186
CASP3	Caspase 3, apoptosis-related cysteine peptidase	ENSP00000311032
CASP6	Caspase 6, apoptosis-related cysteine peptidase	ENSP00000265164
CASP7	Caspase 7, apoptosis-related cysteine peptidase	ENSP00000298700
CASP8	Caspase 8, apoptosis-related cysteine peptidase	ENSP00000351273
CASP9	Caspase 9, apoptosis-related cysteine peptidase	ENSP00000330237
CFLAR	CASP8 and FADD-like apoptosis regulator	ENSP00000312455
CHUK	Conserved helix-loop-helix ubiquitous kinase	ENSP00000359424
CIDEA	Cell death-inducing DFFA-like effector a	ENSP00000339344
CIDEB	Cell death-inducing DFFA-like effector b	ENSP00000258807
CSF2RB	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	ENSP00000384053
DFFA	DNA fragmentation factor, 45kDa, alpha polypeptide	ENSP00000366237
DFFB	DNA fragmentation factor, 40kDa, beta polypeptide (caspase-activated DNase)	ENSP00000367454
ENDOG	Endonuclease G	ENSP00000361725
FADD	Fas (TNFRSF6)-associated via death domain	ENSP00000301838
FAS	Fas (TNF receptor superfamily, member 6)	ENSP00000347979
FASLG	Fas ligand (TNF superfamily, member 6)	ENSP00000356694
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	ENSP00000339151
IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	ENSP00000358622
IL1A	Interleukin 1, alpha	ENSP00000263339
IL1B	Interleukin 1, beta	ENSP00000263341
IL1R1	Interleukin 1 receptor, type I	ENSP00000233946
IL1RAP	Interleukin 1 receptor accessory protein	ENSP00000072516
IL3	Interleukin 3 (colony-stimulating factor, multiple)	ENSP00000296870

IL3RA	Interleukin 3 receptor, alpha (low affinity)	ENSP00000327890
IRAK1	Interleukin-1 receptor-associated kinase 1	ENSP00000358997
IRAK2	Interleukin-1 receptor-associated kinase 2	ENSP00000256458
IRAK3	Interleukin-1 receptor-associated kinase 3	ENSP00000261233
IRAK4	Interleukin-1 receptor-associated kinase 4	ENSP00000349096
MAP3K14	Mitogen-activated protein kinase kinase kinase 14	ENSP00000342059
MYD88	Myeloid differentiation primary response gene (88)	ENSP00000379625
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	ENSP00000226574
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	ENSP00000189444
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	ENSP00000216797
NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	ENSP00000312988
NFKBIE	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	ENSP00000275015
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	ENSP00000325663
NGF	Nerve growth factor (beta polypeptide)	ENSP00000358525
NTRK1	Neurotrophic tyrosine kinase, receptor, type 1	ENSP00000351486
PAWR	PRKC, apoptosis, WT1, regulator	ENSP00000328088
PPP3CA	Protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	ENSP00000378323
PPP3CB	Protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	ENSP00000378306
PPP3CC	Protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform	ENSP00000240139
PPP3R1	Protein phosphatase 3 (formerly 2B), regulatory subunit B, alpha isoform	ENSP00000234310
PPP3R2	Protein phosphatase 3 (formerly 2B), regulatory subunit B, beta isoform	ENSP00000363939
PRKCA	Protein kinase C, alpha	ENSP00000284384
RELA	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	ENSP00000384273
RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	ENSP00000259808
SMG7	Smg-7 homolog, nonsense mediated mRNA decay factor (C. elegans)	ENSP00000340766
TNF	Tumor necrosis factor (TNF superfamily, member 2)	ENSP00000392858
TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a	ENSP00000221132
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	ENSP00000276431
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	ENSP00000162749

TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	ENSP00000241261
TP53	Tumor protein p53	ENSP00000269305
TRADD	TNFRSF1A-associated via death domain	ENSP00000341268
TRAF2	TNF receptor-associated factor 2	ENSP00000247668

Supplementary table S5.

Node (Gene symbol)	**Ensembl ID** (ENSP)	**Stress**	**Connectivity (degree)**	**Betweenness centrality**	**Closeness centrality**	**Subnetwork contribution/s**
ADAM10	ENSP00000260408	4376	10	0.00104299	0.34473198	**AD**
ADAM17	ENSP00000309968	220	5	2.53E-04	0.32748025	**AD NOT**
AIFM1	ENSP00000287295	3784	2	0.00536193	0.30800991	**APO**
AKT1	ENSP00000270202	39280	33	0.02669291	0.41675978	**AD APO**
APAF1	ENSP00000353059	4216	13	0.00163344	0.35557674	**AD APO**
APBA1	ENSP00000265381	176	5	1.61E-04	0.33452915	**AD**
APBA2	ENSP00000219865	22	3	6.30E-06	0.32921447	**AD**
APBA3	ENSP00000315136	0	2	0	0.31161236	**AD**
APBB1	ENSP00000299402	6412	7	0.00387928	0.34283088	**AD**
APBB1IP	ENSP00000365411	0	2	0	0.3163698	**AD**
APBB2	ENSP00000295974	64	2	5.82E-05	0.30548731	**AD**
APBB3	ENSP00000346378	2128	3	8.11E-04	0.3057377	**AD**
APC	ENSP00000257430	12230	28	0.0059543	0.39512712	**WNT**
APH1A	ENSP00000358105	1800	9	3.46E-04	0.35056391	**AD**
APH1B	ENSP00000261879	9620	17	0.00305574	0.36712598	**AD**
APLP1	ENSP00000221891	16	3	1.41E-05	0.26510306	**AD**
APLP2	ENSP00000263574	4920	8	0.00316447	0.32690622	**AD**
APOE	ENSP00000252486	760	4	3.85E-04	0.31423757	**AD**
APP	ENSP00000284981	165316	40	0.11865233	0.43221321	**AD**
APPBP2	ENSP00000083182	0	1	0	0.30202429	**AD**
ATF6	ENSP00000356919	0	2	0	0.29346971	**AD**
ATM	ENSP00000278616	254	5	8.76E-05	0.33543165	**APO**
ATP2A1	ENSP00000349595	0	2	0	0.29673827	**AD**
AXIN1	ENSP00000262320	14978	29	0.00699894	0.39596603	**WNT**

AXIN2	ENSP00000302625	930	13	5.36E-04	0.35188679	**WNT**
BACE1	ENSP00000318585	646	6	7.15E-04	0.32719298	**AD**
BACE2	ENSP00000332979	0	1	0	0.30202429	**AD**
BAD	ENSP00000309103	5894	16	0.00377838	0.36143411	**AD APO**
BAMBI	ENSP00000364683	0	1	0	0.22348712	**WNT**
BAX	ENSP00000293288	11616	16	0.0084067	0.3865285	**APO**
BCL2	ENSP00000329623	15288	21	0.00812957	0.39512712	**APO**
BCL2L1	ENSP00000302564	6700	16	0.00294129	0.36712598	**APO**
BID	ENSP00000318822	5934	21	0.00389901	0.36143411	**AD APO**
BIRC2	ENSP00000227758	1992	17	7.68E-04	0.33244207	**APO**
BIRC3	ENSP00000263464	3314	20	0.00103258	0.35188679	**APO**
BTRC	ENSP00000359206	10586	24	0.00380282	0.3938754	**WNT**
CABIN1	ENSP00000263119	5138	13	0.00278468	0.34126258	**AD WNT**
CACNA1C	ENSP00000266376	32318	6	0.00928438	0.28692308	**AD**
CACNA1D	ENSP00000288139	0	4	0	0.227162	**AD**
CACNA1F	ENSP00000365441	0	4	0	0.227162	**AD**
CACNA1S	ENSP00000355192	14	5	3.22E-05	0.22757779	**AD**
CACYBP	ENSP00000356652	94	7	3.33E-05	0.34569045	**WNT**
CALM1	ENSP00000349467	109204	38	0.04628466	0.3972311	**AD**
CALM2	ENSP00000272298	1570	6	7.05E-04	0.30399348	**AD**
CALM3	ENSP00000291295	0	4	0	0.30177994	**AD**
CAMK2A	ENSP00000381412	12174	16	0.00255578	0.33878292	**WNT**
CAMK2B	ENSP00000258682	8060	14	8.72E-04	0.32127476	**WNT**
CAMK2D	ENSP00000339740	50926	16	0.00721693	0.33214604	**WNT**
CAMK2G	ENSP00000319060	17570	11	0.00165961	0.32406603	**WNT**
CAPN1	ENSP00000279247	2612	9	0.00201641	0.34283088	**AD APO**
CAPN2	ENSP00000295006	230	5	1.58E-04	0.3131822	**AD APO**
CASP10	ENSP00000286186	4204	21	0.00162665	0.36003861	**APO**
CASP3	ENSP00000311032	144652	36	0.10875593	0.44351962	**AD APO**

CASP6	ENSP00000265164	2926	16	0.00141878	0.36712598	**APO**
CASP7	ENSP00000298700	5380	17	0.002725	0.34729981	**AD APO**
CASP8	ENSP00000351273	18592	36	0.00859504	0.40809628	**AD APO**
CASP9	ENSP00000330237	4206	16	0.00191302	0.3508937	**AD APO**
CCND1	ENSP00000227507	11998	24	0.00586873	0.39139559	**WNT**
CCND2	ENSP00000261254	518	10	3.18E-04	0.3552381	**WNT**
CCND3	ENSP00000362082	104	7	3.63E-05	0.33755656	**WNT**
CDC42	ENSP00000314458	7676	17	0.00485	0.39304531	**WNT**
CDK5	ENSP00000297518	2412	10	0.00245968	0.35557674	**AD**
CDK5R1	ENSP00000318486	1752	6	0.00157386	0.35288553	**AD**
CER1	ENSP00000370297	65810	20	0.00564041	0.28758674	**WNT**
CFLAR	ENSP00000312455	7542	23	0.00305805	0.37983707	**APO**
CHD8	ENSP00000406288	176	3	6.56E-05	0.33543165	**WNT**
CHRNA2	ENSP00000240132	0	8	0	0.23592663	**AD**
CHRNA3	ENSP00000315602	0	8	0	0.23592663	**AD**
CHRNA4	ENSP00000359285	5974	9	0.00536193	0.23607595	**AD**
CHRNA5	ENSP00000299565	0	8	0	0.23592663	**AD**
CHRNA6	ENSP00000276410	0	8	0	0.23592663	**AD**
CHRNA7	ENSP00000303727	53622	9	0.04721958	0.30649137	**AD**
CHRNB2	ENSP00000357461	0	8	0	0.23592663	**AD**
CHRNB3	ENSP00000289957	0	8	0	0.23592663	**AD**
CHRNB4	ENSP00000261751	0	8	0	0.23592663	**AD**
CHUK	ENSP00000359424	38632	49	0.01687821	0.41124587	**APO**
CIB1	ENSP00000333873	54	3	7.01E-05	0.30826446	**AD**
CIDEA	ENSP00000339344	0	1	0	0.19157678	**APO**
CIDEB	ENSP00000258807	8544	3	0.00536193	0.2368254	**APO**
CIR1	ENSP00000339723	0	5	0	0.29416404	**NOT**
COX4I1	ENSP00000253452	0	20	0	0.29027237	**AD**
COX5A	ENSP00000317780	0	20	0	0.29027237	**AD**

COX5B	ENSP00000258424	0	20	0	0.29027237	**AD**
COX6B1	ENSP00000246554	0	20	0	0.29027237	**AD**
COX6C	ENSP00000297564	0	20	0	0.29027237	**AD**
COX7B	ENSP00000417656	0	20	0	0.29027237	**AD**
COX7C	ENSP00000247655	0	20	0	0.29027237	**AD**
COX8A	ENSP00000321260	0	20	0	0.29027237	**AD**
CSF2RB	ENSP00000384053	1874	7	0.00177738	0.33038087	**APO**
CSNK1A1	ENSP00000261798	3024	11	9.92E-04	0.35796545	**WNT**
CSNK1A1L	ENSP00000369126	0	3	0	0.32776801	**WNT**
CSNK1D	ENSP00000324464	13948	14	0.00771451	0.39221872	**WNT**
CSNK1E	ENSP00000352929	2160	11	5.42E-04	0.3549001	**WNT**
CSNK1G1	ENSP00000305777	970	9	2.52E-04	0.34729981	**WNT**
CSNK1G2	ENSP00000255641	0	1	0	0.28914729	**WNT**
CSNK1G3	ENSP00000353904	0	1	0	0.28914729	**WNT**
CSNK2A1	ENSP00000217244	32200	22	0.01332479	0.41722595	**AD WNT**
CSNK2A2	ENSP00000262506	18652	16	0.0051692	0.40411701	**WNT**
CSNK2B	ENSP00000415615	7226	13	0.00159143	0.38453608	**WNT**
CTNNB1	ENSP00000344456	308690	75	0.1719886	0.48129032	**WNT**
CTNNBIP1	ENSP00000366466	14	6	7.05E-06	0.33970856	**WNT**
CUL1	ENSP00000326804	6804	23	0.00212711	0.37753036	**WNT**
CYC1	ENSP00000317159	36724	60	0.01199983	0.31423757	**AD**
CYCS	ENSP00000307786	24070	10	0.01450503	0.34251607	**AD**
DAAM1	ENSP00000247170	160	5	5.41E-05	0.31771721	**WNT**
DFFA	ENSP00000366237	9700	4	0.00589617	0.3092869	**APO**
DFFB	ENSP00000367454	7484	3	0.00481512	0.30903065	**APO**
DKK1	ENSP00000363081	7964	11	0.00300205	0.35659656	**WNT**
DKK2	ENSP00000285311	2	3	8.50E-07	0.27527675	**WNT**
DKK4	ENSP00000220812	58	4	4.79E-05	0.3312611	**WNT**
DLK1	ENSP00000340292	0	1	0	0.29650238	**NOT**

DLL1	ENSP00000355718	2072	14	0.00107966	0.33363148	**NOT**
DLL3	ENSP00000205143	0	3	0	0.29697452	**NOT**
DLL4	ENSP00000249749	18	9	2.02E-05	0.29935795	**NOT**
DTX1	ENSP00000257600	1528	9	0.00105286	0.33214604	**NOT**
DTX2	ENSP00000322885	0	3	0	0.29863891	**NOT**
DTX3	ENSP00000338050	0	3	0	0.29863891	**NOT**
DTX4	ENSP00000227451	0	1	0	0.29650238	**NOT**
DVL1	ENSP00000368169	65626	42	0.02010073	0.39470899	**NOT WNT**
DVL2	ENSP00000005340	97036	45	0.03307946	0.41169978	**WNT**
DVL3	ENSP00000316054	65998	43	0.02237074	0.39807898	**WNT**
EIF2AK1	ENSP00000199389	0	3	0	0.29416404	**AD**
EIF2AK2	ENSP00000233057	3836	12	0.0024823	0.36390244	**AD**
EIF2AK3	ENSP00000307235	230	5	1.07E-04	0.31907613	**AD**
EIF2AK4	ENSP00000263791	0	2	0	0.27815063	**AD**
ENDOG	ENSP00000361725	0	1	0	0.23562855	**APO**
EP300	ENSP00000263253	57910	48	0.02604835	0.4121547	**NOT WNT**
ERN1	ENSP00000401445	222	5	1.39E-04	0.31344538	**AD**
FADD	ENSP00000301838	5564	27	0.00269456	0.35388994	**AD APO**
FAS	ENSP00000347979	4326	13	0.00271297	0.33603604	**AD APO**
FASLG	ENSP00000356694	3312	19	0.00214402	0.34188818	**APO**
FOSL1	ENSP00000310170	334	9	2.20E-04	0.33303571	**WNT**
FRAT1	ENSP00000360060	160	8	9.18E-05	0.34794776	**WNT**
FRAT2	ENSP00000360058	0	6	0	0.32519616	**WNT**
FZD1	ENSP00000287934	61846	35	0.00632286	0.3437788	**WNT**
FZD10	ENSP00000229030	58718	33	0.00493779	0.34314627	**WNT**
FZD2	ENSP00000323901	65628	34	0.00980126	0.35255198	**WNT**
FZD3	ENSP00000240093	58718	33	0.00493779	0.34314627	**WNT**
FZD4	ENSP00000311581	58718	36	0.00493779	0.34409594	**WNT**
FZD5	ENSP00000354607	111182	42	0.03119338	0.38139059	**WNT**

FZD6	ENSP00000351605	58718	34	0.00493779	0.34346225	**WNT**
FZD7	ENSP00000286201	58718	36	0.00493779	0.34409594	**WNT**
FZD8	ENSP00000363826	60234	37	0.00542682	0.34441367	**WNT**
FZD9	ENSP00000345785	58718	33	0.00493779	0.34314627	**WNT**
GAPDH	ENSP00000229239	2756	7	0.00164891	0.35865385	**AD**
GNAQ	ENSP00000286548	2118	17	1.74E-04	0.33422939	**AD**
GRIN1	ENSP00000360616	1458	9	8.33E-05	0.32547993	**AD**
GRIN2A	ENSP00000332549	1094	13	4.87E-04	0.32238548	**AD**
GRIN2B	ENSP00000279593	1586	14	9.15E-04	0.31825939	**AD**
GRIN2C	ENSP00000293190	612	10	1.00E-04	0.31291946	**AD**
GRIN2D	ENSP00000263269	640	11	1.18E-04	0.3131822	**AD**
GSK3B	ENSP00000324806	68694	40	0.03808322	0.45321993	**AD WNT**
HDAC1	ENSP00000362649	37362	38	0.02064376	0.39180672	**NOT**
HDAC2	ENSP00000381331	6776	16	0.00279316	0.35388994	**NOT**
HES1	ENSP00000232424	784	5	3.07E-04	0.32099828	**NOT**
HES5	ENSP00000367714	0	7	0	0.30954357	**NOT**
HEY1	ENSP00000338272	0	8	0	0.30980066	**NOT**
HR	ENSP00000370826	0	2	0	0.281935	**NOT**
HSD17B10	ENSP00000168216	0	1	0	0.30202429	**AD**
IDE	ENSP00000265986	0	1	0	0.30202429	**AD**
IKBKB	ENSP00000339151	36300	50	0.01874801	0.40809628	**APO**
IKBKG	ENSP00000358622	29312	46	0.01253613	0.39180672	**APO**
IL1A	ENSP00000263339	212	10	2.56E-04	0.28803089	**APO**
IL1B	ENSP00000263341	3234	13	0.00127541	0.32834507	**AD APO**
IL1R1	ENSP00000233946	5580	15	0.00383746	0.34473198	**APO**
IL1RAP	ENSP00000072516	1278	11	5.60E-04	0.31109258	**APO**
IL3	ENSP00000296870	288	4	3.05E-04	0.29370079	**APO**
IL3RA	ENSP00000327890	186	3	7.45E-05	0.28604294	**APO**
IRAK1	ENSP00000358997	5418	21	0.00242481	0.34473198	**APO**

IRAK2	ENSP00000256458	1750	14	4.49E-04	0.32294372	**APO**
IRAK3	ENSP00000261233	628	11	1.58E-04	0.31291946	**APO**
IRAK4	ENSP00000349096	1450	13	3.24E-04	0.31825939	**APO**
ITPR1	ENSP00000405934	48366	14	0.0097698	0.34794776	**AD**
ITPR2	ENSP00000370744	88	10	2.36E-05	0.32547993	**AD**
ITPR3	ENSP00000363435	94	11	2.67E-05	0.32633421	**AD**
JAG1	ENSP00000254958	46	9	6.73E-05	0.29911788	**NOT**
JAG2	ENSP00000328169	0	7	0	0.29863891	**NOT**
JUN	ENSP00000360266	20832	27	0.01306543	0.39978564	**WNT**
KAT2A	ENSP00000225916	3412	14	8.44E-04	0.37151394	**NOT**
KAT2B	ENSP00000263754	10624	26	0.00358149	0.38533058	**NOT**
KFYI	ENSP00000377770	0	37	0	0.27048586	**AD**
LEF1	ENSP00000265165	50588	17	0.01596053	0.38453608	**WNT**
LFNG	ENSP00000222725	0	2	0	0.29673827	**NOT**
LPL	ENSP00000309757	348	4	4.31E-04	0.30177994	**AD**
LRP1	ENSP00000243077	3892	6	0.00214502	0.3300885	**AD**
LRP5	ENSP00000294304	11636	18	0.00329986	0.34665428	**WNT**
LRP6	ENSP00000261349	24044	28	0.00802832	0.36604514	**WNT**
MAML1	ENSP00000292599	1100	19	3.15E-04	0.34283088	**NOT**
MAML2	ENSP00000412394	1100	19	3.15E-04	0.34283088	**NOT**
MAML3	ENSP00000408478	990	18	2.72E-04	0.34157509	**NOT**
MAP3K1	ENSP00000382423	25004	32	0.01319844	0.39596603	**AD**
MAP3K14	ENSP00000342059	6460	26	0.00277795	0.36712598	**APO**
MAP3K7	ENSP00000358335	17108	26	0.00811384	0.36930693	**WNT**
MAPK8	ENSP00000353483	8408	16	0.00478894	0.38493292	**WNT**
MAPKAPK2	ENSP00000356070	0	1	0	0.29439621	**AD**
MAPT	ENSP00000340820	10982	15	0.00613928	0.40455531	**AD**
MFNG	ENSP00000349490	0	3	0	0.29697452	**NOT**
MME	ENSP00000353679	0	1	0	0.29462875	**AD**

MMP2	ENSP00000219070	1870	8	0.00114941	0.35056391	**WNT**
MMP7	ENSP00000260227	470	5	2.47E-04	0.3384755	**WNT**
MMP9	ENSP00000361405	910	9	4.01E-04	0.35659656	**WNT**
MT-CO1	ENSP00000354499	0	20	0	0.29027237	**AD**
MT-CO2	ENSP00000354876	242990	22	0.09435199	0.36532811	**AD**
MT-CO3	ENSP00000354982	7906	21	0.00754915	0.32238548	**AD**
MT-CYB	ENSP00000354554	26610	59	0.00573093	0.30649137	**AD**
MYC	ENSP00000367207	52216	47	0.03057477	0.41957255	**WNT**
MYCBP	ENSP00000380702	0	1	0	0.29579699	**WNT**
MYD88	ENSP00000379625	3272	16	0.00110454	0.33482944	**APO**
NCOR2	ENSP00000348551	21790	23	0.00915498	0.37638749	**NOT**
NCSTN	ENSP00000294785	3604	11	0.0014446	0.36284047	**AD NOT**
NDUFA1	ENSP00000360492	784	47	2.31E-05	0.2762963	**AD**
NDUFA10	ENSP00000252711	784	47	2.31E-05	0.2762963	**AD**
NDUFA11	ENSP00000311740	784	47	2.31E-05	0.2762963	**AD**
NDUFA12	ENSP00000330737	784	47	2.31E-05	0.2762963	**AD**
NDUFA13	ENSP00000380364	784	47	2.31E-05	0.2762963	**AD**
NDUFA2	ENSP00000252102	784	47	2.31E-05	0.2762963	**AD**
NDUFA3	ENSP00000398290	784	47	2.31E-05	0.2762963	**AD**
NDUFA4	ENSP00000339720	784	47	2.31E-05	0.2762963	**AD**
NDUFA5	ENSP00000347988	784	47	2.31E-05	0.2762963	**AD**
NDUFA6	ENSP00000330937	784	47	2.31E-05	0.2762963	**AD**
NDUFA7	ENSP00000301457	784	47	2.31E-05	0.2762963	**AD**
NDUFA8	ENSP00000362873	784	47	2.31E-05	0.2762963	**AD**
NDUFA9	ENSP00000266544	784	47	2.31E-05	0.2762963	**AD**
NDUFAB1	ENSP00000007516	784	47	2.31E-05	0.2762963	**AD**
NDUFB1	ENSP00000330787	784	47	2.31E-05	0.2762963	**AD**
NDUFB10	ENSP00000268668	784	47	2.31E-05	0.2762963	**AD**
NDUFB11	ENSP00000276062	784	47	2.31E-05	0.2762963	**AD**

NDUFB2	ENSP00000247866	784	47	2.31E-05	0.2762963	**AD**
NDUFB3	ENSP00000237889	784	47	2.31E-05	0.2762963	**AD**
NDUFB4	ENSP00000184266	784	47	2.31E-05	0.2762963	**AD**
NDUFB5	ENSP00000259037	784	47	2.31E-05	0.2762963	**AD**
NDUFB6	ENSP00000369176	56318	48	0.0331944	0.32576419	**AD**
NDUFB7	ENSP00000215565	784	47	2.31E-05	0.2762963	**AD**
NDUFB8	ENSP00000299166	784	47	2.31E-05	0.2762963	**AD**
NDUFB9	ENSP00000276689	784	47	2.31E-05	0.2762963	**AD**
NDUFC2	ENSP00000281031	784	47	2.31E-05	0.2762963	**AD**
NDUFS1	ENSP00000233190	98352	48	0.07135624	0.34283088	**AD**
NDUFS2	ENSP00000356972	784	47	2.31E-05	0.2762963	**AD**
NDUFS3	ENSP00000263774	784	47	2.31E-05	0.2762963	**AD**
NDUFS4	ENSP00000296684	784	47	2.31E-05	0.2762963	**AD**
NDUFS5	ENSP00000362058	784	47	2.31E-05	0.2762963	**AD**
NDUFS6	ENSP00000274137	784	47	2.31E-05	0.2762963	**AD**
NDUFS7	ENSP00000233627	784	47	2.31E-05	0.2762963	**AD**
NDUFS8	ENSP00000315774	784	47	2.31E-05	0.2762963	**AD**
NDUFV1	ENSP00000322450	784	47	2.31E-05	0.2762963	**AD**
NDUFV2	ENSP00000327268	0	37	0	0.27048586	**AD**
NDUFV3	ENSP00000346196	784	47	2.31E-05	0.2762963	**AD**
NFATC1	ENSP00000327850	7424	14	0.00412396	0.35969142	**WNT**
NFKB1	ENSP00000226574	42738	45	0.02087124	0.41536748	**APO**
NFKB2	ENSP00000189444	1680	14	8.47E-04	0.3463324	**APO**
NFKBIA	ENSP00000216797	38876	40	0.01668362	0.38453608	**AD APO**
NFKBIB	ENSP00000312988	9002	17	0.00307034	0.3569378	**APO**
NFKBIE	ENSP00000275015	2052	9	7.37E-04	0.33573357	**APO**
NFKBIZ	ENSP00000325663	0	1	0	0.29370079	**APO**
NGF	ENSP00000358525	12286	19	0.00684588	0.38139059	**APO**
NKD1	ENSP00000268459	32	4	1.12E-05	0.30623974	**WNT**

NKD2	ENSP00000296849	0	3	0	0.29603175	**WNT**
NLK	ENSP00000384625	354	7	2.27E-04	0.33603604	**WNT**
NOS1	ENSP00000320758	74	3	1.61E-05	0.30775578	**AD**
NOTCH1	ENSP00000277541	152598	61	0.09254796	0.42099323	**AD NOT**
NTRK1	ENSP00000351486	0	2	0	0.29140625	**APO**
NUMBL	ENSP00000252891	0	2	0	0.32604895	**NOT**
PAWR	ENSP00000328088	0	4	0	0.31989708	**APO**
PHF17	ENSP00000226319	0	1	0	0.32519616	**WNT**
PLCB1	ENSP00000338185	125438	33	0.01272933	0.37151394	**AD WNT**
PLCB2	ENSP00000260402	174948	36	0.03396956	0.40324324	**AD WNT**
PLCB3	ENSP00000279230	125438	33	0.01272933	0.37151394	**AD WNT**
PLCB4	ENSP00000334105	128872	34	0.01384574	0.37188435	**AD WNT**
PLCG1	ENSP00000244007	34740	34	0.01442894	0.36390244	**AD**
PORCN	ENSP00000322304	1654	19	2.78E-04	0.28714396	**WNT**
PPARD	ENSP00000310928	370	7	1.01E-04	0.35557674	**WNT**
PPP1R1A	ENSP00000257905	36	5	1.44E-05	0.27877429	**AD**
PPP1R1B	ENSP00000254079	690	6	2.94E-04	0.31344538	**AD WNT**
PPP3CA	ENSP00000378323	41098	21	0.01408111	0.37983707	**AD APO WNT**
PPP3CB	ENSP00000378306	18912	15	0.0037641	0.36354776	**AD APO WNT**
PPP3CC	ENSP00000240139	13518	14	0.00113969	0.32748025	**AD APO WNT**
PPP3R1	ENSP00000234310	17686	15	0.00303604	0.34990619	**AD APO WNT**
PPP3R2	ENSP00000363939	9998	9	6.77E-04	0.32238548	**APO WNT**
PRICKLE1	ENSP00000345064	2	5	2.88E-06	0.29650238	**WNT**
PRICKLE2	ENSP00000295902	2	5	2.88E-06	0.29650238	**WNT**
PRKCA	ENSP00000284384	73608	39	0.02698936	0.40367965	**APO WNT**
PRKCB	ENSP00000305355	24102	23	0.00555594	0.36425781	**WNT**
PRKCD	ENSP00000331602	35952	26	0.01064182	0.40676118	**WNT**
PRKCE	ENSP00000306124	6020	14	0.00273646	0.35969142	**WNT**
PRKCG	ENSP00000263431	17278	21	0.00281818	0.35056391	**WNT**

PRKCH	ENSP00000329127	8	5	4.71E-06	0.30349878	**WNT**
PRKCI	ENSP00000295797	15530	18	0.01205483	0.373	**WNT**
PRKCQ	ENSP00000263125	4332	15	0.0019424	0.36640472	**WNT**
PRKCZ	ENSP00000367830	18416	32	0.00901166	0.40989011	**WNT**
PSEN1	ENSP00000326366	25162	28	0.01752636	0.40854326	**AD NOT WNT**
PSEN2	ENSP00000355747	19322	25	0.01465683	0.40280778	**AD NOT**
PSENEN	ENSP00000222266	9620	17	0.00305574	0.36712598	**AD NOT**
PTCRA	ENSP00000304447	0	2	0	0.29721116	**NOT**
RAC1	ENSP00000348461	35110	25	0.02087303	0.41816143	**WNT**
RAC2	ENSP00000249071	2098	11	5.27E-04	0.35221907	**WNT**
RAC3	ENSP00000304283	6512	13	0.00278679	0.36497065	**WNT**
RBPJ	ENSP00000345206	1044	15	6.67E-04	0.32127476	**NOT**
RBX1	ENSP00000216225	12974	21	0.00646565	0.39807898	**WNT**
RCAN1	ENSP00000320768	816	5	4.32E-04	0.34001823	**AD**
RCAN2	ENSP00000329454	0	1	0	0.27548006	**AD**
RELA	ENSP00000384273	48268	49	0.02691498	0.41398446	**AD APO**
RFNG	ENSP00000307971	0	1	0	0.29650238	**NOT**
RHOA	ENSP00000400175	4460	16	0.00251031	0.35188679	**WNT**
RIPK1	ENSP00000259808	3640	30	0.00136881	0.34859813	**APO**
RUVBL1	ENSP00000318297	44	7	5.62E-05	0.33452915	**WNT**
RYR1	ENSP00000352608	18372	4	0.00256963	0.28692308	**AD**
RYR2	ENSP00000355533	67924	8	0.01034793	0.26585887	**AD**
RYR3	ENSP00000373884	0	1	0	0.21014085	**AD**
SDHA	ENSP00000264932	0	3	0	0.20505772	**AD**
SDHB	ENSP00000364649	16672	13	0.0017777	0.25741891	**AD**
SDHC	ENSP00000356953	16672	13	0.0017777	0.25741891	**AD**
SDHD	ENSP00000364699	16672	13	0.0017777	0.25741891	**AD**
SENP2	ENSP00000296257	3886	7	8.19E-04	0.32491289	**WNT**
SFRP1	ENSP00000220772	10570	30	0.00663457	0.31962296	**WNT**

SFRP2	ENSP00000274063	1730	29	2.95E-04	0.2955626	**WNT**
SFRP4	ENSP00000410715	1730	29	2.95E-04	0.2955626	**WNT**
SFRP5	ENSP00000266066	1730	29	2.95E-04	0.2955626	**WNT**
SIAH1	ENSP00000349156	2696	9	0.00121685	0.3678501	**WNT**
SKP1	ENSP00000231487	7076	23	0.00240872	0.37791287	**WNT**
SMAD4	ENSP00000341551	3600	15	0.0020413	0.36178468	**WNT**
SMG1	ENSP00000402515	5962	2	0.00536193	0.27226277	**WNT**
SMG7	ENSP00000340766	0	1	0	0.2141217	**APO**
SNAP91	ENSP00000195649	0	1	0	0.26700072	**AD**
SNCA	ENSP00000338345	153202	19	0.09687142	0.41583055	**AD**
SNW1	ENSP00000261531	2002	21	9.50E-04	0.34409594	**NOT**
SOX17	ENSP00000297316	8	5	6.09E-06	0.32633421	**WNT**
TBL1X	ENSP00000217964	4238	10	0.00252914	0.3532197	**WNT**
TBL1Y	ENSP00000328879	0	2	0	0.32633421	**WNT**
TCF7	ENSP00000340347	456	10	2.10E-04	0.3437788	**WNT**
TCF7L1	ENSP00000282111	576	10	2.82E-04	0.3437788	**WNT**
TCF7L2	ENSP00000358404	4592	24	0.00284292	0.37449799	**WNT**
TLE1	ENSP00000365682	9226	19	0.00541752	0.37676768	**NOT**
TLE2	ENSP00000262953	3974	15	7.69E-04	0.35456274	**NOT**
TLE3	ENSP00000319233	0	1	0	0.28172205	**NOT**
TLE4	ENSP00000365735	1012	12	1.81E-04	0.35591603	**NOT**
TNF	ENSP00000392858	1094	13	3.74E-04	0.33393017	**AD APO**
TNFRSF10A	ENSP00000221132	276	13	9.52E-05	0.3312611	**APO**
TNFRSF10B	ENSP00000276431	276	13	9.52E-05	0.3312611	**APO**
TNFRSF1A	ENSP00000162749	5726	25	0.00245423	0.36319377	**AD APO**
TNFSF10	ENSP00000241261	26	12	1.03E-05	0.32072227	**APO**
TP53	ENSP00000269305	53250	46	0.03380625	0.40631808	**APO WNT**
TRADD	ENSP00000341268	4442	27	0.00148992	0.3463324	**APO**
TRAF2	ENSP00000247668	3414	20	0.00134363	0.34859813	**APO**

UQCR10	ENSP00000332887	26610	59	0.00573093	0.30649137	**AD**
UQCR11	ENSP00000262946	26610	59	0.00573093	0.30649137	**AD**
UQCRB	ENSP00000287022	26610	59	0.00573093	0.30649137	**AD**
UQCRC1	ENSP00000203407	36724	60	0.01199983	0.31423757	**AD**
UQCRC2	ENSP00000268379	99228	60	0.04795027	0.33909091	**AD**
UQCRFS1	ENSP00000306397	26610	59	0.00573093	0.30649137	**AD**
UQCRH	ENSP00000309565	26610	59	0.00573093	0.30649137	**AD**
UQCRQ	ENSP00000367934	26610	59	0.00573093	0.30649137	**AD**
VANGL1	ENSP00000310800	2	5	2.88E-06	0.29650238	**WNT**
VANGL2	ENSP00000357040	2	5	2.88E-06	0.29650238	**WNT**
VSNL1	ENSP00000295156	0	1	0	0.19108607	**AD**
WIF1	ENSP00000286574	1728	21	3.11E-04	0.29416404	**WNT**
WNT1	ENSP00000293549	35482	28	0.00993132	0.3366426	**WNT**
WNT10A	ENSP00000258411	9208	17	1.25E-04	0.28692308	**WNT**
WNT10B	ENSP00000301061	21944	19	0.00392623	0.31962296	**WNT**
WNT11	ENSP00000325526	9208	17	1.25E-04	0.28692308	**WNT**
WNT16	ENSP00000222462	9208	17	1.25E-04	0.28692308	**WNT**
WNT2	ENSP00000265441	9250	22	1.47E-04	0.29462875	**WNT**
WNT2B	ENSP00000358698	9208	17	1.25E-04	0.28692308	**WNT**
WNT3	ENSP00000225512	9250	22	1.47E-04	0.29462875	**WNT**
WNT3A	ENSP00000284523	44252	32	0.01665364	0.36967294	**WNT**
WNT4	ENSP00000290167	9208	17	1.25E-04	0.28692308	**WNT**
WNT5A	ENSP00000264634	22130	28	0.00612741	0.34729981	**WNT**
WNT5B	ENSP00000308887	9208	17	1.25E-04	0.28692308	**WNT**
WNT6	ENSP00000233948	9208	17	1.25E-04	0.28692308	**WNT**
WNT7A	ENSP00000285018	9250	18	1.47E-04	0.28758674	**WNT**
WNT7B	ENSP00000341032	9252	18	1.59E-04	0.28758674	**WNT**
WNT8A	ENSP00000381739	9208	17	1.25E-04	0.28692308	**WNT**
WNT8B	ENSP00000340677	9208	17	1.25E-04	0.28692308	**WNT**

WNT9A	ENSP00000272164	9208	17	1.25E-04	0.28692308	**WNT**
WNT9B	ENSP00000290015	9208	17	1.25E-04	0.28692308	**WNT**

Supplementary table S6.

Node (Gene symbol)	**Stress**	**Connectivity (degree)**	**Betweenness centrality**	**Closeness centrality**	---/NH-B/HB	**Subnetwork contribution/s**
AKT1	39280	33	**0.02669291**	**0.4167598**	NH-B	**AD APO**
APC	12230	28	0.0059543	**0.3951271**	---	**WNT**
APP	**2E+05**	**40**	**0.11865233**	**0.4322132**	HB	**AD**
AXIN1	14978	29	0.00699894	**0.395966**	---	**WNT**
BAX	11616	16	0.0084067	**0.3865285**	---	**APO**
BCL2	15288	21	0.00812957	**0.3951271**	---	**APO**
BTRC	10586	24	0.00380282	**0.3938754**	---	**WNT**
CALM1	**1E+05**	**38**	**0.04628466**	**0.3972311**	HB	**AD**
CAMK2D	**50926**	16	0.00721693	0.332146	---	**WNT**
CASP3	**1E+05**	**36**	**0.10875593**	**0.4435196**	HB	**AD APO**
CASP8	18592	**36**	0.00859504	**0.4080963**	---	**AD APO**
CCND1	11998	24	0.00586873	**0.3913956**	---	**WNT**
CDC42	7676	17	0.00485	**0.3930453**	---	**WNT**
CER1	**65810**	20	0.00564041	0.2875867	---	**WNT**
CFLAR	7542	23	0.00305805	**0.3798371**	---	**APO**
CHRNA7	**53622**	9	**0.04721958**	0.3064914	NH-B	**AD**
CHUK	38632	**49**	0.01687821	**0.4112459**	---	**APO**
CSNK1D	13948	14	0.00771451	**0.3922187**	---	**WNT**
CSNK2A1	32200	22	0.01332479	**0.417226**	---	**AD WNT**
CSNK2A2	18652	16	0.0051692	**0.404117**	---	**WNT**
CSNK2B	7226	13	0.00159143	**0.3845361**	---	**WNT**
CTNNB1	**3E+05**	**75**	**0.1719886**	**0.4812903**	HB	**WNT**
CUL1	6804	23	0.00212711	**0.3775304**	---	**WNT**
CYC1	36724	**60**	0.01199983	0.3142376	---	**AD**

DVL1	**65626**	**42**	0.02010073	**0.394709**	---	**NOT WNT**
DVL2	**97036**	**45**	**0.03307946**	**0.4116998**	HB	**WNT**
DVL3	**65998**	**43**	**0.02237074**	**0.398079**	HB	**WNT**
EP300	**57910**	**48**	**0.02604835**	**0.4121547**	HB	**NOT WNT**
FZD1	**61846**	35	0.00632286	0.3437788	---	**WNT**
FZD10	**58718**	33	0.00493779	0.3431463	---	**WNT**
FZD2	**65628**	34	0.00980126	0.352552	---	**WNT**
FZD3	**58718**	33	0.00493779	0.3431463	---	**WNT**
FZD4	**58718**	**36**	0.00493779	0.3440959	---	**WNT**
FZD5	**1E+05**	**42**	**0.03119338**	**0.3813906**	HB	**WNT**
FZD6	**58718**	34	0.00493779	0.3434623	---	**WNT**
FZD7	**58718**	**36**	0.00493779	0.3440959	---	**WNT**
FZD8	**60234**	**37**	0.00542682	0.3444137	---	**WNT**
FZD9	**58718**	33	0.00493779	0.3431463	---	**WNT**
GSK3B	**68694**	**40**	**0.03808322**	**0.4532199**	HB	**AD WNT**
HDAC1	37362	**38**	0.02064376	**0.3918067**	---	**NOT**
IKBKB	36300	**50**	0.01874801	**0.4080963**	---	**APO**
IKBKG	29312	**46**	0.01253613	**0.3918067**	---	**APO**
JUN	20832	27	0.01306543	**0.3997856**	---	**WNT**
KAT2B	10624	26	0.00358149	**0.3853306**	---	**NOT**
KFYI	0	**37**	0	0.2704859	---	**AD**
LEF1	**50588**	17	0.01596053	**0.3845361**	---	**WNT**
MAP3K1	25004	32	0.01319844	**0.395966**	---	**AD**
MAPK8	8408	16	0.00478894	**0.3849329**	---	**WNT**
MAPT	10982	15	0.00613928	**0.4045553**	---	**AD**
MT-CO2	**2E+05**	22	**0.09435199**	0.3653281	NH-B	**AD**
MT-CYB	26610	**59**	0.00573093	0.3064914	---	**AD**
MYC	**52216**	**47**	**0.03057477**	**0.4195726**	HB	**WNT**
NCOR2	21790	23	0.00915498	**0.3763875**	---	**NOT**

NDUFA1	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFA10	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFA11	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFA12	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFA13	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFA2	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFA3	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFA4	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFA5	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFA6	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFA7	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFA8	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFA9	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFAB1	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFB1	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFB10	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFB11	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFB2	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFB3	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFB4	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFB5	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFB6	**56318**	**48**	**0.0331944**	0.3257642	HB	**AD**
NDUFB7	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFB8	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFB9	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFC2	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFS1	**98352**	**48**	**0.07135624**	0.3428309	HB	**AD**
NDUFS2	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFS3	784	**47**	2.31E-05	0.2762963	---	**AD**

NDUFS4	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFS5	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFS6	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFS7	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFS8	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFV1	784	**47**	2.31E-05	0.2762963	---	**AD**
NDUFV2	0	**37**	0	0.2704859	---	**AD**
NDUFV3	784	**47**	2.31E-05	0.2762963	---	**AD**
NFKB1	42738	**45**	0.02087124	**0.4153675**	---	**APO**
NFKBIA	38876	**40**	0.01668362	**0.3845361**	---	**AD APO**
NGF	12286	19	0.00684588	**0.3813906**	---	**APO**
NOTCH1	**2E+05**	**61**	**0.09254796**	**0.4209932**	HB	**AD NOT**
PLCB1	**1E+05**	33	0.01272933	0.3715139	---	**AD WNT**
PLCB2	**2E+05**	**36**	**0.03396956**	**0.4032432**	HB	**AD WNT**
PLCB3	**1E+05**	33	0.01272933	0.3715139	---	**AD WNT**
PLCB4	**1E+05**	34	0.01384574	0.3718844	---	**AD WNT**
PPP3CA	41098	21	0.01408111	**0.3798371**	---	**AD APO WNT**
PRKCA	**73608**	**39**	**0.02698936**	**0.4036797**	HB	**APO WNT**
PRKCD	35952	26	0.01064182	**0.4067612**	---	**WNT**
PRKCI	15530	18	0.01205483	**0.373**	---	**WNT**
PRKCZ	18416	32	0.00901166	**0.4098901**	---	**WNT**
PSEN1	25162	28	0.01752636	**0.4085433**	---	**AD NOT WNT**
PSEN2	19322	25	0.01465683	**0.4028078**	---	**AD NOT**
RAC1	35110	25	0.02087303	**0.4181614**	---	**WNT**
RBX1	12974	21	0.00646565	**0.398079**	---	**WNT**
RELA	48268	**49**	**0.02691498**	**0.4139845**	HB	**AD APO**
RYR2	**67924**	8	0.01034793	0.2658589	---	**AD**

SKP1	7076	23	0.00240872	**0.3779129**	---	**WNT**
SNCA	**2E+05**	19	**0.09687142**	**0.4158306**	NH-B	**AD**
TCF7L2	4592	24	0.00284292	**0.374498**	---	**WNT**
TLE1	9226	19	0.00541752	**0.3767677**	---	**NOT**
TP53	**53250**	**46**	**0.03380625**	**0.4063181**	HB	**APO WNT**
UQCR10	26610	**59**	0.00573093	0.3064914	---	**AD**
UQCR11	26610	**59**	0.00573093	0.3064914	---	**AD**
UQCRB	26610	**59**	0.00573093	0.3064914	---	**AD**
UQCRC1	36724	**60**	0.01199983	0.3142376	---	**AD**
UQCRC2	**99228**	**60**	**0.04795027**	0.3390909	HB	**AD**
UQCRFS1	26610	**59**	0.00573093	0.3064914	---	**AD**
UQCRH	26610	**59**	0.00573093	0.3064914	---	**AD**
UQCRQ	26610	**59**	0.00573093	0.3064914	---	**AD**

Supplementary table S7.

Drug name	Compound identifier (CID)	Formula	Description	Autism-related reference/s. PMID	AD-related reference/s. PMID
ACETYLCHOLINESTERASE INHIBITORS					
Donepezil	CID 3152	$C_{24}H_{29}NO_3$	Centrally acting reversible acetylcholinesterase inhibitor. Its main therapeutic use is in the palliative treatment of mild to moderate Alzheimer's disease	Hardan and Handen, 2002 (12427297; humans)	Davidsson et al., 2001 (11226635; humans)
Galantamine	CID 9651	$C_{17}H_{21}NO_3$	Used in the treatment of mild to moderate Alzheimer´s disease and various other memory impairments; particularly, those of vascular origin	Hertzman, 2003 (15152789; humans)	Raskind et al., 2000 (10881250; humans)
Rivastigmine	CID 77990	$C_{18}H_{28}N_2O_8$	Parasympathomimetic or cholinergic agent used in the treatment of mild to moderate dementia of the Alzheimers type and dementia due to Parkinson\'s disease. This drug can be administered orally or via a transdermal patch; the latter form reduces the prevalence of side effects, which typically include nausea and vomiting	Chez et al., 2004 (15119476; humans)	Auriacombe et al., 2002 (12094822; humans)
ALLOSTERIC MODULATORS OF METABOTROPIC GLUTAMATE RECEPTORS					
6-methyl-2-(phenylethynyl) pyridine/MPEP	CID 3025961	$C_{14}H_{11}N$	An mglu5 antagonist	Silverman et al., 2010 (20032969; BTBR mice)	---
3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide/ CDPPB	CID 11245456	$C_{23}H_{16}N_4O$	Drug used in scientific research which acts as a positive allosteric modulator selective for the metabotropic glutamate receptor subtype mGluR5. It has antipsychotic effects in animal models, and mGluR5 modulators are under investigation as potential drugs for the treatment of schizophrenia, as well as other applications	Won et al., 2012 (22699620; Shank2-mutant mice)	---

ANTIEPILEPTICS/ANTICONVULSANTS					
Carbamazepine	CID 2554	$C_{15}H_{12}N_2O$	Primarily used in the treatment of epilepsy, bipolar disorder, and trigeminal neuralgia. It is also used off-label for a variety of indications, including attention-deficit hyperactivity disorder (ADHD), schizophrenia, phantom limb syndrome, complex regional pain syndrome, paroxysmal extreme pain disorder, neuromyotonia, intermittent explosive disorder, borderline personality disorder and post-traumatic stress disorder	Donner and Frisk, 1965 (4955094)	Olin et al., 2001 (11739066; humans)
Levetiracetam	CID 5284583	$C_8H_{14}N_2O_2$	A pyrrolidine with antiepileptic activity. The exact mechanism through which levetiracetam exerts its effects is unknown but does not involve inhibitory and excitatory neurotransmitter activity	Rugino and Samsock, 2002 (12177568; humans)	Belcastro et al., 2007 (17880574; humans)
Topiramate	CID 5284627	$C_{12}H_{21}NO_8S$	Second-generation antiepileptic drug used in the treatment of seizure disorders and migraine headaches	Hardan et al., 2004 (15650499; humans)	Shi et al., 2013 (23889921; APPswe/PS1dE9 transgenic mice)
Valproic acid	CID 3121	$C_8H_{16}O_2$	Clinically used as an anticonvulsant and mood-stabilizing drug, primarily in the treatment of epilepsy, bipolar disorder, and, less commonly, major depression. It is also used to treat migraine headaches and schizophrenia	Childs and Blair, 1997 (9307927; humans)	Zhang et al., 2010 (19748552)
ANTIDEPRESANTS- SSRI					
Escitalopram	CID 146570	$C_{20}H_{21}FN_2O$	Antidepressant of the selective serotonin reuptake inhibitor (SSRI) class	Owley et al., 2010 (20020537; humans)	Rao et al., 2006 (16477587; humans)
Fluoxetine	CID 3386	$C_{17}H_{18}F_3NO$	Antidepressant of the selective serotonin reuptake inhibitor (SSRI) class	Hollander et al., 2012 (22193531; humans)	Auchus and Bissey-Black, 1997 (9447502; humans)
Fluvoxamine	CID 5324345	$C_{15}H_{22}F_3N_2O_2^+$	Antidepressant which functions as a selective serotonin	Sugie et al.,	

			reuptake inhibitor (SSRI)	2005 (16119478; humans)	---
Paroxetine	CID 43815	$C_{19}H_{20}FNO_3$	SSRI antidepressant	Posey et al., 1999 (9951204)	Tucker et al., 2005 (15974925; TgCRND8 mice)
Sertraline	CID 63009	$C_{17}H_{18}Cl_3N$	Antidepressant of the selective serotonin reuptake inhibitor (SSRI) class	Hellings et al., 1996 (8778118; humans)	Rosenberg et al., 2010 (20087081; humans)
Venlafaxine	CID 5656	$C_{17}H_{27}NO_2$	Antidepressant of the serotonin-norepinephrine reuptake inhibitor (SNRI) class for the treatment of generalized anxiety disorder, and comorbid indications in certain anxiety disorders with depression	Carminati et al., 2006 (16307837; humans)	Bragin et al., 2005 (15751450; humans)
ANTIDEPRESANTS- TRICYCLIC					
Clomipramine	CID 2801	$C_{19}H_{23}ClN_2$	Tricyclic antidepressant (TCA)	Brasic et al., 1994 (8035936; humans)	Petracca et al., 1996 (8854297; humans)
Desipramine	CID 2995	$C_{18}H_{22}N_2$	Tricyclic antidepressant (TCA). It inhibits the reuptake of norepinephrine and to a lesser extent serotonin	Gordon et al., 1992 (1536276; humans)	---
Imipramine	CID 3696	$C_{19}H_{24}N_2$	Also known as melipramine, is an antidepressant medication, a tricyclic antidepressant of the dibenzazepine group. It is mainly used in the treatment of major depression and enuresis (inability to control urination)	Campbell et al., 1971 (5172531)	Reifler et al., 1989 (2643356; humans)
ANTIOPIOIDS					
Naltrexone	CID 5360515	$C_{20}H_{23}NO_4$	Opioid receptor antagonist used primarily in the management of alcohol dependence and opioid dependence. It is marketed in generic form as its hydrochloride salt, naltrexone hydrochloride	Campbell et al., 1990 (2196621; humans)	Pomara et al., 1985 (3903533; humans)
ANTIPSYCHOTICS					
Aripiprazole	CID 60795	$C_{23}H_{27}Cl_2N_3O_2$	Atypical antipsychotic and antidepressant commonly used in the treatment of schizophrenia, bipolar disorder, and clinical depression	Owen et al., 2009 (19948625; humans)	De Deyn et al., 2005 (16160622; humans)

Chlorpromazine	CID 2726	$C_{17}H_{19}ClN_2S$	Typical antipsychotic and first drug developed with specific antipsychotic action	Alfredsson et al., 1985 (3920702; humans)	Ballard et al., 2008 (18384230; humans)
Clozapine	CID 2818	$C_{18}H_{19}ClN_4$	Antipsychotic medication used in the treatment of schizophrenia, and is also used off-label in the treatment of bipolar disorder	Lambrey et al., 2010 (20166802; humans)	Wirz-Justice et al., 2000 (11186599; humans)
Flupentixol	CID 5281881	$C_{23}H_{25}F_3N_2OS$	Also known as flupenthixol, marketed under brand names such as Depixol and Fluanxol, is a typical antipsychotic drug of the thioxanthene class	De Buck, 1974 (4471032)	---
Gabapentin	CID 3446	$C_9H_{17}NO_2$	A medication that was originally developed for treating epilepsy. Presently, gabapentin is widely used to relieve pain, especially neuropathic pain	Guglielmo et al., 2013 (23422383; human)	Regan and Gordon, 1997 (9004063)
Haloperidol	CID 3559	$C_{21}H_{23}ClFNO_2$	Typical antipsychotic. It is in the butyrophenone class of antipsychotic medications and has pharmacological effects similar to the phenothiazines	Miral et al., 2008 (18080171; humans)	Auchus and Bissey-Black, 1997 (9447502; humans)
Loxapine	CID 3964	$C_{18}H_{18}ClN_3O$	Typical antipsychotic medication, used primarily in the treatment of schizophrenia. It is a member of the dibenzoxazepine class and as a dibenzazepine derivative, it is structurally related to clozapine (which belongs to the chemically closely akin class of dibenzodiazepines)	Reinblatt et al., 2006 (17069553; humans)	---
Olanzapine	CID 4585	$C_{17}H_{20}N_4S$	A synthetic derivative of thienobenzodiazepine with antipsychotic, antinausea, and antiemetic activities. As a selective monoaminergic antagonist, olanzapine binds with high affinity binding to the following receptors: serotoninergic, dopaminergic, muscarinic M1-5, histamine H1, and α-1-adrenergic receptors; it binds weakly to γ-aminobutyric acid type A, benzodiazepine, and β-adrenergic receptors	Fido and Al-Saad, 2008 (18685284; humans)	Vigen et al., 2011 (21572163; humans)
Quetiapine	CID 5002	$C_{21}H_{25}N_3O_2S$	Atypical antipsychotic approved for the treatment of schizophrenia, and bipolar disorder	Golubchik et al., 2011 (21996644; humans)	Vigen et al., 2011 (21572163; humans)

Risperidone	CID 5073	$C_{23}H_{27}FN_4O_2$	Atypical antipsychotic usually used to treat schizophrenia (including adolescent schizophrenia), schizoaffective disorder, the mixed and manic states associated with bipolar disorder, and irritability in people with autism	Miral et al., 2008 (18080171; humans)	Vigen et al., 2011 (21572163; humans)
Ziprasidone	CID 60853	$C_{21}H_{24}Cl_2N_4O_2S$	Atypical antipsychotic approved for the treatment of schizophrenia. The intramuscular injection form of ziprasidone is approved for acute agitation in schizophrenic patients	McDougle et al., 2002 (12164181; humans)	---
COMPLEMENTARY AND ALTERNATIVE MEDICINES					
Magnesium	CID 888	Mg^{+2}	Magnesium ion	Mousain-Bosc et al., 2006 (16846101; humans)	Li et al., 2013 (23658180; APPswe/PS1dE9 mice)
Vitamin B6 (Pyridoxine)	CID 1054	$C_8H_{11}NO_3$	Pyridoxine is one of the compounds that can be called vitamin B6, along with pyridoxal and pyridoxamine	Mousain-Bosc et al., 2006 (16846101; humans)	Douaud et al., 2013 (23690582; humans)
Vitamin B12	CID 16686079	$C_{63}H_{87}CoN_{14}O_{14}P$	Vitamin B12, vitamin B12 or vitamin B-12, also called cobalamin, is a water soluble vitamin with a key role in the normal functioning of the brain and nervous system, and for the formation of blood. It is one of the eight B vitamins	James et al., 2009 (19056591; humans)	Douaud et al., 2013 (23690582; humans)
INHIBITORS OF mTOR					
Luteolin	CID 5280445	$C_{15}H_{10}O_6$	Luteolin is a yellow crystalline compound. It is a flavonoid; more specifically, it is one of the more common flavones	Theoharides et al., 2012 (22697063; humans)	Rezai-Zadeh et al. 2009 (18410522; Tg2576 mice)
Rapamycin	CID 5284616	$C_{51}H_{79}NO_{13}$	Sirolimus, also known as rapamycin, is an immunosuppressant drug used to prevent rejection in organ transplantation; it is especially useful in kidney	Sato et al., 2012	Spilman et al., 2010

			transplants. Sirolimus (a macrolide) was first discovered as a product of the bacterium Streptomyces hygroscopicus in a soil sample from Easter Island an island also known as Rapa Nui, hence the name	(23250422; Tsc2(+/-) mice)	(20376313; PDAPP transgenic mice)
NMDAR AGONISTS					
D-cycloserine	CID 6234	$C_3H_6N_2O_2$	An analogue of the amino acid D-alanine with broad-spectrum antibiotic and glycinergic activities. Positive modulator of NMDA glutamate receptors	Posey et al., 2004 (15514414; humans)	Schwartz et al., 1996 (8614505; humans)
NMDAR ANTAGONISTS					
Amantadine	CID 2130	$C_{10}H_{17}N$	Formally known as 1-adamantylamine or 1-aminoadamantane	King et al., 2001 (11392343; humans)	---
Memantine	CID 4054	$C_{12}H_{21}N$	Memantine is the first in a novel class of Alzheimer's disease medications acting on the glutamatergic system by blocking NMDA glutamate receptors	Erickson et al., 2007 (17016714; humans)	Hellweg et al., 2012 (22513699; humans)
NEUROHORMONES					
Melatonin	CID 896	$C_{13}H_{16}N_2O_2$	Melatonin, also known chemically as N-acetyl-5-methoxytryptamine, is a naturally occurring compound found in animals, plants and microbes. In animals, circulating levels of the hormone melatonin vary in a daily cycle, thereby allowing the entrainment of the circadian rhythms of several biological functions.	Giannotti et al., 2006 (16897403; humans)	Cardinali et al., 2010 (21358972; humans)
Oxytocin (Syntocinon)	CID 439302	$C_{43}H_{66}N_{12}O_{12}S_2$	Oxytocin is a mammalian hormone that acts primarily as a neuromodulator in the brain. Oxytocin has the distinction of being the very first polypeptide hormone to be sequenced and synthesized biochemically, by Vincent du Vigneaud et al. in 1953.	Hollander et al., 2003 (12496956; humans)	---
MOOD STABILIZERS					
Lithium	CID 28486	Li^+	Lithium ion	Kerbeshian et al., 1987 (3429701; humans)	Nunes et al., 2013 (22746245; humans)
PSYCOESTIMULANTS					
Buspirone	CID 2477	$C_{21}H_{31}N_5O_2$	Psychoactive drug primarily used as an anxiolytic,	Realmuto et al.,	Salzman,

			specifically for generalized anxiety disorder	1989 (2723129; humans)	2001 (11520475)
Methylphenidate	CID 4158	$C_{14}H_{19}NO_2$	Norepinephrine-dopamine reuptake inhibitor (NDRI)	Quintana et al., 1995 (7559293; humans)	Herrmann et al., 2008 (18480686; humans)
Pemoline	CID 4723	$C_9H_8N_2O_2$	Used to treat attention-deficit hyperactivity disorder and narcolepsy	King et al., 1993 (19630639; prepubertal male Sprague Dawley rats)	---
SYMPATHOLYTIC MEDICATIONS					
Clonidine	CID 2803	$C_9H_9Cl_2N_3$	Used to treat medical conditions, such as high blood pressure, attention-deficit hyperactivity disorder, and anxiety/panic disorder. It is a direct-acting 2 adrenergic agonist and a guanidine receptor agonist.	Ming et al., 2008 (18280681; humans)	Peskind et al., 1995 (7654129; humans)
Guanfacine	CID 3519	$C_9H_9Cl_2N_3O$	Agonist of the 2A subtype of norepinephrine receptors	Handen et al., 2008 (18552703; humans)	---
Propranolol	CID 4946	$C_{16}H_{21}NO_2$	Sympatholytic non-selective β-blocker	Narayanan et al., 2010 (20502989; humans)	Peskind et al., 2005 (15764868; humans)

Supplementary table S8.

Gene symbol	Number of drugs targeting the network node
GSK3B	6
AKT1	5
CALM1	3
GRIN1	3
GRIN2B	3
CHRNA7	2
GRIN2A	2
JUN	2
ADAM17	1
APP	1
BCL2	1
CALM2	1
CAMK2A	1
CAMK2B	1
CAMK2D	1
CASP3	1
CASP9	1
CCND1	1
CCND3	1
CDC42	1
CHRNA2	1
CHRNA3	1
CHRNA4	1
CHRNA5	1
CHRNA6	1
CHRNB2	1
CHRNB3	1
CHRNB4	1
COX5A	1
CSNK1G2	1
CSNK1G3	1
DFFB	1
EIF2AK2	1
GRIN2C	1
GRIN2D	1
HDAC1	1
MAPK8	1
MAPKAPK2	1
MMP9	1
MT-CO1	1
MT-CO2	1
MT-CO3	1
NDUFS2	1
NDUFS7	1
NDUFS8	1
NDUFV1	1
NGF	1
PLCB2	1

PLCB3	1
RAC1	1
RAC2	1
RAC3	1
RHOA	1
RYR1	1
RYR2	1
RYR3	1
TNFRSF1A	1

Supplementary table S9.

Gene symbol	Target				Selected reference/s. PMID	Number of drugs targeting node
	GLIAL			NEURONAL		
	Astro-glia	Micro-glia	Oligo-dendroglia			
GSK3B	Yes	Yes	Yes	Yes	Beurel and Jope, 2010 (20553816) Yuskaitis and Jope, 2009 (19007880) Ragot et al., 2011 (21575614) Reddy, 2013 (23816568)	6
AKT1	Yes	Yes	Yes	Yes	Neary et al., 2005 (15853465) Li et al., 2006 (16918383) Lu and Wong, 2004 (14761849) Pezet et al., 2005 (15869474)	5
CALM1	Yes	ND	ND	Yes	Lukas et al., 2008 (18613964) Khodosevich et al., 2009 (19668709)	3
GRIN1	Yes	ND	Yes	Yes	Lee et al., 2010 (21152063) Cavaliere et al., 2012 (22297298) Kioussi et al., 2007 (17920452)	3
GRIN2B	Yes	ND	ND	Yes	Krebs et al., 2003 (12716944) Priya et al., 2013 (23085505)	3
CHRNA7	ND	ND	ND	Yes	Adams et al., 2012 (22314319)	2
GRIN2A	Yes	Yes	Yes	Yes	Lee et al., 2010 (21152063) Hwang et al., 2009 (19422891) Cavaliere et al., 2012 (22297298) Bethea and Reddy, 2012 (22154832)	2
JUN	Yes	Yes	Yes	Yes	Dong et al., 2009 (19393026) Waetzig et al., 2005 (15739188) Vollgraf et al., 1999 (10582611)	2

					Raivich et al., 2004 (15233917)	

Supplementary table S10.

Drug	Compound identifier (CID)	Number of drug interactors (Total)	Number of drug interactors (NH-Bs)	Number of drug interactors (HBs)	Interactor with highest betweenness	Interactor with highest connectivity	Number of differentially expressed drug interactors
Chlorpromazine	CID2726	1	0	1 (*CALM1*)	*CALM1* (0.04628466)	*CALM1* (38)	0
Clozapine	CID2818	2	1 (*AKT1*)	1 (*GSK3B*)	*GSK3B* (0.03808322)	*GSK3B* (40)	0
D-cycloserine	CID6234	1	0	0	*GRIN1* (0.00008329)	*GRIN1* (9)	1
Fluoxetine	CID3386	1	0	0	*BCL2* (0.00812957)	*BCL2* (21)	1
Galantamine	CID9651	9	1 (*CHRNA7*)	0	*CHRNA7* (0.04721958)	*CHRNA7* (9)	0
Haloperidol	CID3559	2	0	0	*GRIN2B* (0.0009148)	*GRIN2B* (14)	1
Imipramine	CID3696	1	0	1 (*CALM1*)	*CALM1* (0.04628466)	*CALM1* (38)	0
Lithium	CID28486	2	1 (*AKT1*)	1 (*GSK3B*)	*GSK3B* (0.03808322)	*GSK3B* (40)	0
Luteolin	CID5280445	4	0	1 (*CASP3*)	*CASP3* (0.10875593)	*CASP3* (36)	2
Magnesium	CID888	32	1 (*MT-CO2*)	2 (*GSK3B*, *PCLB2*)	*MT-CO2* (0.09435199)	*NDUFS2*, *NDUFS7*, *NDUFS8*, *NDUFV1* (47)	**8**
Melatonin	CID896	2	0	2 (*APP*, *CALM1*)	*APP* (0.11865233)	*APP* (40)	0
Memantine	CID4054	3	0	0	*GRIN2B* (0.0009148)	*GRIN2B* (14)	1
Olanzapine	CID4585	2	1 (*AKT1*)	1 (*GSK3B*)	*GSK3B* (0.03808322)	*GSK3B* (40)	0
Rapamycin	CID5284616	9	1 (*AKT1*)	1 (*GSK3B*)	*GSK3B* (0.03808322)	*GSK3B* (40)	**2**
Valproic acid	CID3121	3	1 (*AKT1*)	1 (*GSK3B*)	*GSK3B* (0.03808322)	*GSK3B* (40)	0
Ziprasidone	CID60853	1	1 (*CHRNA7*)	0	*CHRNA7* (0.04721958)	*CHRNA7* (9)	0

Supplementary table S11.

Gene Ontology identifier (GO ID)	**NAME**	**Count**	**%**	**Corrected *p*-value (FDR)**	**List total**	**Pop hits**	**Pop total**	**GENES**
GO:0016310	Phosphorylation	12	41.37931	3.50E-05	29	800	13528	*NDUFS7, CSNK1G2, NDUFV1, GSK3B, NDUFS8, CAMK2D, CAMK2B, CSNK1G3, MAPKAPK2, EIF2AK2, NDUFS2, CAMK2A*
GO:0006793	Phosphorus metabolic process	12	41.37931	8.25E-05	29	973	13528	*NDUFS7, CSNK1G2, NDUFV1, GSK3B, NDUFS8, CAMK2D, CAMK2B, CSNK1G3, MAPKAPK2, EIF2AK2, NDUFS2, CAMK2A*
GO:0006796	Phosphate metabolic process	12	41.37931	8.25E-05	29	973	13528	*NDUFS7, CSNK1G2, NDUFV1, GSK3B, NDUFS8, CAMK2D, CAMK2B, CSNK1G3, MAPKAPK2, EIF2AK2, NDUFS2, CAMK2A*
GO:0050905	Neuromuscular process	5	17.24138	0.000269555	29	62	13528	*GRIN2B, RAC3, GRIN2C, GRIN2D, GRIN2A*
GO:0031644	Regulation of neurological system process	6	20.68966	0.00039468	29	153	13528	*GRIN2B, GRIN2C, GRIN2D, GRIN2A, CAMK2A, NGF*

GO:0046777	Protein amino acid autophosphorylation	5	17.24138	0.000631583	29	85	13528	*CSNK1G2*, *CAMK2D*, *CAMK2B*, *EIF2AK2*, *CAMK2A*
GO:0007264	Small GTPase mediated signal transduction	7	24.13793	0.000633286	29	305	13528	*CDC42*, *RAC2*, *RAC3*, *RAC1*, *RHOA*, *MAPKAPK2*, *NGF*
GO:0006120	Mitochondrial electron transport, NADH to ubiquinone	4	13.7931	0.0015444	29	42	13528	*NDUFS7*, *NDUFV1*, *NDUFS8*, *NDUFS2*
GO:0031346	Positive regulation of cell projection organization	4	13.7931	0.001741235	29	47	13528	*CDC42*, *RAC1*, *RHOA*, *NGF*
GO:0007242	Intracellular signaling cascade	11	37.93103	0.001741235	29	1256	13528	*CDC42*, *PLCB3*, *RAC2*, *RAC3*, *GSK3B*, *DFFB*, *RAC1*, *RHOA*, *MAPKAPK2*, *PLCB2*, *NGF*
GO:0060341	Regulation of cellular localization	6	20.68966	0.001741235	29	248	13528	*GRIN2B*, *GSK3B*, *RHOA*, *CAMK2A*, *CALM2*, *NGF*
GO:0050804	Regulation of synaptic transmission	5	17.24138	0.001741235	29	136	13528	*GRIN2B*, *GRIN2C*, *GRIN2A*, *CAMK2A*, *NGF*
GO:0006816	Calcium ion transport	5	17.24138	0.001741235	29	142	13528	*GRIN2B*, *CAMK2D*, *GRIN2A*, *CAMK2B*, *CAMK2A*
GO:0042775	Mitochondrial ATP synthesis coupled electron transport	4	13.7931	0.001741235	29	56	13528	*NDUFS7*, *NDUFV1*, *NDUFS8*, *NDUFS2*
GO:0000082	G1/S transition of mitotic cell cycle	4	13.7931	0.001741235	29	56	13528	*CAMK2D*, *ADAM17*, *CAMK2B*, *CAMK2A*
GO:0042773	ATP synthesis coupled electron transport	4	13.7931	0.001741235	29	56	13528	*NDUFS7*, *NDUFV1*, *NDUFS8*, *NDUFS2*
GO:0015980	Energy derivation by oxidation of organic compounds	5	17.24138	0.001741235	29	144	13528	*NDUFS7*, *NDUFV1*, *GSK3B*, *NDUFS8*, *NDUFS2*
GO:0051969	Regulation of	5	17.24138	0.001779556	29	147	13528	*GRIN2B*, *GRIN2C*, *GRIN2A*,

	transmission of nerve impulse							*CAMK2A*, *NGF*
GO:0048167	Regulation of synaptic plasticity	4	13.7931	0.0021736	29	64	13528	*GRIN2B*, *GRIN2C*, *GRIN2A*, *CAMK2A*
GO:0022904	Respiratory electron transport chain	4	13.7931	0.0021736	29	64	13528	*NDUFS7*, *NDUFV1*, *NDUFS8*, *NDUFS2*
GO:0006468	Protein amino acid phosphorylation	8	27.58621	0.002233524	29	667	13528	*CSNK1G2*, *GSK3B*, *CAMK2D*, *CAMK2B*, *CSNK1G3*, *MAPKAPK2*, *EIF2AK2*, *CAMK2A*
GO:0044057	Regulation of system process	6	20.68966	0.002496	29	309	13528	*GRIN2B*, *GRIN2C*, *GRIN2D*, *GRIN2A*, *CAMK2A*, *NGF*
GO:0015674	Di-, tri-valent inorganic cation transport	5	17.24138	0.002760522	29	176	13528	*GRIN2B*, *CAMK2D*, *GRIN2A*, *CAMK2B*, *CAMK2A*
GO:0001964	Startle response	3	10.34483	0.00282568	29	16	13528	*GRIN2B*, *GRIN2D*, *GRIN2A*
GO:0051130	Positive regulation of cellular component organization	5	17.24138	0.00282568	29	181	13528	*CDC42*, *GSK3B*, *RAC1*, *RHOA*, *NGF*
GO:0030031	Cell projection assembly	4	13.7931	0.0035915	29	83	13528	*CDC42*, *RAC2*, *RAC3*, *RAC1*
GO:0060079	Regulation of excitatory postsynaptic membrane potential	3	10.34483	0.003659741	29	19	13528	*GRIN2B*, *GRIN2C*, *GRIN2A*
GO:0031344	Regulation of cell projection organization	4	13.7931	0.004090821	29	89	13528	*CDC42*, *RAC1*, *RHOA*, *NGF*
GO:0060078	Regulation of postsynaptic membrane potential	3	10.34483	0.004884728	29	23	13528	*GRIN2B*, *GRIN2C*, *GRIN2A*
GO:0045333	Cellular respiration	4	13.7931	0.004884728	29	97	13528	*NDUFS7*, *NDUFV1*, *NDUFS8*, *NDUFS2*
GO:0006119	Oxidative	4	13.7931	0.004884728	29	98	13528	*NDUFS7*, *NDUFV1*, *NDUFS8*,

	phosphorylation							*NDUFS2*
GO:0030036	Actin cytoskeleton organization	5	17.24138	0.005064475	29	226	13528	*CDC42*, *RAC2*, *RAC3*, *RAC1*, *RHOA*
GO:0007215	Glutamate signaling pathway	3	10.34483	0.005142932	29	25	13528	*GRIN2B*, *GRIN2C*, *GRIN2A*
GO:0051329	Interphase of mitotic cell cycle	4	13.7931	0.005142932	29	103	13528	*CAMK2D*, *ADAM17*, *CAMK2B*, *CAMK2A*
GO:0051325	Interphase	4	13.7931	0.005427385	29	106	13528	*CAMK2D*, *ADAM17*, *CAMK2B*, *CAMK2A*
GO:0030029	Actin filament-based process	5	17.24138	0.00570806	29	241	13528	*CDC42*, *RAC2*, *RAC3*, *RAC1*, *RHOA*
GO:0022900	Electron transport chain	4	13.7931	0.006328902	29	114	13528	*NDUFS7*, *NDUFV1*, *NDUFS8*, *NDUFS2*
GO:0019233	Sensory perception of pain	3	10.34483	0.007420609	29	32	13528	*GRIN2B*, *GRIN2A*, *NGF*
GO:0007610	Behavior	6	20.68966	0.008908442	29	469	13528	*GRIN2B*, *RAC2*, *GRIN2D*, *RAC1*, *GRIN2A*, *NGF*
GO:0048168	Regulation of neuronal synaptic plasticity	3	10.34483	0.008908442	29	36	13528	*GRIN2B*, *GRIN2A*, *CAMK2A*
GO:0007613	Memory	3	10.34483	0.010183361	29	39	13528	*GRIN2B*, *GRIN2A*, *NGF*
GO:0044087	Regulation of cellular component biogenesis	4	13.7931	0.010421302	29	142	13528	*CDC42*, *GSK3B*, *RAC1*, *RHOA*
GO:0051899	Membrane depolarization	3	10.34483	0.011772202	29	43	13528	*GRIN2B*, *GRIN2C*, *GRIN2A*
GO:0006091	Generation of precursor metabolites and energy	5	17.24138	0.012065739	29	313	13528	*NDUFS7*, *NDUFV1*, *GSK3B*, *NDUFS8*, *NDUFS2*
GO:0033058	Directional locomotion	2	6.896552	0.012835707	29	2	13528	*GRIN2C*, *GRIN2A*
GO:0009611	Response to wounding	6	20.68966	0.012835707	29	530	13528	*TNFRSF1A*, *GRIN2C*, *RAC1*, *GRIN2A*, *ADAM17*, *NGF*
GO:0006811	Ion transport	7	24.13793	0.012835707	29	768	13528	*GRIN2B*, *GRIN2C*, *GRIN2D*,

								CAMK2D, GRIN2A, CAMK2B, CAMK2A
GO:0044093	Positive regulation of molecular function	6	20.68966	0.019181931	29	586	13528	*CDC42, RAC1, ADAM17, PLCB2, CAMK2A, CALM2*
GO:0030030	Cell projection organization	5	17.24138	0.019203239	29	368	13528	*CDC42, RAC2, RAC3, RAC1, NGF*
GO:0045471	Response to ethanol	3	10.34483	0.022005329	29	64	13528	*TNFRSF1A, GRIN2B, GRIN2A*
GO:0032386	Regulation of intracellular transport	3	10.34483	0.02635679	29	71	13528	*GSK3B, RHOA, CALM2*
GO:0051050	Positive regulation of transport	4	13.7931	0.029384108	29	223	13528	*GRIN2B, GSK3B, RHOA, CALM2*
GO:0032496	Response to lipopolysaccharide	3	10.34483	0.029635968	29	77	13528	*TNFRSF1A, ADAM17, NGF*
GO:0007010	Cytoskeleton organization	5	17.24138	0.031257247	29	436	13528	*CDC42, RAC2, RAC3, RAC1, RHOA*
GO:0043552	Positive regulation of phosphoinositide 3-kinase activity	2	6.896552	0.031554166	29	6	13528	*CDC42, RAC1*
GO:0043551	Regulation of phosphoinositide 3-kinase activity	2	6.896552	0.031554166	29	6	13528	*CDC42, RAC1*
GO:0002237	Response to molecule of bacterial origin	3	10.34483	0.034030152	29	86	13528	*TNFRSF1A, ADAM17, NGF*
GO:0010310	Regulation of hydrogen peroxide metabolic process	2	6.896552	0.034616395	29	7	13528	*RAC2, RAC1*
GO:0060263	Regulation of respiratory burst	2	6.896552	0.034616395	29	7	13528	*RAC2, RAC1*
GO:0030001	Metal ion transport	5	17.24138	0.034616395	29	465	13528	*GRIN2B, CAMK2D, GRIN2A, CAMK2B, CAMK2A*

GO:0043254	Regulation of protein complex assembly	3	10.34483	0.034616395	29	90	13528	*CDC42*, *GSK3B*, *RAC1*
GO:0010033	Response to organic substance	6	20.68966	0.034616395	29	721	13528	*TNFRSF1A*, *GRIN2B*, *GRIN2A*, *ADAM17*, *EIF2AK2*, *NGF*
GO:0042592	Homeostatic process	6	20.68966	0.040032192	29	751	13528	*GRIN2B*, *RAC2*, *GRIN2C*, *RAC1*, *CAMK2D*, *GRIN2A*
GO:0035235	Ionotropic glutamate receptor signaling pathway	2	6.896552	0.040740805	29	9	13528	*GRIN2B*, *GRIN2A*
GO:0007626	Locomotory behavior	4	13.7931	0.040740805	29	274	13528	*RAC2*, *GRIN2D*, *RAC1*, *NGF*
GO:0007265	Ras protein signal transduction	3	10.34483	0.040740805	29	105	13528	*RHOA*, *MAPKAPK2*, *NGF*
GO:0007049	Cell cycle	6	20.68966	0.040740805	29	776	13528	*GSK3B*, *CAMK2D*, *ADAM17*, *CAMK2B*, *CAMK2A*, *CALM2*
GO:0051930	Regulation of sensory perception of pain	2	6.896552	0.040740805	29	10	13528	*GRIN2D*, *GRIN2A*
GO:0043550	Regulation of lipid kinase activity	2	6.896552	0.040740805	29	10	13528	*CDC42*, *RAC1*
GO:0032981	Mitochondrial respiratory chain complex I assembly	2	6.896552	0.040740805	29	10	13528	*NDUFS7*, *NDUFS8*
GO:0010257	NADH dehydrogenase complex assembly	2	6.896552	0.040740805	29	10	13528	*NDUFS7*, *NDUFS8*
GO:0051931	Regulation of sensory perception	2	6.896552	0.040740805	29	10	13528	*GRIN2D*, *GRIN2A*
GO:0007611	Learning or memory	3	10.34483	0.042866367	29	111	13528	*GRIN2B*, *GRIN2A*, *NGF*
GO:0009967	Positive regulation of signal transduction	4	13.7931	0.042866367	29	295	13528	*TNFRSF1A*, *RAC1*, *RHOA*, *ADAM17*
GO:0080010	Regulation of oxygen and reactive oxygen	2	6.896552	0.042866367	29	11	13528	*RAC2*, *RAC1*

	species metabolic process							
GO:0042981	Regulation of apoptosis	6	20.68966	0.042866367	29	804	13528	*GSK3B*, *RAC1*, *RHOA*, *GRIN2A*, *ADAM17*, *NGF*
GO:0007268	Synaptic transmission	4	13.7931	0.042866367	29	298	13528	*GRIN2B*, *GRIN2C*, *GRIN2D*, *GRIN2A*
GO:0043067	Regulation of programmed cell death	6	20.68966	0.043818872	29	812	13528	*GSK3B*, *RAC1*, *RHOA*, *GRIN2A*, *ADAM17*, *NGF*
GO:0010941	Regulation of cell death	6	20.68966	0.043882026	29	815	13528	*GSK3B*, *RAC1*, *RHOA*, *GRIN2A*, *ADAM17*, *NGF*
GO:0033108	Mitochondrial respiratory chain complex assembly	2	6.896552	0.043912526	29	12	13528	*NDUFS7*, *NDUFS8*
GO:0006812	Cation transport	5	17.24138	0.045823639	29	553	13528	*GRIN2B*, *CAMK2D*, *GRIN2A*, *CAMK2B*, *CAMK2A*
GO:0022402	Cell cycle process	5	17.24138	0.048503997	29	565	13528	*GSK3B*, *CAMK2D*, *ADAM17*, *CAMK2B*, *CAMK2A*

PARTE IV----------

DISCUSSÃO GERAL

A maior parte dos casos de autismo é considerada idiopática. Todas as condições englobadas dentro do leque de doenças do PDD são comportamentalmente caracterizadas a partir de três critérios: (i) falhas na interação social, (ii) falhas na comunicação e (iii) a presença de comportamentos estereotipados repetitivos e restritivos em diferentes graus (*American Psychiatric Association*, 2000). Contudo, não existe ainda, na atualidade, um parâmetro característico mensurável, mas sim algumas características neurocitoanatômicas gerais que poderiam potencialmente nos auxiliar na definição do que é o autismo; mais especificamente, (i) anormalidades na proliferação, migração (heterotopias) e diferenciação (displasias) celular em diferentes áreas do encéfalo. Por exemplo, tem sido reportada a existência de heterotopias subcorticais e periventriculares e displasias no cerebelo, córtex e giro dentado (Bailey et al,, 1998; Wegiel et al., 2010), sendo todas estas mudanças uma consequência de alterações na neurogênese e da arquitetura encefálica das crianças (Wegiel et al., 2010). A outra grande característica observada em pacientes autistas é (ii) a presença de ativação neuroglial e neuroinflamação (Vargas et al., 2005). Células microgliais e astrócitos são críticos para um funcionamento normal da atividade neural, plasticidade sináptica, interações neurônio-glia e para uma correta conectividade cerebral (ver "referencial teórico"; capítulo 2). De fato, a presença de mecanismos relacionados com um incremento nas respostas neurogliais é considerada, na literatura, não só no contexto de doenças neurodegenerativas (ex.: doença de Alzheimer), mas também em doenças neuropsiquiátricas (ex.: esquizofrenia e ASD) (Salmina, 2009; Verkhratsky et al., 2013).

No cerebelo, a ativação glial tem sido associada a uma degeneração e perda de células de Purkinje. Finalmente, (iii) existe uma associação entre um aumento exacerbado da medida da cabeça (perímetro encefálico) durante os primeiros anos de vida da criança autista (Courchesne et al., 2003).

O desenvolvimento de organismos vivos é paralelo a um estresse fisiológico intrínseco associado a mudanças internas massivas durante a morfogênese (Kagias et al., 2012). A existência deste estresse fisiológico intrínseco pode estar contribuindo nessa janela de tempo de vulnerabilidade aos estressores ambientais. Além disso, considerando que a ativação glial e neuroinflamação parece persistir até a vida adulta dos pacientes autistas (Vargas et al., 2005), uma pergunta lógica seria se o aumento no volume do encéfalo durante os primeiros anos de vida, e característico da desordem, (Courchesne et al., 2003) poderia ser uma consequência de uma superativação da proliferação neural, como uma resposta adaptativa da criança para compensar a morte celular induzida pela ação conjunta de estressores ambientais e a genética do indivíduo. Um incremento total no número de células poderia levar a uma migração precipitada/não controlada das células neurais, levando, por exemplo, a uma distribuição aberrante dos neurônios nas diferentes áreas encefálicas. Apesar disso, não se conhecem bem os mecanismos moleculares ou "mínimo denominador molecular comum" (MDMC) que poderia estar por trás destes eventos.

Até onde chega nosso conhecimento, o conceito de mínimo denominador comum (ex.: molecular) no contexto do ASD foi mencionado pela primeira vez, na literatura, por Williams & Casanova (2010), salientando a importância de conhecer quais rotas de sinalização podem servir como ponte entre fatores genéticos e ambientais para entender os mecanismos patofisiológicos associados às desordens do espectro autista. De fato, na procura deste denominador, surge uma tendência a combinar

diferentes aspectos etiológicos ("hipóteses acumulativas") para poder explicar a heterogeneidade do autismo. Casanova, por exemplo, com a sua hipótese do "*triple hit*" (2007), propõe que sendo o ASD um transtorno pediátrico, os diferentes graus sintomatológicos observados podem ser uma consequência das combinações entre genética, fatores ambientais em conjunto com o momento exato do desenvolvimento onde a criança é exposta a este(s) estressor(es) externos. A vida pré-natal, a infância (sobretudo os três primeiros anos de vida) e a adolescência são, certamente, os momentos de maior vulnerabilidade (Charmandari et al., 2013). Nosso grupo também fez outra proposta que explicava que doses cumulativas de mercúrio, de diferentes fontes (ex.: poluição, consumo de peixe pelas mães, vacinas e o número de amálgamas dentais das mães) durante o desenvolvimento da criança, unido a uma falha na capacidade de eliminar (metabolizar) o mercúrio de nosso organismo poderia incrementar a probabilidade de desenvolver algum transtorno do espectro (Zeidán-Chuliá et al., 2011) (ver "referencial teórico"; capítulo 1). Então, o primeiro objetivo foi pensar em uma ferramenta suficientemente potente para que pudéssemos lidar com grandes quantidades de informação (literatura existente no tópico do ASD) e tentar criar um modelo de interações gene-ambiente no transtorno que integrasse todo o conhecimento até a data de escrita da tese no tópico. Se fosse possível fazer este modelo, seria possível especular que as aparentemente diferentes hipóteses etiológicas do autismo são, na verdade, diferentes caminhos de fazer alvo na(s) mesma(s) rota(s) bioquímicas e, então, poderiam se caracterizar os processos biológicos, componentes celulares e funções moleculares associadas a este modelo. Elas seriam comuns no ASD, porque a meta-análise prévia consideraria toda a literatura existente sobre o tópico. Além disso, poderíamos elucidar qual é o nó (gene e/ou composto) mais central nesse modelo, o que representaria o MDMC no ASD. No caso de encontrar um gene ou grupo

de genes centrais nunca antes descrito, poderia ser checada a sua expressão em biópsias de pacientes autistas *post mortem*.

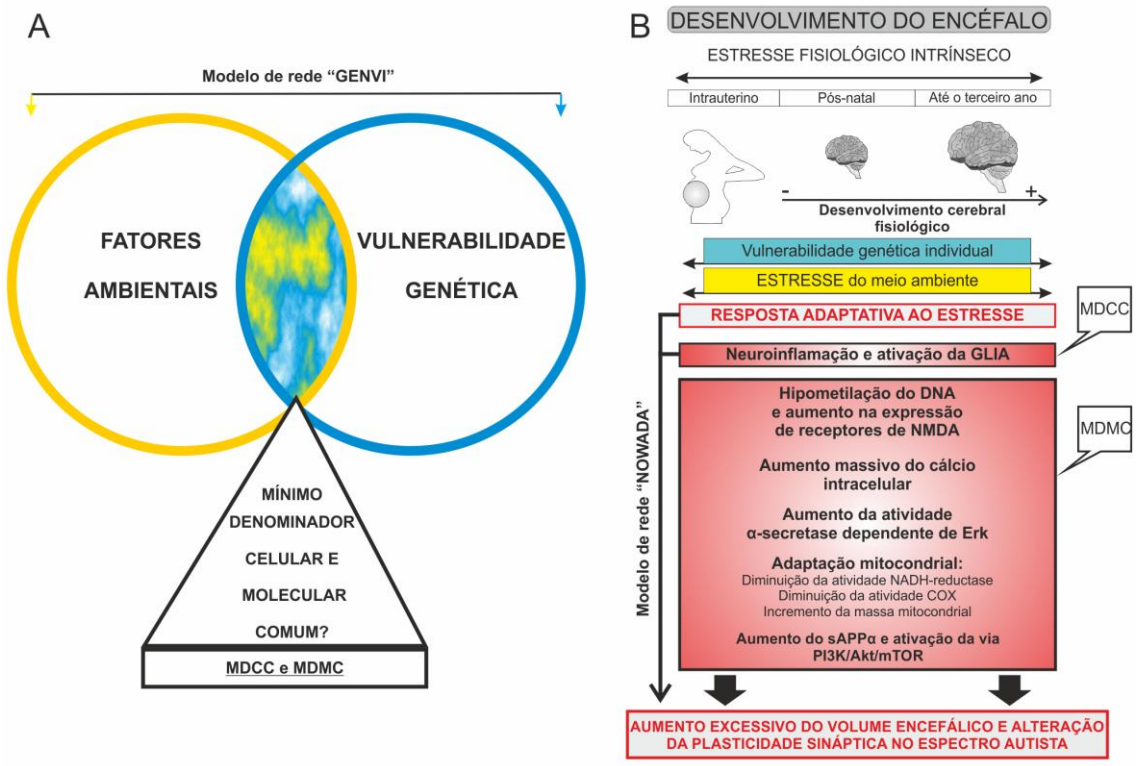


Figura 5. Mínimo denominador comum no espectro autista. A. Na complexa faixa representada por estressores ambientais e as diversas suscetibilidades genéticas no ASD encontra-se o que já foi chamado "mínimo denominador comum" (Williams & Casanova, 2010) a todas as desordens do espectro autista. B. Nossas redes *in silico* e análises de microarranjo propõem um modelo onde a resposta neuroinflamatória por parte da glia (MDCC), induzida por estressores ambientais, levaria a uma perda celular que seria compensada com um incremento na proliferação celular; mecanismo que seria mediado por cálcio (MDMC) e um incremento do sAPPα .

Fonte: elaborado pelo autor.

Nossa análise das interações gene-ambiente (**Figura 5**) no contexto autista mostrou o cálcio como molécula mais central do modelo *in silico* (GENVI). A comunicação celular mediada por cálcio no nosso encéfalo é um elemento característico na comunicação celular mediada por astrócitos. De fato, a neurogênese regulada por astroglia, plasticidade estrutural neuronal e formação de novas sinapses, poderia ter um papel fundamental na dinâmica da interação social (Mercadante et al., 2008). O

desenvolvimento normal do sistema nervoso precisa de um funcionamento organizado de uma série de processos como a migração neuronal, o crescimento de espinhos dendríticos e a formação e a eliminação de sinapses (Bolton & Eroglu, 2009). Porém, em condições estressantes (ex.: privação sensorial, estresse induzido por separação, ação tóxica de xenobióticos, traumas psicológicos) pode ocorrer uma ativação da glia, processos neuroinflamatórios que levem a mudanças na plasticidade estrutural do encéfalo em desenvolvimento (Musholt et al., 2009). As mudanças neurocitoanatómicas observadas no autismo como o crescimento excessivo do encéfalo nos primeiros anos de vida em áreas como cerebelo, lobo frontal e estruturas límbicas são mudanças associadas a uma proliferação celular excessiva, falha na diferenciação neuronal e presença de astrogliose (Pardo et al., 2005). Todas estas evidências sugerem uma desregulação das interações neurônio-glia e da sinalização mediada por cálcio no encéfalo em desenvolvimento de crianças autistas (ver "referencial teórico"; capítulo 2).

Nossa análise das interações gene-ambiente no contexto autista também assinalou a *RAC1*, em particular, e a família de RHO GTPases, em geral, como genes/proteínas relevantes na patologia autista, com o *CDC42* como gene com expressão mais reduzida no cerebelo de pacientes autistas. A família de Rho GTPases (especialmente, RAC, CDC42 e RHOA) é realmente um regulador chave do citoesqueleto de actina em resposta a sinais extracelulares e distúrbios na sua atividade que levam a mudanças drásticas na morfologia dos espinhos dendríticos (Etienne-Manneville & Hall, 2002). Em pacientes com FXS (do inglês, "*Fragile X syndrome*"), que pertence ao grupo de autismo sindrômico, e também no seu modelo animal (camundongos Knockout para *Fmr1*), observou-se um elevado número de espinhos dendríticos imaturos ou espinhos mais longos e mais finos do que o normal (sinal de imaturidade) (Sokol & Edwards-Brown, 2004).

A estrutura dos espinhos é crítica para a funcionalidade da sinapse e se esta estiver alterada, pode resultar em déficits comportamentais e cognitivos (Bear et al., 2004). De fato, neurônios com *CDC42* mutante mostram um cumprimento maior dos dendritos quando são comparados com neurônios normais (Scott et al., 2003).

Em outras palavras, nosso primeiro estudo mostrou, claramente, que o cálcio é a molécula central ou o MDMC e, e pela primeira vez na literatura, que a família de RHO GTPases poderia estar por trás das alterações observadas na morfologia dos espinhos dendríticos em pacientes autistas, tendo um papel crítico na patologia da desordem.

Um dado muito interessante derivado do modelo GENVI (gene-ambiente no contexto autista) ("Resultados"; artigo 1) é que a rede se encontra enriquecida em genes/proteínas da via WNT. A via de sinalização WNT, mais especificamente, a rota GSK-3β/β-catenina, tem sido vinculada a processos relacionados com a neurogênese no SNC (ver "referencial teórico"; capítulo 3). Ela também tem sido relacionada com o desenvolvimento da doença de Alzheimer, mostrando que o Aβ (ver **Figura 3**) pode inibir a via da β-catenina e contribuir ao déficit de neurogênese e reparo associado a estes pacientes (He & Shen, 2009). Os autores propõem que os altos níveis de Aβ aumentariam os níveis protéicos de GSK-3β, resultando na degradação proteossomal da β-catenina e, portanto, uma menor expressão de genes pró-neurais. Aliás, existe um estudo que mostra que o tratamento crônico com lítio (inibidor farmacológico de GSK-3) de pacientes com transtorno bipolar reduz significativamente a prevalência de sofrer a doença de Alzheimer (Nunes et al. 2007). Então, surge a questão se a via WNT e o processamento proteolítico do APP (geralmente associado à patologia da doença de Alzheimer) poderiam explicar alguns dos eventos característicos descritos no ASD. Esta foi a premissa do nosso segundo estudo, que pretendia criar um modelo de rede *in silico* (NOWADA) ("Resultados"; artigo 2), para elucidar se a expressão dos genes que

contribuem às rotas bioquímicas, tipicamente associadas à doença de Alzheimer, poderia estar alterada no autismo. Nossos resultados mostram que, efetivamente, a doença de Alzheimer e o autismo compartilham uma alteração no mesmo conjunto de genes. Porém, os dados sugerem que é a via não amiloidogênica, de caráter anabólico e pró-proliferativo, que é a favorecida em autismo e não a via amilodogênica de produção de Aβ, que é tipicamente vinculada à doença de Alzheimer. Nosso modelo propõe que o crescimento excessivo do encéfalo e falhas na conectividade encefálica poderiam ser explicados por um aumento na densidade de receptores NMDA e que o consequente superinfluxo de cálcio intracelular levaria a uma (i) adaptação mitocondrial para evadir a morte celular por apoptose e (ii) um aumento da atividade α-secretase, que levaria a um aumento do sAPPα. A consequência deste aumento é a ativação da via proliferativa PI3K-Akt-mTOR. Menor taxa de apoptose e maior de proliferação, durante o desenvolvimento encefálico, levam irremediavelmente a um encéfalo de tamanho maior e, portanto, a uma alteração na conectividade geral do encéfalo.

Existe um número de evidências circunstanciais na literatura que apoiam nosso modelo: (i) ao contrário do que se tem observado em pacientes com doença de Alzheimer, os níveis elevados de sAPPα encontraram-se no plasma de crianças autistas (Sokol et al., 2006; Ray et al., 2011); (ii) outros estudos mostram evidências de autoimunidade e ativação persistente do sistema imune de pacientes autistas (Ashwood & Van de Water, 2004); curiosamente, camundongos que superexpressam sAPPα, de fato, apresentam uma ativação do sistema imunitário, alterações na interação social, elevados níveis GFAP e níveis alterados de Notch1 e IL6 (Bailey et al., 2013). Similarmente, no autismo têm sido descritos elevados níveis de GFAP em áreas onde a arquitetura neuronal é alterada, sugerindo a presença de astrogliose, alterações

potenciais na neurogênese e a migração neuronal (Laurence & Fatemi, 2005; Vargas et al., 2005).

Com relação à presença de espinhos dendríticos anormais em pacientes autistas, nosso modelo concorda com resultados de outros grupos que mostram (i) que espinhos dendríticos respondem à estimulação mediada por receptores metabotrópicos de glutamato do grupo 1 em cultura de neurônios hipocampais e crescem (Vanderklish & Edelman, 2002) e que (ii) a ativação da via Raf/ERK inibe o desenvolvimento de espinhos dendríticos maduros em cultura primária de neurônios corticais (Yang et al., 2013).

A ideia clássica de que a função cerebelar é restrita ao controle e à coordenação motora está ficando obsoleta. Estudos recentes em humanos mostram uma contribuição cerebelar na cognição (linguagem), emoções (medo), no sono e até nas respostas viscerais, porque extensas rotas sinápticas conectam o cerebelo ao córtex cerebral, ao hipocampo, à amígdala, hipotálamo e à medula espinhal (Reeber et al., 2013). O cerebelo é funcional e anatomicamente anormal em pacientes com ASD e síndromes que compartilham a mesma sintomatologia cognitiva (autismo sindrômico). Este quadro é, geralmente, associado a mutações genéticas associadas que levam a um desenvolvimento anormal no cerebelo (Rogers et al., 2013). Em outras palavras, o cerebelo representa uma estrutura do SNC comumente alterada nos transtornos do espectro autista e síndromes genéticas associadas ao autismo (**Figura 1**); mas é também a área menos estudada e conhecida no contexto autista, motivo pelo qual decidimos usar biópsias cerebelares humanas de pacientes *post mortem* comparadas com amostras de controle em nossos estudos de microarranjo.

A última parte da tese validou, por farmacologia de sistemas, diferentes drogas que poderiam ser utilizadas para fazer alvo na via representada pela rede NOWADA (e alterada no autismo) e assinalou o magnésio e rapamicina como os melhores candidatos. Certamente, as RHO GTPasas (ex.: CDC42, RHOA e RAC1), que se encontram diferencialmente expressas no cerebelo de pacientes autistas ("Resultados"; artigo 1), usam magnésio como cofator para alcançar eficiência catalítica e especificidade na hidrólise de GTP (Zhang et al., 2000). Concentrações reduzidas de magnésio têm sido descritas no autismo e da mesma forma no sangue e encéfalo de pacientes com doença de Alzheimer (Strambi et al., 2006; Andrási et al., 2005; Barbagallo et al., 2011). Recentemente, tem-se sugerido que um aumento na concentração encefálica de magnésio tem um efeito sinapto-protetor num modelo animal da doença de Alzheimer, sugerindo um potencial terapêutico no tratamento desta neurodegeneração em humanos (Li et al., 2013). No autismo, têm-se descrito efeitos comportamentais positivos quando as crianças são tratadas com magnésio e piridoxina; uma combinação que também tem se mostrado efetiva em pacientes autistas que sofrem de convulsões epiléticas (Lelord et al., 1981; Francis et al., 2013; Frye et al., 2013). Mas o uso terapêutico da rapamicina (inibidor da via mTOR) no autismo é muito atual e ainda discutido. É conhecido o fato de que TSC1 e TSC2 são inibidores da atividade mTOR e estudos em camundongos $TSC2^{+/-}$ (modelo animal de síndrome genética associada ao autismo; ver **figura 1**) mostraram que o tratamento com rapamicina reverte as alterações comportamentais nestes animais (Han & Sagin, 2011; Ehninger et al., 2008). Os presentes resultados unidos aos poucos estudos nesta direção são animadores para futuros ensaios clínicos com inibidores da via mTOR em pacientes autistas. Porém, deixam em aberto uma pergunta lógica: estas drogas (magnésio e rapamicina) representariam tratamentos realmente eficazes em pacientes adultos ou seriam estratégias preventivas úteis se

fossem administradas em crianças ou adolescentes autistas? Ou, até mesmo, a gestantes que estivessem no grupo de risco para mães de autistas?

CONCLUSÕES

Nosso modelo integrativo de rede caracterizando as interações gene-ambiente fez possível a procura de um mínimo denominador comum molecular (MDCM) no ASD entre o complexo leque de interações gene-ambiente. A caracterização deste modelo mostrou que ele é enriquecido em genes importantes para processos biológicos como neurogênese e plasticidade sináptica, entre outros, assinalando o cálcio claramente como o MDCM que procurávamos. Nosso modelo também apresentou, pela primeira vez na literatura, que a expressão de genes da família RHO GTPase está alterada no cerebelo destes pacientes, podendo esta enzima estar envolvida no processo de formação de espinhos dendríticos imaturos, característica que contribui na falha de conectividade presente no encéfalo destas crianças. Nossa revisão sistemática da literatura aponta o papel fundamental das células da glia na neuroinflamação induzida pelos estressores ambientais (MDCC) e na morte celular associada, gatilho que induziria uma resposta adaptativa que ativa a proliferação de células neurais e a neurogênese. Nosso modelo de superestimulação dos receptores NMDA, elevado influxo de cálcio, adaptação mitocondrial para atenuar a apoptose induzida pelo cálcio e aumento de atividade α-secretase levaria a um aumento nos níveis de sAPPα e explicaria por que as crianças autistas apresentam um aumento da massa encefálica nos primeiros anos de vida. A via não amiloidogênica representaria a rota anabólica que levaria a esse estado pró-proliferativo. Esta rota poderia ser potencialmente modulada com o uso de terapias baseadas no magnésio ou na rapamicina. Estes estudos nos

permitiram formular as seguintes conclusões específicas: (i) o modelo *in silico* GENVI caracterizou a complexa interação gene-ambiente em ASD e demonstrou que é possível propor um modelo de rede que possa integrar todo o conhecimento até a data sobre fatores epidemiológicos associados ao autismo caracterizando os processos biológicos, componentes celulares e funções moleculares associados ao transtorno; (ii) a revisão sistemática da literatura e as análises por ferramentas computacionais apontam para o cálcio e as células da glia como MDCM e MDCC, respectivamente, em ASD; (iii) pela primeira vez na literatura, mostrou-se a expressão alterada de genes da família RHO GTPase no cerebelo destes pacientes, podendo estar envolvida no processo de formação de espinhos dendríticos imaturos; (iv) o modelo *in silico* NOWADA explica o crescimento aberrante do encéfalo e falha na conectividade neural em pacientes autistas, com base em uma resposta adaptativa que leva a um aumento dos níveis de sAPPα e incremento na proliferação celular durante o desenvolvimento do SNC; (v) nossa análise por farmacologia de sistemas salientou a exploração do magnésio e da rapamicina como drogas com potencial terapêutico ou preventivo em autismo.

PERSPECTIVAS

1- Mediante o uso de abordagens computacionais, procurar um marcador no sangue que permita o diagnóstico bioquímico do autismo;

2- Explorar o uso do transplante de células de Sertoli em modelos animais de autismo (ex.: LPS) como terapia potencial para o transtorno.

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ANEXOS

Communication Disorders, Deaf Studies & Hearing Aids

Zeidán-Chuliá and Fonseca Moreira, Commun Disord Deaf Stud Hearing Aids 2013, 1:1
http://dx.doi.org/10.4172/jcdsha.1000101

Letter to the Editor Open Access

Clostridium Bacteria and its Impact in Autism Research: Thinking "Outside The Box" of Neuroscience

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Keywords: Environmental factor; Microorganism; Infection; Toxin; Neurodevelopmental disorder; Psychiatric disorder

With a prevalence of 1 in every 50 children in the United States and an incidence that seems to be increasing with time [1,2], there is concern worldwide (not only within the society but also among the scientific community) about the etiologic cause/s of autism. The literature is full of hypotheses dealing with numerous environmental factors and genes accounting for its apparently higher prevalence and associated neuropathology, respectively [3-5].

Considering this multifactorial scenario, elucidation of routes that could potentially serve as point/s of crosstalk between genetic and environmental contributions, may be a priority to better comprehend the pathological basis of the disorder [6]. With this goal, our group recently published a network model able to integrate 112 genes/proteins and 191 environmental factors, already reported in the literature together with potential candidates in the context of autism, where calcium (Ca^{2+}) was shown to be its most relevant (central) node [3].

In addition to Ca^{2+}, the Rho GTPase RAC1 was shown to be among the most central nodes within the *in silico* model with no previous autism-related report in the literature. Furthermore, genes belonging to the Ca^{2+}-RHO family of GTPases interactome network revealed a differential gene expression in the cerebellum of autistic patients. Therefore, this family may indeed represent one of these points of crosstalk commonly altered in autism spectrum conditions.

A number of anaerobic bacteria are pathogenic to humans and their virulence is based on secreted toxins, which are mainly produced by species from the *Clostridium* genus [7]. Particularly, these are not invasive bacteria but their secreted active molecules can exert deleterious effects at a distance from the microorganism. Bolte [8] published a hypothetical paper postulating that a subgroup of children diagnosed with autism could be suffering from *Clostridium tetani* colonization of the intestinal tract and that the neurological symptoms were the direct result of *in vivo* production of tetanus neurotoxin.

Four years later, Finegold et al. [9] reported that autistic children had nine species of *Clostridium* not found in control children, whereas controls yielded just three species not found in children with autism. In an elegant study, Parracho et al. [10] demonstrated that the faecal flora of autism spectrum disorders (ASD) patients was enriched in *Clostridium histolyticum* group (*Clostridium* clusters I and II) of bacteria than that of healthy children; a particular bacteria group that are recognized to be toxin-producers. Ras and Rho family GTPases are specifically targeted by clostridial toxins [11].

For instance, specific inhibition of Rho, Rac, and Cdc42 by *Clostridium difficile* toxin B induces apoptosis of granule neurons [12] and can induce changes in spine and density morphology [13]. Thus, the centrality displayed by RAC1 in our *in silico* model of gene-environment interactions in the autistic context and the differential expression of the Rho family of small GTPases found in the cerebellum of patients [3] is consistent with reports supporting clostridial spores as key elements in the etiology of autism [14].

Moreover, higher concentrations of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA), a compound produced by different species of the *Clostridium* genus, have been found in urine samples of children with autism and seems to be also increased. In this study, the authors postulated it as a probable metabolite of m-tyrosine (or a tyrosine analog) able to deplete brain catecholamines and lead to typical autism-related symptomatology [15].

Nowadays, a number of researchers are paying attention to "gut dysbiosis" or a state of imbalance in the gut microbial ecosystem that includes excessive proliferation of specific organisms and loss of others, as a potential cause for several diseases and disorders like autism, obesity, and even diabetes [16-19]. With these examples, our aim is to emphasize the use of multidisciplinary research approaches, in addition to neuroscientific ones, to unravel the etiological causes and pathological events associated to autism; perhaps, the best example of multifactorial disorder.

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Received July 11, 2013; **Accepted** September 22, 2013; **Published** September 24, 2013

Citation: Zeidán-Chuliá F, Fonseca Moreira JC (2013) *Clostridium* Bacteria and its Impact in Autism Research: Thinking "Outside The Box" of Neuroscience. Commun Disord Deaf Stud Hearing Aids 1: 101. doi: 10.4172/jcdsha.1000101

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Citation: Zeidán-Chuliá F, Fonseca Moreira JC (2013) *Clostridium* Bacteria and its Impact in Autism Research: Thinking "Outside The Box" of Neuroscience. Commun Disord Deaf Stud Hearing Aids 1: 101. doi: 10.4172/jcdsha.1000101

Effective Clinical Care

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Critical review

Cell-based therapy proposals for the treatment of autism: Sertoli cells included in the 'tool box'?

F Zeidán-Chuliá*, JCF Moreira

Abstract

Introduction

Autism is a neurodevelopmental condition characterised by severe abnormalities in communication, social awareness and skills, and the presence of restrictive as well as stereotyped patterns of behaviours. Its aetiology is not exactly known to date. Without any definitive cure and just a few effective biomedical interventions, cell-based therapy approaches seems to be gaining interest by researchers in the topic. In this critical review, we summarised the current proposals and speculated whether testis-derived Sertoli cells could be used as an additional tool for cell therapy in such a context, considering their immune-privileged characteristics, safety and efficacy already reported when transplanted into the mammalian brain.

Conclusion

We believe that transplants of Sertoli cells, alone or in combination with other cell types, have potential to be a very useful tool (in addition to other cell sources), not only for acute and chronic neurodegenerative conditions, but also for neuropsychiatric disorders.

Introduction

Autism can be defined as a complex neurodevelopmental disorder characterised by impaired social interaction and communication, repetitive patterns of behaviour and unusual stereotyped interests[1]. Multiple putative causes (genetic and environmental) have been associated with this syndrome, which exhibits variable ranges of symptoms among patients[2,3]. It is currently estimated that autism affects one in 50 children in the United States[4]. However, it is still under debate whether such increasing numbers are due, at least in part, to clearer diagnostic concepts, diagnostic switching from other developmental disabilities to pervasive developmental disorders, service availability and/or increasing awareness about autistic spectrum disorders (ASDs) in general[5]. With all, there is no curative treatment for autism. Currently, proposed pharmacological therapies include tricyclic antidepressants, anticonvulsants, neurotransmitter reuptake inhibitors, acetylcholinesterase inhibitors and atypical antipsychotics with the aim of improving behavioural symptoms[6,7].

Cell-based therapy to the injured or diseased brain (e.g. stroke, Parkinson's disease and Alzheimer's disease) is still the approach that seems to keep higher expectations, not only in the scientific community but also within our society, since the discovery of stem cells as immature cells able to proliferate, self-renew and differentiate into diverse committed cellular types and tissues[8]. For instance, both neurons and glial cells have been shown to be successfully generated from embryonic stem cells, induced pluripotent stem cells, adult germ line stem cells, mesenchymal stem cells (MSCs) and, of course, neural stem cells[9–11], raising the question whether cell-based therapy (especially, with the use of MSCs) may be considered as an option in the future to correct the neurobiological deficits in autistic children. In this critical review, we summarise the promising proposals concerning this approach in the context of autism and suggest Sertoli cells (SCs) as an additional candidate source for future cellular transplantation.

Discussion

The 'tool box': cell-based therapy proposals in the context of ASD

In this article, it would be extremely difficult, if not virtually impossible, to describe in detail the aberrant synaptic functioning and brain connectivity in ASD at the genetic, molecular, cellular, regional and system levels due to space limitations; but the literature is already rich in studies that well illustrate the neuropathological events that characterise ASD[1,12–14]. Nevertheless, there are a number of characteristic features we would like to briefly highlight: (I) There are accumulating data suggesting that a common characteristic in autism cases may be oxidative stress; and such an event would represent the mechanism through which environmental factors may exert their deleterious effects (either prenatally, perinatally or postnatally), further exacerbated by the interaction of genetically susceptible alleles, and lead to the developmental abnormalities observed in the disorder[3,15]. Furthermore, evidence of reactive oxygen species-mediated damage to mitochondrial DNA in children with autism has also been reported[16]. (II) It has been demonstrated that GABA-A receptors are decreased in three brain regions (parietal cortex,

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FOR CITATION PURPOSES: *Zeidán-Chuliá F, Moreira JCF.* Cell-based therapy proposals for the treatment of autism: Sertoli cells included in the 'tool box'? OA Autism 2013 Apr 01;1(1):9.

Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to the conception, design, and preparation of the manuscript, as well as read and approved the final manuscript.
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superior frontal cortex and cerebellum) that have previously been implicated in the pathogenesis of the disorder, suggesting widespread GABAergic dysfunction in the brains of these subjects[17]. In contrast, significantly higher glutamate levels have been observed in the hippocampus, frontal regions and serum of autistic patients when compared to controls[18,19]. Glutamate is the primary excitatory neurotransmitter produced in the central nervous system, and overactivity of glutamate and its receptors lead to excitotoxicity that can account for the neuronal dysfunction observed in these patients[20]. (III) Other studies have described neuropathological changes in brain tissues of autistic patients (especially, in the cerebellum) in the form of extensive neuroglial responses characterised by both microglial and astroglial activation. Moreover, the results suggest that ASD subjects carrying more microglial activation correlates with higher impairment in their cognitive skills[21–23]. Activated microglia and astrocytes in postmortem brain tissue represent evidence for neuroinflammation. As a matter of fact, a number of studies have shown that the levels of glial fibrillary acidic protein are increased in autism. For instance, Purkinje cell loss in ASD was sometimes found to be accompanied by gliosis and increased expression of glial fibrillary acidic protein[22,23]. Cao et al.[24] reported astrocytosis in the frontal cortex with decrease in Wnt and β-catenin proteins in autistic subjects. In that study, branching processes, total branching length and cell body sizes of astrocytes from autistic patients were significantly reduced. (IV) Additionally, the neuroinflammatory process is characterised by the increase of transforming growth factor-β1 (TGF-β1), interleukin-6 (IL6) and IL10 in the brain of patients together with higher levels of inflammatory cytokines, such as tumour necrosis factor-α, interferon-γ (IFNγ), IL1, IL6, IL8 and IL12 in blood mononuclear cells, serum, plasma and cerebrospinal fluid of autistic subjects, as well as higher plasma heat shock protein-70, TGF-β2, caspase 7 and IFNγ when compared to age- and gender-matched controls[25]. (V) Dendritic spines are the major sites of information processing in the brain, and as for other cognitive disorders, aberrant spine morphology seems to be a characteristic feature in autism[26,27]. Abnormalities in dendritic spines have additionally been observed in brain specimens from epilepsy patients and animal models of epilepsy[28]. Interestingly enough, a significant overlap between epilepsy and ASD has been detected but it is not clear how often ASD may impact epilepsy and the effect of epilepsy on social cognition[29]. (VI) Autism has been considered as a paediatric autoimmune neuropsychiatric disorder since some studies have found serum antineuronal antibodies (e.g. serum levels of antiganglioside M1) in a subgroup of autistic children, which significantly correlated with the severity of the disorder[30,31]. (VII) A number of studies have reported cerebral hypoperfusion in autistic patients, correlated with certain symptomatology such as repetitive behaviours, desire for sameness, impairments in processing facial expressions and emotions as well as decreased language development[32–34].

To the best of our knowledge, one can basically find four elegant proposals in the form of 'review article' and 'letter to the editor' that suggest the potential use of cellular transplantation for the treatment of ASD (Table 1). Ghanizadeh[35,36] proposed two different (but complementary) alternatives for such an approach, based on the use of a GABAergic cell line or c-Kit+ cells with the aim of compensating the existing imbalance in the ratio of GABA to glutamate in autistic brains[17–20,37]. Unfortunately, no preclinical trials on animal models of autism have been performed to date, in order to check the plausibility of these promising hypotheses. The other two proposals presented MSCs as the most adequate candidate cell source for cell therapy in these patients due to their peculiar characteristics[38,39]. MSCs are multipotent adult stem cells, able to differentiate into cells not only of mesodermal origin (e.g. adipocytes, chondrocytes and osteocytes) but also of representative lineages of the three embryonic layers. These stem cells can be isolated from several and perhaps most postnatal organs and tissues such as umbilical cord, adipose

Table 1 Current proposals for the use of cell-based therapy in the context of autism

Cell source	Aim	Year	PMID
Cord blood expanded CD34+ cells together with MSCs	To compensate immune abnormalities and neural hypoperfusion	2007	17597540
GABAergic cell line	To compensate the GABA inhibitory neurotransmitter deficiency	2010	20934920
c-Kit+ cells	To prevent excitotoxicity by targeting the hyperglutamatergic state with the increase of GLT-1 transporter levels	2011	21225454
MSCs	To inhibit immune alterations To rescue the loss of Purkinje cells, cortical and synaptic plasticity To restore brain-damaged functions	2012	22496609

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tissue, placenta, amnion, dental pulp, cord blood and especially, bone marrow that seems to be the most often utilised source[11]. MSCs have become very popular among other cell sources belonging to what we could call 'the tool box' of cell-based therapy, probably due to their ability to modulate the immune response and thus, to exert immunosuppressive effects and their well-described potential to differentiate into neurons, hepatocytes, myocytes, chondrocytes, osteocytes and adipocytes[40]. In contrast to embryonic stem cells, which are obtained from the inner cell mass of the blastocyst, are associated with tumourigenesis, and their use involves ethical and legal considerations; the use of MSCs seems to be less problematic with regard to these issues[41]. Perhaps, the main reason why MSCs were suggested as a candidate source for cell therapy in these subjects[38,39] was their strong immunosuppressive activity and the capability to inhibit the release of proinflammatory cytokines that makes them a powerful tool for autologous and heterologous transplantations. Ichim et al.[39] additionally proposed the combined use of MSCs together with CD34+ cells with a potential to produce angiogenic factors and to give rise to endothelial cells themselves, for the treatment of hypoperfusion defect that has been associated with autism. MSCs are not prone to tumour formation and have a decade record of biosafety data *in vivo*[42] making these cells an exciting therapeutic option for the future. Nevertheless, as for Ghanizadeh's[35,36] proposals, it still remains to be elucidated the real potential of these cells when transplanted in different *in vivo* models of autism, and the tissue source from where these MSCs should be isolated for such a purpose, since it has been shown that many genes were differentially expressed in MSCs from different ontogenetic sources or from different culture conditions and they even posses different proliferative and differentiation potential[43,44].

The potential therapeutic use of SCs in ASD: why not?

It is generally accepted that the use of stem cells could potentially provide benefits when transplanted into a patient by integrating, differentiating and restoring functional and behavioural deficits in the central nervous system. On the other hand, major concerns still reside in avoiding tumourigenesis and graft rejection due to cellular transplantation[45,46]. Since the use of pharmacological immunosuppression has been associated with toxicity issues in both human and animal models[47,48], the search and election of safe immunoprivileged cell sources, like MSCs, appears to be mandatory for ensuring long-term viability of future clinical trials in humans.

Testis is an example of immune privilege site, and such a characteristic is critical for preventing a detrimental immune response against the autoimmunogenic germ cells. It is also critical for the tolerance of neo-antigens from developing germ cells that appear after the constitution of self-tolerance, but imposes the paradoxical challenge of providing sufficient and enough protection against pathogens and tumourigenic cells[49]. Somatic SCs are considered the main structural component of the seminiferous tubule, they give rise to the blood–testis barrier and their functions are essential for the generation of spermatozoa since these cells produce growth factors that stimulate self-renewal (GDNF and FGF2) and differentiation (activin A, BMP4 and SCF) of spermatogonial stem cells[50,51]. They also secrete TGF-β1, which is a potent immunosuppressive factor that suppresses the secretion of IFN-γ and tumour necrosis factor-α by immune cells[52,53]. These characteristics could actually confer local immunoprotection when transplanted alone or in combination with other cells. For instance, preclinical trials with positive results have been performed in animal models of Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis[54–58]. Furthermore, different research groups are developing novel protocols to genetically engineer SCs for providing continuous delivery of therapeutic proteins of interest[59]. Then, considering the autoimmune characteristics of autism with evidence of neuroinflammation, oxidative stress and both increased microglial and astroglial activation, could SCs represent an interesting candidate cell source to be tested for transplantations in the brain of animal models of autism? Already, one can find different successful studies in the literature that inspire optimism. For instance: (I) It has been demonstrated that transplanted testis-derived SCs into adult rat brains survive, produce localised immunoprotection and suppress microglial response when co-transplanted with bovine adrenal chromaffin cells (xenograft), and without the administration of systemic immunosuppressive drugs[60]. (II) In addition, it has been shown that intravenous infusion with SCs in islet transplantation induced systemic immune tolerance, reduced the peripheral blood lymphocyte and cytokine levels and effectively prolonged the survival of islet grafts[61]. (III) Besides, the survival and immunoprotective capability of SCs isolated from neonatal pigs have also been tested after transplantation in humans, in a type 1 diabetes mellitus patient with an islet xenotransplant[62]. This study showed evidence that co-xenotransplantation of these cells into a subcutaneous autologous collagen pouch could be considered a safe alternative for the control of type 1 diabetes.

Conclusion

We believe that transplants of SCs, alone or in combination with other cell types, have the potential to be a very useful tool (in addition to other cell sources), not only for acute and

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chronic neurodegenerative conditions, but also for neuropsychiatric disorders. In general, a major challenge for developing cell therapies is the use of appropriate animal models for testing both safety and efficacy of the approach and to collect the most complete preclinical data. In the context of autism, we face the same obstacle because the available *in vivo* models are of several, quite different, kinds and sometimes parallels with autism are uncertain. Optimal approaches to select animal models for either neurodegenerative or neuropsychiatric conditions require a deep understanding of the nature of the disease/disorder. We need a better understanding of the pathophysiologic mechanisms underlying autism and its symptoms. In other words, we have a putative tool, but where do we test it?

Acknowledgements

We apologise to all our colleagues whose studies were not cited due to limitation of space. We thank the Brazilian research funding agencies FAPERGS (PqG 1008860, PqG 1008857, ARD11/1893-7, PRONEX 1000274), CAPES (PROCAD 066/2007), CNPq, PROPESQ-UFRGS and IBN-Net #01.06.0842-00 for providing us financial support.

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Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to the conception, design, and preparation of the manuscript, as well as read and approved the final manuscript.
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Competing interests: none declared. Conflict of interests: none declared.
All authors contributed to the conception, design, and preparation of the manuscript, as well as read and approved the final manuscript.
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