UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

IOHANNA DECKMANN

ALTERAÇÃO DE PERMEABILIDADE DE BARREIRA HEMATOENCEFÁLICA E POSSÍVEIS MECANISMOS SUBJACENTES NO CONTEXTO DO TRANSTORNO DO ESPECTRO AUTISTA: EFEITO PREVENTIVO DO RESVERATROL

TESE DE DOUTORADO

Porto Alegre
2022

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Doutora em Bioquímica.

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SUMÁRIO

PARTE I

Resumo

O Transtorno do Espectro Autista (TEA) pode apresentar uma infinidade de condições clínicas associadas, como maior volume encefálico nos primeiros anos de vida em uma parcela dos pacientes; dessa forma, nos propusemos a avaliar, em modelo animal de autismo, processos possivelmente associados à formação de macrocefalia, bem como revisar na literatura os possíveis mecanismos subjacentes a essa dinâmica. No Capítulo I, avaliamos o conteúdo encefálico de água, a permeabilidade da barreira hematoencefálica (BHE), a expressão das aquaporina (AQP) 1 e 4 e da proteína ácida fibrilar glial (GFAP, um marcador de astrócitos) no modelo animal de TEA por exposição ao ácido valproico (VPA), e o potencial efeito do resveratrol (RSV). O tratamento com RSV preveniu a maior permeabilidade da BHE e o edema encefálico presentes no grupo VPA. A exposição ao VPA diminuiu os níveis da AQP1 no plexo coroide, na área somatossensorial primária, na região da amígdala e no córtex pré-frontal medial, reduziu os níveis de AQP4 no córtex pré-frontal medial e aumentou AQP4 na área somatossensorial primária (com prevenção pelo RSV), além de aumentar o número de astrócitos e a imunomarcação de GFAP na área somatossensorial primária e no córtex pré-frontal medial, com melhora funcional promovida pelo RSV. No Capítulo II, fizemos uma extensa revisão da literatura e observamos a translacionalidade das alterações imunológicas entre pacientes com TEA e o modelo de TEA por exposição ao VPA. As alterações neuroimunológicas são uma marca registrada no TEA; nesse sentido, hipotetizamos que a indução do modelo de TEA pelo VPA possivelmente envolve mecanismos de ativação imunitária materna e que as alterações neuroimunológicas podem estar por trás das alterações observadas no Capítulo I, uma vez que são relacionadas com disfunções de BHE. Finalmente, no Capítulo III, revisamos a contribuição das alterações no metabolismo das purinas na fisiopatologia do TEA, pois, apesar da etiologia desse transtorno permanecer desconhecida, algumas rotas biológicas já foram associadas ao desencadeamento e/ou progressão do TEA, como a sinalização purinérgica. Em resumo, demonstramos a importante contribuição do modelo VPA também nos estudos de disfunções de barreiras neurais – ampliando os conhecimentos e a aplicabilidade desse modelo –, bem como reforçamos o caráter neuroprotetor preventivo do RSV nas disfunções da BHE devido ao seu papel tanto na manutenção da integridade da BHE quanto na prevenção de alterações subjacentes observadas. Finalmente, a partir de investigações translacionais, postulamos que as disfunções neuroimunológicas podem promover as alterações tanto em barreiras neurais quanto em vias biológicas associadas ao TEA, como a sinalização purinérgica, o que pode contribuir, não só na fisiopatologia, mas também na manutenção das características associadas a esse transtorno.

Palavras-chave: transtorno do espectro autista; ácido valproico; resveratrol; barreiras neurais; barreira hematoencefálica; aquaporina; astrócito; sistema nervoso central.

Abstract

Autism Spectrum Disorder (ASD) can present a plethora of associated clinical conditions, such as increased brain volume in the first years of life in a portion of patients; therefore, we proposed to evaluate, in an animal model of autism, processes possibly associated with the formation of macrocephaly, as well as to review, in the literature, the possible mechanisms underlying this dynamic. In Chapter I, we evaluated brain water content, blood-brain barrier (BBB) permeability, aquaporin (AQP) 1 and 4, and glial fibrillary acidic protein (GFAP, a marker of astrocytes) expression in the animal model of ASD by exposure to valproic acid (VPA), and the potential effect of resveratrol (RSV). Treatment with RSV prevented the increased permeability of the BBB and the brain edema present in the VPA group. Exposure to VPA decreased AQP1 levels in the choroid plexus, primary somatosensory area, amygdala region, and medial prefrontal cortex, reduced AQP4 levels in the medial prefrontal cortex, and increased AQP4 in the primary somatosensory area (with prevention by RSV), in addition to an increase in the number of astrocytes and GFAP immunostaining in the primary somatosensory area and in the medial prefrontal cortex, with functional improvement promoted by RSV. In Chapter II, we revised the literature and looked at the translationality of immunological changes between ASD patients and the VPA exposure model of ASD. Neuroimmunological changes are a hallmark of ASD; in this sense, we hypothesized that the induction of the ASD model by the VPA possibly involves mechanisms of maternal immune activation and that neuroimmunological alterations may be behind the alterations observed in Chapter I, since they are related to BBB dysfunctions. Finally, in Chapter III, we also reviewed the contribution of changes in purine metabolism to the pathophysiology of ASD, because, although the etiology of this disorder remains unknown, some biological pathways have already been associated with the triggering and/or progression of ASD, such as purinergic signaling. In summary, we demonstrated the important contribution of the VPA model also in the studies of neural barriers dysfunctions – expanding the knowledge and applicability of this model –, as well as reinforcing the preventive neuroprotective character of RSV in BBB dysfunctions due to its role both in maintaining the BBB integrity and prevention of underlying changes observed. Finally, from translational investigations, we postulated that neuroimmunological dysfunctions could promote changes both in neural barriers and in biological pathways associated with ASD, such as purinergic signaling, which can contribute not only to the pathophysiology but also to the maintenance of the characteristics associated with this disorder.

Keywords: autism spectrum disorder; valproic acid; resveratrol; neural barriers; blood-brain barrier; aquaporin; astrocyte; central nervous system.

Lista de abreviaturas

ABC – lista de verificação de comportamento aberrante, do inglês *aberrant behavior checklist*

ADORA2A/ADORA3 – receptores de adenosina A2A e A3

AIM – ativação imunitária materna

AKT – proteína cinase B ou serina/treonina cinase

AMPc – monofosfato de adenosina cíclico, do inglês *cyclic adenosine monophosphate*

AP-1 – proteína ativadora 1, do inglês *activator protein-1*

AQP – aquaporina

ATP – trifosfato de adenosina, do inglês *adenosine triphosphate*

BA – Área de Brodmann, do inglês *Brodmann Area*

BDNF – fator neurotrófico derivado do cérebro, do inglês *brain derived neurotrophic factor*)

BHE – barreira hematoencefálica

BHL – barreira hematoliquórica

BTBR – cepa de camundongo BTBR $T^{+}Itpr3^{tf}/J$, um modelo genético de TEA

CDC – Centro de Controle e Prevenção de Doenças, do inglês *Centers for Disease Control and Prevention*

CGI – impressão clínica global, do inglês *clinical global impression*

CHIP28 –proteína integral formadora de canal de 28 kDa, do inglês *channel-forming integral protein of 28 kDa*, nome inicial da AQP1

COX – ciclo-oxigenase

CX – conexina

CXCL1 – ligante de quimiocina 1, do inglês C-X-C *motif chemokine ligand 1*

DNA – ácido desoxirribonucleico, do inglês *deoxyribonucleic acid*

DSM-5 – Manual de Diagnóstico e Estatística de Transtornos Mentais, do inglês *Diagnostic and Statistical Manual of Mental Disorders*

Egr3 – fator de resposta de crescimento precoce 3, do inglês *early growth response factor 3*

ERβ – receptor de estrogênio β, do inglês *estrogen receptor beta*

ESAM – moléculas de adesão seletivas de endotélio, do inglês *endothelial cell-selective adhesion molecule*

FMR1 – regulador translacional FMRP 1, gene humano que codifica a proteína relacionada ao retardo mental do X frágil 1 (FMRP)

Foxp3 – fator de transcrição *forkhead box P3*

GABA – neurotransmissor ácido γ-aminobutírico, do inglês *gamma aminobutyric acid*

GABA-T – GABA-transaminase

GAT – transportador de GABA, do inglês *GABA transporter*

GATA3 – fator de transcrição que reconhece sequências de nucleotídeos G-A-T-A em promotores de genes alvo, ativando-os ou reprimindo-os

GETTEA – Grupo de Estudos Translacionais em Transtorno do Espectro Autista

GFAP – proteína ácida fibrilar glial, do inglês *glial fibrillary acidic protein*

GLAST/EAAT1 – transportador de glutamato/aspartato ou transportador de aminoácido excitatório 1, do inglês *glutamate/aspartate transporter/excitatory aminoacid transporter 1*

Glt-1 – transportador de glutamato 1, do inglês *glutamate transporter-1*

GM-CSF – fator estimulador de colônias de granulócitos e macrófagos, do inglês *granulocyte-macrophage colony-stimulating factor*

GS – glutamina sintetase

GSH – glutationa (L-gama-glutamil-cisteinil-glicina)

GSK-3β – glicogênio sintase cinase 3 beta, do inglês *glycogen synthase kinase 3 beta*

GWAS – estudos de associação ampla do genoma, do inglês *genome wide association studies*

HDAC – desacetilases de histonas, do inglês *histone deacetylase*

HLA – antígeno leucocitário humano, do inglês *human leukocyte antigen*)

IFN-γ – interferon gamma

IL – interleucina

iNOS – óxido nítrico sintase, do inglês *inducible nitric oxide synthase*

JAM – moléculas juncionais de adesão, do inglês *junctional adhesion molecules*

KCC4 – cotransportador 4 de potássio e cloreto

Kir – canais retificadores internos de K^+

LCR – líquido cefalorraquidiano ou líquor

MAPK – proteína cinase ativada por mitogênio, do inglês *mitogen-activated protein kinase*

MCP-1 – proteína quimioatrativa para monócitos 1, do inglês *monocyte chemoattractant protein-1*

MECP2 – gene codificador da proteína ligante de metil-CpG 2 (MeCP2), associado à Síndrome de Rett e ao Transtorno do Desenvolvimento Intelectual

miR – microRNA

MMP-9 – metaloproteinase de matriz-9, do inglês *matrix metalloproteinase-9*

mTOR – alvo da rapamicina em mamíferos, do inglês *mammalian target of rapamycin*

NBCe2 – cotransportador eletrogênico de sódio/bicarbonato

NDD – distúrbios do neurodesenvolvimento, do inglês *neurodevelopmental disorders*

NFκB – fator nuclear kappa B (NF-κb), do inglês *nuclear factor kappa b*

NO – óxido nítrico, do inglês *nitric oxid*

NOTCH – via biológica caracterizada pelo receptor *notch*

NPA – sequência asparagina-prolina-alanina conservado ao longo da sequência de aminoácidos das aquaporinas

Nrf2 – fator nuclear 2 relacionado ao eritroide-2, do inglês, *nuclear factor erythroid 2–related factor 2*

PBMCs – células mononucleares de sangue periférico, do inglês *peripheral blood mononuclear cells*

PDE – fosfodiesterase, do inglês *phosphodiesterase*

PDGFβR – receptor β do fator de crescimento derivado de plaquetas, do inglês *platelet-derived growth factor (PDGF) β receptor*

PGC-1α – coativador-1 'alfa' do receptor ativado por proliferador do peroxissoma, do inglês *peroxisome proliferator-activated receptor-gamma coactivator 1-alpha*

PI3K – fosfoinositídeo 3-cinase

PTEN – fosfatase homóloga à tensina, do inglês *phosphatase and tensin homologue*

RhoA – membro da família Ras homólogo A, do inglês *Ras homolog family member A*

RNAm – ácido ribonucleico mensageiro, do inglês *messenger ribonucleic acid*

RORγt – receptor órfão gama relacionado ao receptor de ácido retinoico (RAR), do inglês *retineic-acid-receptor-related orphan nuclear receptor gamma*

RSV – *trans*-resveratrol (3,4′,5-triidroxiestilbeno)

SIRT – sirtuína

SNC – sistema nervoso central

SNP – polimorfismos de nucleotídeo único, do inglês *single nucleotide polymorphism*

SOCS-1 – supressor da sinalização de citocina 1, do inglês *suppressor of cytokine signaling 1*

SOD – superóxido dismutase

SSADH – semialdeído succinato desidrogenase, do inglês *succinic semialdehyde dehydrogenase*

STAT – transdutores de sinal e ativadores de transcrição, do inglês *signal transducers and activators of transcription*

T-bet – fator 21 de transcrição da T-Box, do inglês *T-Box transcription factor 21*

TDAH – transtorno de déficit de atenção e hiperatividade

TEA – transtorno do espectro autista

TGFβ1 – fator de crescimento transformador beta 1, do inglês *transforming growth factor beta 1*

TNF-α – fator de necrose tumoral alfa, do inglês *alpha tumor necrosis factor*

TSC 1 e 2 – complexo da esclerose tuberosa 1 e 2, do inglês *tuberous sclerosis complex*, genes associados à Esclerose Tuberosa

VPA – ácido valproico (ácido 2-propilpentanoico), do inglês *valproic acid*

Wnt – via biológica cuja sigla em inglês contém letras extraídas das palavras *Wingless* (drosófilas mutantes sem asas) e *Int* (denominação do gene mutante que causa a ausência de asas em drosófilas)

ZO – proteínas da zônula de oclusão

Lista de figuras

1. INTRODUÇÃO

1.1. Transtorno do Espectro Autista

Os distúrbios do neurodesenvolvimento (NDDs, do inglês *neurodevelopmental disorders*) são descritos ao longo dos anos mesmo antes dessa terminologia moderna (MORRIS-ROSENDAHL; CROCQ, 2020). Os NDDs, segundo a 5ª edição do Manual de Diagnóstico e Estatística de Transtornos Mentais (DSM-5, do inglês *Diagnostic and Statistical Manual of Mental Disorders*), agrupam condições de início precoce – cujas características incluem deficiências na linguagem, cognição, entre outros – tais como distúrbios de aprendizagem específicos, deficiência intelectual, distúrbios motores e de comunicação, transtorno de déficit de atenção e hiperatividade (TDAH) e transtorno do espectro autista (TEA) (AMERICAN PSYCHIATRIC ASSOCIATION, 2013).

O termo “autismo” deriva do grego αὐτός (autos) e significa “de si mesmo, em relação a si mesmo”. Foi cunhado por Paul Bleuler (em 1911) para designar o retraimento social, considerado um dos sintomas mais graves em pacientes com esquizofrenia (KUHN, 2004). Creditava-se a Leo Kanner o pioneirismo na descrição do TEA ao relatar um distúrbio complexo definido por parâmetros comportamentais presente em um grupo de crianças com dificuldades em comum, como prejuízo de interação social (KANNER, 1943). Na mesma década, Hans Asperger também relatou um grupo de crianças com deficiências sociais e de comunicação, porém com alta inteligência não-verbal (ASPERGER, 1944; PEARCE, 2005).

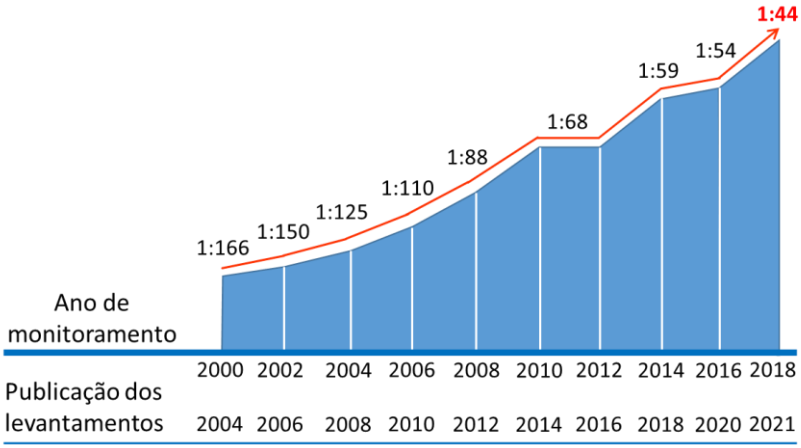
Graças a crescentes esforços com o objetivo de dar os devidos créditos às mulheres por sua contribuição nas mais diversas áreas, recentemente a comunidade científica reconheceu a psiquiatra russa/soviética Grunya Ssucharewa como pioneira no contexto do TEA pela descrição de 6 crianças com características autistas (ZELDOVICH, 2018), cujos sintomas incluíam “falta de expressividade facial e de movimentos expressivos, afastamento de seus semelhantes, fala estereotipada e interesses exclusivos” (SSUCHAREWA, 1926).

1.1.1. Prevalência

O TEA é uma desordem altamente prevalente e de incidência crescente (Figura 1). Levantamentos epidemiológicos mais recentes estimam 1 caso a cada 44 crianças até 8 anos de idade nos Estados Unidos, afetando 4,2 vezes mais o sexo masculino em relação ao feminino (MAENNER et al., 2021). Essa alta prevalência é associada a

diversos investimentos públicos em saúde e elevado custo econômico e social, incluindo custos em relação à perda de produtividade dos pais/responsáveis por indivíduos com TEA, custos médicos, terapêuticos e com educação (especial), sendo esse último um dos principais componentes (ROGGE; JANSSEN, 2019).

Figura 1 – Prevalência de TEA nos Estados Unidos no século XXI.



Adaptado de Centro de Controle e Prevenção de Doenças (CDC, do inglês *Centers for Disease Control and Prevention*).

1.1.2. Diagnóstico

O TEA agrupa transtornos anteriormente separados (síndrome de Asperger, autismo clássico, transtorno desintegrativo da infância e transtornos invasivos do desenvolvimento não especificados) e é caracterizado por prejuízos em dois domínios fundamentais para o diagnóstico (AMERICAN PSYCHIATRIC ASSOCIATION, 2013):

a) Prejuízo na comunicação e interação social em múltiplos contextos, o que compreende déficits em reciprocidade social, na comunicação não-verbal utilizada para interação social e em habilidades para iniciar, manter e entender relacionamentos, dificuldade de contato visual, entre outros;

b) Comportamentos repetitivos, estereotipias, atividades e interesses restritos, como repetir palavras ou frases continuamente (ecolalia), rigidez comportamental (dificuldade com mudanças de rotina, interesses obsessivos), hiper ou hiporresponsividade sensoriais a estímulos (não nocivos e nocivos, respectivamente), interesses exagerados em aspectos sensoriais do ambiente, entre outros.

Além dos sintomas centrais, esses indivíduos podem apresentar ainda uma ampla gama de sintomas e comorbidades associadas, como irritabilidade (MCGUIRE

et al., 2016), prejuízo intelectual (BAUMAN, 2010; MEFFORD; BATSHAW; HOFFMAN, 2012; SRIVASTAVA; SCHWARTZ, 2014), TDAH (LAU-ZHU; FRITZ; MCLOUGHLIN, 2019), transtornos de ansiedade (ZABOSKI; STORCH, 2018), problemas de conduta (HERVAS; RUEDA, 2018) e do sono (DEVNANI; HEGDE, 2015), epilepsia (GESCHWIND, 2009; SPENCE; SCHNEIDER, 2009), problemas gastrointestinais (WASILEWSKA; KLUKOWSKI, 2015), além de alterações sensoriais em mais de 90% do pacientes (CHANG et al., 2014).

Apesar dos critérios diagnósticos serem bem delimitados, ainda há certo atraso no reconhecimento das alterações comportamentais. Essa dificuldade de identificação se dá, também, pela ausência de biomarcadores, além da apresentação altamente heterogênea e multifatorial desse transtorno (GOTTFRIED et al., 2013). O diagnóstico que, idealmente, deveria ocorrer até os 2 anos de idade, comumente ocorre quando a criança apresenta a sintomatologia característica da díade comportamental e/ou não apresenta habilidades típicas da sua idade (DOVER; LE COUTEUR, 2007).

Levando-se em conta esse contexto, as técnicas de diagnóstico por imagem têm sido úteis na pesquisa sobre alterações eletrofisiológicas, anatômicas e funcionais uma vez que fornecem um robusto material acerca do encéfalo *in vivo* (TCHACONAS; ADESMAN, 2013) tanto de pessoas diagnosticadas com TEA quanto com elevado risco genético para o transtorno. No início dos anos 2000, foi conduzido o primeiro estudo preditivo a partir dessas técnicas: analisando o cerebelo, mais de 95% dos casos de TEA (dentre 52 pacientes) e 92% dos casos controle foram classificados corretamente (AKSHOOMOFF et al., 2004). Outros parâmetros neuroanatômicos já foram utilizados como métricas preditivas, como excesso de líquido cefalorraquidiano (LCR ou líquor) entre o espaço subaracnoide e a superfície cortical do encéfalo (extra-axial) (SHEN et al., 2013, 2017, 2018), aumento da espessura e área de superfície cortical (HAZLETT et al., 2017; XIAO et al., 2017), redes de conectividade de substância branca (JIN et al., 2015) e conectividade inter-hemisférica mais fraca em áreas de linguagem (DINSTEIN et al., 2011; LOMBARDO et al., 2015). Recentemente, um estudo avaliando 59 bebês de 6 meses de idade com alto risco para desenvolver o transtorno, apresentou 82% de sensibilidade (predizendo corretamente um diagnóstico de TEA aos 24 meses) e 100% de especificidade (prevendo um diagnóstico negativo para TEA) a partir da identificação de conexões funcionais em

regiões relacionadas com comportamento social e repetitivo, linguagem, desenvolvimento motor – tipicamente associadas ao TEA (EMERSON et al., 2017).

Apesar dos exames por imagem se mostrarem uma alternativa diagnóstica interessante e com maior precisão na predição precoce de TEA (comparativamente a métodos de triagem comportamentais) em bebês de alto risco, somente a análise de alterações neuroanatômicas ainda não é uma métrica de predição forte o suficiente para ser extrapolada para a rotina clínica (GENG; KANG; WONG, 2020), mesmo utilizando outras técnicas complementares, como o eletroencefalograma (EEG), que aumenta para 95% o valor preditivo positivo (BOSL; TAGER-FLUSBERG; NELSON, 2018; GABARD-DURNAM et al., 2019) do diagnóstico. Porém, a avaliação do desenvolvimento inicial do encéfalo em crianças de alto risco genético pode ser uma estratégia promissora na investigação de biomarcadores e de subtipos de TEA.

1.1.3. Fatores de risco

O TEA é uma desordem de causa idiopática; no entanto, acredita-se que o gatilho etiológico envolve não somente fatores genéticos e ambientais, mas também suas interações (GOTTFRIED et al., 2015).

Especula-se que 5-30% dos casos de TEA podem ser explicados por mutação de um único gene ou por rearranjo genômico (duplicações ou deleções) (SCHAAF et al., 2020). Já estudos com gêmeos dizigóticos e monozigóticos demonstram concordância genética de 31% e 88%, respectivamente. Ainda, quando considerado o sexo, a concordância é de 100% para gêmeas monozigóticas e de 86% quando os irmãos são do sexo masculino (ROSENBERG et al., 2009). Essa alta concordância não deixa dúvidas de que a herança genética é um fator determinante. Além disso, já foram descritos genes que desencadeiam condições genéticas com alta sobreposição ao TEA, como a Síndrome do X Frágil (regulador translacional FMRP 1 – FMR1), a Esclerose Tuberosa (complexo da esclerose tuberosa 1 e 2 – TSC1 e 2), a Síndrome de Cowden (fosfatase homóloga à tensina – PTEN), a Síndrome de Rett (gene codificador da proteína ligante de metil-CpG 2 – MECP2) (BELMONTE; BOURGERON, 2006; BROWN et al., 1982; FELICIANO et al., 2013; NUMIS et al., 2011). Complementarmente, alguns fatores epigenéticos parecem estar envolvidos no desencadeamento do TEA, como a idade avançada dos genitores (DURKIN et al.,

2008), o que poderia ter relação com alguns fatores como metilação de DNA, diminuição na fertilidade e mudança do estilo de vida (OLSEN; ZHU, 2009).

Levando-se em conta fatores ambientais, sabe-se que o Sistema Nervoso Central (SNC) em desenvolvimento é particularmente vulnerável a danos externos. Nesse sentido, observações epidemiológicas sugerem que a exposição a substâncias teratogênicas pode ser um dos fatores envolvidos no desencadeamento do TEA. Entre os fatores ambientais já descritos como possíveis gatilhos, estão os fatores relacionados diretamente à mãe, como nutrição materna, equilíbrio hormonal, estresse e a ativação imunitária materna (AIM), e a exposição materna a substâncias químicas, incluindo poluentes atmosféricos, pesticidas, derivados de plásticos e metais (CHERONI; CAPORALE; TESTA, 2020), além de substâncias como a talidomida (IMAI et al., 2014) e o ácido valproico (VPA) (CHRISTENSEN et al., 2013; ROULLET; LAI; FOSTER, 2013; SMITH; BROWN, 2014).

1.1.4. Ácido valproico como fator de risco e indutor do modelo animal de TEA

O VPA (ácido 2-propilpentanoico) é um ácido carboxílico de cadeia ramificada, altamente lipossolúvel e permeável tanto à barreira hematoencefálica (BHE) quanto à placentária (BRUNI; WILDER, 1979). Foi utilizado como solvente orgânico para outras moléculas até que sua propriedade anticonvulsivante foi descoberta (BURTON, 1882; MEUNIER et al., 1963). Desde então, tem sido utilizado na indústria farmacêutica como um dos principais medicamentos no tratamento de epilepsia, transtorno bipolar e profilático de enxaqueca (REYNOLDS; SISK; RASGON, 2007) devido a sua rápida absorção no trato gastrointestinal, alta biodisponibilidade, curto tempo de meia-vida e rápida eliminação do organismo (BRUNI; WILDER, 1979; SILVA et al., 2008).

Já foram descritos alguns mecanismos prováveis pelos quais o VPA exerce seu papel anticonvulsivante, como aumento dos níveis do neurotransmissor ácido γ-aminobutírico (GABA) (REYNOLDS; SISK; RASGON, 2007), favorecimento da transcrição gênica por meio da inibição de desacetilases de histonas (HDAC, do inglês *histone deacetylase*) (PHIEL et al., 2001) e na ativação das sirtuínas (REID et al., 2005). Em relação ao TEA, apesar de ainda não se conhecerem os mecanismos fisiopatológicos desencadeados pela exposição intraútero ao VPA (NICOLINI; FAHNESTOCK, 2018), acredita-se que envolva mecanismos de AIM (FONTES-DUTRA et al., 2020).

No final dos anos 90, foram feitos os primeiros relatos de associação entre TEA e exposição ao VPA durante o neurodesenvolvimento tanto em indivíduos cujas mães utilizaram esse fármaco durante o período gestacional (CHRISTIANSON; CHESTER; KROMBERG, 1994; MOORE et al., 2000; WILLIAMS et al., 2001; WILLIAMS; HERSH, 1997) quanto em modelos animais de roedores com a indução de características do tipo autista na prole de ratas expostas ao teratógeno, os quais apresentaram alterações morfológicas no cerebelo, no tronco encefálico e nos nervos cranianos (semelhantes às descritas em indivíduos com TEA) (INGRAM et al., 2000; RODIER et al., 1997, 1996). Em meados dos anos 2000, o pesquisador Tomasz Schneider foi pioneiro na padronização de testes capazes de avaliar características do tipo autista em roedores e na demonstração dessas alterações (SCHNEIDER; PRZEWŁOCKI, 2005), validando o modelo animal de autismo por exposição pré-natal ao VPA.

Além dos déficits em comportamentos equivalentes ao comportamento social em humanos com TEA (BAMBINI-JUNIOR et al., 2011; SCHNEIDER; PRZEWŁOCKI, 2005; YOCHUM et al., 2008), também já foram descritos prejuízos em outros domínios comportamentais, como aumento de estereotipias (SCHNEIDER et al., 2008), rigidez comportamental (BAMBINI-JUNIOR et al., 2011), menor sensibilidade a estímulos nocivos (SCHNEIDER et al., 2008; WANG et al., 2016), atividade exploratória aumentada (SCHNEIDER et al., 2008; TSUJINO et al., 2007), comportamentos do tipo ansioso (MARKRAM et al., 2008; SCHNEIDER et al., 2008), entre outros. No Brasil, nosso grupo de pesquisa foi o primeiro a ampliar os estudos pré-clínicos para investigação dos possíveis gatilhos etiológicos através da impressão digital deixada pela exposição intraútero ao VPA (BAMBINI-JUNIOR et al., 2011). Ainda, em 2013, foi publicado o estudo mais contundente em relação à predisposição ao TEA pela exposição ao VPA: a utilização desse fármaco durante a gestação – especialmente no primeiro trimestre – eleva em quase 5x o risco de ter filhos com diagnóstico de TEA (CHRISTENSEN et al., 2013).

Sabe-se que o primeiro trimestre gestacional é especialmente vulnerável a quaisquer eventos danosos uma vez que compreende o período de neurogênese, uma conhecida janela biológica de risco para diversos transtornos neuropsiquiátricos, inclusive o TEA (COURCHESNE et al., 2018). Como já mencionado, o modelo animal VPA reproduz a circunstância que leva ao transtorno (validade de construto), replica parâmetros comportamentais, moleculares e morfológicos similares aos encontrados

na condição humana (validade de face), além de possibilitar o estudo de estratégias terapêuticas eficazes para o tratamento da sintomatologia associada (validade preditiva) (MABUNGA et al., 2015). Dessa forma, modelos animais são importantes ferramentas de estudo que possibilitam a obtenção dos mais diversos tipos de amostras biológicas, bem como permitem a análise de vias biológicas potencialmente envolvidas na fisiopatologia do transtorno, facilitando a aquisição de conhecimentos que podem auxiliar no manejo clínico – principalmente no caso de desordens como o TEA, cujo diagnóstico se dá exclusivamente por análise comportamental.

1.2. Bases neurobiológicas e mecanismos adjacentes

Apesar de ainda não se conhecer exatamente o que leva ao surgimento do TEA, são descritas diversas alterações que podem tanto ter relação com o gatilho etiológico quanto com a manutenção/progressão dos sintomas. Há muitos anos são relatadas alterações histoanatômicas no contexto do TEA, como anormalidades em regiões encefálicas como hipocampo, subículo, córtex entorrinal, núcleos da amígdala, córtex neocerebelar, núcleos do teto do cerebelo (BAUMAN; KEMPER, 1985), alterações em regiões como os núcleos da base, como aumento no núcleo caudado (LANGEN et al., 2007), hiperativação em processos sensório-motores (TAKARAE et al., 2007), déficits executivos decorrentes da ativação reduzida nas regiões frontal, estriatal e parietal (SHAFRITZ et al., 2008), entre outras.

Uma das primeiras estruturas associadas ao TEA foi o cerebelo, uma vez que indivíduos com anormalidades nessa estrutura apresentam comportamentos do tipo autista e maior diagnóstico de TEA comparado à população sem essas alterações (PARISI; DOBYNS, 2003). Já foram descritas alterações em células de *Purkinje* (FATEMI et al., 2002; INGRAM et al., 2000; PETER et al., 2016; TSAI et al., 2012), alterações funcionais (FERNÁNDEZ et al., 2021), malformações cerebelares (MURAKAMI et al., 1989; OTAZU et al., 2021; RUMSEY et al., 1988), aumento de células da microglia (GIFFORD et al., 2021), entre outras.

Outra região bastante associada ao TEA é a amígdala. Estudos mostraram que macacos *Rhesus* submetidos à ablação de amígdala apresentavam menor resposta a estímulos sociais e menor interesse por convivência com seus coespecíficos (KLING; BROTHERS, 1992; KLING; STEKLIS, 1976); de forma complementar, pacientes com lesões nessa estrutura mostram redução no número de neurônios (AVINO et al., 2018;

SCHUMANN; AMARAL, 2006; WILKINSON, 2006) e conectividade atípica (FISHMAN et al., 2018), além de prejuízos no julgamento social (ADOLPHS et al., 1994; YOUNG et al., 1996) – o que foi chamado de “autismo adquirido”–, ao passo que modelos animais enfatizam alterações na sinalização de cálcio (BARRETT et al., 2017) e aumento da razão excitação/inibição sináptica nessa estrutura (LIN et al., 2013). Ainda, enquanto que a amígdala apresenta alterações de tamanho durante a infância em indivíduos com TEA, em adolescentes, essas mudanças parecem persistir no hipocampo, associadas a ativação astroglial e desbalanço excitatório/inibitório (GROEN et al., 2010; ROJAS et al., 2004; SCHUMANN et al., 2004).

O córtex cerebral como um todo também apresenta mudanças morfofuncionais. Já foi observado dobramento cortical atípico em crianças com TEA principalmente no lobo frontal (HARDAN et al., 2004; LEVITT et al., 2003), alterações na conectividade inter-hemisférica (FENLON et al., 2015), redução da massa cinzenta e da conectividade funcional entre o hemisfério esquerdo e o córtex cingulado posterior em pacientes com alto funcionamento (PEREIRA et al., 2018), hiperplasia cortical (SPARKS et al., 2002) e conectividade neural alterada em circuitos corticais excitatórios e inibitórios (ZIKOPOULOS; BARBAS, 2013). A nível de córtex frontal, crianças com TEA apresentam 67% mais neurônios e um aumento em 17,6% do peso do cérebro (COURCHESNE et al., 2011). A mesma região em modelos animais apresentou redução da transmissão sináptica excitatória em neurônios piramidais das camadas 2/3 (SACAI et al., 2020). Ainda, já foram demonstradas alterações na organização neuronal nas camadas corticais e na citoarquitetura laminar, especialmente em córtex somestésico e córtex pré-frontal medial (CASANOVA et al., 2002, 2006; KATAOKA et al., 2013; STONER et al., 2014). O córtex somestésico, além de alterações na citoarquitetura (FONTES-DUTRA et al., 2018), também possui anormalidades de conectividade funcional (KHAN et al., 2015), bem como atividade cerebral atípica durante o processamento sensorial (MARCO et al., 2012).

1.3. Alterações na dinâmica do volume encefálico

Considerando a) a ausência de biomarcadores que antecipem o diagnóstico; b) o perímetro cefálico ligeiramente menor do que a média em mais de 90% de neonatos com alto risco familiar (e posteriormente diagnosticados com TEA) (COURCHESNE; CARPER; AKSHOOMOFF, 2003; DEMENTIEVA et al., 2005); e c) macrocefalia na

primeira infância em cerca de 20% das crianças com TEA (CHAWARSKA et al., 2011; HAZLETT et al., 2005), alguns estudos investigaram o neurodesenvolvimento em crianças com alto risco familiar de TEA a partir de técnicas de diagnóstico por imagem. Foi demonstrado o crescimento encefálico excessivo na infância (2-5 anos de idade) seguido por um decréscimo no final da infância/início da adolescência e uma aparente normalização na idade adulta (o tamanho geral do encéfalo está próximo da média considerada neurotípica) (AYLWARD et al., 2002; COURCHESNE; CARPER; AKSHOOMOFF, 2003; HA et al., 2015; HAZLETT et al., 2011; LANGE et al., 2015; LIN et al., 2015), ao passo que apenas 5% dos casos de macrocefalia ainda permanecem na adolescência e vida adulta (REDCAY; COURCHESNE, 2005).

Se por um lado é consolador pensar nesse conjunto de alterações como algo transitório, por outro, é necessário vislumbrar possíveis consequências funcionais desencadeadas pela alteração da dinâmica do crescimento encefálico no TEA. Esse "supercrescimento" ocorre durante anos iniciais de vida, os quais são críticos para o desenvolvimento e aquisição das habilidades de linguagem, social, emoção e atenção; coincidentemente, é o momento temporal em que os sintomas e os sinais do TEA estão se apresentando como perda de habilidades de linguagem e sociais (BARON-COHEN; ALLEN; GILLBERG, 1992; LANDA; GARRETT-MAYER, 2006; WETHERBY et al., 2004; ZWAIGENBAUM et al., 2005). No final da infância/início da adolescência, ocorre a desaceleração ou estagnação do crescimento craniano, quando o esperado seria um crescimento típico da puberdade e a maturação de habilidades relacionadas à sociabilidade. Esses dados em conjunto suportam a ideia de que a relação temporal entre aceleração de crescimento (concomitante à habilidade de linguagem) e desaceleração (concomitante à habilidade de sociabilidade) interfere na formação da citoarquitetura que dá suporte ao surgimento das mesmas (AYLWARD et al., 2002).

Alguns fatores como mutações na PTEN (gene de risco para TEA) (ABGHARI; MORADI; AKOUCHEKIAN, 2019; BUSCH et al., 2019; KAYMAKCALAN et al., 2021), na sinalização pelas proteínas fosfoinositídeo 3-cinase/proteína cinase B/proteína alvo da rapamicina em mamíferos (via PI3K/AKT/mTOR) (KLEIN et al., 2019; YEUNG et al., 2017; ZHANG et al., 2020) e síndromes altamente sobrepostas ao TEA como a Síndrome do X Frágil (OKAZAKI et al., 2021) já foram associados à macrocefalia em pacientes com TEA. Aumento de celularidade transitória, espessura de regiões corticais e presença de edema encefálico (e, possivelmente, disfunções nas barreiras

neurais) são algumas hipóteses relacionadas com esse fenômeno; no entanto, pouco se sabe sobre os mecanismos fisiopatológicos envolvidos na dinâmica do volume encefálico em pacientes com TEA.

1.4. Barreiras neurais

Durante muito tempo, o SNC foi considerado um local imunologicamente privilegiado (TAMBUR; ROITBERG, 2005) devido a sua aparente impenetrabilidade a substâncias oriundas da periferia (o que, posteriormente, descobriu-se ser devido à barreira hematoencefálica – BHE) e a falta de vasos linfáticos no parênquima do SNC (ENGELHARDT et al., 2016). No entanto, estudos recentes propõem uma perspectiva revisada sobre o paradigma do privilégio imunológico do SNC, com especial relevância no estudo etiológico de diferentes distúrbios neurológicos (LOUVEAU; HARRIS; KIPNIS, 2015), com impacto na BHE e barreira hematoliquórica (BHL).

O SNC é protegido por outras barreiras neurais, pelo LCR e pelas meninges que cercam o encéfalo e a medula espinal (LOUVEAU; HARRIS; KIPNIS, 2015). Complementarmente, a BHE possui um caráter seletivo de passagem de moléculas; apesar das junções de oclusão impedirem a passagem paracelular, mecanismos como transportadores e canais especializados permitem a entrada molecular seletiva via transcelular. Além da BHE e da BHL, outras barreiras atuam na interface sangue/tecido neural: hematomedular, hemato-labiríntica, hemato-retiniana e hemato-nervosa (CHOI; KIM, 2008). A integridade dessas barreiras é vital para a homeostase encefálica, uma vez que disfunções, principalmente na BHE, fragilizam as defesas físicas do encéfalo, deixando-o vulnerável a substâncias periféricas danosas (OBERMEIER; DANEMAN; RANSOHOFF, 2013; ZHAO et al., 2015b).

1.4.1. Barreira hematoliquórica

A BHL é uma barreira entre o sangue e o LCR formada pela monocamada de células epiteliais do plexo coroide localizado principalmente nos ventrículos (principal local de produção do LCR), mas também nas estruturas epiteliais subaracnoides. Ao contrário da BHE, a BHL presente no estroma do plexo coroide apresenta fenestras nos capilares e vesículas de pinocitose, que formam um macrofiltro para proteínas (OREŠKOVIĆ; RADOŠ; KLARICA, 2017; TUMANI; HUSS; BACHHUBER, 2017), além de apresentar diferentes tipos de células imunológicas associadas ao plexo coroide,

tanto no estroma quanto aderindo à membrana das células epiteliais voltadas para o LCR (GHERSI-EGEA et al., 2018).

A BHL é mantida por junções de oclusão na superfície apical e por sistemas de transporte nas células epiteliais, permitindo a passagem de água, íons e nutrientes para compor o LCR (ENGELHARDT; SOROKIN, 2009). Três fatores favorecem as trocas entre o sangue e o LCR através do plexo coroide: 1) alto fluxo sanguíneo local coroidal devido a uma extensa rede de capilares em todo o estroma coroide; 2) alta permissividade do endotélio coroidal – apesar da via paracelular ser vedada – a uma grande variedade de moléculas através das fenestrações que permitem uma difusão facilitada de moléculas com peso molecular até ~ 800 kDa; 3) presença de dobras basolaterais e microvilosidades apicais nas membranas das células epiteliais coroidais, aumentando a área de superfície para transferência entre o estroma e o LCR; isso implica em alta demanda energética - por isso, a presença de numerosas mitocôndrias nas células epiteliais do plexo coroide (GHERSI-EGEA et al., 2018).

De forma complementar, as células epiteliais do plexo coroide expressam uma ampla gama de transportadores e moléculas que regulam a produção e a composição do LCR. O gradiente osmótico criado pela secreção de Na^+ e Cl^- pelos transportadores Na^+,K^+,-ATPase, cotransportador eletrogênico NBCe2 (transporte sódio/bicarbonato) e cotransportador 4 de potássio e cloreto KCC4 conduz o influxo transepitelial de água através da aquaporina (AQP) 1 para a formação do LCR (BENARROCH, 2016). Assim, o plexo coroide desempenha um papel fundamental na manutenção da homeostase encefálica (DEMEESTERE; LIBERT; VANDENBROUCKE, 2015).

1.4.2. Barreira hematoencefálica

A BHE é uma barreira seletiva formada por células com atividade altamente coordenada que, juntas, formam a unidade neurovascular: a) células endoteliais especializadas, unidas por junções de oclusão; b) membrana basal; c) pericitos, os quais cobrem as células endoteliais compartilhando da mesma membrana basal; e d) pés terminais astrocíticos, os quais contribuem para as regulações dinâmicas de todo sistema neural (ABBOTT, 2013). Digno de nota, os pericitos são células contráteis devido à presença de actina de músculo liso em sua estrutura; exercem importante papel no controle do fluxo sanguíneo, na regulação da permeabilidade juncional, bem como na formação de contatos focais com o endotélio através da N-caderina e

conexinas, permitindo trocas de íons, metabólitos, segundos mensageiros e ácidos ribonucleicos entre os dois tipos de células (CARDOSO; BRITES; BRITO, 2010; LAI; KUO, 2005; LIU et al., 2012; REINHOLD; RITTNER, 2016). Neurônios e microglia também são observados na região perivascular (CHOI; KIM, 2008).

A passagem paracelular entre as células endoteliais é limitada pela presença das proteínas constituintes das junções de oclusão – claudinas, ocludinas, moléculas juncionais de adesão (JAMs), moléculas de adesão seletivas de endotélio (ESAM) e proteínas da zônula de oclusão (ZOs) –, que unem as células endoteliais evitando a passagem de substâncias maiores que 0,4 kDa (ABBOTT et al., 2010; ABBOTT; RÖNNBÄCK; HANSSON, 2006). Além disso, a lâmina basal (formada por proteínas da matriz extracelular como colágeno e laminina) auxilia na estabilização da BHE fornecendo suporte para as células endoteliais e os pericitos (ZHAO et al., 2015b).

1.4.3. Astrócitos

Os astrócitos são o tipo de célula mais abundante no SNC, desempenhando diversos papéis anatômicos e funcionais (XIE; YANG, 2015). Essas células gliais, que ficam em contato com a superfície externa do endotélio da BHE por meio dos seus prolongamentos usualmente chamados de “pés” (CARDOSO; BRITES; BRITO, 2010), podem se apresentar nas formas protoplasmática (presente na substância cinzenta, com numerosos prolongamentos curtos e muitas ramificações) e fibrosa (presente na substância branca, com menos ramificações e prolongamentos mais longos) (OBERHEIM; GOLDMAN; NEDERGAARD, 2012). Além de auxiliar na manutenção da BHE, fornecendo suporte estrutural por meio da formação de matriz extracelular (síntese de proteoglicanos), os astrócitos mantêm a homeostase fisiológica do SNC, fornecendo um microambiente estável por meio da regulação de processos como regulação imunológica, suporte metabólico, tamponamento de potássio, neurogênese, modulação da transmissão e da plasticidade sináptica, e homeostase de neurotransmissores (BLANCHETTE; DANEMAN, 2015; PALMER; OUSMAN, 2018).

A homeostase de neurotransmissores se dá por recaptura e metabolismo; enquanto a maior parte do glutamato é recaptada pelos astrócitos pelo transportador de glutamato/aspartato (GLAST) e transportador de glutamato 1 (Glt-1), grande parte do GABA é recaptado pelos neurônios GABAérgicos pré-sinápticos pelo transportador de GABA (GAT). Dentre outras funções, o glutamato nos astrócitos pode ser utilizado

para a síntese de glutationa (GSH) – um importante antioxidante biológico não-enzimático. Já o GABA, pode ser convertido em glutamato a partir das enzimas GABA-transaminase (GABA-T) e semialdeído succinato desidrogenase (SSADH), e servir de precursor para glutamina via glutamina sintetase (GS) (SCHOUSBOE; BAK; WAAGEPETERSEN, 2013).

Astrócitos e microglia são as principais células imunológicas inatas no SNC (FARINA; ALOISI; MEINL, 2007; RANSOHOFF; BROWN, 2012). Em condições normais, essas células são cruciais para o controle do processo inflamatório no encéfalo em desenvolvimento através do reconhecimento de fatores pró-inflamatórios capazes de perturbar a homeostase, os quais desempenham um papel importante na etiologia de vários distúrbios neurológicos e neuropsiquiátricos, incluindo o TEA (PETRELLI; PUCCI; BEZZI, 2016). Em condições de estresse celular, os astrócitos são capazes de se proliferar e adquirir uma forma reativa, caracterizada por hipertrofia, presença de numerosos prolongamentos e aumento na produção da proteína ácida fibrilar glial (GFAP, do inglês *glial fibrilar acidic protein*, proteína do citoesqueleto de astrócitos). Além disso, essa conformação reativa pode exacerbar o dano tecidual, uma vez que leva à redução na captação de glutamato, dificultando a ação dos astrócitos na homeostase de neurotransmissores (JAIN; KUMAR WADHWA; RAMANLAL JADHAV, 2015), bem como libera citocinas pró-inflamatórias como o TNF-α, a IL-1β e a IL-6, produzem e liberam óxido nítrico (NO) e espécies reativas de oxigênio (LIBERTO et al., 2004; LIDDELOW; BARRES, 2017).

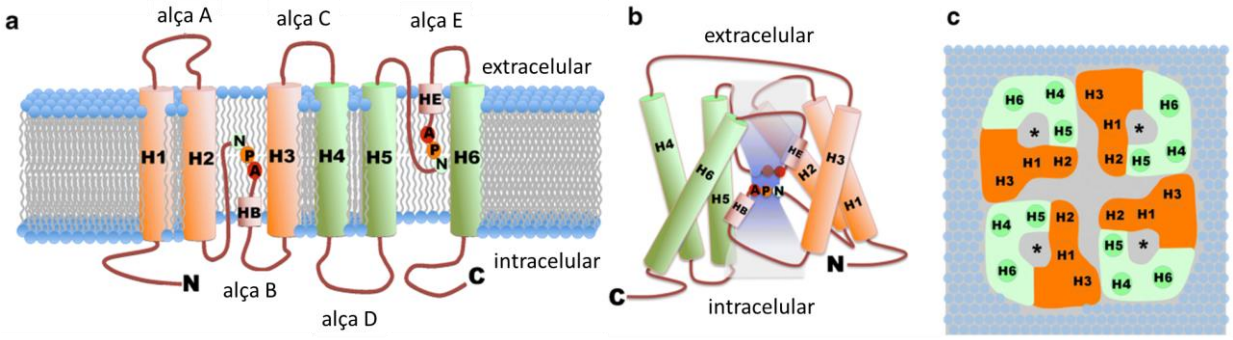
O TEA, assim como outros transtornos psiquiátricos, tem sua fisiopatologia associada a disfunções astrocitárias (MONY et al., 2016; ZEIDÁN-CHULIÁ et al., 2014), como aumento de gliose reativa e proliferação de células da glia no encéfalo de indivíduos com o TEA (PETRELLI; PUCCI; BEZZI, 2016), associação entre o TEA e genes relacionados à ativação de células da glia e do sistema imunológico (VOINEAGU et al., 2011). Apesar de alguns estudos não demonstrarem alterações nos parâmetros de astrócitos em tecidos *post mortem* de pacientes com TEA (LEE et al., 2017; MORGAN et al., 2014), previamente observamos alterações no metabolismo do glutamato no hipocampo em diferentes idades (BRISTOT SILVESTRIN et al., 2013), bem como aumento da expressão proteica de GFAP em diferentes estruturas encefálicas (BRISTOT SILVESTRIN *et al.*, *dados não publicados*) em modelo animal VPA, corroborando com a literatura que demonstra a ativação neuroglial em pacientes

com TEA e modelos animais (EDMONSON; ZIATS; RENNERT, 2014; VARGAS et al., 2005; ZHAO et al., 2019). Além disso, os modelos animais da Síndrome do X Frágil apresentam uma perturbação específica na constituição das camadas mais profundas, além de apresentar um número aumentado de astrócitos (LEE et al., 2019).

1.4.4. Aquaporinas

Todas as membranas plasmáticas de mamíferos são permeáveis à água; os tecidos mais permeáveis recebem essa propriedade das AQP, família de proteínas canais presentes em praticamente todos os organismos. Possuem aproximadamente 30 kDa de peso molecular e sua principal função é permitir a passagem de água através das membranas celulares. São proteínas integrais de membrana que formam poros que permitem a passagem de água através das membranas enquanto bloqueiam a passagem de íons e solutos carregados (FILIPPIDIS; CAROZZA; REKATE, 2017) (Figura 2).

Figura 2 – Vista esquemática da estrutura das aquaporinas



A) A estrutura primária das aquaporinas contêm porções N- e C-terminais citosólicas e seis domínios transmembrana (H1 a H6) conectados por cinco alças (A, C e E extracelulares e B e D intracelulares). Os *motifs* NPA estão localizados nas alças B (HB) e E (HE), formam hélices hidrofóbicas curtas que se dobram em direção à membrana para lados opostos. B) Modelo esquemático de arranjo dos domínios transmembrana, mostrando HB e HE, bem como o motivo NPA ao redor do poro condutor de água (mostrado em azul claro). É representada a obstrução do poro central pelos motivos NPA de acordo com o modelo da ampulheta. C) Vista de cima da superfície extracelular na parte superior de um tetrâmero de AQP. O arranjo da transmembrana que abrange os segmentos H1 a H3 (em laranja) e H4 a H6 (em verde) é mostrado. Os asteriscos destacam o poro de água de cada monômero AQP. Adaptado de (WITTEKINDT; DIETL, 2018).

Em geral, os monômeros de AQP apresentam seis domínios transmembrana com as porções carboxila (C) e amino terminal (N) intracelulares (NAGELHUS;

OTTERSEN, 2013; PAPADOPOULOS; VERKMAN, 2013). A organização das AQP em homotetrâmeros dá o formato de “ampulheta” ao poro central (BADAUT et al., 2014), por meio da interação tridimensional do motivo asparagina-prolina-alanina (Asn-Pro-Ala ou NPA), conservado ao longo da sequência de aminoácidos (BADAUT et al., 2014; ERIKSSON et al., 2013; NAGELHUS; OTTERSEN, 2013; PAPADOPOULOS; VERKMAN, 2013; VERKMAN; MITRA, 2000).

A primeira AQP descrita (AQP1) foi descoberta por acaso em um experimento para isolamento de peptídeos e inicialmente chamada de CHIP28 (proteína integral formadora de canal de 28 kDa, do inglês *channel-forming integral protein of 28 kDa*) (BROWN, 2017; PRESTON et al., 1992). Desde então, já foram descritas 13 AQP, algumas permeáveis somente à água (AQP1, AQP2, AQP4, AQP5 e AQP8) e outras – as aquagliceroporinas – (AQP3, AQP6, AQP7, AQP9 e AQP10), com a habilidade de transportar moléculas pequenas e sem carga, como glicerol, ureia e amônia. Ainda, foi proposto o subgrupo de superaquaporinas (AQP11 e AQP12), que são distintas devido à sua baixa homologia para o restante da família, apesar de também possuírem a sequência NPA (WITTEKINDT; DIETL, 2018). As AQP expressas em maiores concentrações no SNC são a 1 e a 4 (FILIPPIDIS; CAROZZA; REKATE, 2017).

AQP1 é expressa principalmente nas células ependimais das superfícies apical e basolateral do plexo coroide (BENGA; HUBER, 2012; OSHIO et al., 2003), promovendo movimento transcelular da água e contribuindo com cerca de 25% da produção de LCR (FILIPPIDIS; CAROZZA; REKATE, 2017). Também já foi descrita em neurônios sensoriais no corno dorsal da medula espinhal e gânglios trigêmeos (contribuindo possivelmente para a sinalização da dor) (OSHIO et al., 2006; SHIELDS et al., 2007), em filamentos neuronais após lesão encefálica traumática (FUKUDA et al., 2012), em astrócitos na substância branca, na glia limitante e neurônios que inervam os vasos sanguíneos piais em primatas (ARCIENEGA et al., 2010).

A extensa distribuição de AQP4 em vários locais no encéfalo não deixa dúvidas do papel crucial da AQP4 na homeostase encefálica (NAGELHUS; OTTERSEN, 2013; PAPADOPOULOS; VERKMAN, 2007, 2013; XIAO; HU, 2014). Especula-se que essa proteína possa estar envolvida no desenvolvimento e maturação da BHE (NICO et al., 2001). AQP4 é o principal canal de água no SNC, expresso principalmente em processos perivasculares dos pés astrocíticos, mas também no espaço subaracnoide (BLOCH; MANLEY, 2007; HUBBARD et al., 2015), medula espinal (OSHIO et al.,

2004), retina e nervo óptico (NAGELHUS et al., 1998), células ependimárias (JUNG et al., 1994), giro denteado (HSU et al., 2011) e córtex temporal (HOSHI et al., 2017). Interessantemente, astrócitos reativos aumentam a expressão de AQP4, deslocando a expressão dos pés terminais para o soma do astrócito (ILIFF et al., 2014; KRESS et al., 2014). De forma complementar, alguns estudos em modelos nocaute para AQP4 demonstraram importantes alterações no padrão de disparo neuronal, sugerindo que essa proteína também exerça papel na manutenção da excitabilidade neural (KONG et al., 2008; NAGELHUS; OTTERSEN, 2013) e no tamponamento de potássio (BENGA; HUBER, 2012) devido à sua colocalização com o canal de potássio 4.1 (Kir4.1) (AMIRY-MOGHADDAM et al., 2003).

Em geral, poucos estudos abordam alterações de barreiras neurais e de canais de água no contexto do TEA. Em relação a alterações de permeabilidade encefálica, animais do modelo VPA apresentaram (macroscopicamente) maior permeabilidade da BHE ao corante azul de Evans no cerebelo (KUMAR; SHARMA; SHARMA, 2015; KUMAR; SHARMA, 2016a, 2016b). Em relação às AQP, foi demonstrado diminuição na expressão de AQP4 no cerebelo *post mortem* de indivíduos com o transtorno (FATEMI et al., 2008), ao passo que outro estudo demonstrou não haver alterações nos níveis séricos da mesma proteína em pacientes com TEA (KALRA et al., 2015).

1.5. Alterações neuroimunológicas

Existem várias maneiras pelas quais o ambiente gestacional pode afetar o neurodesenvolvimento da criança, incluindo a AIM, um dos fatores relacionados ao desencadeamento de TEA (MELTZER; VAN DE WATER, 2017). Já foi demonstrado aumento de citocinas pró-inflamatórias na prole de modelos animais de AIM (CHOI et al., 2016), associação entre anticorpos anti-fetais na mãe e déficits relacionados ao TEA na criança (BRAUNSCHWEIG et al., 2012; WARREN et al., 1990), além de autoimunorreatividade intensa (WILLS et al., 2009; ZIMMERMAN et al., 2007) principalmente às células progenitoras neuronais (MAZUR-KOLECKA et al., 2014).

Mais recentemente, diversos estudos têm mostrado uma associação entre os membros da família do antígeno leucocitário humano (HLA, do inglês *human leukocyte antigen*) e a incidência de TEA (AL-HAKBANY; AWADALLAH; AL-AYADHI, 2014; CHIEN et al., 2012; GUERINI et al., 2015; MOSTAFA; SHEHAB; AL-AYADHI, 2013; TORRES; WESTOVER; ROSENSPIRE, 2012). Ademais, a partir de estudos de

associação ampla do genoma (GWAS, do inglês *genome wide association studies*), foi observada a regulação positiva de diversos genes enriquecidos para funções de resposta imunológica e inflamatória, domínios de imunoglobulina e outras ontologias imunorreguladoras em amostras de pacientes com TEA (GARBETT et al., 2008; GREGG et al., 2008; LINTAS; SACCO; PERSICO, 2012; VOINEAGU et al., 2011).

O desequilíbrio imunológico pós-natal é amplamente descrito e uma marca registrada no TEA (GOTTFRIED et al., 2015). Há relatos de neuroinflamação encefálica (VARGAS et al., 2005), diminuição de células T reguladoras $CD4^{+}CD25^{+}$ (MOSTAFA; AL SHEHAB; FOUAD, 2010), resposta diferencial de monócitos (ENSTROM et al., 2010) e de células mononucleares de sangue periférico (PBMCs, do inglês *peripheral blood mononuclear cell*) (ASHWOOD et al., 2011c) isolados de pacientes sob estímulo em cultivos celulares, alteração na expressão de células T em modelos animais (PONZIO et al., 2007), redução na atividade de células NK (WARREN; FOSTER; MARGARETTEN, 1987) – mas não na contagem geral (DELISI et al., 1983), maior expressão de genes de citotoxicidade NK (ENSTROM et al., 2009) e expressão diferencial de células NK $CD57^{+}CD3^{-}$ (SINISCALCO et al., 2016).

Também são reportados aumentos tanto nos níveis de citocinas anti-inflamatórias IL-4, IL-5 e IL-13 em PBMCs após estímulo (KRAKOWIAK et al., 2017; MOLLOY et al., 2006) quanto pró-inflamatórias: 1) em sangue, IL-1RA, IL-1β, IL-6, IL-8, IL-12p40, IL-17, IFN-γ, GM-CSF (AL-AYADHI; MOSTAFA, 2012; ASHWOOD et al., 2011b; CROONENBERGHS et al., 2002), quimiocinas MCP-1, RANTES e eotaxina (ASHWOOD et al., 2011a); 2) tecido cerebral, IL-6, GM-CSF, TNF-α, MCP-1, TGF-β (LI et al., 2009; VARGAS et al., 2005); 3) PBMCs, TNF-α, IFN-γ e IL-17 (AKINTUNDE et al., 2015; JYONOUCHI et al., 2005; JYONOUCHI; SUN; ITOKAZU, 2002).

Considerando a etiologia desconhecida do TEA, ao longo dos anos, diversos estudos têm proposto que alterações em vias biológicas específicas – principalmente associadas ao sistema imunológico – teriam relação com alguns dos mecanismos fisiopatológicos envolvidos nesse transtorno. Nesse contexto, o sistema purinérgico emerge como forte candidato para modulação fenotípica. Por exemplo, camundongos nocaute para o receptor A1A apresentaram aumento de neuroinflamação e a atividade microglial (SYNOWITZ et al., 2006) ao passo que o receptor A2A tem função anti-inflamatória e pode ser regulado negativamente pelo microRNA (miR) miR-214, que promove a liberação das citocinas inflamatórias TNF-α e IL-6 (ZHAO et al., 2015a).

Além disso, a microglia desempenha um papel crucial na neuroinflamação, tanto promovendo a vigilância necessária para manter a homeostase do SNC quanto espalhando ainda mais a inflamação quando ativada, facilitando o afrouxamento da BHE, permitindo infiltrado inflamatório da periferia em direção ao SNC. De qualquer forma, é um elemento suscetível à modulação da sinalização purinérgica (FUMAGALLI; LECCA; ABBRACCHIO, 2011), uma vez que durante eventos lesivos, há grande liberação de ATP local e consequente ativação de microglia residente por meio de receptores purinérgicos (MELANI et al., 2005), principalmente o P2X7 que é capaz de aumentar as respostas inflamatórias generalizadas, principalmente pela liberação de IL-1β (FERRARI et al., 1997; GRAHAMES et al., 1999), bem como promover liberação de fatores neurotróficos (SUZUKI et al., 2004). O ATP liberado e a ativação de P2X7 também são capazes de ativar os astrócitos, mediando a sinalização para a síntese de citocinas pró-inflamatórias (SOLLE et al., 2001) e neurotransmissores como o glutamato (DUAN et al., 2003), diminuindo sua captação e reduzindo a atividade da GS (LO et al., 2008).

No contexto do TEA, poucos estudos descrevem a modulação inflamatória mediada pela sinalização purinérgica. O tratamento de camundongos BTBR com um antagonista do receptor A2A aumentou a expressão de GATA3, T-bet (AHMAD et al., 2017b) e RORγt, diminuindo Foxp3 e IL-10 (ANSARI et al., 2017b) modulou a expressão de receptores *toll-like* (AHMAD et al., 2017a), promoveu a expressão de quimiocinas (AHMAD et al., 2018b), aumentou a resposta Th1, diminuiu a resposta Th2 , aumentando também os níveis de citocinas pró-inflamatórias (ANSARI et al., 2017a). Esses dados demonstram o importante papel anti-inflamatório do receptor A2A, uma vez que a inibição da ligação de adenosina via antagonismo foi capaz de replicar déficits comumente descritos no TEA.

1.6. Resveratrol

Considerando o contexto neuroimunológico envolvido no TEA, moléculas com efeito anti-inflamatório emergem como potenciais alvos de estudo para compreensão de vias biológicas envolvendo ativação imunológica que possam estar relacionadas com a fisiopatologia do TEA, bem como para novas estratégias farmacológicas em pacientes diagnosticados com o transtorno. Nesse sentido, os polifenóis –

especialmente o *trans*-resveratrol (RSV, 3,4′,5-triidroxiestilbeno) – vêm ganhando cada vez mais atenção da comunidade científica.

O RSV é um polifenol da família dos estilbenos encontrado naturalmente em pinhas, amendoins, casca da uva (consequentemente, no vinho tinto), entre outros (KOUSHKI et al., 2018). Os efeitos protetores e terapêuticos da utilização do RSV como nutracêutico têm sido descritos em diversas patologias, incluindo câncer, obesidade, diabetes tipo 2, doenças cardiovasculares e neurais (SHAYGANFARD, 2020; VANG et al., 2011). Além disso, também são amplamente descritos e revisados seus efeitos anti-inflamatórios, antioxidantes e neuroprotetores (MENG et al., 2021; QUINCOZES-SANTOS; GOTTFRIED, 2011; VANG et al., 2011; ZHANG et al., 2021).

Vang e colaboradores (VANG et al., 2011), em uma robusta revisão da literatura, descrevem amplamente os mais diversos papéis modulatórios do RSV, tais como proliferação celular, apoptose e danos ao DNA, angiogênese, atividade mitocondrial, metabolismo do glutamato, entre outros. Têm sido propostos diversos mecanismos por meio dos quais o RSV provavelmente desempenha seu papel protetor, envolvendo principalmente modulação de processos inflamatórios, transcrição gênica e sinalização celular (SHAYGANFARD, 2020). Sabe-se que o RSV é capaz de contrapor os efeitos pró-inflamatórios e pró-oxidantes observados em modelos animais de diversas condições patológicas, como obesidade, diabetes e hipertensão, diminuindo os níveis de TNFα, IL-1β, IL-6, proteína quimioatrativa para monócitos 1 (MCP-1) e óxido nítrico sintase (iNOS) (VANG et al., 2011). Além disso, o efeito supressor da inflamação já foi associado tanto com a inibição da ciclo-oxigenase 2 (COX-2) (BAUR; SINCLAIR, 2006), quanto com a ativação do supressor da sinalização de citocina 1 (SOCS-1) (MALHOTRA; BATH; ELBARBRY, 2015). Também foi relatada a atuação inibitória da proteína p53 (processo relacionado a sobrevivência celular) provavelmente devido ao (bem descrito) papel do RSV na ativação da SIRT1 (BAUR; SINCLAIR, 2006; MALHOTRA; BATH; ELBARBRY, 2015), um membro da família de HDACs. Ainda, o RSV também promove a diferenciação de fatores neurotróficos, como o fator neurotrófico derivado do cérebro (BDNF, do inglês *brain derived neurotrophic factor*) (MALHOTRA; BATH; ELBARBRY, 2015).

No contexto do TEA, nosso grupo de pesquisa é pioneiro na utilização do RSV como estratégia terapêutica. Demonstramos que o tratamento pré-natal com RSV entre os dias embrionários (E) E6,5 e E18,5 foi capaz de prevenir os déficits de

sociabilidade na prole (BAMBINI-JUNIOR et al., 2014; HIRSCH et al., 2018), além de alterações sensoriais, celulares (FONTES-DUTRA et al., 2018; SANTOS-TERRA et al., 2021) e moleculares (HIRSCH et al., 2018). Ainda em modelos animais de TEA, também já foi demonstrado que o RSV é capaz de contrapor a desregulação do receptor de estrogênio β (ERβ) (XIE et al., 2018), aumentar a expressão gênica de SIRT1 e do fator de resposta de crescimento precoce 3 (Egr3, do inglês *early growth response factor 3*) na amígdala dos camundongos (HIDEMA et al., 2020), agindo principalmente em animais machos (JUYBARI et al., 2020), além de suprimir neuroinflamação (BHANDARI; KUHAD, 2017), atenuar citocinas pró-inflamatórias e ativação de JAK1-STAT3 (AHMAD et al., 2018c), atenuar a expressão de receptores de quimiocinas (BAKHEET et al., 2016), inibir receptores *toll-like* neuronais e sinalização por COX-2 (AHMAD et al., 2018a) e melhorar a desregulação da sinalização de células Treg e respostas Th1, Th2 e Th17 (BAKHEET et al., 2017) em modelo genético BTBR.

Além disso, recentemente, demonstramos que o uso de RSV em crianças diagnosticadas com TEA foi capaz de reduzir os índices de irritabilidade medidos pela lista de verificação de comportamento aberrante (ABC, do inglês *aberrant behavior checklist*), favorecendo, assim, a melhora na sociabilidade (MARCHEZAN *et al*., *manuscrito submetido*). Outros estudos mostram que fibroblastos de pacientes com aumento nas concentrações de acil-carnitina e comprometimento da β-oxidação do ácido graxo mitocondrial também responderam satisfatoriamente ao tratamento com RSV, sendo a melhora proporcional ao nível de comprometimento dos pacientes na escala de responsividade social (BARONE et al., 2021). Dessa forma, nosso conjunto de dados prévios evidencia o papel terapêutico do RSV enquanto ferramenta de estudo em múltiplos contextos biológicos envolvendo a pesquisa acerca do TEA, bem como lança luz na compreensão dos mecanismos fisiopatológicos envolvidos tanto no gatilho e manutenção do TEA quanto na prevenção promovida pelo RSV.

1.7. Justificativa e hipóteses

O TEA, inicialmente descrito na década de 20, atrai cada vez mais atenção da comunidade científica devido à sua incidência crescente. Apesar do aumento exponencial nos estudos acerca desse transtorno nos últimos anos, sua etiologia ainda permanece um quebra-cabeças longe de ser solucionado. Nesse sentido, os

modelos animais emergem como importantes ferramentas de estudo, especialmente no caso de transtornos como o TEA cujo diagnóstico é exclusivamente pela análise de alterações comportamentais.

Além da sintomatologia central necessária para o diagnóstico, os pacientes com TEA podem apresentar condições associadas, como aumento do perímetro cefálico. Sabe-se que o TEA possui um forte componente neuroimunológico na sua fisiopatologia, o que pode estar por trás das alterações comportamentais, bem como pode ter associação com disfunções de barreiras neurais. Assim, a hipótese é que a AIM durante o período embrionário não só contribua para o fenótipo do tipo autista, como também resulte em disfunções de barreiras neurais por meio da desorganização cortical, aumento da permeabilidade da BHE, entrada de infiltrado inflamatório da periferia em direção ao SNC, alteração no perfil de células neurais, bem como utilize mecanismos subjacentes para a manutenção e progressão desse *status* neuroinflamatório, como a sinalização purinérgica.

Dessa forma, considerando:

- O relato de macrocefalia em pacientes pediátricos com TEA, bem como o amplo histórico de neuroinflamação envolvendo esse transtorno;
- A excelente translacionalidade do modelo VPA, uma vez que preenche os quesitos das validades de face e construto e possui valor preditivo;
- Os importantes efeitos preventivos do RSV no contexto do TEA, prevenindo comportamentos do tipo autista, alterações celulares e moleculares;
- A associação de alterações pontuais na sinalização purinérgica e outras rotas metabólicas com o desencadeamento e a manutenção do TEA.

A presente tese visou avaliar a integridade de barreiras neurais no modelo VPA, bem como revisar as publicações existentes na literatura acerca do componente neuroinflamatório presente no TEA e da contribuição da sinalização purinérgica nesse contexto.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar a integridade de barreiras neurais e seus mecanismos subjacentes em um modelo animal de TEA pela exposição pré-natal ao VPA, bem como o possível efeito preventivo da administração pré-natal do RSV sobre os mesmos parâmetros propostos, além de revisar a contribuição da sinalização purinérgica no TEA.

2.2. Objetivos específicos

- Capítulo I: avaliação das barreiras neurais e mecanismos subjacentes em modelo animal de TEA
    - Avaliar o conteúdo encefálico de água (medida de edema);
    - Analisar a integridade da barreira hematoencefálica;
    - Analisar a expressão e distribuição das aquaporinas 1 e 4 em diferentes regiões do SNC;
    - Analisar parâmetros astrocíticos.
- Capítulo II: revisão do estudo do componente neuroinflamatório presente no TEA
    - Realizar um levantamento das principais alterações neuroimunológicas descritas tanto em modelos animais quanto em pacientes com TEA;
    - Revisar os mecanismos envolvidos no contexto imunológico no desencadeamento do fenótipo autista a partir da exposição ao fator de risco VPA;
    - Estudar as vias comuns translacionais envolvendo as alterações imunológicas descritas em humanos e animais.
- Capítulo III: revisão do estudo sobre o envolvimento da sinalização purinérgica no TEA
    - Entender como o sistema purinérgico está envolvido na fisiopatologia do TEA;
    - Revisar as estratégias terapêuticas propostas que se utilizam dessa sinalização como mecanismo para modulação dos comportamentos do tipo autista.

PARTE II

Capítulo I: artigo publicado.

"*Resveratrol prevents brain edema, blood–brain barrier permeability, and altered aquaporin profile in autism animal model*"

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RESVERATROL PREVENTS BRAIN EDEMA, BLOOD-BRAIN BARRIER PERMEABILITY AND ALTERED AQUAPORIN PROFILE IN AUTISM ANIMAL MODEL

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Author contributions: ID, JS-T, MF-D, MK-R, GB-N, GBS, RR, VB-J and CG: experimental design and intellectual contribution. RR, VB-J and CG: acquisition of financial resources. ID, GB-N and GBS: edema and Evans blue analyses. ID, JS-T, MF-D and MK-R: immunofluorescence and western blotting analyses. ID, JS-T, MF-D, VB-J and CG: data discussion and manuscript preparation.

Abbreviations: aCC: anterior cingulate cortex (in the medial prefrontal cortex); AmR: amygdala region; AQP: aquaporin; ASD: autism spectrum disorder; BBB: blood-brain barrier; CA: cornu ammonis (CA1-CA3, in the hippocampus); CNS: central nervous system; GFAP: glial fibrillary acidic protein; IL: infralimbic cortex (in the medial prefrontal cortex); MIA: maternal immune activation; mPFC: medial prefrontal cortex; PrL: prelimbic cortex (in the medial prefrontal cortex); pSSA: primary somatosensory area; RSV: resveratrol; VPA: valproic acid.

Highlights:

- Autism Spectrum Disorder (ASD) is hallmarked by neuroimmune background;

- ASD animal model replicates the greater brain volume described in ASD patients;
- Resveratrol (RSV) prevents blood-brain barrier alterations and edema formation;
- RSV restore AQP4 levels to control levels;
- RSV promotes a functional amelioration in astrocytes.

Abstract

Autism Spectrum Disorder can present a plethora of clinical conditions associated with the disorder, such as greater brain volume in the first years of life in a significant percentage of patients. We aimed to evaluate the brain water content, the blood-brain barrier permeability, and the expression of aquaporin 1 and 4, and GFAP in a valproic acid-animal model, assessing the effect of resveratrol. On postnatal day 30, Wistar rats of the valproic acid group showed greater permeability of the blood-brain barrier to the Evans blue dye and a higher proportion of brain water volume, prevented both by resveratrol. Prenatal exposition to valproic acid diminished aquaporin 1 in the choroid plexus, in the primary somatosensory area, in the amygdala region and in the medial prefrontal cortex, reduced aquaporin 4 in medial prefrontal cortex and increased aquaporin 4 levels in primary somatosensory area (with resveratrol prevention). Valproic acid exposition also increased the number of astrocytes and GFAP fluorescence in both primary somatosensory area and medial prefrontal cortex. In medial prefrontal cortex, resveratrol prevented the increased fluorescence. Finally, there was an effect of resveratrol *per se* on the number of astrocytes and GFAP fluorescence in the amygdala region and in the hippocampus. Thus, this work demonstrates significant changes in blood-brain barrier permeability, edema formation, distribution of aquaporin 1 and 4, in addition to astrocytes profile in the animal model of autism, as well as the use of resveratrol as a tool to investigate the mechanisms involved in the pathophysiology of Autism Spectrum Disorder.

Keywords: Autism spectrum disorder. Valproic acid. Resveratrol. Water content. Blood-brain barrier. Aquaporin.

1. INTRODUCTION

Autism Spectrum Disorder (ASD) was first characterized in the 1920s by the Russian psychiatrist Grunya Sukharewa, who described 6 children with autistic characteristics (Ssucharewa, 1926; Zeldovich, 2018). Currently, ASD is a highly prevalent neurodevelopmental disorder characterized by 1) deficits in communication and social interaction and 2) the presence of repetitive behaviors and restricted interests/activities (APA, 2013), affecting 1:54 children up to 8 years old in the USA (Maenner et al., 2020).

Despite research advances on this disorder, the etiology of ASD remains unknown. However, epidemiological observations suggest that environmental factors, such as valproic acid (VPA), are closely related to the onset of ASD (Christensen et al., 2013; Roullet et al., 2013; Smith & Brown, 2014). Besides the core symptoms, several conditions associated with ASD are described, including greater brain volume in the first years of life (Aylward et al., 2002) affecting around 20% of ASD patients (Sacco et al., 2015).

For a long time, it was hypothesized that the Central Nervous System (CNS) was an immunologically privileged location (Tambur & Roitberg, 2005) due to blood-brain barrier (BBB), a selective barrier composed, among others, by astrocytes (Abbott, 2013). Several studies relate astrocytic dysfunctions to psychiatric disorders, including ASD (Mony et al., 2016; Zeidán-Chuliá et al., 2014). Increased reactive gliosis, proliferation of glial cells in the brain of ASD individuals (Petrelli et al., 2016) as well as the association between ASD and genes related to the activation of glia and the immune system (Voineagu et al., 2011), stressing the role of astrocytes in ASD and in BBB impairments.

Besides astrocytes, the BBB's dynamic can be affected by the water channels aquaporins (AQP). AQP1 is expressed mainly in the apical membrane of the choroid plexus (involved in the production of cerebrospinal fluid), in addition to glial membranes, astrocytes and ependymal cells (Benga & Huber, 2012; Oshio et al., 2003). On the other hand, AQP4 is the most common water channel in the CNS, present in greater quantity in terminal feet of astrocytes that surround blood vessels (essential constituent of BBB) (Nagelhus & Ottersen, 2013; Papadopoulos & Verkman, 2007; Xiao & Hu, 2014), playing an important role in removing water from the cerebral parenchyma, in addition to assisting the potassium buffering (Benga & Huber, 2012).

Few studies have been conducted related to BBB and AQP alteration in ASD individuals, indicating decreased cerebellar AQP4 (*postmortem*) (Fatemi et al., 2008), and no alteration in serum (Kalra et al., 2015), as well as a huge cerebellar permeability in the VPA-animal model of ASD (Kumar & Sharma, 2016b, 2016a; Kumar et al., 2015). Considering the increased brain volume and the possible impairment of neural barrier systems, as well as the pro-inflammatory and pro-oxidant processes already observed in ASD individuals, molecules with antioxidant and anti-inflammatory properties become important targets for the study of neuroprotective mechanisms in ASD.

The *trans*-resveratrol (RSV, 3,5,4'-trihydroxystilbene) is a naturally occurring polyphenolic compound present in grapes, peanuts and red wine, having several biological effects (Frémont, 2000; Vang et al., 2011). Several studies emphasizing the protective and therapeutic roles of RSV in several pathologies (Berman et al., 2017; Koushki et al., 2020), highlighting the anti-inflammatory (J.-A. Lee et al., 2015; Sánchez-Fidalgo et al., 2010), antioxidant (Mohammadshahi et al., 2014) and neuroprotective effects (Quincozes-Santos & Gottfried, 2011; Tang, 2010) of RSV.

Our research group have been demonstrated the preventive effect of prenatal treatment with RSV in sociability and sensory deficits in the offspring of VPA-animal model (Bambini-Junior et al., 2014; Fontes-Dutra et al., 2018), as well as in microRNA levels (Hirsch et al., 2018). These results enable the use of RSV as both a reliable method for understanding the pathophysiology of ASD and an assisting tool in the study of biological routes and structures involved in its etiology.

The mechanisms that mediate the brain volume dynamics are largely unknown; thus, we proposed an investigation of factors possibly associated with the formation of brain edema in ASD in an animal model of autism. Thus, we aimed to evaluate the proportion of brain fluid volume, the BBB permeability, as well as to analyze AQP 1 and 4 and GFAP+-astrocytes in 30-day-old animals of the animal model of autism induced by prenatal exposure to VPA, evaluating the possible therapeutic effect of RSV.

2. EXPERIMENTAL PROCEDURE

2.1 Animals

Wistar rats from the Center for Reproduction and Experimentation of Laboratory Animals (CREAL-UFRGS) of the Federal University of Rio Grande do Sul (UFRGS) were used, kept in standard animal facilities conditions, with food and water *ad libitum*,

light/dark cycle 12 hours, constant temperature (22ºC ± 1ºC) and a maximum of four animals per housing box. All procedures were approved by the local Ethics Commission on the Use of Animals (CEUA-UFRGS 36229) and performed according to ethical principles in accordance with the NIH Guide for the Care and Use of Laboratory Animals, as well as Brazilian Arouca Law (11,794, of October 8, 2008).

The animals' euthanasia procedure followed the Euthanasia Practice Guidelines of the National Council for Animal Experimentation Control (Normative Resolution N. 13, 2013). Euthanasia was performed by anesthetic overdose with ketamine and xilasine, supplied in concentrations three times higher (300 mg/kg and 40 mg/kg, respectively) than the concentration required to obtain an anesthetic-surgical plan.

2.2 Animal model and RSV treatment

The animals were mated overnight and, in the next morning, the presence of sperm was verified in the vaginal canal of the females; when fertilization was confirmed, the embryonic day 0.5 (E0.5) was determined. From E6.5 to E18.5, pregnant rats received RSV (trans-resveratrol, Fluxome, Stenløse, Denmark - 3.6 mg/kg) or dimethylsulfoxide (DMSO P.A. - vehicle, equivalent volume of RSV injection in a proportion 1:1 of DMSO and saline) subcutaneously. At E12.5, pregnant rats received a single injection of VPA (sodium valproate, Sigma-Aldrich, USA - 600mg/kg) or saline solution (0.9% - vehicle) via intraperitoneal (i.p.), as previously described (Bambini-Junior et al., 2014). On postnatal day 21 (P21), the litter was weaned and, at P30, male rats were euthanized by anesthetic overdose. The total number of animals used in the study was 24 control, 28 RSV, 25 VPA, and 22 RSV+VPA divided randomly among experiments, generated from the following number of dams: 5 control, 7 RSV, 14 VPA, and 12 RSV+VPA. As we use a maximum of 1 male from the same litter in each group, the excess offspring per litter was destined to other projects in the lab). The litters were randomly divided so that sibling animals were not part of the same experiment (n of animals is n of litters). The loss rate for the VPA groups was 50% in this protocol.

2.3 Tissue preparation and analysis

2.3.1 Brain water content

Immediately after the euthanasia, the brains were removed, weighed, and placed in a drying oven at 60ºC. After 72h, brains were reweighted and the brain water

content was measured considering the difference between wet tissue weight (w) and dry weight (d) (Wei et al., 2015). We considered the animal body weight since the animals prenatally VPA-exposed presented lower body weight throughout the development as described by Schneider et al., 2005 (Schneider & Przewłocki, 2005) as follows: {[(wet weight - dry weight)/wet weight]×100/animal body weight}.

2.3.2 Evans blue dye permeability

The animals were injected via i.p. with Evans blue 2% solution (4 mg/kg) diluted in saline solution 0,9% (Kumar et al., 2015) and, after 2 hours, were anesthetized and subjected to transcardiac perfusion with saline solution and 4% paraformaldehyde. The brain was removed, post-fixed in 4% paraformaldehyde and preserved in sucrose (15% and 30%). The tissues were kept in an ultrafreezer (-80ºC) until coronal slices (25 µm) were made in cryostat (Leica Microsystems). The brain coordinates following Paxinos Atlas (5th edition): bregma 3.72/3.24 (medial prefrontal cortex - mPFC) and -2.92/-3.00 (primary somatosensory area - pSSA, amygdala region - AmR, hippocampus and choroid plexus).

The slices were also incubated with DAPI solution for marking nuclei (diluted in 1:10,000 in saline solution 0.9%) for 10 minutes, followed by 5 washes with 0.1 M pH 7.4 PBS (3 minutes each) and adding the Fluoshield® mounting medium and the coverslip. The images were obtained in a 20x magnification using a confocal microscope (Olympus FV1000 – Olympus FluoView 4.0 Viewer) at the Center for Microscopy and Microanalysis (CMM-UFRGS) and the fluorescence was analyzed using the ImageJ® software.

2.3.3 Immunofluorescence

After anesthesia, the animals were euthanized by transcardiac perfusion with saline solution 0.9% and 4% paraformaldehyde, the brain was removed, preserved and cut as already described.

The technique was performed according to a previous protocol (Fontes-Dutra et al., 2018) and followed the following steps: 1) tissue permeabilization with PBS-Triton 0.1% or 0.3% (according to primary antibody); 2) 3 washes with PBS; 3) blocking with PBS-Triton 0.1% (or 0.3%) containing 5% bovine serum albumin (BSA); 4) incubation with primary antibodies for 48h at 4ºC in PBS-Triton 0.1% (or 0.3%) BSA 1% solution; 5) 5 washes with PBS buffer; 6) incubation with secondary antibodies for

2 hours at room temperature; 7) 5 washes with PBS; 8) incubation with DAPI solution (1:10,000 - 10 minutes); 9) 5 washes with PBS followed by addition of Fluoshield® mounting medium and coverslip. The images were obtained as previously described. Two trained researchers did manual analysis of both fluorescence distribution and cell counting in the brain regions and subregions, blinded for the experimental groups, using the ImageJ® software.

All primary antibodies were chosen according to the previous data from references cited in the manufacturer datasheets. All reagent information were detailed in Supplementary Table 1. Representative images of the AQP1 and AQP4 labeling can be seen in Supplementary Figure 1 and Supplementary Figure 2, respectively.

2.3.4 Western blotting

Following to the euthanasia, the brain was removed and the mPFC, pSSA, AmR and hippocampus were dissected.

The samples were homogenized in a buffer containing protease inhibitor, 10% SDS, EDTA 100 mM, TRIS/HCl buffer 500 mM pH 8. The total proteins were quantified by the Lowry method (Lowry et al., 1951) and the samples prepared in a buffer containing glycerol, bromophenol blue, TRIS/HCl buffer and β-mercaptoethanol. 40µg of protein was applied in 10% polyacrylamide gel, separated by one-dimensional electrophoresis and transferred to nitrocellulose membranes for the detection of the AQP1 and AQP4 immunocontents. The membranes were blocked in 5% BSA dissolved in a pH 7.5 TRIS buffer (TBS) with 0.1% Tween-20 (TTBS) and incubated overnight at 4ºC with the primary antibodies.

After incubation, the membranes were washed with TTBS and incubated with secondary antibodies, followed by 3 washes. The substrate SuperSignal® West Pico (Thermo Fisher Scientific) was used on the membranes and the chemiluminescent signal was detected using ImageQuant™ LAS 4000 (GE HealthCare Life Sciences). The quantification of the relative immunocontent was performed with the ImageJ software (v. 1.51) and the data was normalized by the housekeeping protein β-actin. All reagent information were detailed in Supplementary Table 1.

2.4 Statistical analysis

The data were analyzed using the IBM SPSS Statistics 20 program (IBM SPSS, Armonk, NY, USA). Kolmogorov-Smirnov and Shapiro-Wilk tests of normality were

applied to determine data distribution. The data of "edema" and "BBB permeability to Evans blue dye" had a non-normal distribution; therefore, a non-parametric test was performed for independent samples (Kruskal-Wallis). Immunofluorescence and western blotting data showed normal data distribution, using the two-way ANOVA test followed by Sidak's post-test. When there was an interaction effect, pairwise comparison was analyzed in the post hoc; when there was no effect, the effect of exposure to factors (VPA or RSV) was analyzed.

The graphs were made using the GraphPad Prism 6 program. Data were reported as median ± interquartile range (IQR) for the non-parametric test and mean ± standard deviation for the parametric test. $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Prenatal administration of RSV prevents the alterations induced by prenatal exposure to VPA in body weight and in proportional brain water content at P30

Prenatal exposure to VPA increased brain water content ($p = 0.003$, Figure 1A), decreased body weight ($p = 0.001$, Figure 1B) and increased proportion of brain fluid ($p = 0.002$, Figure 1C). In this experiment, prenatal treatment with RSV was able to prevent these alterations. Detailed statistics are shown in Table 1.

3.2. Prenatal administration of RSV prevents the increased BBB permeability induced by VPA at P30

The VPA group had increased BBB permeability to Evans blue dye (representative images in Figure 2) in the choroid plexus ($p = 0.009$), in the pSSA, both in layers II/III ($p = 0.004$) and IV/V ($p = 0.005$), in subregions of the mPFC when compared to the control and/or RSV group) anterior cingulate cortex (aCC) (II/III: $p = 0.001$; IV/V: $p = 0.013$); 2) prelimbic cortex (PrL) (II/III: $p = 0.019$; IV/V: $p = 0.022$); and 3) infralimbic cortex (IL) (II/III: $p = 0.028$; IV/V: $p = 0.016$). RSV was able to prevent the permeability alterations in these regions. No significant difference was observed in dye permeability in the hippocampus ($p = 0.134$) or in the AmR ($p = 0.050$). Detailed statistics are shown in Table 2.

3.3. Prenatal exposure to VPA changes choroid plexus morphology and decreases AQP1 distribution at P30

The choroid plexus from both groups exposed to VPA had a huge morphological alteration (indicated by the white arrow in Figure 3A) and decreased AQP1 labeling (Figure 3A-3B, control: 1244±279.3; RSV: 1053±131.9; VPA: 867.1±76.11; RSV+VPA: 798.6±221.3; F (3, 16) = 5.345, p VPA = 0.0022). These alterations were not prevented by RSV.

3.4. Prenatal administration of RSV prevents the VPA-induced increase in AQP4-immunocontent and distribution in pSSA at P30.

Prenatal exposure to VPA decreased AQP1 content in pSSA (Figure 4A, Control: 1.594±0.073; RSV: 1.342±0.210; VPA: 1.148±0.180; RSV+VPA: 0.834±0.185; F (1, 12) = 31.39; p VPA = 0.0001) in deep layers (II/III; p interaction = 0.9098; IV/V: p VPA = 0.0005) with no preventive effect by RSV (Table 3). Interestingly, the VPA group had increased AQP4 content in pSSA (Figure 4E, control: 1.445±0.0256; RSV: 1.086±0.149; VPA: 2.391±0.368; RSV+VPA: 1.457±0.314; F (1, 12) = 5.128; p interaction = 0.0429) in deep layers (II/III: p interaction = 0.4255; IV/V: p interaction = 0.0011) with a significant preventive effect of RSV. Detailed statistics of immunofluorescence are shown in Table 3.

3.5. mPFC has decreased levels of both AQP1 and AQP4 induced by prenatal exposure to VPA

Despite no change was observed in the AQP1 immunocontent in mPFC from VPA group (Figure 4C, control: 1.221±0.185; RSV: 1.124±0.182; VPA: 1.081±0.389; RSV+VPA: 0.975±0.195; F (1, 12) = 0.00095; p interaction = 0.9759), both groups exposed prenatally to VPA presented decreased distribution of this protein in all subregions of the mPFC, with no preventive effect of RSV: aCC – layers II/III (p VPA = 0.0043) and IV/V (p VPA = 0.0010); PrL – layers II/III (p VPA = 0.0010) and IV/V (p VPA < 0.0001); IL – layers II/III (p VPA = 0.0009) and IV/V (p VPA = 0.0004) (Table 4).

The VPA group had decreased content of AQP4 (Figure 4G, control: 0.816±0.196; RSV: 1.019±0.130; VPA: 0.387±0.164; RSV+VPA: 0.946±0.507; F (1, 12) = 6.808; p RSV = 0.0228) with decreased distribution of this protein in all subregions of the mPFC: aCC – layers II/III (p interaction = 0.0421) and IV/V (p interaction = 0.0125); PrL – layers II/III (p interaction = 0.0449) and IV/V (p interaction= 0.0167); IL – layers II/III (p interaction = 0.0412) and IV/V (p interaction = 0.0171). Detailed statistics of immunofluorescence are shown in Table 4.

3.6. Intrauterine exposure to VPA induces a decrease in AQP1 in the AmR

Despite no differences were observed in AQP1 immunocontent in the AmR among experimental groups (Figure 4B, Control: 0.918±0.214; RSV: 1.089±0.399; VPA: 0.871±0.113; RSV+VPA: 0.842±0.277; F (1, 12) = 0.6424; p interaction = 0.4384), both groups that received VPA had decreased distribution of AQP1 (p VPA = 0.0004) (Table 5). No changes were observed in both AQP4 content (Figure 4F, Control: 1.192±0.457; RSV: 1.288±0.333; VPA: 0.943±0.108; RSV+VPA: 1.512±0.521; F (1, 12) = 1.484; p interaction = 0.2466) and in distribution among groups (p interaction = 0.5988). Detailed statistics of immunofluorescence are shown in Table 5.

3.7. Prenatal exposure to VPA does not alter the expression profile of AQP1 and AQP4 in the hippocampus, but the RSV treatment had a *per se* effect

No changes were observed in the hippocampal content of AQP1 (Figure 4D, Control: 1.031±0.275; RSV: 0.927±0.273; VPA: 0.941±0.214; RSV+VPA: 0.977±0.238; F (1, 12) = 0.3097; p interaction = 0.5881). However, a *per se* effect of RSV was observed, with decreased AQP1 distribution in dentate gyrus and CA2 region: Dentate gyrus (p RSV = 0.0412); CA1 (p RSV = 0.0518); CA2 (p RSV = 0.0330); CA3 (p RSV = 0.0810) (Table 6).

No changes were observed in both AQP4 content (Figure 4H, Control: 0.533±0.271; RSV: 0.422±0.221; VPA: 0.291±0.106; RSV+VPA: 0.360±0.116; F (1, 12) = 0.8866; p interaction = 0.365) and distribution among groups: dentate gyrus (p interaction = 0.0387); CA1 (p interaction = 0.0786); CA2 (p interaction = 0.1113); CA3 (p interaction = 0.0490). Detailed statistics of immunofluorescence are shown in Table 6.

3.8 RSV treatment improves the functional but not morphological aspect of astrocytic changes induced by prenatal exposure to VPA

3.8.1 Primary somatosensory area

An increased number of $GFAP^+$-astrocytes was observed in layers II/III (p VPA = 0.0038) and in layers IV/V (p VPA = 0.0038) in both groups that were prenatally exposed to VPA (representative image in Figure 5). As expected, this data was

reflected by increased GFAP immunofluorescence per area (II/III: p VPA = 0.0270; IV/V: p RSV = 0.0380). Detailed statistics are shown in Table 7.

3.8.2 Medial prefrontal cortex

This region showed different effects between the upper (II/III) and deeper (IV/V) layers. In upper layers, exposition to VPA increases the number of astrocytes, with no preventive effect of RSV (aCC: p VPA = 0.0135; PrL: p VPA = 0.0269; IL: p VPA = 0.0278), which was reflected in the GFAP immunofluorescence values (aCC: p VPA = 0.0379; PrL: p VPA = 0.0604; IL: p interaction = 0.5621).

In deeper layers, both groups exposed to VPA increased the number of $GFAP^+$-astrocytes (aCC: p VPA = 0.0091; PrL: p VPA = 0.0004; IL: p VPA = 0.0018). However, we observed increased GFAP immunofluorescence in the VPA group, with a significantly preventive effect of RSV (aCC: p interaction = 0.0171; PrL: p interaction = 0.2853; IL: p interaction = 0.0455). Detailed statistics are shown in Table 8.

3.8.3 Amygdala region

In this region, we observed a *per se* effect of RSV, with decreased number $GFAP^+$-astrocytes (p interaction = 0.0017) and GFAP immunofluorescence (p interaction = 0.0450) in RSV group. Detailed statistics are shown in Table 9.

3.8.4 Hippocampus

Another *per se* effect of RSV was observed in number of $GFAP^+$-astrocytes (dentate gyrus: p interaction = 0.0162; CA1: p interaction = 0.1926; CA2: p RSV = 0.0001; CA3: p interaction = 0.0461) and GFAP immunofluorescence values (dentate gyrus: p interaction = 0.0096; CA1: p interaction = 0.0172; CA2: p interaction = 0.0214; CA3: p interaction = 0.0058). Detailed statistics are shown in Table 10.

4. DISCUSSION

A significant percentage of ASD patients presents increased brain volume in the first years of life, followed by an apparent normalization of this volume in late childhood (Aylward et al., 2002; Bartholomeusz et al., 2002; Emerson et al., 2017; Hazlett et al., 2011). Recent evidence in animal models has been highlighting the association of maternal inflammatory processes during critical embryonic development with excessive brain growth and with the triggering of ASD-associated behavior in the

offspring (Le Belle et al., 2014). In fact, maternal immune activation (MIA) contributes to the onset of several neuropsychiatric disorders, including ASD (Estes & McAllister, 2016). Thus, we postulate that the fingerprint caused by prenatal exposure to VPA could involve mechanisms of MIA since animals from VPA-animal model present enhanced levels of IL-1β, IL-6, and TFN-α in the hippocampus and other brain regions (Deckmann et al., 2018), besides enhanced TFN-α levels and microglial activation after prenatal VPA exposure (Zamberletti et al., 2019).

In the present study, we demonstrated that the prenatal exposure to VPA increased the absolute brain water content, providing a clearer overview of the higher liquid volume in the brain of the VPA group even though the body structure was smaller. The preventive effect of RSV against these changes, in addition to the alterations in the proteins AQP1 and AQP4 according to brain region, opens new clues about the mechanisms associated to the brain volume changes in ASD patients.

The VPA group presented evident BBB permeability in brain regions related to the neocortex: choroid plexus (directly in contact with neocortex), pSSA (layers II/III and IV/V) and all subregions of the mPFC (aCC, PrL and IL, in superficial and deeper layers). In all of these regions, RSV treatment was able to prevent the BBB permeability. BBB damage can be a pivotal event for brain edema development, which could be both initiated and regulated by several pro-inflammatory mediators (among them cytokines and chemokines) that coordinate the extent of leukocyte entry to the brain parenchyma, causing loosening of the tight junctions and vasogenic edema (Stamatovic et al., 2006). RSV, known by its antioxidants and anti-inflammatory properties, could be acting as a neuroprotective molecule in different pathways during embryonic development. In a model of cerebral ischemia-reperfusion, RSV attenuates BBB dysfunctions and reverses the brain water accumulation by the regulation of matrix metallopeptidase 9 (MMP-9) (Wei et al., 2015). MMP-9 is an enzyme with zinc-dependent proteolytic activity that has the ability to break down collagen IV (which composes basal lamina) and whose increased levels were associated with neurodevelopmental disorders, including ASD (Reinhard et al., 2015). Both VPA and RSV are able to act in an epigenetic way, mainly modulating the histone activity. The histone deacetylase inhibition (HDACi) - an effect of VPA - is able to induce several effects on the BBB stabilization by the deregulation of important transcription factors associated with BBB formation like SOX7, SOX18, TAL1, and ETS1 (Roudnicky et al., 2020), as well as is known to interfere in the immune system, inducing increased

transcription of proinflammatory genes associated with the NFkappaB pathway, for example (Rahman et al., 2004). This is important since inflammatory mediators are known to increase the BBB permeability, leading to an inflammatory infiltrate in the CNS, and are associated with neurodevelopmental disorders, such as ASD. Complementary, VPA-prenatal exposure promotes systemic inflammation, and is known that the maternal immune activation (MIA) animal model is associated with BBB disruption (Simões et al., 2018). The early treatment with RSV (beginning at E6.5) probably reduced BBB alterations by the attenuating HDACi induced by VPA through the activating Sirt and promoting modulations of proteins like MMP9 and TIMP1 (Moussa et al., 2017; Sawda et al., 2017; Wei et al., 2015). Therefore, RSV acts as a stabilizer of the transcription, preventing both BBB cell alterations (directly) and the shift for a proinflammatory status in the immune system (indirectly).

We investigated the expression and distribution of aquaporins, important water channels, in different regions of CNS. These proteins perform several roles, but one of the main ones is to facilitate the movement of water, both in and out of the CNS (Rosu et al., 2019). The VPA group decreased the AQP1 distribution in the choroid plexus, in the deeper layers of pSSA, in the AmR and in all of the subregions of mPFC analyzed. Choroid plexus is crucial given its role in the production and release of CSF and it has been shown that in animals knocked out for AQP1 there was a reduction of up to 25% in the rate of cerebrospinal fluid secretion (Oshio et al., 2003). In addition, a morphological alteration was observed in the insertion of the choroid plexus in the third ventricle. A similar lesion was observed in a model of cerebral ischemic edema (Akdemir et al., 2016), but here in the present study, we do not consider any association between this morphological alteration and the pathophysiology of ASD, which may simply be a teratogenic effect of VPA *per se*.

The VPA group increased the AQP4 content in deeper layers of the pSSA (prevented by RSV) whereas the mPFC presented decreased levels without prevention by the RSV. Alterations on the AQP4 profile in *postmortem* brain tissue of ASD individuals have already been reported (Fatemi et al., 2008), including a discrete reduction in Broadmann area 9 (BA9 - equivalent of the frontal cortex) and an increase in the BA40 (parietal cortex, where is the pSSA). They also showed increased connexin 43 levels (a protein present in astrocytic gap junction) in BA9, representing an increase in neuroglial signaling and an improvement of cell-cell communication in the frontal lobe (an integrative area) (Fatemi et al., 2008). Moreover, *AQP4* knockout mice have

reduced brain swelling in cytotoxic edema, whilst there is a significantly worse result in the case of vasogenic brain edema (Papadopoulos & Verkman, 2007). In the VPA model, it is still not elucidated what type of brain edema is present. Lastly, there are findings linking AQP4 with neuroimmune modulation (Ikeshima-Kataoka, 2016), which represents an important clue in the ASD pathophysiology, since the immune component of this disorder is both relevant and well established (Gottfried et al., 2015).

Noteworthy, as there are few studies on the dynamics of AQP1 and AQP4 in ASD, we think it is important to clarify some points. Despite the common sense that AQP4 is more widely expressed in the brain than AQP1, we observed higher fluorescence levels in AQP1 compared to AQP4 in the control groups in some regions (pSSA and AmR). In astrocytes, the distribution of AQP4 is predominantly in the endfeet projections surrounding vessels; however, its brain concentration varies according to region, with higher levels in the cerebellum and lower expression in the hippocampus, diencephalons, and cortex (Hubbard et al., 2015). Undoubtedly, AQP1 is mainly expressed in the choroid plexus; nevertheless, it is also expressed under normal conditions in other brain structures, such as the brain stem, cerebellum, brain cortex, hippocampus, hypothalamus, and olfactory bulb (Li et al., 2020; Qiu et al., 2014). Beyond that, studies demonstrate neuronal localization of AQP1 in mouse cortical slices, as well as increased cortical levels of AQP1 in Alzheimer's disease models. Moreover, this same work also demonstrates that wild-type animals at 60 days old, present higher amounts of AQP1 protein than AQP4 in cortical homogenates (Park et al., 2021). Finally, in the amygdala region, the human protein atlas demonstrates the mRNA expression of AQP1 (The Human Protein Atlas, n.d.). These studies indicate that the concentration and distribution of AQP 1 and 4 vary according to cell type/domain and brain region, which corroborates our present study regarding the brain region.

The increased GFAP-immunofluorescence and number of $GFAP^+$-astrocytes in mPFC and pSSA by prenatal exposure to VPA corroborate with previous studies showing neuroglial activation in both ASD patients and animal models (Bristot Silvestrin et al., 2013; Edmonson et al., 2014; Vargas et al., 2005; Zhao et al., 2019). Here, we observed an important preventive effect of RSV in the mPFC, demonstrated in the decrease of GFAP immunofluorescence. Based on previous data, that indicate the neuroprotective effect of lower doses of RSV in hippocampus slices (emphasizing the important role of RSV in improving glutamate uptake by astrocytes and modulate

the synaptic plasticity) (Bobermin et al., 2012; De Almeida et al., 2008; Quincozes-Santos et al., 2013; Quincozes-Santos & Gottfried, 2011), and an improvement in neuroinflammation (an ASD hallmark) in an ASD-animal model (Ahmad et al., 2018; Bhandari & Kuhad, 2017), it would be possible that also in this context, we have a beneficial effect on astrocyte metabolism and function, since this treatment is effective in ameliorates several behavioral impairments in VPA animal model (Bambini-Junior et al., 2014; Fontes-Dutra et al., 2018; Hirsch et al., 2018).

Despite some studies demonstrated no alterations in astrocyte parameters in ASD post mortem tissues (T. T. Lee et al., 2017; Morgan et al., 2014), animal models of fragile X syndrome (a disorder with a high prevalence of ASD) present a specific disruption in the constitution of the deeper layers, besides presenting an increased number of astrocytes (F. Lee et al., 2019). Therefore, alterations in the laminar constitution could influence directly the distribution of astrocytes. The dynamics of cortical disorganization is widely described in ASD. An event like acute neuroinflammation, with increased levels of brain cytokines, may contribute to synaptic reorganization, which results in long-term alterations regarding hyperexcitability of the whole neural circuitry (Clarkson et al., 2017). One of the most relevant findings in patients with ASD is the identification of disturbance in the organization of the minicolumns (Casanova, 2007) and the presence of patches with loss of layer delimitation in the cortex (DeNardo et al., 2015; Stoner et al., 2014), being the deeper cortical layers the most affected.

In hippocampus and AmR, we observed only effects of RSV treatment. A possible explanation for this effect observed in the amygdala region is that the amygdalar nuclei originate at different times between embryonic day E10-E12 in rats, before the induction of the animal model (in E12.5), and during the prenatal treatment with RSV (between E6.5 to E18.5) (Soma et al., 2009). Although the embryonary origin of the hippocampus starting from E15 (Hayashi et al., 2015), the effects of prenatal exposure to VPA seem to be progressive and of late-onset (Santos-Terra, *unpublished observations*). Despite being a molecule with important neuroprotective effects already described, RSV was able to cause changes in the hippocampus at P30. Considering the progressive effect of VPA in adulthood, maybe RSV develops an earlier cellular background to better support the progressive damage induced by VPA.

One of the major theories regarding ASD pathophysiology refers to the electrophysiological changes, mainly the imbalance between excitation and inhibition.

The presence of epilepsy or seizure episodes in approximately 30% of individuals with ASD reinforces the excitatory profile predominant in ASD (Spence & Schneider, 2009). In response to this hyperexcitability and chronic neuroinflammation, might be observed proliferation and hypertrophy of the astrocytes, which acquire a reactive profile (Poskanzer & Molofsky, 2018), due to several roles, including K + buffering. The extracellular K^+ is critical for defining the resting potential of neurons and astrocyte membranes, and mechanisms for removing this ion from the synaptic cleft are vital to maintaining cerebral homeostasis (Bellot-Saez et al., 2017). One mechanism of K^+ uptake by glial cells is through the action of internal rectifying channels of K^+ (Kir) (Olsen et al., 2015). This is particularly important since AQP4 and Kik4.1 are highly overlapping channels in the astrocytic end-feet (Strohschein et al., 2011) and probably AQP4 is required to sustain efficient K+ clearance, considering the association of water flux alteration and increasing intensity of epileptic seizures (Amiry-Moghaddam et al., 2003), and a delay in K+ buffering in *AQP4*-null mice (Lu et al., 2008).

Considering all data, we hypothesized that the brain impairments induced by the VPA model include a neuroinflammation background triggered in the developing brain of the embryo, which contributes to the increased BBB permeability (and consequently edema due to the entry of water and inflammatory infiltrate). In consequence, there is a decrease in the levels of AQP1 and AQP4 to maintain water homeostasis in the brain. In parallel, neuroinflammation triggers the excitotoxicity process, leading to a reactive astrocytic phenotype. The increased astrocytic activity leads to an increased need for K+ buffering, which in turn increases Kir4.1 and, consequently, AQP4 levels in a region-specific manner (which, in turn, could be the main onset to brain edema formation). The fingerprinting caused by VPA happens in multiple regions; since pSSA is a primary processing area, both the impact caused by VPA and the prevention mechanisms by RSV may be more expressive and less complex than those occurring in mPFC, an associative and more complex region. Here, RSV prevents successfully the impairments regarding BBB permeability and the increase of AQP4 in the pSSA, as well as decreases GFAP antibody labeling in the mPFC, indicating a lower glial reactivity. Thereby, based on several shreds of evidence that point to RSV as a stabilizer of the neural environment, RSV could also normalize K+ levels and restructure synaptic connections in pSSA considering the co-localization of AQP4 and Kir4.1 channels.

5. CONCLUDING REMARKS

In summary, we demonstrated that prenatal exposure to VPA alters the bodyweight of the animals, as well as induces brain edema, and increases the permeability of BBB. In addition, there was an altered AQP profile in region-dependent in VPA-exposed animals and GFAP augmented expression. RSV was able to prevent important changes in GFAP+ astrocytes and in AQP4 in the pSSA. The neuroprotective role of the RSV in this model shed some light on pathways possibly associated with the alterations induced by VPA along with neuroimmune changes also observed in ASD individuals. Taken together, the present data emphasize the investigation of the mechanisms involved in the neuroimmunological issues as a promising strategy in the understanding of biological pathways in ASD pathophysiology.

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List of Figures

$N_{RSV+VPA}$: 5. GFAP is marked in green, AQP4 in red and DAPI nuclear dye in blue. Scale bar: 50 µm.

Supplementary Figure 1 - Representative images of the AQP1 labeling. Illustrative images of DAPI (blue) and AQP1 (green) staining in the pSSA (layers IV/V), aCC (mPFC – layers II/III), DG (hippocampus) and AmR in the different experimental groups. Scale bar: 50 µm.

Supplementary Figure 2 - Representative images of the AQP4 labeling. Illustrative images of DAPI (blue), GFAP (green) and AQP4 (red) staining in the pSSA (layers IV/V), aCC (mPFC – layers II/III), DG (hippocampus) and AmR in the different experimental groups. Scale bar: 50 µm.

List of Tables

Table 1 – Descriptive statistics of body weight and in proportional brain water content

	Median (IQR)	Chi-Square, df	p Value	Mean rank	Pairwise comparisons	
%	CON: 78.27 (75.10±78,74) RSV: 77.44 (74.89±77.56) VPA: 79.41 (79.23±79.73) RSV+VPA: 77.91 (77.74±77.93)	14.019, 3	0.003**	CON: 10.00 RSV: 6.86 VPA: 20.50 RSV+VPA: 11.25	CON vs RSV:	>0.999
					CON vs VPA:	0.044*
					CON vs RSV+VPA:	>0.999
					RSV vs VPA:	0.002**
					RSV vs RSV+VPA:	>0.999
					VPA vs RSV+VPA:	0.208
g	CON: 73.50 (59.00±83.50) RSV: 73.00 (61.00±76.00) VPA: 45.50 (43.00±47.75) RSV+VPA: 83.50 (77.25±107.00)	15.659, 3	0.001**	CON: 9.50 RSV: 10.86 VPA: 20.50 RSV+VPA: 5.00	CON vs RSV:	>0.999
					CON vs VPA:	0.030*
					CON vs RSV+VPA:	>0.999
					RSV vs VPA:	0.064#
					RSV vs RSV+VPA:	>0.999
					VPA vs RSV+VPA:	0.002**
%/g	CON: 1.052 (0.914±1.347) RSV: 1.025 (0.998±1.268) VPA: 1.749 (1.663±1.847) RSV+VPA: 0.934 (0.737±1.009)	14.699, 3	0.002**	CON: 14.33 RSV: 12.79 VPA: 3.50 RSV+VPA: 19.88	CON vs RSV:	>0.999
					CON vs VPA:	0.034*
					CON vs RSV+VPA:	>0.999
					RSV vs VPA:	0.082#
					RSV vs RSV+VPA:	0,570
					VPA vs RSV+VPA:	0.001**

%: brain water content percentage; **g:** body weight; **%/g:** proportion of brain fluid. p <0.05 was considered significant. *p<0.05, **p<0.01. Statistical analyses: Kruskal-Wallis. N_{CON}: 5, N_{RSV}: 6, N_{VPA}: 6, $N_{RSV+VPA}$: 4.

Table 2 – Descriptive statistics of Evans blue dye

	Median (IQR)	Chi-Square, df	p Value	Mean rank	Pairwise comparisons	
CP	CON: 98.08 (75.07±103,4) RSV: 77.09 (56.18±91.46) VPA: 253.6 (150.4±393.0) RSV+VPA: 72.00 (62.16±88.70)	11.674, 3	0.009**	CON: 12.60 RSV: 7.64 VPA: 19.50 RSV+VPA: 7.30	CON vs RSV: CON vs VPA: CON vs RSV+VPA: RSV vs VPA: RSV vs RSV+VPA: VPA vs RSV+VPA:	>0.999 0.584 >0.999 0.014* >0.999 0.020*
HIP	CON: 100.6 (81.16±122.5) RSV: 83.16 (63.64±97.31) VPA: 107.4 (57.4±180.5) RSV+VPA: 68.5 (55.03±75.59)	5.572, 3	0.134	CON: 14.90 RSV: 10.36 VPA: 13.25 RSV+VPA: 6.20	CON vs RSV: CON vs VPA: CON vs RSV+VPA: RSV vs VPA: RSV vs RSV+VPA: VPA vs RSV+VPA:	>0.999 >0.999 0.149 >0.999 >0.999 0.542
AmR	CON: 97.14 (90.83±115.4) RSV: 79.56 (66.98±94.11) VPA: 108.7 (46.77±159.2) RSV+VPA: 66.17 (44.93±83.03)	7.812, 3	0.050	CON: 15.60 RSV: 8.86 VPA: 14.75 RSV+VPA: 6.40	CON vs RSV: CON vs VPA: CON vs RSV+VPA: RSV vs VPA: RSV vs RSV+VPA: VPA vs RSV+VPA:	0.381 >0.999 0.114 0.778 >0.999 0.269
pSSA (II/III)	CON: 123.00 (113.7±146.1) RSV: 88.71 (78.22±106.4) VPA: 838.6 (624.3±1019.0) RSV+VPA: 86.19 (78.83±112.4)	13.541, 3	0.004**	CON: 13.8 RSV: 6.79 VPA: 19.50 RSV+VPA: 7.30	CON vs RSV: CON vs VPA: CON vs RSV+VPA: RSV vs VPA: RSV vs RSV+VPA: VPA vs RSV+VPA:	0.321 >0.999 0.585 0.006** >0.999 0.020*
pSSA (IV/V)	CON: 124.6 (115.4±142.0) RSV: 105.9 (62.22±118.5) VPA: 483,9 (321.5±578.7) RSV+VPA: 95.09 (91.75±120.7)	12.855, 3	0.005**	CON: 13.40 RSV: 7.00 VPA: 19.50 RSV+VPA: 7.40	CON vs RSV: CON vs VPA: CON vs RSV+VPA: RSV vs VPA: RSV vs RSV+VPA: VPA vs RSV+VPA:	0.468 0.856 0.757 0.008** >0.999 0.022*
aCC (II/III)	CON: 100.9 (78.63±112.2) RSV: 77.78 (74.32±90.65) VPA: 370.1 (279.2±416.7) RSV+VPA: 71.57 (68.05±95.45)	11.670, 3	0.001**	CON: 11.50 RSV: 8.21 VPA: 18.50 RSV+VPA: 5.25	CON vs RSV: CON vs VPA: CON vs RSV+VPA: RSV vs VPA: RSV vs RSV+VPA: VPA vs RSV+VPA:	>0.999 0.465 0.690 0.033* >0.999 0.009**
aCC (IV/V)	CON: 86.73 (81.27±99.31) RSV: 78.16 (77.02±107.5) VPA: 423.9 (335.2±447.6) RSV+VPA: 80.25 (78.98±92.59)	10.801, 3	0.013*	CON: 11.20 RSV: 6.86 VPA: 18.50 RSV+VPA: 8.00	CON vs RSV: CON vs VPA: CON vs RSV+VPA: RSV vs VPA: RSV vs RSV+VPA: VPA vs RSV+VPA:	>0.999 0.391 >0.999 0.010* >0.999 0.071#
PrL (II/III)	CON: 78.56 (71.57±100.8) RSV: 79.78 (74.39±106.5) VPA: 378.2 (230.0±422.7) RSV+VPA: 73.94 (64.44±89.42)	9.926, 3	0.019*	CON: 8.40 RSV: 9.71 VPA: 18.50 RSV+VPA: 6.50	CON vs RSV: CON vs VPA: CON vs RSV+VPA: RSV vs VPA: RSV vs RSV+VPA: VPA vs RSV+VPA:	>0.999 0.065# >0.999 0.106 >0.999 0.024*
PrL (IV/V)	CON: 82.80 (78.15±99.0) RSV: 86.23 (71.51±108.7) VPA: 364.9 (172.0±378.0) RSV+VPA: 69.02 (63.39±89.87)	9.653, 3	0.022*	CON: 9.70 RSV: 9.71 VPA: 18.00 RSV+VPA: 5.38	CON vs RSV: CON vs VPA: CON vs RSV+VPA: RSV vs VPA: RSV vs RSV+VPA: VPA vs RSV+VPA:	>0.999 0.219 >0.999 0.152 >0.999 0.015*

IL (II/III)	CON: 81.6 (80.25±93.42) RSV: 79.34 (71.30±104.2) VPA: 232.9 (141.5±351.4) RSV+VPA: 78.77 (68.97±106.5)	9.060, 3	0.028*	CON: 9.40 RSV: 7.93 VPA: 18.38 RSV+VPA: 8.50	CON vs RSV:	>0.999
					CON vs VPA:	0.141
					CON vs RSV+VPA:	>0.999
					RSV vs VPA:	0.029*
					RSV vs RSV+VPA:	>0.999
					VPA vs RSV+VPA:	0.109
IL (IV/V)	CON: 87.79 (86.19±104.8) RSV: 81.65 (76.40±108.2) VPA: 275.0 (153.4±336.0) RSV+VPA: 87.05 (66.13±98.72)	10.289, 3	0.016*	CON: 10.80 RSV: 7.21 VPA: 18.50 RSV+VPA: 7.88	CON vs RSV:	>0.999
					CON vs VPA:	0.313
					CON vs RSV+VPA:	>0.999
					RSV vs VPA:	0.014*
					RSV vs RSV+VPA:	>0.999
					VPA vs RSV+VPA:	0.066#

II/III: upper cortical layers; **IV/V**: deeper cortical layers; **aCC**: anterior cingulate cortex; **AmR**: amygdala region; **CP**: choroid plexus; **HIP**: hippocampus; **IL**: infralimbic cortex; **IQR**: interquartile range; **PrL**: prelimbic cortex; **pSSA**: primary somatosensory area. p <0.05 was considered significant. *p<0.05, **p<0.01. Statistical analyses: Kruskal-Wallis. N_{CON}: 5, N_{RSV}: 7, N_{VPA}: 4, $N_{RSV+VPA}$: 4.

Table 3 – Descriptive statistics of the AQP1 and AQP4 distribution profile in pSSA

	Mean ± SD	F (DFn, DFd); p Value	Pairwise comparisons	
AQP1 (II/III)	CON: 438.3±105.9 RSV: 455.8±121.3 VPA: 373.7±89.17 RSV+VPA: 380.2±111.2	Interaction: F (1, 17) = 0,013; p = 0.909 VPA: F (1, 17) = 2,171; p = 0.158 RSV: F (1, 17) = 0,064; p = 0.804	CON vs RSV:	>0.999
			CON vs VPA:	0.934
			CON vs RSV+VPA:	0.966
			RSV vs VPA:	0.744
			RSV vs RSV+VPA:	0.838
			VPA vs RSV+VPA:	>0.999
AQP1 (IV/V)	CON: 556.3±140.8 RSV: 507.6±82.91 VPA: 337.6±80.89 RSV+VPA: 365.4±83.67	Interaction: F (1, 17) = 0.821; p = 0.377 VPA: F (1, 17) = 18.28; p = 0.0005*** RSV: F (1, 17) = 0.0607; p = 0.808	CON vs RSV:	0.969
			CON vs VPA:	0.015*
			CON vs RSV+VPA:	0.049*
			RSV vs VPA:	0.039*
			RSV vs RSV+VPA:	0.139
			VPA vs RSV+VPA:	0.997
AQP4 (II/III)	CON: 154.1±32.47 RSV: 194.3±55.26 VPA: 194.4±52.50 RSV+VPA: 196.8±69.81	Interaction: F (1, 18) = 0.6649; p = 0.4255 VPA: F (1, 18) = 0.8502; p = 0.3687 RSV: F (1, 18) = 0.8440; p = 0.3704	CON vs RSV:	0.801
			CON vs VPA:	0.799
			CON vs RSV+VPA:	0.789
			RSV vs VPA:	>0.999
			RSV vs RSV+VPA:	>0.999
			VPA vs RSV+VPA:	>0.999
AQP4 (IV/V)	CON: 109.9±23.35 RSV: 147.5±21.48 VPA: 173.8±27.00 RSV+VPA: 123.7±28.31	Interaction: F (1, 19) = 14.75; p = 0.0011** VPA: F (1, 19) = 2.573; p = 0.1252 RSV: F (1, 19) = 0.7357; p = 0.4017	CON vs RSV:	0.2348
			CON vs VPA:	0.0052**
			CON vs RSV+VPA:	0.9966
			RSV vs VPA:	0.4338
			RSV vs RSV+VPA:	0.5920
			VPA vs RSV+VPA:	0.0252*

II/III: upper cortical layers; **IV/V**: deeper cortical layers; **pSSA**: primary somatosensory area; **SD**: standard deviation. p <0.05 was considered significant. *p<0.05, **p<0.01, ***p<0.001. Statistical analyses: two-way ANOVA parametric test followed by Sidak. N_{CON}: 5, N_{RSV}: 6, N_{VPA}: 6, $N_{RSV+VPA}$: 5. "Mean" represents de mean of fluorescence.

Table 4 – Descriptive statistics of the AQP1 and AQP4 distribution profile in mPFC

	Mean ± SD	F (DFn, DFd); p Value	Pairwise comparisons	
AQP1 aCC (II/III)	CON: 340.0±20.61 RSV: 317.6±59.90 VPA: 237.3±34.16 RSV+VPA: 240.3±89.26	Interaction: F (1, 14) = 0.232; p = 0.637 VPA: F (1, 14) = 11.59; p = 0.0043** RSV: F (1, 14) = 0.136 ; p = 0.717	CON vs RSV:	0.989
			CON vs VPA:	0.091#
			CON vs RSV+VPA:	0.105
			RSV vs VPA:	0.264
			RSV vs RSV+VPA:	0.301
			VPA vs RSV+VPA:	>0.999
AQP1 aCC (IV/V)	CON: 413.4±89.15 RSV: 384.2±68.89 VPA: 213.5±60.90 RSV+VPA: 256.6±107.4	Interaction: F (1, 14) = 0.839; p = 0.375 VPA: F (1, 14) = 17.29; p = 0.0010*** RSV: F (1, 14) = 0.0311; p = 0.863	CON vs RSV:	0.995
			CON vs VPA:	0.017*
			CON vs RSV+VPA:	0.079#
			RSV vs VPA:	0.049*
			RSV vs RSV+VPA:	0.207
			VPA vs RSV+VPA:	0.979
AQP1 PrL (II/III)	CON: 440.8±84.40 RSV: 400.3±110.8 VPA: 226.7±39.04 RSV+VPA: 249.5±114.8	Interaction: F (1, 14) = 0.512; p = 0.486 VPA: F (1, 14) = 17.01; p = 0.0010** RSV: F (1, 14) = 0.039; p = 0.845	CON vs RSV:	0.985
			CON vs VPA:	0.024*
			CON vs RSV+VPA:	0.050#
			RSV vs VPA:	0.086#
			RSV vs RSV+VPA:	0.168
			VPA vs RSV+VPA:	0.999
AQP1 PrL (IV/V)	CON: 444.4±79.13 RSV: 383.0±66.96 VPA: 262.1±52.99 RSV+VPA: 189.3±85.19	Interaction: F (1, 14) = 0.028; p = 0.869 VPA: F (1, 14) = 30.04; p < 0.0001**** RSV: F (1, 14) = 3.832; p = 0.0705	CON vs RSV:	0.739
			CON vs VPA:	0.013*
			CON vs RSV+VPA:	0.0007***
			RSV vs VPA:	0.146
			RSV vs RSV+VPA:	0.0080**
			VPA vs RSV+VPA:	0.686
AQP1 IL (II/III)	CON: 438.1±84.61 RSV: 365.1±95.19 VPA: 205.0±41.25 RSV+VPA: 238.9±122.0	Interaction: F (1, 14) = 1.550; p = 0.2336 VPA: F (1, 14) = 17.51; p = 0.0009*** RSV: F (1, 14) = 0.2064 ; p = 0.6565	CON vs RSV:	0.780
			CON vs VPA:	0.0108*
			CON vs RSV+VPA:	0.0324*
			RSV vs VPA:	0.111
			RSV vs RSV+VPA:	0.294
			VPA vs RSV+VPA:	0.996
AQP1 IL (IV/V)	CON: 459.4±80.84 RSV: 418.4±89.54 VPA: 247.2±30.44 RSV+VPA: 229.1±138.2	Interaction: F (1, 14) = 0.069; p = 0.796 VPA: F (1, 14) = 21.20; p = 0.0004*** RSV: F (1, 14) = 0.459; p = 0.5089	CON vs RSV:	0.982
			CON vs VPA:	0.0236*
			CON vs RSV+VPA:	0.0132*
			RSV vs VPA:	0.086#
			RSV vs RSV+VPA:	0.0489*
			VPA vs RSV+VPA:	>0.999
AQP4 aCC (II/III)	CON: 522.8±86.07 RSV: 400.5±94.07 VPA: 298.3±119.1 RSV+VPA: 433.3±213.3	Interaction: F (1, 16) = 4.879; p = 0.0421* VPA: F (1, 16) = 2.709; p = 0.1193 RSV: F (1, 16) = 0.01204; p = 0.9140	CON vs RSV:	0.586
			CON vs VPA:	0.064#
			CON vs RSV+VPA:	0.880
			RSV vs VPA:	0.787
			RSV vs RSV+VPA:	0.999
			VPA vs RSV+VPA:	0.589
AQP4 aCC (IV/V)	CON: 555.4±160.3 RSV: 354.4±76.63 VPA: 254.0±42.98 RSV+VPA: 366.8±167.8	Interaction: F (1, 16) = 7.910; p = 0.0125* VPA: F (1, 16) = 6.711; p = 0.0197* RSV: F (1, 16) = 0.6246 ; p = 0.4409	CON vs RSV:	0.093
			CON vs VPA:	0.0058**
			CON vs RSV+VPA:	0.172
			RSV vs VPA:	0.769
			RSV vs RSV+VPA:	>0.999
			VPA vs RSV+VPA:	0.722
AQP4 PrL (II/III)	CON: 428.0±68.79 RSV: 354.1±90.40 VPA: 213.2±45.47 RSV+VPA: 314.6±144.4	Interaction: F (1, 16) = 4.736; p = 0.0449* VPA: F (1, 16) = 9.964; p = 0.0061** RSV: F (1, 16) = 0.1171 ; p = 0.7367	CON vs RSV:	0.717
			CON vs VPA:	0.0064**
			CON vs RSV+VPA:	0.337
			RSV vs VPA:	0.134
			RSV vs RSV+VPA:	0.987
			VPA vs RSV+VPA:	0.500
AQP4	CON: 482.0±107.3	Interaction: F (1, 16) = 7.142; p = 0.0167*	CON vs RSV:	0.216

PrL (IV/V)	RSV: 339.8±92.58 VPA: 227.3±49.74 RSV+VPA: 338.6±157.5	VPA: F (1, 16) = 7.278; p = 0.0158* RSV: F (1, 16) = 0.1057 ; p = 0.7493	CON vs VPA:	0.0061**
			CON vs RSV+VPA:	0.266
			RSV vs VPA:	0.501
			RSV vs RSV+VPA:	>0.999
			VPA vs RSV+VPA:	0.576
AQP4 IL (II/III)	CON: 466.8±100.4 RSV: 356.7±105.0 VPA: 193.8±27.21 RSV+VPA: 267.8±111.5	Interaction: F (1, 16) = 4.930; p = 0.0412* VPA: F (1, 16) = 19.05; p = 0.0005*** RSV: F (1, 16) = 0.1895 ; p = 0.6692	CON vs RSV:	0.331
			CON vs VPA:	0.0009***
			CON vs RSV+VPA:	0.0237*
			RSV vs VPA:	0.0736#
			RSV vs RSV+VPA:	0.668
			VPA vs RSV+VPA:	0.817
AQP4 IL (IV/V)	CON: 446.3±70.78 RSV: 333.9±87.51 VPA: 235.7±52.18 RSV+VPA: 315.0±108.4	Interaction: F (1, 16) = 7.073; p = 0.0171* VPA: F (1, 16) = 10.13; p = 0.0058** RSV: F (1, 16) = 0.2106 ; p = 0.6525	CON vs RSV:	0.184
			CON vs VPA:	0.0029**
			CON vs RSV+VPA:	0.122
			RSV vs VPA:	0.351
			RSV vs RSV+VPA:	0.999
			VPA vs RSV+VPA:	0.643

II/III: upper cortical layers; **IV/V**: deeper cortical layers; **aCC**: anterior cingulate cortex; **IL**: infralimbic cortex; **PrL**: prelimbic cortex; **SD**: standard deviation. p <0.05 was considered significant. *p<0.05, **p<0.01, ***p<0.001, ***p<0.0001. Statistical analyses: two-way ANOVA parametric test followed by Sidak. N_{CON}: 5, N_{RSV}: 6, N_{VPA}: 6, $N_{RSV+VPA}$: 5. "Mean" represents de mean of fluorescence.

Table 5 – Descriptive statistics of the AQP1 and AQP4 distribution profile in AmR

	Mean ± SD	F (DFn, DFd); p Value	Pairwise comparisons	
AQP1	CON: 394.4±13.92 RSV: 354.3±46.69 VPA: 291.2±67.83 RSV+VPA: 242.8±67.61	Interaction: F (1, 17) = 0.0279; p = 0.869 VPA: F (1, 17) = 18.95; p = 0.0004*** RSV: F (1, 17) = 3.226; p = 0.090	CON vs RSV:	0.8603
			CON vs VPA:	0.0621#
			CON vs RSV+VPA:	0.0049**
			RSV vs VPA:	0.3386
			RSV vs RSV+VPA:	0.0249*
			VPA vs RSV+VPA:	0.6718
AQP4	CON: 112.9±15.93 RSV: 124.6±11.77 VPA: 119.7±10.60 RSV+VPA: 123.0±26.89	Interaction: F (1, 16) = 0,2881; p = 0.5988 VPA: F (1, 16) = 0.1118; p = 0.7425 RSV: F (1, 16) = 0.9222; p = 0.3512	CON vs RSV:	0.887
			CON vs VPA:	0.991
			CON vs RSV+VPA:	0.939
			RSV vs VPA:	0.998
			RSV vs RSV+VPA:	>0.999
			VPA vs RSV+VPA:	0.999

AmR: amygdala region; **SD**: standard deviation. p <0.05 was considered significant. *p<0.05, **p<0.01, ***p<0.001. Statistical analyses: two-way ANOVA parametric test followed by Sidak. N_{CON}: 5, N_{RSV}: 6, N_{VPA}: 6, $N_{RSV+VPA}$: 5. "Mean" represents de mean of fluorescence.

Table 6 – Descriptive statistics of the AQP1 and AQP4 distribution profile in hippocampus.

	Mean ± SD	F (DFn, DFd); p Value	Pairwise comparisons	
AQP1 DG	CON: 420.2±68.76 RSV: 315.9±121.7 VPA: 387.9±154.8 RSV+VPA: 260.1±123.2	Interaction: F (1, 18) = 0.049; p = 0.826 VPA: F (1, 18) = 0.697; p = 0.415 RSV: F (1, 18) = 4.837; p = 0.041*	CON vs RSV:	0.694
			CON vs VPA:	0.999
			CON vs RSV+VPA:	0.287
			RSV vs VPA:	0.905
			RSV vs RSV+VPA:	0.976
			VPA vs RSV+VPA:	0.482
AQP1 CA1	CON: 418.9±72.71 RSV: 316.8±114.7 VPA: 341.8±107.7 RSV+VPA: 246.9±136.7	Interaction: F (1, 18) = 0.0057; p = 0.940 VPA: F (1, 18) = 2.413; p = 0.137 RSV: F (1, 18) = 4.340; p = 0.052#	CON vs RSV:	0.607
			CON vs VPA:	0.841
			CON vs RSV+VPA:	0.136
			RSV vs VPA:	0.999
			RSV vs RSV+VPA:	0.892
			VPA vs RSV+VPA:	0.679
AQP1 CA2	CON: 424.1±50.26 RSV: 340.3±107.3 VPA: 356.3±126.8 RSV+VPA: 226.6±124.3	Interaction: F (1, 18) = 0.245; p = 0.626 VPA: F (1, 18) = 3.858; p = 0.065 RSV: F (1, 18) = 5.331; p = 0.033*	CON vs RSV:	0.768
			CON vs VPA:	0.895
			CON vs RSV+VPA:	0.057
			RSV vs VPA:	>0.999
			RSV vs RSV+VPA:	0.465
			VPA vs RSV+VPA:	0.327
AQP1 CA3	CON: 366.6±54.55 RSV: 316.9±105.5 VPA: 335.5±120.2 RSV+VPA: 217.8±124.0	Interaction: F (1, 18) = 0.564; p = 0.462 VPA: F (1, 18) = 2.071; p = 0.167 RSV: F (1, 18) = 3.418; p = 0.081#	CON vs RSV:	0.972
			CON vs VPA:	0.997
			CON vs RSV+VPA:	0.212
			RSV vs VPA:	0.999
			RSV vs RSV+VPA:	0.592
			VPA vs RSV+VPA:	0.404
AQP4 DG	CON: 297.6±56.51 RSV: 338.4±50.03 VPA: 361.9±96.46 RSV+VPA: 251.4±95.38	Interaction: F (1, 19) = 4.936; p = 0.0387* VPA: F (1, 19) = 0.1120; p = 0.7415 RSV: F (1, 19) = 1.048; p = 0.3187	CON vs RSV:	0.967
			CON vs VPA:	0.718
			CON vs RSV+VPA:	0.929
			RSV vs VPA:	0.997
			RSV vs RSV+VPA:	0.437
			VPA vs RSV+VPA:	0.135
AQP4 CA1	CON: 300.7±44.61 RSV: 346.4±66.26 VPA: 343.9±107.4 RSV+VPA: 267.2±65.51	Interaction: F (1, 19) = 3.456; p = 0.0786# VPA: F (1, 19) = 0.2992; p = 0.5908 RSV: F (1, 19) = 0,2202; p = 0.6442	CON vs RSV:	0.935
			CON vs VPA:	0.929
			CON vs RSV+VPA:	0.982
			RSV vs VPA:	>0.999
			RSV vs RSV+VPA:	0.505
			VPA vs RSV+VPA:	0.447
AQP4 CA2	CON: 307.7±73.17 RSV: 351.1±40.68 VPA: 336.3±79.70 RSV+VPA: 285.2±62.61	Interaction: F (1, 19) = 2.790; p = 0.1113 VPA: F (1, 19) = 0.4353; p = 0.5173 RSV: F (1, 19) = 0.01884; p = 0.8923	CON vs RSV:	0.901
			CON vs VPA:	0.979
			CON vs RSV+VPA:	0.995
			RSV vs VPA:	0.999
			RSV vs RSV+VPA:	0.540
			VPA vs RSV+VPA:	0.717
AQP4 CA3	CON: 277.4±48.82 RSV: 342.3±27.86 VPA: 302.4±68.88 RSV+VPA: 272.6±51.07	Interaction: F (1, 19) = 4.422; p = 0.0490* VPA: F (1, 19) = 0.9866; p = 0.3331 RSV: F (1, 19) = 0.6090; p = 0.4448	CON vs RSV:	0.353
			CON vs VPA:	0.967
			CON vs RSV+VPA:	>0.999
			RSV vs VPA:	0.770
			RSV vs RSV+VPA:	0.238
			VPA vs RSV+VPA:	0.909

DG: dentate gyrus; **SD**: standard deviation. p <0.05 was considered significant. *p<0.05. Statistical analyses: two-way ANOVA parametric test followed by Sidak. N_{CON}: 5, N_{RSV}: 6, N_{VPA}: 6, $N_{RSV+VPA}$: 5. "Mean" represents de mean of fluorescence.

Table 7 – Descriptive statistics of the GFAP fluorescence and number of GFAP⁺-astrocytes in pSSA

	Mean ± SD	F (DFn, DFd); p Value	Pairwise comparisons	
GFAP (II/III)	CON: 153.1±38.39 RSV: 177.3±49.76 VPA: 201.6±35.43 RSV+VPA: 247.4±93.58	Interaction: F (1, 18) = 0.1940; p = 0.6649 VPA: F (1, 18) = 5.797; p = 0.0270* RSV: F (1, 18) = 2.023; p = 0.1721	CON vs RSV:	0.983
			CON vs VPA:	0.6985
			CON vs RSV+VPA:	0.105
			RSV vs VPA:	0.978
			RSV vs RSV+VPA:	0.306
			VPA vs RSV+VPA:	0.746
GFAP (IV/V)	CON: 109.6±45.17 RSV: 146.5±32.95 VPA: 128.4±8.990 RSV+VPA: 189.9±91.40	Interaction: F (1, 18) = 0.3125; p = 0.5831 VPA: F (1, 18) = 2.004; p = 0.1740 RSV: F (1, 18) = 5.017; p = 0.0380*	CON vs RSV:	0.822
			CON vs VPA:	0.991
			CON vs RSV+VPA:	0.133
			RSV vs VPA:	0.991
			RSV vs RSV+VPA:	0.695
			VPA vs RSV+VPA:	0.324
Number (II/III)	CON: 66.40±19.51 RSV: 65.00±28.09 VPA: 89.00±22.12 RSV+VPA: 103.0±8.485	Interaction: F (1, 18) = 0.7098; p = 0.4106 VPA: F (1, 18) = 10.99; p = 0.0038** RSV: F (1, 18) = 0.4752; p = 0.4994	CON vs RSV:	>0.999
			CON vs VPA:	0.459
			CON vs RSV+VPA:	0.082#
			RSV vs VPA:	0.341
			RSV vs RSV+VPA:	0.051#
			VPA vs RSV+VPA:	0.875
Number (IV/V)	CON: 39.40±18.42 RSV: 63.00±18.45 VPA: 74.50±28.47 RSV+VPA: 86.60±11.67	Interaction: F (1, 18) = 0.4240; p = 0.5232 VPA: F (1, 18) = 11.05; p = 0.0038** RSV: F (1, 18) = 4.086; p = 0.0584#	CON vs RSV:	0.373
			CON vs VPA:	0.067#
			CON vs RSV+VPA:	0.0117*
			RSV vs VPA:	0.922
			RSV vs RSV+VPA:	0.373
			VPA vs RSV+VPA:	0.921

II/III: upper cortical layers; **IV/V**: deeper cortical layers; **pSSA**: primary somatosensory area; **SD**: standard deviation. p <0.05 was considered significant. *p<0.05, **p<0.01. Statistical analyses: two-way ANOVA parametric test followed by Sidak. N_{CON}: 5, N_{RSV}: 6, N_{VPA}: 6, $N_{RSV+VPA}$: 5.

Table 8 – Descriptive statistics of the GFAP fluorescence and number of GFAP⁺-astrocytes in mPFC

	Mean ± SD	F (DFn, DFd); p Value	Pairwise comparisons	
GFAP aCC (II/III)	CON: 316.5±48.96 RSV: 366.6±87.41 VPA: 522.5±157.7 RSV+VPA: 397.2±130.6	Interaction: F (1, 14) = 2.882; p = 0.1117 VPA: F (1, 14) = 5.252; p = 0.0379* RSV: F (1, 14) = 0.5305 ; p = 0.4784	CON vs RSV:	0.980
			CON vs VPA:	0.0789#
			CON vs RSV+VPA:	0.869
			RSV vs VPA:	0.269
			RSV vs RSV+VPA:	0.998
			VPA vs RSV+VPA:	0.554
GFAP aCC (IV/V)	CON: 307.0±30.66 RSV: 319.9±44.57 VPA: 422.3±49.83 RSV+VPA: 299.3±81.86	Interaction: F (1, 14) = 7.310; p = 0.0171* VPA: F (1, 14) = 3.549; p = 0.0805 RSV: F (1, 14) = 4.806; p = 0.0458*	CON vs RSV:	0.999
			CON vs VPA:	0.0348*
			CON vs RSV+VPA:	>0.999
			RSV vs VPA:	0.071#
			RSV vs RSV+VPA:	0.993
			VPA vs RSV+VPA:	0.0321*
GFAP PrL (II/III)	CON: 297.9±35.88 RSV: 313.1±61.58 VPA: 406.1±79.69 RSV+VPA: 345.0±106.4	Interaction: F (1, 14) = 1.234; p = 0.2853 VPA: F (1, 14) = 4.171; p = 0.0604# RSV: F (1, 14) = 0.4471; p = 0.5146	CON vs RSV:	0.999
			CON vs VPA:	0.231
			CON vs RSV+VPA:	0.923
			RSV vs VPA:	0.377
			RSV vs RSV+VPA:	0.987
			VPA vs RSV+VPA:	0.825
GFAP PrL (IV/V)	CON: 312.7±31.30 RSV: 288.5±44.65 VPA: 416.3±48.23 RSV+VPA: 283.2±80.47	Interaction: F (1, 14) = 4.820; p = 0.0455* VPA: F (1, 14) = 3.930; p = 0.0674# RSV: F (1, 14) = 10.05; p = 0.0068**	CON vs RSV:	0.979
			CON vs VPA:	0.061#
			CON vs RSV+VPA:	0.960
			RSV vs VPA:	0.0158*
			RSV vs RSV+VPA:	>0.999
			VPA vs RSV+VPA:	0.0173*
GFAP IL (II/III)	CON: 338.2±49.43 RSV: 297.2±55.84 VPA: 405.2±106.1 RSV+VPA: 322.0±87.58	Interaction: F (1, 14) = 0.3527; p = 0.5621 VPA: F (1, 14) = 1.659; p = 0.2186 RSV: F (1, 14) = 3.036; p = 0.1034	CON vs RSV:	0.955
			CON vs VPA:	0.746
			CON vs RSV+VPA:	0.999
			RSV vs VPA:	0.265
			RSV vs RSV+VPA:	0.997
			VPA vs RSV+VPA:	0.593
GFAP IL (IV/V)	CON: 316.5±24.27 RSV: 270.5±60.38 VPA: 398.6±70.81 RSV+VPA: 278.4±50.56	Interaction: F (1, 14) = 2.159; p = 0.1638 VPA: F (1, 14) = 3.182; p = 0.0962# RSV: F (1, 14) = 10.84; p = 0.0053**	CON vs RSV:	0.723
			CON vs VPA:	0.204
			CON vs RSV+VPA:	0.886
			RSV vs VPA:	0.0176*
			RSV vs RSV+VPA:	>0.999
			VPA vs RSV+VPA:	0.0383*
Number aCC (II/III)	CON: 109.2±25.58 RSV: 139.8±61.92 VPA: 170.0±22.97 RSV+VPA: 192.3±54.32	Interaction: F (1, 15) = 0.04255; p = 0.8393 VPA: F (1, 15) = 7.828; p = 0.0135* RSV: F (1, 15) = 1.705; p = 0.2113	CON vs RSV:	0.869
			CON vs VPA:	0.240
			CON vs RSV+VPA:	0.075
			RSV vs VPA:	0.876
			RSV vs RSV+VPA:	0.451
			VPA vs RSV+VPA:	0.975
Number aCC (IV/V)	CON: 85.00±21.71 RSV: 113.6±48.03 VPA: 170.0±25.50 RSV+VPA: 148.8±70.04	Interaction: F (1, 15) = 1.542; p = 0.2334 VPA: F (1, 15) = 8.959; p = 0.0091** RSV: F (1, 15) = 0.033; p = 0.8572	CON vs RSV:	0.897
			CON vs VPA:	0.0443*
			CON vs RSV+VPA:	0.243
			RSV vs VPA:	0.303
			RSV vs RSV+VPA:	0.818
			VPA vs RSV+VPA:	0.979
Number PrL (II/III)	CON: 111.4±45.53 RSV: 114.0±54.61 VPA: 156.2±30.54 RSV+VPA: 191.5±81.46	Interaction: F (1, 15) = 0.4302; p = 0.5218 VPA: F (1, 15) = 6.018; p = 0.0269* RSV: F (1, 15) = 0.5779; p = 0.4589	CON vs RSV:	>0.999
			CON vs VPA:	0.756
			CON vs RSV+VPA:	0.232
			RSV vs VPA:	0.801
			RSV vs RSV+VPA:	0.262
			VPA vs RSV+VPA:	0.921
Number	CON: 93.20±19.65	Interaction: F (1, 15) = 0.291; p = 0.5978	CON vs RSV:	0.998

PrL (IV/V)	RSV: 105.0±39.21 VPA: 188.2±21.46 RSV+VPA: 180.0±70.15	VPA: F (1, 15) = 20.99; p = 0.0004*** RSV: F (1, 15) = 0.010; p = 0.9240	CON vs VPA:	0.0120*
			CON vs RSV+VPA:	0.0342*
			RSV vs VPA:	0.0307*
			RSV vs RSV+VPA:	0.081#
			VPA vs RSV+VPA:	0.999
Number **IL (II/III)**	CON: 103.6±31.94 RSV: 106.6±79.16 VPA: 158.0±49.46 RSV+VPA: 187.0±70.94	Interaction: F (1, 15) = 0.2208; p = 0.6452 VPA: F (1, 15) = 5.935; p = 0.0278* RSV: F (1, 15) = 0.3345; p = 0.5716	CON vs RSV:	>0.999
			CON vs VPA:	0.678
			CON vs RSV+VPA:	0.292
			RSV vs VPA:	0.729
			RSV vs RSV+VPA:	0.328
			VPA vs RSV+VPA:	0.980
Number **IL (IV/V)**	CON: 82.20±33.26 RSV: 89.00±36.88 VPA: 166.4±50.58 RSV+VPA: 173.5±70.11	Interaction: : F (1, 15) = 4.55e-005; p = 0.9947 VPA: F (1, 15) = 14.41; p = 0.0018** RSV: F (1, 15) = 0.097; p = 0.7587	CON vs RSV:	>0.999
			CON vs VPA:	0.084#
			CON vs RSV+VPA:	0.074#
			RSV vs VPA:	0.128
			RSV vs RSV+VPA:	0.118
			VPA vs RSV+VPA:	>0.999

II/III: upper cortical layers; **IV/V**: deeper cortical layers; **aCC**: anterior cingulate cortex; **IL**: infralimbic cortex; **PrL**: prelimbic cortex; **SD**: standard deviation. p <0.05 was considered significant. *p<0.05, **p<0.01, ***p<0.001. Statistical analyses: two-way ANOVA parametric test followed by Sidak. N_{CON}: 5, N_{RSV}: 6, N_{VPA}: 6, $N_{RSV+VPA}$: 5.

Table 9 – Descriptive statistics of the GFAP fluorescence and number of GFAP$^+$-astrocytes in AmR

	Mean ± SD	F (DFn, DFd); p Value	Pairwise comparisons	
GFAP	CON: 88.98±30.23 RSV: 75.56±14.83 VPA: 82.66±14.10 RSV+VPA: 149.6±74.12	Interaction: F (1, 16) = 4.728; p = 0.0450* VPA: F (1, 16) = 3.357; p= 0.0856 RSV: F (1, 16) = 2.095; p = 0.1670	CON vs RSV:	0.996
			CON vs VPA:	>0.999
			CON vs RSV+VPA:	0.187
			RSV vs VPA:	>0.999
			RSV vs RSV+VPA:	0.069#
			VPA vs RSV+VPA:	0.119
Number	CON: 33.80±7.463 RSV: 11.40±5.225 VPA: 18.60±10.53 RSV+VPA: 39.80±21.74	Interaction: F (1, 16) = 14.26; p = 0.0017** VPA: F (1, 16) = 1.307; p = 0.2697 RSV: F (1, 16) = 0.01080; p = 0.9185	CON vs RSV:	0.083#
			CON vs VPA:	0.398
			CON vs RSV+VPA:	0.978
			RSV vs VPA:	0.948
			RSV vs RSV+VPA:	0.0185*
			VPA vs RSV+VPA:	0.111

AmR: amygdala region; **SD**: standard deviation. p <0.05 was considered significant. *p<0.05, **p<0.01. Statistical analyses: two-way ANOVA parametric test followed by Sidak. N_{CON}: 5, N_{RSV}: 6, N_{VPA}: 6, $N_{RSV+VPA}$: 5.

Table 10 – Descriptive statistics of the GFAP fluorescence and number of GFAP⁺-astrocytes in hippocampus.

	Mean ± SD	F (DFn, DFd); p Value	Pairwise comparisons	
GFAP DG	CON: 458.6±93.64 RSV: 642.1±62.81 VPA: 553.7±135.4 RSV+VPA: 435.8±162.8	Interaction: F (1, 19) = 8.292; p = 0.0096** VPA: F (1, 19) = 1.130; p = 0.3012 RSV: F (1, 19) = 0.3929; p = 0.5382	CON vs RSV:	0.170
			CON vs VPA:	0.751
			CON vs RSV+VPA:	0.999
			RSV vs VPA:	0.806
			RSV vs RSV+VPA:	0.0753#
			VPA vs RSV+VPA:	0.484
GFAP CA1	CON: 445.0±121.7 RSV: 623.1±140.4 VPA: 509.1±168.7 RSV+VPA: 392.4±81.06	Interaction: F (1, 19) = 6.811; p = 0.0172* VPA: F (1, 19) = 2.175; p = 0.1567 RSV: F (1, 19) = 0.2951; p = 0.5933	CON vs RSV:	0.262
			CON vs VPA:	0.964
			CON vs RSV+VPA:	0.988
			RSV vs VPA:	0.656
			RSV vs RSV+VPA:	0.0611#
			VPA vs RSV+VPA:	0.578
GFAP CA2	CON: 409.5±170.2 RSV: 635.5±73.25 VPA: 453.4±109.4 RSV+VPA: 438.6±86.79	Interaction: F (1, 19) = 6.286; p = 0.0214* VPA: F (1, 19) = 2.539; p = 0.1276 RSV: F (1, 19) = 4.842; p = 0.0403*	CON vs RSV:	0.0323*
			CON vs VPA:	0.987
			CON vs RSV+VPA:	0.998
			RSV vs VPA:	0.077#
			RSV vs RSV+VPA:	0.059#
			VPA vs RSV+VPA:	>0.999
GFAP CA3	CON: 427.0±97.55 RSV: 682.6±36.56 VPA: 446.3±119.8 RSV+VPA: 455.9±87.03	Interaction: F (1, 19) = 9.676; p = 0.0058** VPA: F (1, 19) = 6.882; p = 0.0167* RSV: F (1, 19) = 11.25; p = 0.0033**	CON vs RSV:	0.0023**
			CON vs VPA:	0.999
			CON vs RSV+VPA:	0.996
			RSV vs VPA:	0.0023**
			RSV vs RSV+VPA:	0.0047**
			VPA vs RSV+VPA:	>0.999
Number DG	CON: 80.80±11.73 RSV: 120.8±28.44 VPA: 104.4±22.65 RSV+VPA: 93.83±24.41	Interaction: F (1, 19) = 6.965; p = 0.0162* VPA: F (1, 19) = 0.03032; p = 0.8636 RSV: F (1, 19) = 2.353; p = 0.1416	CON vs RSV:	0.069#
			CON vs VPA:	0.441
			CON vs RSV+VPA:	0.928
			RSV vs VPA:	0.798
			RSV vs RSV+VPA:	0.333
			VPA vs RSV+VPA:	0.959
Number CA1	CON: 71.00±19.25 RSV: 99.60±29.59 VPA: 81.71±19.49 RSV+VPA: 84.67±21.86	Interaction: F (1, 19) = 1.825; p = 0.1926 VPA: F (1, 19) = 0.04939; p = 0.8265 RSV: F (1, 19) = 2.762; p = 0.1129	CON vs RSV:	0.307
			CON vs VPA:	0.965
			CON vs RSV+VPA:	0.908
			RSV vs VPA:	0.720
			RSV vs RSV+VPA:	0.869
			VPA vs RSV+VPA:	>0.999
Number CA2	CON: 76.40±12.40 RSV: 118.8±16.63 VPA: 84.29±20.77 RSV+VPA: 116.3±19.82	Interaction: F (1, 19) = 0.4575; p = 0.5069 VPA: F (1, 19) = 0.1254; p = 0.7272 RSV: F (1, 19) = 23.66; p = 0.0001***	CON vs RSV:	0.0093**
			CON vs VPA:	0.977
			CON vs RSV+VPA:	0.0107*
			RSV vs VPA:	0.0253*
			RSV vs RSV+VPA:	>0.999
			VPA vs RSV+VPA:	0.0299*
Number CA3	CON: 88.20±17.75 RSV: 132.4±18.50 VPA: 84.29±21.88 RSV+VPA: 91.67±22.23	Interaction: F (1, 19) = 4.553; p = 0.0461* VPA: F (1, 19) = 6.695; p = 0.0181* RSV: F (1, 19) = 8.936; p = 0.0075**	CON vs RSV:	0.0174*
			CON vs VPA:	0.999
			CON vs RSV+VPA:	0.999
			RSV vs VPA:	0.0045**
			RSV vs RSV+VPA:	0.0232*
			VPA vs RSV+VPA:	0.988

DG: dentate gyrus; **SD**: standard deviation. p <0.05 was considered significant. *p<0.05, **p<0.01, ***p<0.001. Statistical analyses: two-way ANOVA parametric test followed by Sidak. N_{CON}: 5, N_{RSV}: 6, N_{VPA}: 6, $N_{RSV+VPA}$: 5.

List of Supplementary Tables

Supplementary Table 1 – Immunofluorescence and western blotting reagents information

Immunofluorescence			
Reagent	**Supplier**	**Code**	**Dilution**
Anti-AQP1 (mouse)	Santa Cruz Biotechnology	sc-32737	1:500 in blocking solution – PBS-Triton 0.1% BSA 1%
Anti-AQP4 (rabbit)	Santa Cruz Biotechnology	sc-20812	1:500 in blocking solution – PBS-Triton 0.3% BSA 1%
Anti-GFAP (mouse)	Cell Signaling mAb	#3670	1:500 in blocking solution – PBS-Triton 0.3% BSA 1%
Anti-IgG (mouse) - Alexa 488	Molecular Probes	A11029	1:2000 in blocking solution – PBS-Triton 0.1% BSA 1%
Anti-IgG (rabbit) - Alexa 546	Molecular Probes	A11036	1:2000 in blocking solution – PBS-Triton 0.1% BSA 1%
DAPI Nucleic Acid Stain (4′,6-diamidino-2-phenylindole)	Invitrogen	MP01306	1:10000 in saline solution 0.9%
Mounting Medium Fluorshield	Sigma-Aldrich	F6182-20ML	

Western blotting			
Reagent	**Supplier**	**Code**	**Dilution**
Anti-AQP1 (mouse)	Santa Cruz Biotechnology	sc-32737	1:50 in blocking solution – 5% BSA-TTBS
Anti-AQP4 (rabbit)	Santa Cruz Biotechnology	sc-20812	1:50 in blocking solution – 5% BSA-TTBS
Anti-β-actin (mouse)	SIGMA Aldrich	A1978	1:3000 in TTBS
IgG-HRP (mouse)	Santa Cruz Biotechnology	sc-2314	1:1000 in 5% milk-TTBS
IgG-HRP (rabbit)	Santa Cruz Biotechnology	sc-2004	1:1000 in TTBS

Common reagents	
PBS	0.1 M pH 7.4 Phosphate-buffered saline
SDS 10%	sodium dodecyl sulfate
EDTA 100 mM	ethylenediamine tetraacetic acid
TRIS/HCl buffer 500 mM pH 8	tris(hydroxymethyl)aminomethane and chloridric acid
TBS	TRIS buffer

Graphical abstract:

Autism Spectrum Disorder (ASD) animal model replicates the high brain volume described in ASD patients. Resveratrol (RSV) prevents changes in blood-brain barrier, aquaporin profile, edema formation, as well as promotes a functional amelioration in astrocytes.

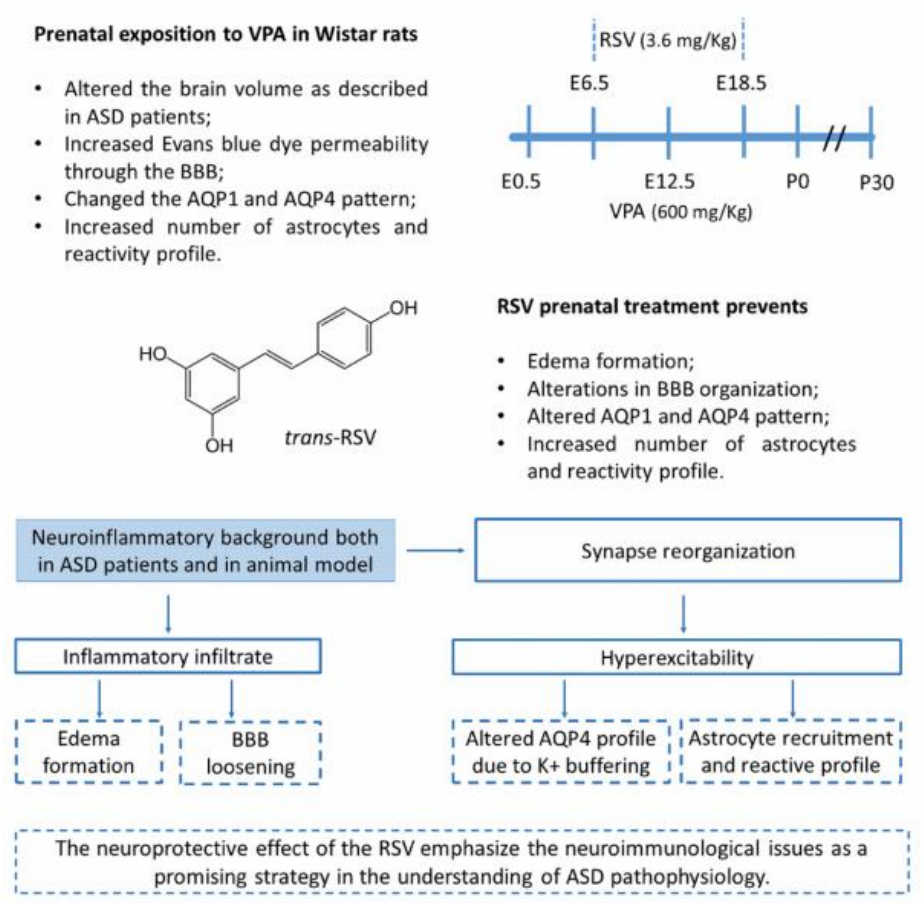


Figure 1

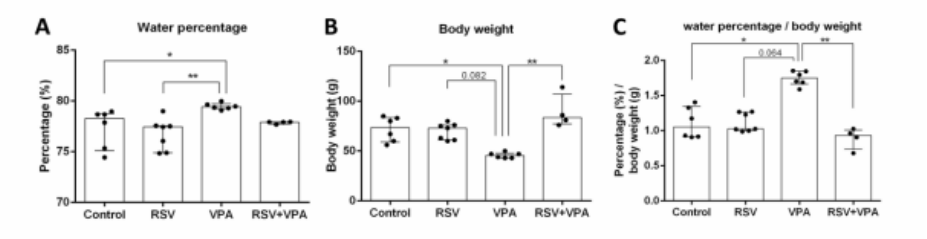
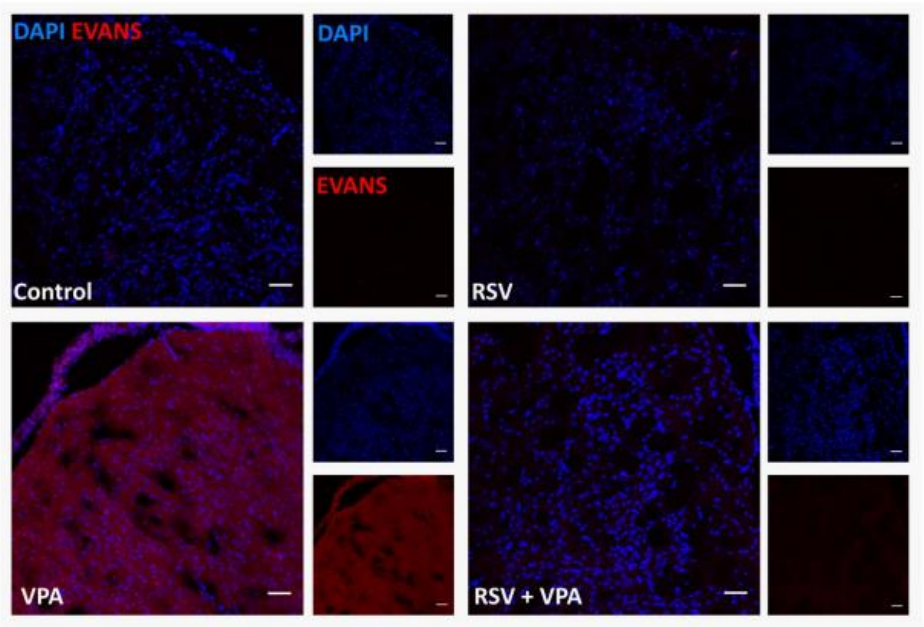

A
Water percentage
Percentage (%)
85
80
75
70
*
**
Control
RSV
VPA
RSV+VPA
B
Body weight
Body weight (g)
150
100
50
0
*
0.082
**
Control
RSV
VPA
RSV+VPA
C
water percentage / body weight
Percentage (%) / body weight (g)
2.0
1.5
1.0
0.5
0.0
*
0.064
**
Control
RSV
VPA
RSV+VPA

Figure 2



47

Figure 3

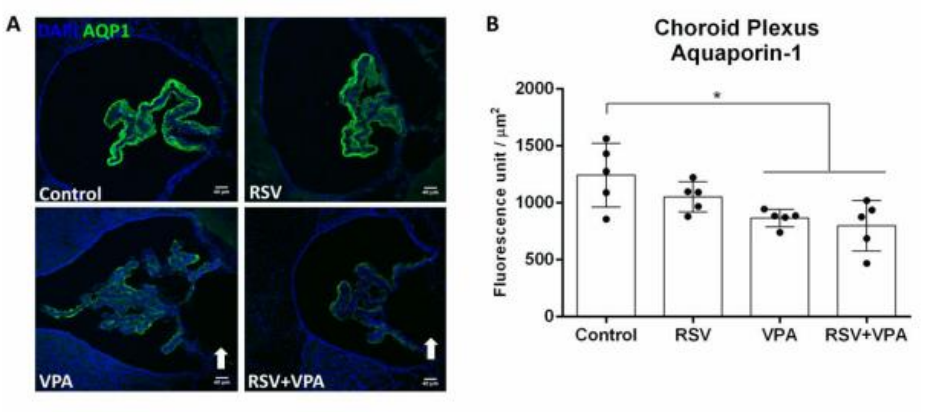

A
DAPI AQP1
Control
RSV
VPA
RSV+VPA
B
Choroid Plexus
Aquaporin-1
Fluorescence unit / μm²
2000
1500
1000
500
0
*
Control
RSV
VPA
RSV+VPA

Figure 4

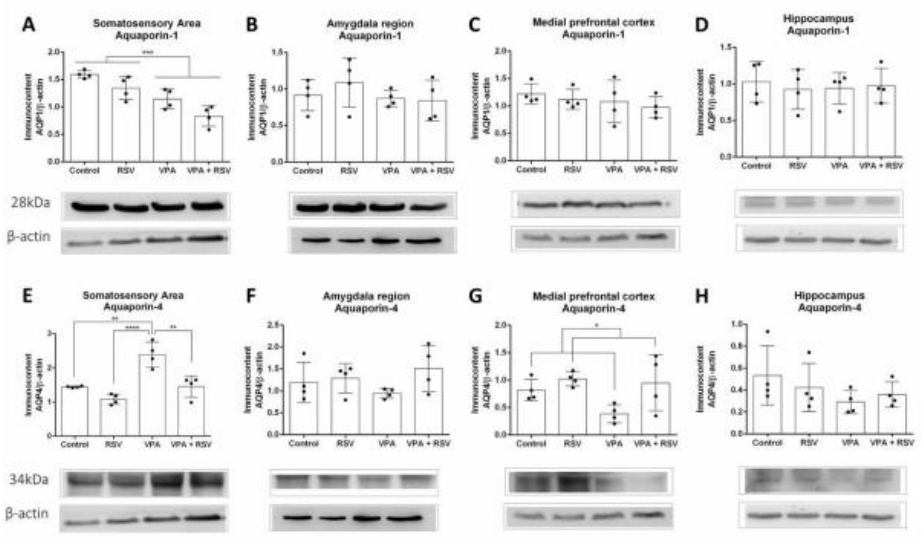
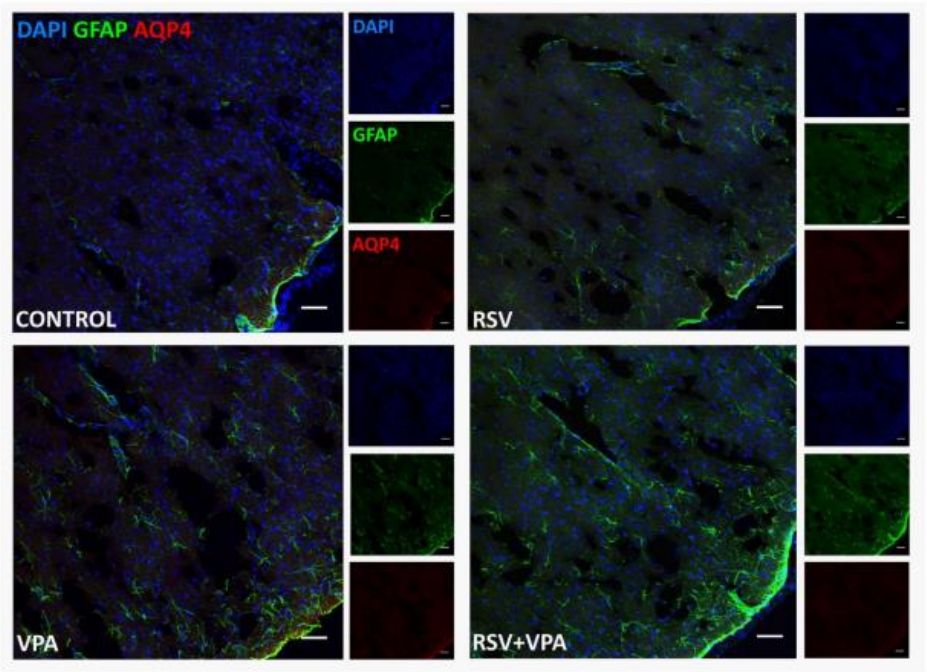
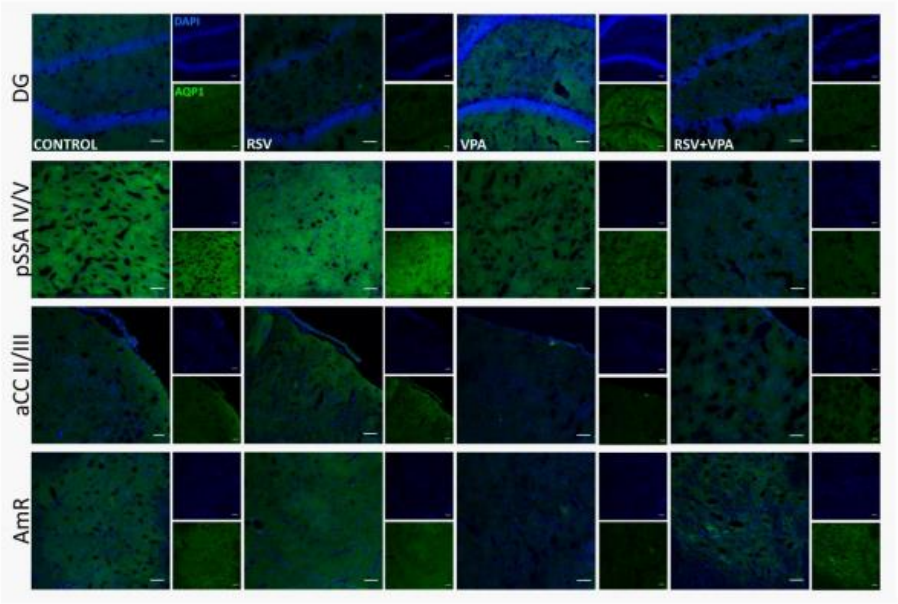

A
Somatosensory Area
Aquaporin-1
B
Amygdala region
Aquaporin-1
C
Medial prefrontal cortex
Aquaporin-1
D
Hippocampus
Aquaporin-1
Control
RSV
VPA
VPA + RSV
28kDa
β-actin
E
Somatosensory Area
Aquaporin-4
F
Amygdala region
Aquaporin-4
G
Medial prefrontal cortex
Aquaporin-4
H
Hippocampus
Aquaporin-4
34kDa
β-actin

Figure 5



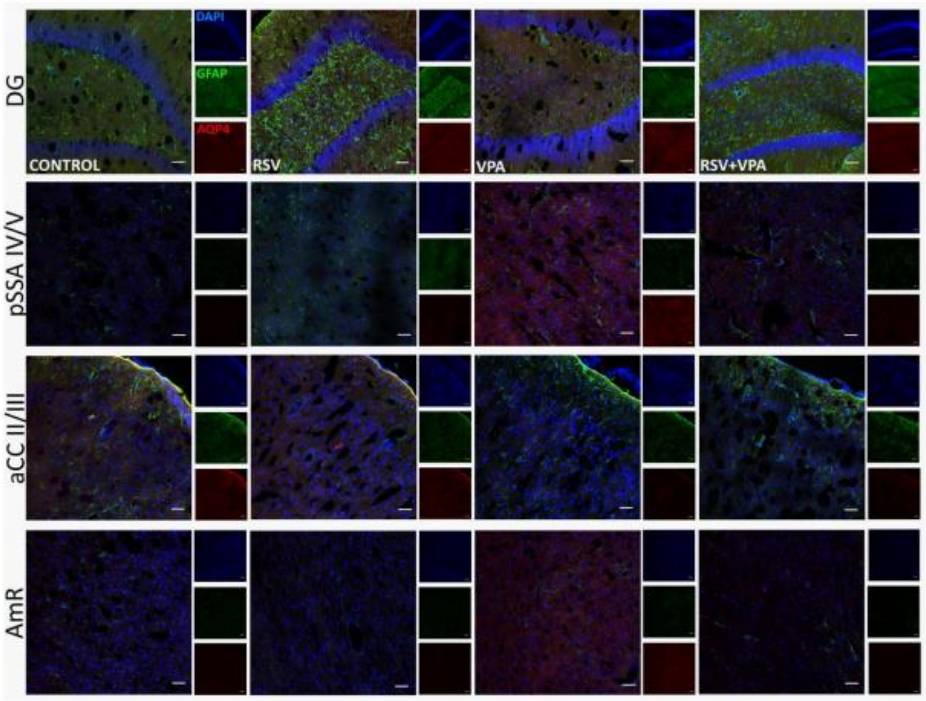
50

Supplementary Figure 1



Supplementary Figure 1 - Representative images of the AQP1 labeling. Illustrative images of DAPI (blue) and AQP1 (green) staining in the pSSA (layers IV/V), aCC (mPFC - layers II/III), DG (hippocampus) and AmR in the different experimental groups. Scale bar: 50 μm.

Supplementary Figure 2



Supplementary Figure 2 - Representative images of the AQP4 labeling. Illustrative images of DAPI (blue), GFAP (green) and AQP4 (red) staining in the pSSA (layers IV/V), aCC (mPFC - layers II/III), DG (hippocampus) and AmR in the different experimental groups. Scale bar: 50 μm.

Capítulo II: revisão publicada

"*Neuroimmune Alterations in Autism: A Translational Analysis Focusing on the Animal Model of Autism Induced by Prenatal Exposure to Valproic Acid*"

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Neuroimmune alterations in autism: a translational analysis focusing on the animal model of autism induced by prenatal exposure to valproic acid

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ABSTRACT

Autism Spectrum Disorder (ASD) is a highly prevalent developmental disorder characterized by deficits in communication and social interaction and in stereotyped or repetitive behaviors. Besides the classical behavioral dyad, several comorbidities are frequently present in patients with ASD, such as anxiety, epilepsy, sleep disturbances and gastrointestinal tract dysfunctions. Although the etiology of ASD remains unclear, there is supporting evidence for the involvement of both genetic and environmental factors. Valproic acid (VPA) is an anticonvulsant and mood stabilizer that, when used during the gestational period, increases the risk of ASD in the offspring. The animal model of autism induced by prenatal exposure to VPA demonstrates important structural and behavioral features that can be observed in individuals with autism, being an excellent tool for testing new drug targets and developing novel behavior and drug therapies. In addition, immunological alterations during pregnancy could affect the developing embryo, since immune molecules can pass through the placental barrier. In fact, exposure to pathogens during the pregnancy is a known risk factor for ASD, and maternal immune activation can lead to autistic-like features in animals. Interestingly, neuroimmune alterations are common in both autistic individuals and in animal models of ASD. Thus, we summarize in the present review important alterations on inflammatory markers, such cytokines and chemokines in patients with ASD and in the VPA animal model.

Keywords

ASD, neuroimmune, cytokine, animal model, valproic acid

INTRODUCTION

Since the first descriptions, in the early 1940's by Leo Kanner and Hans Asperger, new data has been shared to the scientific community about Autism Spectrum Disorder (ASD) [1]. Currently, ASD is diagnosed by changes in two behavioral domains: a) communication and social interaction impairments in multiple contexts, including deficits in social reciprocity, non-verbal communication used for social interaction and in skills to initiate, maintain and understand relationships; and b) Repetitive behaviors, restricted and stereotyped activities [2].

There is no clinical marker or quantitative examination in peripheral tissues that can be used for an early diagnosis of this disorder [3]. Even though there are many well accepted surveys for behavioral diagnosis, ASD is a highly complex and heterogeneous disorder, presenting distinct manifestations, in which two individuals hardly share the same set of symptoms [4,5]. The large heterogeneity of the symptoms could potentially be explained by individual differences, for example in the immune system. Alterations in cytokines levels are common in autistic individuals, with a frequent observation of elevated levels of pro inflammatory cytokines [6,7].

Genome-wide association studies (GWAS) have already described interesting relations between immune system disruptions and neurological disorders like autism and schizophrenia [8]. Specifically in ASD, an interesting example is the dysregulated genes reported, as IL-1β and IL-12, both involved in cytokine-cytokine receptor interaction [9]. One study relating ASD and neuroimmune genetic disruption shows an alteration on glutamate receptor metabotropic 5 (GRM5) single nucleotide polymorphisms (SNPs) [10], which is

not exactly a neuroimmunological alteration, but this gene is highly expressed in many neuronal regions implicated in ASD, besides acting on synaptic plasticity, modulating innate immunity and microglia activation. When occurs a GRM5-positive allosteric modulation, several negative behaviors described in ASD are rescued, including stereotypies [10]. Taken together, the evidences showing genes interaction and ASD diagnosis demonstrate important genetic contribution in neuroimmunological imbalance in ASD.

According to the most recent epidemiological survey conducted in United States, the current incidence of ASD is 1:68 [11]. Although the etiology of ASD remains unknown, it is hypothesized that the onset of this disorder depends on the interplay between genetic and environmental factors. Epidemiological observations suggest that exposure to teratogens - especially in the first trimester of pregnancy - could be closely related to ASD development. An important example is the prenatal exposure to valproic acid (VPA) [12,13].

Valproic acid (VPA) and VPA animal model

The compound VPA is a drug widely used as an anticonvulsant and mood stabilizer in the treatment of epilepsy and bipolar disorder [13,14]. Although VPA is well tolerated and safe in adults, there is evidence of its teratogenicity [14]. Clinical studies over the years have shown that intrauterine exposure to VPA is associated with birth defects, cognitive impairments, and increased risk of autism [13]. In recent years, animal studies have investigated the anatomical, behavioral, molecular, immunological and physiological outcomes related to exposure to VPA [13].

Epidemiological observations demonstrate a strong correlation between prenatal exposure to VPA and ASD [15–18]. Based on these observations, an

animal model for study of autism prenatally induced by VPA was established [19–21]. Behavioral studies show that exposure to VPA in rats and mice leads to several autistic-like behaviors in male offspring, including social behavior deficits, increased repetitive behaviors, and communication deficits similar to those found in ASD subjects [19–23], pointing out the animal model's translationality, as the diagnosis of ASD is given through behavioral evaluation.

Since current diagnostic criteria for ASD are exclusively clinical and resulted from behavioral analyses, the study of ASD in humans prior to the onset of symptoms becomes a very challenging task. Animal models provide the opportunity for analyzing the developmental changes that can trigger ASD-like features [24,25]. They provide the possibility to study and manipulate biological pathways for understanding and even preventing or reversing the appearance of the morphological, functional and behavioral alterations found in ASD. In addition, studies with animals can reveal some new important factors involved in the etiology of this disorder.

Histone-deacetylases inhibitors (HDACi) and neuroimmune alterations

Autism and many other psychiatric disorders, like schizophrenia, bipolar disorder and major depression present not only susceptibility to environmental risk factors, but also a high genetic influence [26,27]. In the last years, there is growing evidence indicating that epigenetic alterations may have an important role in several psychiatric disorders.

Epigenetic regulation includes long-term changes, as DNA methylation, and short-term changes, as modifications in histone proteins associated with DNA [28]. Histones are small basic proteins that act as spools around which DNA winds, regulating the packaging of DNA and allowing or inhibiting gene

expression. When the histone is acetylated by histone acetyltransferases (HATs), this local alteration leads to chromatin decondensation, promoting gene expression by the activation of the transcription machinery. On the other hand, histone deacetylation - mediated by histone deacetylases (HDACs), results in inhibition of transcription promoting a controlled gene expression [28,29].

Substantial epigenetic alterations were found in the regulatory regions of many candidate genes for ASD, such as GABAergic genes, GAD67, Reelin, Oxytocin receptor, BDNF, showing that the epigenetic component in ASD has been widely studied [26]. The histone post-translational modifications, as acetylation and methylation, play a key role in the gene expression regulation [30]. These characteristics are crucial for important biological processes like the action of immune system, in which HDACs modulate gene expression of toll-like receptors and interferon signaling pathways [31].

The HDAC inhibitors drugs play an important role in immune context. Studies showed an increased transcription of the major histocompatibility complex (MHC) class II, located in the tumor cell surface in mouse and humans [32], indicating an interesting effect on several immune cells. It leads to less viability of T CD4 cells and decreases the production of pro-inflammatory cytokines, making the T CD8 cells increase the secretion of pro-inflammatory cytokines, modulating the activity of natural killer (NK), as well in cells and Treg cells [33].

Hence, several drugs used as antidepressants and mood stabilizer are characterized as HDAC inhibitors class. Valproate, a well-known HDAC inhibitor drug, induces important delays in the neuronal maturation [34], already described in ASD [35]. Moreover, VPA prenatal exposure alters the postnatal histone 3 (H3)

acetylation levels in cerebellum [36], stimulates glial cell proliferation in the developing rat brain [37] and also induces changes in acetylation levels in astrocytes of hippocampus and cortex in cell culture, more than other antidepressants and mood stabilizer [38]. These unique effects of VPA, especially in comparison to similar HDAC inhibitor drugs, indicate that the VPA molecule might have exclusive properties which are still unclear, although some evidence indicates a possible VPA binding in the catalytic center of HDACs [39]. Those epigenetics alterations occur before the well described neuroimmune alterations, and, thus, epigenetics mechanisms may be involved in the immune disturbance [36]. These data highlight the role of the valproic acid and HDAC inhibitors as epigenetic modulators that could be underpinning the immunological alteration, as well as the neurological outcomes, in psychiatric disorders.

The intimate relationship between central nervous system and immune system

For a long time, immune and central nervous systems were considered compartments that operate separately and independently. However, recent studies demonstrate an active communication between these two systems, modulating bi-directly each other with neurotransmitters and neuromodulators in periphery. In addition, in a landmark study, lymphatic vessels were discovered in central nervous system, putting in check the current view of the brain as an "immune privileged site" and raising new possibilities for the crosstalk between brain and immune system [40]. Anatomically the central nervous system (CNS) is bathed by the cerebrospinal fluid (CSF) and surrounded by the meninges, which contain lymphatic and blood vessels [41]. The brain parenchyma is separated from the circulating blood by a blood-brain barrier (BBB), which

prevents the entry of pathogens, circulating immune cells, and other substances from the blood.

The BBB is defined as a semipermeable membrane that separates the circulating blood from the brain and extracellular fluid in the central nervous system [42]. CNS blood vessels interact with different peripheral and brain-resident immune cell populations, as perivascular macrophages and microglial cells, respectively. The BBB is formed by the concerted action of endothelial and glial cells. During development, at embryonic day 10 (E10), initial clues for angiogenesis lead to the early properties of BBB in CNS by activation of the Wnt/b-Catenin canonical pathway [43–45]. There is no consensus about the exact time when the BBB is fully formed [46]. Nevertheless, at E15, pericytes, which have crucial roles in BBB formation and maintenance, begin to interact intimately with endothelial cells (EC) in the capillary walls [47]. In postnatal life, endothelial cells from brain capillaries are covered up by mature pericytes, sharing their basement membrane with endothelial cells [48]. Moreover, the astrocytes project cellular terminations called “end feet” toward the capillaries, providing the outer layer of the BBB. Pericytes and astrocytes also secrete proteins involved in extracellular matrix formation and deposition of the basement membrane [48,49].

The presence of this limiting barrier allows the CNS to control and fine tune the flow of a variety of molecules from periphery, regulating its permeability to seek homeostasis. In CNS physiology, there are extensive vessels where monocytes, granulocytes and dendritic cells circulate [50]. In addition, the brain parenchyma is populated with microglia, resident-cells from the immune lineage that play crucial roles in brain surveillance and response against multiple types

of damage. Studies with rodents showed that, during neurodevelopment, specific monocytes emerge at E7 and infiltrate the CNS at E9.5 as pre-macrophages, expressing the chemotactic factor CX3C chemokine-receptor 1 (CRXCR1) [50]. The presence of cytokines as interleukin-1 beta (IL-1β) and tumor growth factor beta (TGF-β) allows the differentiation of pre-macrophages in early microglia at E14.5, which then generate mature microglia at P14. In fact, TGF-β seems to be crucial for microglial specification in CNS [51,52].

Microglial cells are capable to interact with almost all cell types in the CNS modulating cell maturation during development and promoting tissue repair and homeostasis. Moreover, in postnatal life, microglia play crucial roles in sensing perturbations in encephalic environment, actively responding to even minor pathological changes in CNS [53,54] by altering their shape and gene expression profile. The term “microglial activation” has been considered as a shift from a “resting” stage to an “activated” state when disturbance of tissue homeostasis is detected or upon experimental stimulation. However, this term implies the understanding of an “inactivated” phenotype when brain tissue is not facing any changes in homeostasis. In fact, microglial cells are never inactive, showing highly dynamic surveillance functions in CNS [50,55,56]. Many authors are suggesting to rename this surveillance state of microglial cells to “surveying microglia”, instead of “resting microglia” [50]. These cells can shift from their “surveying” or “resting” state to “activated” or “alerted” state when facing chances in CNS homeostasis, as infections recognized by toll-like receptors [57], cell damage or trauma. Recent studies have demonstrated that the lipopolysaccharide (LPS) exposure downregulates the transcriptional factor Sal-like protein 1 (SALL1) and promotes several alterations in microglial identity, with

a concomitant upregulation of genes associated to other resident macrophages, indicating that SALL1 might be important for maintenance of microglial identity in response to immune challenge [50,58]. Once activated, microglial cells can commit to different phenotypes called “reactive”, having a large functional and molecular diversity. These changes in microglia profile are correlated with the type of challenge faced by the CNS. They can shift to a pro-inflammatory state also called “M1 phenotype” [59] presenting highly phagocytic and neurotoxic activities and releasing pro-inflammatory chemokines and cytokines in response to an immune challenge, such as a microorganism invasion [60] or the presence of pro inflammatory signals [61–63]. Once the immune stimulator is controlled, microglial cells are able to shift to a more neuroprotective profile called “M2 phenotype” which involves anti-inflammatory responses [59,64]. Nonetheless, the activated pro-inflammatory profile can progress in pathological conditions. Although the immune challenge and the brain environment are responsible for the early microglial responses, signals from CNS resident and infiltrating immune cells can shape reactive profiles of microglial cells and play important roles in many brain diseases [65–69]. All these stimuli could direct microglia’s fate to alternative states, including microglial cell death, but there’s still scarce information about the course of microglial activation, their reversibility to the surveying state [70] or the preservation of molecular memory of previous stimuli. Moreover, cells that infiltrate from the blood and differentiated into microglia could also return to the periphery [65,71].

There is a low basal entry of immune cells from blood periphery into the CNS in normal conditions. Studies have shown that, although microglial cells play major roles in brain surveillance, the perivascular macrophages represent a

crucial immune regulator and sensor of perturbations in CNS and periphery. These cells are derived from bone marrow and are intimately associated with the bloodstream since they reside between endothelial cells and astrocyte's end feet [72–74]. This privileged location of perivascular macrophages allow them to simultaneously monitor the blood and the brain interstitial fluid, providing a fine control of brain homeostasis and BBB integrity [72,75]. Although macrophages display different locations, they can perform specific roles in these microenvironments. In addition to perivascular space, macrophages can be located within choroid plexus and meningeal space. In choroid plexus, which is considered the major site of CNS immune surveillance, there are tissue-resident macrophages called epiplexus cells disposed alongside the fourth ventricle with dendritic cells (DC), monocytes and mast cells [76,77]. Referred by many authors as the "immune regulatory gate", the choroid plexus is capable to induce specific immune responses and allows cell migration between blood and CSF [78,79]. The meningeal macrophages are positioned in the subdural meninges and act as sentinel cells for damage and infection in brain tissue, surveying the cerebrospinal fluid (CSF) and the extracellular lumen of meningeal blood vessels [80,81]. Thus, macrophages play critical roles in CNS surveillance, homeostasis and disease. Nonetheless, there is a variety of other immune cell types in the brain environment. In physiological condition, studies have observed the presence of monocytes in meningeal spaces, although more evidence is still needed [82]. Granulocytes (neutrophils, mast cells, eosinophils, and basophils) can be found in meningeal spaces with mast cells also present in brain parenchyma [72,83]. These cells are highly phagocytic and play important roles in response to brain infections and tissue damage [72,84,85]. Dendritic cells (DC),

the main antigen-presenting cells in periphery, can also be found in CNS. They are located in the choroid plexus, meningeal space, and are specially abundant in lymphatic vessels in meninges [86–88]. The presence around these vessels suggests important roles for DC in inflammatory diseases and brain infections [40].

During inflammatory condition, there is extensive infiltration of immune cells in the CNS. The barriers that regulate cellular entry are the blood-brain barrier (BBB) within the CNS parenchyma, and the blood-cerebrospinal fluid (blood-CSF) barrier within the choroid plexus" [89]. When brain homeostasis is compromised, immune cells can infiltrate from the periphery to the brain parenchyma due to the elevation in BBB permeability. This is generally observed and investigated in the context of a pathological CNS inflammatory response [90–92]. Under pathological conditions, microglia activation can lead to BBB disruption, allowing a substantial cellular infiltrate and amplifying the inflammatory response [93,94]. One of the key mediators in these processes is the release of cytokines and chemokines by periphery and brain-resident immune cells. This novel view of the immune system as an active player in brain function is modifying our current view of neuropsychiatric disorders. Immune alterations are now seen as central for the pathophysiology of many brain diseases and further understanding of this neuroimmune axis can result in new therapies and diagnostic tools.

Neuroimmune alterations in ASD: from patients to the VPA animal model

In the last decade, the immune system has caught the attention of neuroscientists for the interplay between neurons and immune mediators, not only in disease, but also in the homeostasis of the brain. In the past, the central

nervous system was called “an immune-privileged region”, once the blood brain barrier controls the cross talking between brain and the periphery. However, recent findings demonstrated that this privilege is not related to the absence of immune modulation in brain activity and homeostasis, but a time-dependent specific modulation in many regions during brain development [95]. Immune cells and immune molecules, such as cytokines and chemokines, can modulate cognitive, emotional and behavioral processes, triggering different responses in neuronal and glial cells [96]. Cytokines are small signaling-molecules acting as mediators of communication between immune cells. Their roles include stimulation and regulation of cell development, maturation and response against immune challenges [97,98]. Chemokines can be characterized as a vast group of 8-10 kDa molecules from the super family of cytokines that induce chemotaxis of immune cells. Once bound in their receptor, the complex chemokine-receptor can activate signaling cascades that induce immune cell trafficking to the target area. Also, this complex plays important roles as molecular signal in crosstalk among neuronal and glial cells and immune resident cells in nervous system, as microglia [99,100]. Since chemokines are capable to target different types of receptor, they can modulate different cell processes, including cell adhesion, proliferation, phagocytosis, apoptosis, angiogenesis, cytokine secretion and T cell activation [101].

Lymphocytes are cells capable of recognizing any foreign antigens displayed by antigen-presenting cells, constituting the main cells of adaptive immunity [102]. Lymphocytes respond by proliferating and differentiating in effector cells, whose function is the elimination of the pathogen and creation of an immunological memory [103]. When naïve CD4+ T cells encounter specific

antigens, they can differentiate into a range of effector subgroups. Several transcription factors are individually required for T-cell differentiation, generating a specific lineage that express characteristic cytokines. That is, once specific transcription factors are activated, they promote differentiation of naïve T cells, which differentiate into specific immunological responses: Th1, Th2 and Th17. In the presence of IFN-γ and IL-12, Signal transducer and activator of transcription (STAT) 1 and STAT4 signal for the expression of the transcription factor T box expressed in T cells (T-bet) and promotes response Th1. On the other hand, Th2 cell commitment occurs when IL-4 and STAT6 increase expression of GATA-binding protein (GATA3) transcription factor. The presence of TGF-β associated with IL-6 signaling via STAT3 generating the expression of retinoid-related orphan receptor (RORγt) transcription factor, results in the differentiation of Th17 cells. Also, TGF-β, with IL-2 signaling via STAT5 is known to generate, at least in vitro, inducible Treg cells, which express Foxp3 transcription factor (See Figure 1) [104].

The modulation of cytokine levels can alter significantly the brain physiology and behavior. Recent studies highlight a link between immune dysfunction and behavioral impairments [105]. For example, the relation between IL-6 and several altered behaviors has already been established in the literature [106–108]. Signs of neuroinflammation and altered inflammatory response are seen in ASD subjects throughout life [109]. Therefore, some authors hypothesize that the neuroimmune disturbances could be causal for ASD [110]. Below, we will detail the main neuroimmunological findings (summarized in Tables 1 and 2) in ASD subjects and in VPA animal model of autism:

IL-1β

IL-1β is a cytokine produced by fibroblasts, monocytes, tissue macrophages, dendritic cells (DCs), B lymphocytes, epithelial cells, and natural killer (NK) cells [111] that promotes inflammation by indirectly stimulating lymphocyte function and activating macrophages [112,113]. IL-1β has the ability to increase the expression of adhesion molecules such as VCAM-1 and ICAM-1, supporting the infiltration of inflammatory cells from the circulation into the tissue and resulting in chronic IL-1-induced inflammation [112,113]. IL-1β stimulates expression of inflammatory mediators and induces T-helper type 17 (Th17) response. Furthermore it can also play important roles as a mediator of the anti-inflammatory response [112,113].

Both elevation and reduction in IL-1β levels have already been reported in ASD subjects. Increased levels of IL-1β were found in plasma [114,115], serum [116,117], and peripheral blood mononuclear cells (PBMCs) [118–120] whereas decreased levels were described in neonatal dried blood samples (n-DBSS) [121]. In VPA animal model, IL-1β was increased in hippocampus [122,123], in LPS-exposed hippocampus [109] and in whole brain homogenate [124]. Increased levels of this cytokine are associated with increased stereotypy [120], one of the main characteristics of ASD.

IL-2

Interleukin-2 has an important role in controlling the survival of immature and mature T cells [125] and is mainly secreted by CD8+ and CD4+ T cells after recognition of the antigen and co-stimulators [111]. IL-2 is the most important cytokine for promoting the clonal expansion of antigen-activated T cells [126]. The only report in ASD is a reduction of IL-2 levels in neonatal dried blood samples (n-DBSS) [121].

IL-4

IL-4 is the main cytokine of Th2 response and is primarily produced by T cells and mast cells. IL-4 promotes proliferation of B cells and cytotoxic T cells and stimulates IgG and IgE production [97], besides stimulating leukocytes recruitment and promoting the expression of adhesion molecules [127]. Increased levels of this cytokine were associated with greater impairments in non-verbal communication [120]. In ASD subjects, reduced level of IL-4 in n-DBSS [121] and elevated levels in amniotic fluid [128] have been reported.

IL-5

IL-5 is a cytokine produced by T cells that acts as an activator of eosinophils [129]. IL-5 promotes eosinophil proliferation and maturation, stimulating IgA and IgM production [97]. In ASD patients, a decrease in IL-5 in n-DBSS [121] and an increase in plasma samples [115] were described.

IL-6

The main source of IL-6 are T-helper cells, macrophages and fibroblasts. IL-6 targets activated B-cells and plasma cells, promoting differentiation into plasma cells and IgG production [97]. IL-6 is also involved in induction of Th17 response and has a dual profile pro- and anti-inflammatory [112,113]. Studies have demonstrated essential involvement of IL-6 in triggering core symptoms related to pro-inflammatory response in autistic model of maternal immune activation (MIA) [130].

Increased levels of IL-6 are associated with increased stereotypy in ASD [120], impaired cognitive abilities, abnormal anxiety and decreased social interactions [107]. Here, we review the main findings about IL-6 levels in ASD: IL-6 is elevated in brain tissue (cerebellum, frontal cortex and anterior cingulated

gyrus) [7,131,132], and in serum and PBMC [116–120], while it is reduced in plasma and n-DBSS [114,121]. In the VPA animal model of autism, higher levels of IL-6 were reported in hippocampus [123], hippocampus and spleen after LPS challenge [109] and whole brain homogenate [124].

IL-8

Interleukin-8 is a chemoattractant cytokine produced mainly by macrophages that specifically targets neutrophils, promoting their activation [133]. So, its major functions result from its chemotactic and pro-inflammatory activities [97]. Elevated levels of this cytokine were associated with increased hyperactivity, stereotypy, and lethargy [120]. Higher levels of IL-8 was described in frontal cortex [132], plasma [115], cerebrospinal fluid (CSF) [134], PBMCs [120] and n-DBSS [121] of ASD subjects.

IL-10

This cytokine can be produced by several cellular types including DCs, macrophages, mast cells, NK cells, eosinophils, neutrophils and B cells [135], and is able to regulate growth and/or differentiation of B cells, NK cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells, exerting a primarily anti-inflammatory activity [97,135]. IL-10 is important to fine tune the immune response against invading pathogens, maintaining the homeostatic state [135]. In ASD patients, increased levels to IL-10 were found in anterior cingulated gyrus and amniotic fluid [128,134], while IL-10 levels is decreased in PBMCs [96].

IL-12

IL-12 is produced by T cell and acts in naïve T-cells and NK cells, activating them [97], and inducing IFNγ production, which is critical for the

induction of Th1 cells [136]. Plasma, PBMCs and serum of ASD subjects show higher levels of IL-12 [115,117,120] whereas n-DBSS show lower IL-12 levels [121]. Increased IL-12 levels were associated with increased stereotypy and lethargy in ASD patients [120].

IL-13

Similarly to IL-4, IL-13 is involved in type-2 immunity and is produced by T-cells. However, basophils, eosinophils and NK cells can also produce IL-13 [137]. The only report in autistic patients shows increased plasma levels of IL-13 [115].

IL-17

Interleukin-17 has an important role in immunity against intra and extracellular pathogens [138]. IL-17-producing cells including natural killer T cells and innate lymphoid cells play crucial roles in inflammation-associated diseases, such as infection, autoimmunity and tumors [139]. Also was described the effector role of IL-17a in onset of offspring behavioral abnormalities of mothers MIA-induced, showing the important crosstalk between the neuroinflammatory state and behavioral manifestations [140]. Increase levels of IL-17 have been reported in plasma and serum [115,141] of patients with ASD.

IL-23

Considered a pro-inflammatory cytokine essential for the differentiation of Th17 lymphocytes [142], IL-23 is produced by macrophages, dendritic cells, keratinocytes and other antigen-presenting cells after recognition of microorganisms [143]. IL-23 is critically involved in autoimmune diseases responses [144]. In autistic patients, elevated IL-23 levels in serum samples were reported [117].

TNF-α

The tumor necrosis factor alpha (TNFα) is an endotoxin-induced serum factor promoting phagocyte cell activation [97], whose main targets and producers are macrophages. TNFα is in higher levels both in patients (frontal cortex [132], PBMC [96,118,119,145], serum [117] and amniotic fluid [128]) and in the VPA animal model of autism (hippocampus and spleen responding to LPS [109] and whole brain tissue [124]).

IFN-γ

Interferon-γ (IFN-γ) plays an important role in host defense against intracellular pathogens. It is produced by NK T cells, CD8+ T cells, and T-helper 1 (Th1) CD4+ T cells and its functions include supporting Th1 differentiation [146], and macrophage activation and increasing neutrophil and monocyte function [97]. Patients with ASD have increased levels of IFN-γ in frontal cortex [132], plasma [147], CSF [134] and PBMC [96] and reduced levels in n-DBSS [121].

TGFβ1

TGF-β is primarily secreted by T cells and B cells, and acts in activated T and B cells. The major function of this cytokine is to inhibit hematopoiesis and T and B cell proliferation [97]. Higher levels to TGFβ1 were reported in anterior cingulated gyrus and CSF [134] of ASD subjects.

MCP-1

Monocyte Chemoattractant Protein-1 (MCP-1) or C-C chemokine ligand 2 (CCL2) signals to cells that contain the specific CCR2 receptor, stimulating their migration to sites where CCL2 is produced and facilitating the amplification of neuroinflammation [148]. Higher levels of MCP-1 were observed in plasma [149], CSF [134] and amniotic fluid [128] of autistic subjects. Increased levels in plasma

were associated with greater impairments in visual reception, fine motor skills and expressive language [149].

GM-CSF

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced by T cells, macrophages and fibroblasts and targets stem cells. Its major function is to stimulate production of granulocyte, monocyte and eosinophils [97]. Diminished levels of GM-CSF were described in n-DBSS of ASD pediatric subjects [121].

G-CSF

The main source of granulocyte colony-stimulating factor (G-CSF) are fibroblasts and endothelial cells and its targets are stem cells in the bone marrow. G-CSF has a hematopoietic function and stimulates granulocyte production [97]. Higher levels of this cytokine were described in plasma of autistic patients [114].

EGF

Epidermal growth factor (EGF) is a small chemoattractant peptide produced by activated T cells that is involved with wound healing by attracting fibroblasts and epithelial cells [114]. Higher levels of this chemokine were reported in plasma samples from autistic patients [114].

RANTES

Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) chemokine or CCL5 is involved in immune cell transport to the inflammation site, promoting polarization towards an Th1 response [150]. Higher levels were associated with increased severity of lethargy, stereotypy and hyperactivity [149] in ASD patients.

Eotaxin

The CC chemokine eotaxin/CCL11 is known to bind to the receptor CCR3 on eosinophils and Th2-type lymphocytes [151]. Increased levels of Eotaxin were associated with increased severity of lethargy, stereotypy and hyperactivity in ASD subjects [149].

Final considerations

Autistic Spectrum Disorder has a high prevalence and a growing incidence over the last few years. This has driven investments in public health and mobilized researchers and health professionals worldwide. There has been a significant progress in ASD research since the disorder was first described, but to date, its etiology remains unclear. An interesting hypothesis is that dysregulation of neuroimmune communication is involved in the onset of ASD. In this review, we summarized the main neuroimmune alterations found both in ASD subjects and in the VPA animal model of autism. Noticeably, several changes in the VPA model reflect the alterations found in patients with ASD (Figure 2). Animal models that present face and construct validity, such as the VPA model, can be an effective tool for the investigation of pathways and tissue alterations involved with the pathogenesis of ASD.

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Legend of figures

Figure 1. Th1, Th2, Th17 commitment lineage from naïve CD4+ T cells. The main functions of each immune response and the signature cytokine are highlighted in the boxes. APC: antigen-presenting cell; NK: natural killer cell; T-bet: T box expressed in T cells; GATA: GATA-binding protein; ROR: Retinoid-related orphan receptor; IL: Interleukin; IFN: Interferon; TGF: Transforming growth factor.

Figure 2. Main results of cytokines altered both in ASD subjects and in VPA animal model. At the interface of the columns and rows are shown the common findings both to humans and to animal model in different biological sources. The references are already cited in Table 1.

Table 1. Main cytokines with altered levels in autism subjects

DSM	Severity	Described comorbidities	Age (years)	Source	Outcome	Analysis method	Reference
ASD	ND	ND	neonatal	amniotic fluid	↑ MCP-1, IL-4, IL-10, TNF-α and TNF-β	Flow cytometry	[128]
ASD	ND	ND	neonatal	n-DBSS	↓ IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, GM-CSF, IFN-γ ↑ sIL-6Rα, IL-8	Flow cytometry	[121]
ASD (DSM-IV)	Mild, moderate and Severe	ND	2-21	Serum	↑ IL-1, IL-6, IL-12, IL-23, TNF-α	ELISA	[117]
ASD (DSM-5)	ND	ND	3-11	PBMCs	↓ CD4+, FOXP3+, T cells ↓ mRNA and protein expression FoxP3 ↑ Tbet, ↑ STAT3, ↑ GATA3	Flow cytometry, PCR and Western Blotting	[152]
ASD (DSM-5)	ND	ND	3-11	PBMCs	↑ ROR-γt in CD4	PCR and Western Blotting	[152]
ASD (DSM-IIIR/DSM-IV)	ND	GI issues	2-16	Duodenal Lamina Propria	↑ CD3+/TNFα+ ↓ CD3+/IL-10+	Flow cytometry	[153]
ASD (DSM-IIIR/DSM-IV)	ND	GI issues	2-16	Epithelium	↑ CD3+/TNFα+ ↓ CD3+/IL-10+	Flow cytometry	[153]
ASD (DSM-IV)	ND	ND	1-17	PBMCs	↑ TNF-α	ELISA	[145]
ASD (DSM-IV)	ND	GI issues	4-15	PBMCs	↑ TNF-α, IFN-γ ↓ IL-10	Flow cytometry	[96]
ASD (DSM-IV)	Severe (nonverbal adult pacients)	ND	18-44	Serum	↑ IL-1β, IL-6	ELISA	[116]

ASD (DSM-IV)	ND	ND	2.9-4.3	PBMCs	↑ IL-1β, IL-6, IL-8, IL-12 p40	Multiplexing bead immunoassays	[120]
ASD (DSM-IV)	ND	ND	2-14	PBMCs	↑ TNF-α, TNFRI, TNFRII, IL-6, IL-1β	ELISA	[119]
ASD (DSM-IV)	ND	ND	2.2-5	PBMCs	↑ 1L-1β, IL-6, TNF-α	Flow citometry	[154]
ASD (DSM-IV)	ND	ND	5-44	*post mortem* brain tissue	↑ IL-6, IL10, TGFβ1 (anterior cingulated gyrus)	Human cytokine array kits	[7]
ASD (DSM-IV)	ND	ND	5-44	CSF	↑ IFNγ, TGFβ2, IL-8, MCP1	Human cytokine array kits	[7]
ASD (DSM-IV)	ND	ND	4-37	*post mortem* brain tissue	↑ IFNγ, IL-6, IL-8, TNF-α (frontal cortex)	Multiplex Bead Immunoassays	[132]
ASD (DSM-IV)	ND	ND	4-14	*post mortem* brain tissue	↑ IL-6 (cerebellum)	Immunohistochemistry	[131]
ASD (DSM-IV)	ND	ND	7-15	Plasma	↑ IL-1β, IL-1RA, IL-5, IL-8, IL-12 (p70), IL-13, IL-17	ELISA	[115]
ASD (DSM-IV)	ND	ND	3-4.5	Plasma	↑ MCP-1, RANTES, Eotaxin	Multiplexing bead immunoassays	[149]
ASD (DSM-IV)	ND	ND	4.7-10.1	Plasma	↑ IFN-γ	ELISA	[147]
ASD (DSM-IV)	Mild to moderate and Severe	ND	6-11	Serum	↑ IL-17A (proportional increase to severity of autism)	ELISA	[141]
ASD (DSM-IV)	ND	ND	5-10	Plasma	↑ IL-1a ↓ IL-6, G-CSF, EGF	ELISA	[114]

DSM: Diagnostic and Statistical Manual of Mental Disorders; **CSF**: cerebrospinal fluid; **ELISA**: enzyme-linked immunosorbent assay; **IFN**: interferon; **IL**: interleukin; **ND**: not described; **n-DBSS**: neonatal dried blood samples; **PBMC**: peripheral blood mononuclear cells; **PCR**: polymerase chain reaction; **TNF**: tumor necrosis factor.

Table 2. Main cytokines with altered levels in the valproic acid animal model of autism

Animal	Dosage	Embryonic day	Administration via	Source	Age	Outcome	Analysis method	References
BALB/c	600 mg/Kg	E11	Subcutaneous	Dorsal hippocampus	P28	↑ IL-1β	PCR	[122]
BALB/c	400 mg/Kg and 600 mg/Kg	E12.5	Subcutaneous	Spleen	8-10 weeks	Only VPA did not onset inflammatory response, but showed exacerbated response to a LPS challenge: ↑ IL-1β, IL-6 and TNF-α expression	PCR	[109]
BALB/c	400 mg/Kg and 600 mg/Kg	E12.5	Subcutaneous	Hippocampus/ Cerebellum	8-10 weeks	↑ IL-6 and TNF-α expression in VPA animals exposed to a LPS challenge	PCR	[109]
Wistar	600 mg/Kg	E12.5	Intraperitoneal	Hippocampus	P40	↑ IL-6, ↑ IL-1β	ELISA	[123]
Wistar	800 mg/Kg	E12.5	Gavage	Whole brain	P21	↑ IL-1β, IL-6, TNF-α	ELISA	[124]

IL: interleukin; **PCR**: polymerase chain reaction.

Figure 1

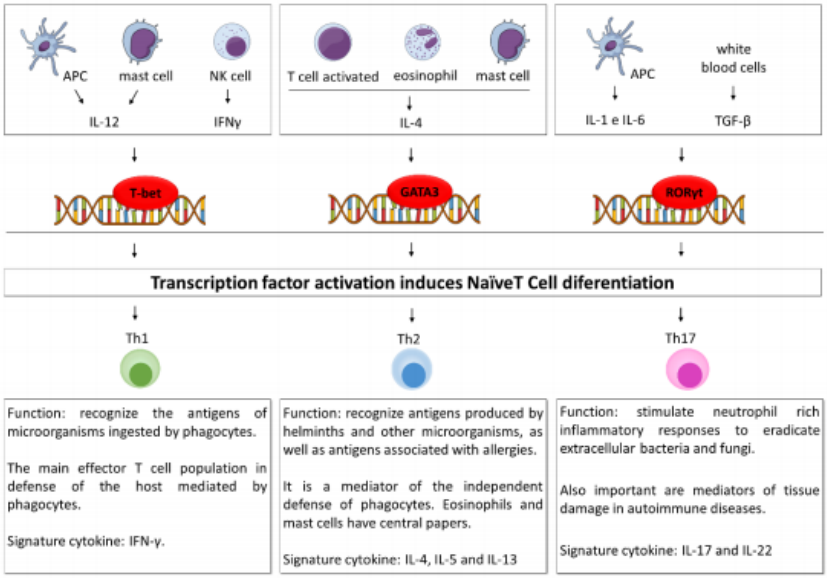
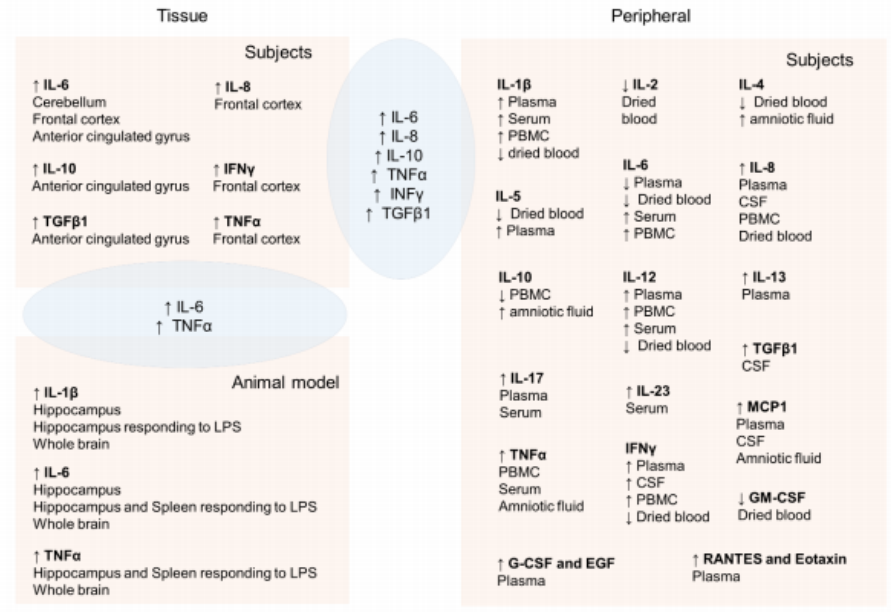

APC
mast cell
NK cell
IL-12
IFNγ
T cell activated
eosinophil
mast cell
IL-4
APC
white blood cells
IL-1 e IL-6
TGF-β
T-bet
GATA3
RORγt
Transcription factor activation induces NaïveT Cell diferentiation
Th1
Th2
Th17
Function: recognize the antigens of microorganisms ingested by phagocytes.
The main effector T cell population in defense of the host mediated by phagocytes.
Signature cytokine: IFN-γ.
Function: recognize antigens produced by helminths and other microorganisms, as well as antigens associated with allergies.
It is a mediator of the independent defense of phagocytes. Eosinophils and mast cells have central papers.
Signature cytokine: IL-4, IL-5 and IL-13
Function: stimulate neutrophil rich inflammatory responses to eradicate extracellular bacteria and fungi.
Also important are mediators of tissue damage in autoimmune diseases.
Signature cytokine: IL-17 and IL-22

Figure 2


Tissue
Peripheral
Subjects
↑ IL-6
Cerebellum
Frontal cortex
Anterior cingulated gyrus
↑ IL-8
Frontal cortex
↑ IL-10
Anterior cingulated gyrus
↑ IFNγ
Frontal cortex
↑ TGFβ1
Anterior cingulated gyrus
↑ TNFα
Frontal cortex
↑ IL-6
↑ IL-8
↑ IL-10
↑ TNFα
↑ INFγ
↑ TGFβ1
↑ IL-6
↑ TNFα
Animal model
↑ IL-1β
Hippocampus
Hippocampus responding to LPS
Whole brain
↑ IL-6
Hippocampus
Hippocampus and Spleen responding to LPS
Whole brain
↑ TNFα
Hippocampus and Spleen responding to LPS
Whole brain
Subjects
IL-1β
↑ Plasma
↑ Serum
↑ PBMC
↓ dried blood
↓ IL-2
Dried blood
IL-4
↓ Dried blood
↑ amniotic fluid
IL-5
↓ Dried blood
↑ Plasma
IL-6
↓ Plasma
↓ Dried blood
↑ Serum
↑ PBMC
↑ IL-8
Plasma
CSF
PBMC
Dried blood
IL-10
↓ PBMC
↑ amniotic fluid
IL-12
↑ Plasma
↑ PBMC
↑ Serum
↓ Dried blood
↑ IL-13
Plasma
↑ TGFβ1
CSF
↑ IL-17
Plasma
Serum
↑ IL-23
Serum
↑ MCP1
Plasma
CSF
Amniotic fluid
↑ TNFα
PBMC
Serum
Amniotic fluid
IFNγ
↑ Plasma
↑ CSF
↑ PBMC
↓ Dried blood
↓ GM-CSF
Dried blood
↑ G-CSF and EGF
Plasma
↑ RANTES and Eotaxin
Plasma

Capítulo III: capítulo de livro aceito para publicação na editora *Springer Nature*

"Purinergic signaling in autism spectrum disorder"

Tópico de pesquisa "*Purinergic Signaling in Neurodevelopment, Neuroinflammation and Neurodegeneration*"

Purinergic signaling in autism spectrum disorder

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Abstract

Autism spectrum disorder (ASD) is a highly prevalent neurodevelopmental disorder estimated to affect 1:54 individuals, characterized by impairments in the social approach and stereotyped behavior patterns. The etiology of this disorder is still unclear; however, disturbances in the regulation of purinergic signaling contribute to establishing the ASD phenotype. Purinergic signaling is a system that involves second extracellular messengers able to triggers responses in several biological processes, activating the immune system, modulating bioenergetics, and promoting neuromodulation, by activation of specific receptors subtypes: metabotropic P1, ionotropic P2X, and metabotropic P2Y. In addition, several psychiatric disorders, including epilepsy, schizophrenia, Alzheimer and, in recent years, ASD, present altered components associated with the purinergic system, such as mitochondrial dysfunction, polymorphisms in genes that encode purinergic receptors, abnormalities in intermediaries of purine metabolism, among others. This chapter summarizes the contribution of purinergic signaling in ASD phenotype and hypothesizes how this intricate puzzle involves extracellular messengers and ASD pathophysiology, focusing on pathways associated with metabolism, neuroimmune modulation, and neurodevelopment. **Keywords:** autism spectrum disorder; purinergic signaling; immunomodulation; neurodevelopment; microRNA; second messengers; metabolism; cell signaling.

1. Introduction

Autism spectrum disorder (ASD) is one of the neuropsychiatric disorders with the highest incidence nowadays. As it is a condition that originates during early neurodevelopment, involving epigenetic changes, several experimental approaches shed light on possible mechanisms involved in the ASD triggering. In this context, the purinergic system emerges as a strong candidate for its phenotype modulation.

2. ASD

ASD is a neurodevelopmental disorder characterized by two sets of characteristic behavioral alterations: communication/social interaction impairments and stereotyped/repetitive behaviors (American Psychiatric Association, 2013). Although the ASD etiology remains unclear, it is already known that both genetic and environmental risk factors can contribute to the onset (Gottfried et al., 2015). Furthermore, the most recent prevalence data of ASD from the USA shows that 1:54 8-years-old children are affected and that the ratio of males/females is 4:1 (Maenner et al., 2020), demonstrating a high prevalence that has been rising in the last decades. Besides that, the absence of biomarkers and the heterogeneity of the disorder challenge the diagnosis and, consequently, the implementation of adequate therapeutic strategies (Masi et al., 2017).

Beyond the core behavioral dyad, ASD individuals may also experience a series of comorbidities, including epilepsy, anxiety, perception alterations, gastrointestinal disturbance, sleep impairments, and many others (Doshi-Velez et al., 2014). The high incidences of electrophysiological (8%-30% of the individuals with ASD) (Spence and Schneider, 2009; Bolton et al., 2011; Lukmanji et al., 2019) and sensory alterations (more than 90% of the individuals) (Chang et al., 2014) support the connectivity theory in ASD: the brain, in this case, presents an excitatory/inhibitory imbalance added to impaired connectivity of different brain regions, leading to local hyper processing, resulting in the impaired interpretation of the different stimuli.

Several pathways have already been described as altered in ASD, both in the brain and in peripheral tissues. Immune system alterations stand out in ASD, including descriptions of increased content of pro-inflammatory cytokines, altered lymphocyte profile, and high levels of autoimmune diseases (Deckmann et al., 2018). In the brain,

the highlights are for routes associated with glutamate and GABA neurotransmission (Horder et al., 2018), as well as pathways involved in synaptic plasticity (Bourgeron, 2015) and the activation and reactivity of glial cells such as astrocytes and microglia (Petrelli et al., 2016).

Purinergic signaling studies in ASD are emerging in recent years, demonstrating several roles that will be discussed in this chapter. In an overview, this system can be the link that unites immune and brain dysfunctions, helping to understand the pathophysiology of autism.

3. Purinergic System and autism-like features

As reviewed by Ulrich *et al*., (2012) and Fumagalli *et al*., (2017), brain development in embryonic life is finely regulated by a range of biological processes. It has been demonstrated that purinergic signaling plays an essential role in organizing embryonic and fetal development and organogenesis in a time-dependent manner, controlling purinergic signaling molecules, such as adenosine (ADO) triphosphate (ATP), the Ca^{2+} release from radial glia, differential receptor subtypes expression, among others (Ulrich et al., 2012; Fumagalli et al., 2017). Here, we present an overview of the contribution of the dysfunction of purines and pyrimidines metabolism in ASD. A summary of the main findings can be seen in Figure 1.

3.1 Humans

The neurobiological bases of ASD-like features (social impairments and stereotyped behavior) are still a challenge for science due to the dynamics and crosslinking of distinct biological pathways, including purinergic signaling throughout development. Single nucleotide polymorphisms (SNP) in the ADO A2A receptor gene (*ADORA2A*) were already associated with ASD (rs2236624-CC and rs2298383) and phenotypic variability, including impaired scores in behavioral assessments (rs3761422, rs5751876 and, rs35320474) (Freitag et al., 2009). Interestingly, ASD patients also demonstrated mutations in the ADO A3 receptor gene (*ADORA3*), identified as rs77883500 and rs139935750, and, in an *in-vitro* assay; the presence of the first SNP induced enhanced levels of cGMP, resulting in increased activity of the serotonin transporter (Campbell et al., 2013).

A *postmortem* analysis of cerebellum from idiopathic ASD patients demonstrated that the cluster of genes associated with impaired social behavior had gene ontology enrichment for purinergic-signaling genes, demonstrating an important overlap (Ginsberg et al., 2012). In a recent study, gene set enrichment analysis of a polygenic risk score in ASD individuals demonstrated an association (R2=0.064; β, −5.30; SE 1.30; P < .001) of adenylyl cyclase activity and cyclic ADO monophosphate (cAMP) concentration with ASD traits (Takahashi et al., 2020). cAMP pathways were also associated with variants identified in ASD in an *in-silico* study (Luo et al., 2018). Finally, a differential presence of metabolites derived from purine metabolism was found in ASD subjects' urine, indicating the general impact of purinergic metabolism in this disorder (Gevi et al., 2016).

According to the region evaluated in a *postmortem* analysis of ASD individuals, the expression of phosphodiesterases (PDE) demonstrated differential results: reduced expression of PDE4A5, PDE4B1, PDE4B3, PDE4B4, and PDE4B2 in the cerebellum and enhanced expression of PDE4AX, PDE4A1, and PDE4B2 in the encephalic region BA9. These alterations are relevant in this context because these enzymes regulate the levels of cAMP, a second messenger in purinergic signaling (Braun et al., 2007). The fragile X syndrome is a genetic disorder with an important overlap of symptoms with ASD, including poor eye contact, difficulties with peer relationships, social withdrawal, repetitive behaviors (Kaufmann et al., 2017). A study using three different fragile X syndrome model systems containing a non-functional *FMR1* gene (human neural progenitor cells, mice, and drosophila), pointed to a general lowered cAMP production in human neural progenitor cells and mice brain, even suggesting that cAMP metabolism can be used as a biomarker in fragile X syndrome (Kelley et al., 2007).

Overall, the data present important roles of purinergic signaling in ASD, especially regarding ADO metabolism and associated pathways. In the next section, the discussion of animal models will clarify these pathways, demonstrating how a purinergic imbalance could influence the severity of ASD traits.

3.2 Animal Models

Several animal models are used in the study of ASD, including a) knockout (KO) for specific ASD-associated genes; b) maternal immune activation (MIA),

especially induced by prenatal exposure to polyinosinic:polycytidylic acid (poly(I:C)) or lipopolysaccharides (LPS); and c) prenatal exposure to valproic acid, among others. In this section, we will discuss the evidence of purinergic involvement in the pathophysiology of ASD models and models of associated psychiatric disorders.

In the offspring from the MIA model induced by prenatal exposure to LPS, purinergic signaling disturbance probably contributed to the impaired astrocyte function due to microglial activation. In two articles, the increased opening of Cx43 and Panx1 channels in hippocampal astrocytes was associated with the release of ATP and glutamate (GLU), which enhanced Ca^{2+} levels after interacting with their receptors (ATP: P2X7 and P2Y1; GLU: mGLUR5), leading to an activated profile in astrocytes and cell death in neurons (Avendaño et al., 2015; Chávez et al., 2019).

Injection of intraperitoneal ATP in postnatal life induces a broad spectrum of alterations, including global reduction of mitochondrial function, altered profile of metabolites, increased levels of corticosterone and cytokines (IL-10, IL-6, and CXCL10), and impaired behavioral performances in exploratory and locomotor features (ASD-like traits). Moreover, when this approach is performed in the offspring of the poly(I:C) model, the alteration in body temperature lasted much longer when compared to controls. This evidence demonstrates the general issues of a hyperpurinerigic condition and highlights that animals exposed to MIA are more susceptible to postnatal dysregulation of purinergic signaling (which can be induced by several environmental factors) (Zolkipli-Cunningham et al., 2021).

Interestingly, purinergic receptors are pivotal for the ASD features derived from the MIA model induced by poly(I:C): the offspring originated from a) dams heterozygous or KO for P2X7 and b) females treated with a specific P2X7 antagonist (JNJ47965567) did not present any ASD-associated characteristics, while wild-types (WT) submitted to MIA had several alterations including impaired sociability, increased stereotyped behavior, increased IL-6, CXCL1, and TNF-α brain content, synapse malformation, disruption of cortical lamination and others. Postnatal administration of JNJ47965567 attenuated the altered parameters in WT exposed to MIA, while administration of ATP elicited ASD traits in animals that were not submitted to any prenatal intervention (Horváth et al., 2019). Inhibition of P2X7 also improved social deficits, dendritic spine dynamics, and other parameters in a model of Rett Syndrome (Garré et al., 2020). Conversely, the P2X4 receptor demonstrated a completely different association: heterozygous or KO animals displayed reduced social interaction

and maternal separation-induced ultrasonic vocalizations. Moreover, only KO animals had sensory impairments, anxiety-like behavior, and altered expression of GLU receptors (prefrontal cortex: reduced GluN2A and Glu2B; hippocampus: reduced GluN1 and enhanced GluA1 and GluA2) (Wyatt et al., 2013).

The distinctive effects of P2X7 and P2X4 are intriguing, while lack of P2X7 protected against MIA-derived impairments, the loss of P2X4 induced ASD-like symptoms. We hypothesize that this difference occurs because P2X7 is a significant inductor of Ca^{2+} release when activated, which could cause excitotoxicity. Activation of P2X7 was already associated with apoptosis/necrosis in neural progenitor cells in the hippocampus (Khan et al., 2018). On the other hand, P2X4 is pivotal for microglial function, especially brain-derived neurotrophic factor (BDNF) release and regulation of synaptic formation and plasticity, processes already associated with ASD pathophysiology (Trang et al., 2009; Montilla et al., 2020). Strikingly, animals KO for A2A, another purinergic receptor, demonstrated enhanced sociability and increased anxiety, implying the broad behavioral factors associated with each receptor (López-Cruz et al., 2017).

Regarding other animal models, the inbred BTBR T+tf/J (BTBR) mouse strain, a genetic model that replicates repetitive behaviors and social deficits seen in ASD individuals, has decreased striatal function of A2A receptor, probably contributing to impairments of dopaminergic neurons (Squillace et al., 2014). The deletion of a PDE4 regulator, named CC2D1A, considered an ASD model, induced spatial memory deficits associated with PDE4 hyperactivity and cAMP reduced levels in the hippocampus (Zamarbide et al., 2019). In the valproic acid embryological exposure model, zebrafish presented increased AMP hydrolysis, A2R1 mRNA expression, and ATP/ADP catabolism in the brain (Zimmermann et al., 2017). In murine, valproic acid increased the AMP/ATP ratio in the midbrain and reduced it in the cortex, possibly inducing a purinergic signaling imbalance (Hegazy et al., 2015), as well as hippocampal upregulation of P2X4 and P2Y2 receptor expressions, and P2X4 receptor expression in the medial prefrontal cortex (Hirsch et al., 2020).

In this section, we discussed how elements from the purinergic signaling universe are associated with ASD or ASD-like features. The major highlight is the important roles of receptors A, P2X, and P2Y, which will lead the discussion in the section “7. Therapeutic Approaches and Purinergic System in ASD”.

4. Purinergic System and Metabolism in ASD

4.1 ADO

Purines and pyrimidines metabolism intermediates are essential signaling molecules for various biological processes, such as providing energy for cell functions by the Na^+/K^+-ATPase activity, a membrane-active pump that requires a huge amount of ATP produced in central nervous system (CNS) (Fumagalli et al., 2017). ADO is a product of ATP hydrolysis and a key molecule involved in purinergic signaling, energetic metabolism, folate metabolism, and other pathways. Studies already demonstrated that ADO might mediate the beneficial effects of the ketogenic diet (Masino et al., 2009), improving electrophysiological impairments (which are common in ASD) (Masino et al., 2013). Besides that, indirect stimulation of ADO production improved behavioral alterations in ASD children (Masino et al., 2011).

The modulation of oligodendrocytes development is one of the major functions of ADO. Shen et al., (2018) postulate that this molecule interacts with oligodendrocyte progenitor cells in different times, stimulating proliferation, differentiation, and myelination (Shen et al., 2018). Interestingly, it was demonstrated in the Lesch-Nyan syndrome, characterized by a deficiency in the hypoxanthine-guanine phosphoribosyltransferase enzyme (HPRT) - which leads to impairments in purine biosynthesis - altered patterns of ADO receptors (ADORA1A and ADORA2A) (Bertelli et al., 2006), besides altered expression of oligodendrocyte transcription factor 2 and myelin basic protein, important oligodendrocyte markers (Kang et al., 2013), which may be associated with the ADO roles in the development of oligodendrocytes. HPRT deficiency also resulted in reduced mRNA expression of P2X3, P2X5, P2Y2, P2Y4, P2Y12, P2Y13, and P2Y14 receptors, which was associated with impaired Ca^{2+} signaling in neuroblastoma cells (Erdorf et al., 2011). In addition, another study demonstrated that HPRT silencing reduced P2Y1 mRNA expression and induced other relevant alterations like reduced pCREB expression, altered activation of ERK1/2 MAP kinases, and decreased phosphorylation of β-catenin (Mastrangelo et al., 2012). This disorder is marked by self-injury behavior (among other behavioral features), a characteristic that is also found in 42% of ASD individuals (Steenfeldt-Kristensen et al., 2020).

The ADO deaminase enzyme (ADA), which is necessary for ADO conversion to inosine and further excretion in the form of uric acid, has been studied in the context of ASD for decades. The first work demonstrated that ASD individuals presented lower levels of ADA activity in the blood (Stubbs et al., 1982). Following that, several studies clarified this difference: the presence of ADA2 alleles, which cause 15-20% reduction in the catalytic reduction, was significantly more frequent in ASD subjects (Persico et al., 2000). The polymorphism named Asp8As induced a reduction of 35% in ADA activity and was associated with ASD in an Italian study (Bottini et al., 2001); however, the same association was not found in a North American study (Hettinger et al., 2008). As hypothesized by Cheffer et al., (2018), this conflict may occur because ADA reduction probably overstimulates A1 receptors, which was described as beneficial in ASD (Cheffer et al., 2018).

Throughout this chapter, ADO will be discussed in specific sections, especially in "7. Therapeutic Approaches and Purinergic System in ASD". Here, we can already observe that ADO metabolism is an integrative topic, mediating the ketogenic diet's effects and modulating important cells like the oligodendrocytes. The alterations described in ADA in humans add relevance to investigate possible pharmacological approaches.

4.2 Mitochondria

The neural signaling requires high mitochondrial energy demand due to ion transport by pumps such as Na^+/K^+-ATPase, and the mitochondria are key organelles for energy support through ATP synthesis. Here, we discuss how dysfunctions, even subtle, in mitochondrial energy production are involved in the pathophysiology of disorders such as ASD.

4.2.1 Data from Humans

Altered mitochondrial metabolism has been studied in ASD since the 1980s, when lactic and pyruvic acidosis with additional structural mitochondrial alterations were reported in Rett syndrome patients (a condition with a huge overlap of symptoms with ASD) (Philippart, 1986). From there, several articles reported issues in this context and, in this section, we will focus on alterations that most likely impair the

content of ADO-associated mediators (ATP, ADP, AMP, and others), possibly disturbing the purinergic signaling.

An analysis of maternal serum from women exposed to high levels of pollution during pregnancy demonstrated an important relation with ASD increased risks in their children. The primary enriched metabolites were associated with mitochondrial dysfunction (MD), especially tricarboxylic acid cycle, carnitine shuttle, and others, suggesting a possible involvement in the onset of ASD (Kim et al., 2021). A meta-analysis of ASD studies highlighted 5% of MD prevalence in the ASD population, much higher than the general average of MD (0.01%). Moreover, these alterations were associated with impaired behavioral parameters and increased presence of developmental regression and motor delay (Rossignol and Frye, 2012). Interestingly, a recent study clarified mitochondrial damage in DR: mitochondria derived from peripheral blood mononuclear cells had increased respiration rates, lower reserve capacity (associated with more susceptibility to reactive species), and more damage in the mtDNA compared to ASD-no DR subjects (Singh et al., 2020).

Serum microRNA (miR) interfered in the MT respiration in peripheral blood mononuclear cells from different ASD subpopulations (defined by the IL-1β/IL-10 ratio) (Jyonouchi et al., 2019); complementary, the mitochondria from platelets and neutrophils presented impaired respiration and decreased reactive oxygen species (ROS) production (only in neutrophils, indicating the reduced function of these cells) (Abdel-Rahman et al., 2020). Finally, fibroblasts derived from ASD individuals demonstrated increased mitochondrial respiratory profile, altered mitochondrial dynamics (biogenesis, fusion, and fission), aberrant anti-inflammatory defenses, , and morphological alterations (Pecorelli et al., 2020).

Metabolomic analysis of urine from ASD individuals demonstrated increased levels of 3-hydroxy-3-methylglutaric acid, 3-methylglutaconic acid, and ethylmalonic acid, indicating impairments in oxidative phosphorylation (Stathopoulos et al., 2020). Using a similar approach, several groups of altered mitochondria-associated metabolites were identified in ASD patients, suggesting that the differential clusters observed can be used not only as biomarkers but also as indicators for personalized therapeutic approaches (Smith et al., 2020).

Postmortem analysis of the cerebral cortex from ASD subjects showed downregulation of gene clusters related to mitochondrial function and synaptic plasticity (Schwede et al., 2018). In the temporal lobe of ASD patients, altered protein

levels and decreased activity of respiratory chain protein complexes were observed, in addition to a disturbance in the biogenesis, dynamics, and antioxidant defense. In this work, the authors highlight that mitochondrial issues may be associated with the huge synaptic impairments in this brain region (Tang et al., 2013). Besides, a decreased expression of electron transport chain complexes in the cerebellum and the frontal and temporal regions were observed in the brain from autistic children (Chauhan et al., 2011). mitochondria plays important roles in synaptic Ca^{2+} buffering and ATP supply necessary to synaptic plasticity (Rossi and Pekkurnaz, 2019); interestingly, proteins that stimulate synaptic increase, like Bcl-xL, also stimulate mitochondrial proliferation, demonstrating an important connection between these structures (Eltokhi et al., 2021).

This evidence indicates a prominent role of mitochondrial impairments in the pathophysiology of ASD. The disturbances in MT can induce oxidative stress, leading to cell damage and apoptosis, impairment of synaptic plasticity, ATP production decrease, and many other effects. In all of these contexts, purinergic signaling may also be involved, suggesting an important field in further studies.

4.2.2 Data from Animal Models

Fragile X syndrome animal models provide an interesting approach for ASD studies due to several points in common between these disorders, including behavioral traits. In *Fmr1* KO mice, mitochondrial transcription factor NRF1; ATP synthase subunits ATP5A and ATPB, and mitochondrial anion channel VDAC1 (a component of the outer membrane) were reduced in extracellular vesicles in the cortex and astrocytes (Ha et al., 2021), inducing several mitochondrial disturbances. In another study, astrocytes from *Fmr1* KO mice demonstrated enhanced ROS production with no change in respiration rates (Vandenberg et al., 2021). Disturbances in astrocyte function, as already discussed in section 3, are extremely deleterious, especially when metabolic features are altered due to the direct impairment of neurons. In neurons of *Fmr1* KO, the presence of “leak” in the c subunit of ATP-synthase was associated with dendritic and synaptic impairments (Licznerski et al., 2020).

MIA has been linked to a higher risk for the child to develop autism or schizophrenia-related symptoms. The pathogen-free poly(I:C) has been used to

induce an important MIA model for the study of neurodevelopmental disorders, which shows relatively high construct and face validity. This ASD animal model decreased ATP production, associated with lower activity of complex I in leukocytes, suggesting a long-term immune dysfunction associated with mitochondrial impairments (Giulivi et al., 2013). In BTBR animals, mitochondria isolated from the brain demonstrated decreased oxidative phosphorylation (probably associated with reduced complex I activity), increased fragmentation, and activation of fission proteins, which were attenuated by ketogenic diet (Ahn et al., 2020). These two consolidated ASD models illustrate that mitochondria can contribute to both immune and brain alterations, key factors in the pathophysiology of the disorder.

More recently, studies demonstrated that loss or altered expression of parvalbumin (PV) induces MT alterations. PV is an important marker of a specific type of GABAergic interneurons, with several alterations already described in ASD (Fontes-Dutra et al., 2018; Filice et al., 2020). Besides, PV comprises a family of proteins with calcium-binding properties, influencing biological properties, such as neural function (Antonoudiou et al., 2020) and immune response (Beers et al., 2001). In the first study with PV KO mice, the loss of PV resulted in an increased number and length of MT and increased dendritic branching in the neocortex (Janickova et al., 2020). In the second study, the same group observed that, according to age, the absence of PV enhanced the generation of ROS and increased mitochondrial length (Janickova and Schwaller, 2020). Interestingly, purinergic signaling impairments during pregnancy were able to induce interneuron alterations: exposure to caffeine delayed the migration of somatostatin-positive interneurons (Silva et al., 2013), indicating that PV, which originates from the same area (ganglionic eminence), may also be affected.

Most of all, this data demonstrates that mitochondria are a pivotal piece in the intricate ASD puzzle. In animal models, we can observe the direct impact of MT dysfunction in synaptic plasticity, immune cell function, neuronal survival, and other features. Although not described directly, the purinergic signaling alterations already observed in these conditions are likely associated, at least in part, with mitochondrial impairments.

5. Purinergic System and Neuroimmune Aspects in ASD

A component intimately involved with the pathophysiology of ASD is the immune system, associated with a pro-inflammatory condition. Neuroinflammation has a key role in a wide plethora of CNS diseases, whose pathophysiology can be studied by the modulation of the neuroimmune processes by molecules such as ADO. ADO is capable of modulating A1AR and A2AAR receptors, which can negatively modulate the excitatory transmission and promote synaptic plasticity, respectively (Martí Navia et al., 2020). This is particularly important in the ASD context, considering the excitatory/inhibitory imbalance in brain cortical structures. KO mice to A1AR have increased neuroinflammation and microglial activity (Synowitz et al., 2006). The A2AR, on the other hand, has an anti-inflammatory role and can be negatively regulated by the miR-214, which promotes the release of inflammatory cytokines TNF-α and IL-6. The anti-inflammatory effect was exacerbated upon the combination of miR-214 antagomiR and A2AR agonist (Zhao et al., 2015).

Owing to their role in both releasing pro-inflammatory mediators and responding to immunological signaling released from other cells, microglia and astrocytes are the brain cells that react to the alterations in the neural environment, migrating to the site of the damage, phagocytosing, and secreting inflammatory mediators (Fumagalli et al., 2011). The microglia perform a crucial role in neuroinflammation. On the one hand, it performs the necessary surveillance to maintain the CNS homeostasis; on the other, when activated, it further spreads inflammation. Besides, the neuroinflammation facilitates the loosening of the blood-brain barrier, allowing inflammatory infiltrate from the periphery towards the CNS. Anyway, it is a cell susceptible to the modulation of purinergic signaling (Fumagalli et al., 2011). During injury events, such as cerebral ischemia, the injury site receives a large amount of ATP, which activates resident microglia via purinergic receptors (Melani et al., 2005). In this case, the P2X7 receptor has a double-edged role: both increasing generalized inflammatory responses, mainly by the IL-1β release (Ferrari et al., 1997; Grahames et al., 1999), and promotes a neurotrophic factors release counteracting the neuronal death (Suzuki et al., 2004). P2Y receptors are also recruited, especially, the involvement of P2Y12 in microglial chemotaxis was already described (Ohsawa et al., 2007). In a complementary way, the astrocytes also perform an important role in the neuroinflammation, becoming hypertrophic and acquiring a reactive profile to form the glial scar. The astrogliosis can be triggered by a set of factors, including the ATP released by stressed cells, which activates the P2X7

receptor, mediating the signaling for the synthesis of pro-inflammatory cytokines (Solle et al., 2001) and neurotransmitters such as glutamate (Duan et al., 2003), diminishing their uptake and reducing the glutamine synthetase activity (Lo et al., 2008). Moreover, P2Y2 stimulation modulates the astrocyte migration (Wang et al., 2005) whilst P2Y1 receptors signaling astrogliosis via the PI3-K/Akt signaling pathway (Franke et al., 2009).

In the context of autism, few studies describe the inflammatory modulation mediated by purinergic signaling. The treatment of BTBR mouse strain with SCH 5826 (SCH, an A2AR antagonist) showed increased levels of $CD4^+IL\text{-}21^+$, $CD4^+IL\text{-}22^+$, $CD4^+GATA3^+$, and $CD4^+T\text{-}bet^+$ and decreased $CD4^+CTLA\text{-}4^+$ expression in splenic cells, besides increased mRNA and protein expression levels of IL-21, IL-22, GATA3, and T-bet in brain tissue (Ahmad et al., 2017b). Regarding toll-like receptors (TLRs), the same treatment increased the percentage of splenic $CD14^+TLR2^+$ cells, $CD14^+TLR3^+$ cells, $CD14^+TLR4^+$ cells, and decreased the percentage of $CD14^+IL\text{-}27^+$ cells, in addition to TLR2, TLR3, TLR4, and NF-κB p65 mRNA and protein expression increased in brain tissue, and decreased IL-27 and IκBα expression (Ahmad et al., 2017a). In addition, the treatment of BTBR mouse strain with SCH promoted, in the brain, (a) increased mRNA of $CCR3^+$, $CCR4^+$, $CCR5^+$, $CCR6^+$, $CCR7^+$, $CXCR3^+$, $CXCR4^+$, and $CXCR5^+$ (Ahmad et al., 2018), (b) increased the Th1 response, (c) decreased Th2 response, and (d) increased protein levels of IL-2+, IL-6+, IL-9+, IFN-γ+, and TNF-α+ and decreased $TGF\text{-}\beta^+$ (Ansari et al., 2017a). Another study in the same model demonstrated, in $CD4^+$ cells, increased positive cells of $IL\text{-}17A^+$ and $ROR\gamma t^+$ and decreased of $Foxp3^+$ and $IL\text{-}10^+$ (Ansari et al., 2017b). These data demonstrate the important anti-inflammatory role of the A2A receptor since the inhibition of ADO binding via SCH-mediated antagonism was able to replicate deficits commonly described in ASD. More details on the role of this receptor can be read in the section "7. Therapeutic Approaches and Purinergic System in ASD" in the subtopic related to the A2AR agonist CGS 21680.

6. Purinergic System and Molecular Modulation in ASD

The purinergic system either can modulate or be modulated by molecular changes. For example, several SNPs have been identified in the P2X7 receptor, generating many complex outcomes (Di Virgilio et al., 2017). In addition, P2X7 can be

regulated by DNA methylation (Liu et al., 2017), transcriptions factors, such as Specificity protein 1 (Sp1) (whose affinity to the P2X7 promoter increases during *status epilepticus*) (Engel et al., 2017), alternative splicing and post-translational regulation, such as phosphorylation as reviewed by Jimenez-Mateos (Jimenez-Mateos et al., 2019).

In the context of ASD, since the diagnostic criterion is purely clinical, there is a growing interest in blood-altered molecules, with potential to be used as molecular markers to anticipate diagnosis, such as miR. These molecules are small non-coding RNAs that regulate the translation of messenger RNAs into their corresponding proteins, essential in controlling several cellular processes during development and in adulthood. As expected, the purinergic signaling can also be modulated by miR; these molecules influence purinergic receptors, ectonucleotidases, and the expression of other molecular components of the purinergic signaling network (Ferrari et al., 2016; Jimenez-Mateos et al., 2019).

Considering that epilepsy is present in 8%-30% of individuals with ASD (Spence and Schneider, 2009; Bolton et al., 2011; Lukmanji et al., 2019), studies with animal models of epilepsy are essential in understanding the pathophysiological mechanisms of ASD (in the absence of studies on this disorder). Important electrophysiological alterations were demonstrated in a unilateral mouse model of *status epilepticus*, with suppression of P2X7 receptor-gated inward currents in the contralateral hippocampus. Besides, the inhibition of miR-22 increased P2X7 receptor expression and cytokine levels in the contralateral hippocampus, causing spontaneous seizures more frequently in these mice. In addition, $P2rx7^{-/-}$ mice do not exhibit the effects of miR-22 inhibition, however, had transiently suppressed spontaneous seizures when microRNA-22 was injected. These data support the role of miR-22 in targeting P2X7 receptors, preventing both seizures and neuroinflammation (Jimenez-Mateos et al., 2015).

In a complementary way, transcription factors are also able to modulate both miR and purinergic receptors expression. *In-vitro* studies demonstrated that Sp1, a transcription factor highly expressed in the brain, induces the transcription of the P2X7 receptor (García-Huerta et al., 2012). Moreover, it was demonstrated, both in neuronal activity induce *in-vitro* and in a mouse model of *status epilepticus*, that Sp1 can induce the transcription of both miR-22 and P2X7 receptor, as well as induces the

transcription of miR-22 in a calcium-sensitive way, shedding some light on neuronal activity-dependent P2X7 receptor expression (Engel et al., 2017). Besides miR-22, other miR are associated with the P2X7 receptor in hippocampi from WT and P2X7 receptor KO mice following *status epilepticus*. Wild-type mice presented up-regulation of 50 miR and down-regulation of 35 miR after lack of the P2X7 receptor (involved in signaling pathways and inflammation), whereas in *status epilepticus* mice, the P2X7 receptor deficiency led to the up-regulation of 44 miR while 13 miR were down-regulated (associated with cell death), emphasizing the impact of P2X7 receptor alterations in the maintenance of normal cellular homeostasis and pathological processes via distinct patterns of miR expression (Conte et al., 2020).

Lastly, due to their importance as regulatory molecules, new molecules with miR-mimicking or miR antagonist (antagomiR) activity have been received increasing attention. For example, traumatic spinal cord injury is worsened by a massive release of glutamate and ATP, which could be generated by an over-expression and activation of purinergic receptors, especially P2X7, producing excitotoxicity in neurodegenerative diseases. In that regard, the miR-135a-5p was identified to be a post-transcriptional modulator of P2X7, over-expressing the P2X7 receptor when in decreased levels. Besides, the antagomiR-135a increased the P2X7 expression whereas the miR-135a-mimicked reduced the P2X7 expression protecting cells from excitotoxic death (Reigada et al., 2019). Therapeutic approaches involving purinergic signaling will be discussed in more detail in the following topic. Thus, the interplay between purinergic signaling and miR modulation in ASD should be elucidated in future investigations. An overview of metabolism, neuroimmune aspects and molecular modulation can be seen in Figure 2.

7. Therapeutic Approaches and Purinergic System in ASD

Regarding therapeutic approaches, several human disorders still do not have a specific treatment, as in the case of ASD, whose treatment involves medications that help with the associated symptoms. However, several studies bring new therapeutic opportunities for the treatment of different pathologies: for example, the stimulation or inhibition of specific purinergic receptors P1 or P2 to improve issues related to the dysregulation of purinergic signaling. An overview can be seen in Table 1 and Figure 3.

7.1. ADO and P1 receptors modulators

7.1.1. CGS 21680

The CGS-21680 (CGS) is a drug agonist selective of ADO A2A subtype receptor. The treatment of BTBR mouse strain with CGS changes gene and protein expression levels of several molecules in spleen and brain: a) in splenic cells, decreased production of positive cells to $CD4^+IL\text{-}21^+$, $CD4^+IL\text{-}22^+$, $CD4^+GATA3^+$, and $CD4^+T\text{-}bet^+$, and increased $CD4^+CTLA\text{-}4$ (Ahmad et al., 2017b); b) in splenic cells and brain tissue, decreased $CD14^+TLR2^+$ cells, $CD14^+TLR3^+$ cells, $CD14^+TLR4^+$ cells and TLR2, TLR3, TLR4, and NF-κB mRNA and protein expression, respectively (Ahmad et al., 2017a); c) in splenic $CD8^+$ T cells, decreased positive cells of $CCR3^+$, $CCR4^+$, $CCR5^+$, $CCR6^+$, $CCR7^+$, $CXCR3^+$, $CXCR4^+$, and $CXCR5^+$, and decreased mRNA of C-C and C-X-C chemokine receptors in the brain tissue (Ahmad et al., 2018); d) in total spleen and splenic $CD4^+$ T cells, diminished positive cells of $IL\text{-}2^+$, $IL\text{-}6^+$, $IL\text{-}9^+$, $IFN\text{-}\gamma^+$, and $TNF\text{-}\alpha^+$ and increased $TGF\text{-}\beta^+$, and improvement in respective mRNA and protein expression in brain tissue of BTBR mouse strain (Ansari et al., 2017a); e) in $CD4^+$ cells, diminished the IL-17A, RORγt, Stat3, and pStat3 levels and elevates the $Foxp3^+$ and $IL\text{-}10^+$ protein and expression levels (Ansari et al., 2017b). In addition to the molecular improvements, CGS also ameliorated the learning deficits, attenuated the self-grooming behavior and the response to the hot plate test in BTBR mice (Ansari et al., 2017b; Amodeo et al., 2018).

In C58 mice, a lineage that exhibits a robust repetitive behavior phenotype (one of the diagnostic criteria for ASD), a combination of three drugs (CGS, L-741,626 (a dopamine D2 receptor antagonist), and glutamate mGlu5 positive allosteric modulator) reduced repetitive motor behavior in C58 mice and four-fold increase in BDNF mRNA expression (Muehlmann et al., 2020). The combination of two ADO A1 (N6-cyclopentyladenosine - CPA) and A2A (CGS) receptor agonists also reduced repetitive behavior in both male and female C58 mice (in a dose-dependent manner), and increased the number of Fos transcripts and Fos positive cells in the dorsal striatum (Lewis et al., 2019). This same drug combination (CPA and CGS) also attenuated stereotypy behavior in a dose-dependent manner in deer mice, animals with elevated rates of spontaneous stereotypy (Tanimura et al., 2010).

7.1.2. Propentofylline

Propentofylline is a xanthine phosphodiesterase inhibitor and ADO reuptake blocker. A double-blind and placebo-controlled clinical trial demonstrated that the association with propentofylline (initiating at 300 mg/d) and risperidone (initiating at 0.5 mg/d) was able to diminish the irritability in ASD patients evaluated by Childhood Autism Rating Scale, demonstrating the propentofylline as a promising adjunctive treatment probably due to their anti-inflammatory and anti-excitatory properties (Behmanesh et al., 2019).

Taken together, these data suggest the important contribution of the A2AR modulation in the improvement of neuroimmune and behavioral dysfunction observed in animal models.

7.2. P2 receptors modulators

7.2.1. Suramin

Suramin is a P2 non-selective purinergic antagonist. A pilot study double-blind, placebo-controlled, translational in male subjects with ASD showed, in a preliminary way, that a single dose of 20 mg/kg of suramin improved the language and social interaction, as well as decrease the repetitive behaviors (Naviaux et al., 2017). Indeed, the same dose in the valproic acid animal model of ASD restored sociability and decreased anxiety-like behavior, as well as normalized the increase of IL-6 expression in the medial prefrontal cortex in juvenile rats (Hirsch et al., 2020).

In the ASD-mouse by gestational exposure to poly(I:C), a single dose of suramin again restored, in a transitory manner, the social behavior and the novelty preference, beyond normalize metabolic pathways disturbed in the mouse model, related mainly to purine metabolism (Naviaux et al., 2014). The weekly injection of suramin (10 or 20 mg/kg) reverted the social behavior and sensorimotor coordination deficits, rescued the mitochondrial respiratory chain hyperactivity abnormalities (decreasing the enzymatic activity of respiratory chain Complex I activity and Complex IV activity), corrected the reduction in the phosphorylation of ERK1 and 2, and in the phosphorylation of calcium/calmodulin-dependent protein kinase II, as well prevented

Purkinje cell loss and restores the diminished levels of P2X7 and P2Y2 immunocontent (Naviaux et al., 2013).

Another study in fragile X syndrome model induced by *Fmr1* KO mice demonstrated that a purinergic antagonist strategy improved the animal performance in novelty preference, marble burying, and social behavior, normalizing abnormal synaptosomes and metabolomics alteration, particularly related to purine metabolism (Naviaux et al., 2015).

7.2.2. Brilliant Blue G

Brilliant Blue G is a specific P2X7 receptor (P2X7R) antagonist. Pregnant mice received 45 mg/kg of this molecule on gestation day 17 (E17) before administration of lipopolysaccharide (LPS), an inductor of maternal immune activation. The inhibition of P2X7R reduced the preterm birth rate, improved the performance on neuromotor tests of the offspring, and rescued the density of cortical neurons (Tsimis et al., 2017).

7.2.3. JNJ-47965567, A438079, OxATP, and A740003

Owing to the presence of convulsive episodes in a considerable percentage of ASD patients, data obtained in epilepsy studies are a great contribution in this sense.

The JNJ-47965567, a specific P2X7 receptor antagonist, reduced spontaneous seizures during continuous video-EEG monitoring, microgliosis, and astrogliosis in a 30 mg/kg dose (Jimenez-Pacheco et al., 2016). Another P2X7R antagonist with a promising therapeutic approach is A438079, able to reduce both the seizure duration and the neuronal death after intracerebroventricular microinjection of 1.75 nmol (Engel et al., 2012).

On the other hand, P2X7R antagonists can increase seizure susceptibility induced by pilocarpine. Both OxATP (a nonselective P2X7R antagonist) and A438079 and A740003 (selective P2X7R antagonists) infusion increased pilocarpine-induced seizure susceptibility (Kim and Kang, 2011).

7.3. Adenylate cyclase modulators

Adenylate cyclase (EC 4.6.1.1) is an enzyme that catalyzes the ATP into cyclic ADO monophosphate (cAMP) and its modulation can direct the level of secondary messengers.

7.3.1. NB001

NB001 is an experimental compound that suppresses type 1 adenylate cyclase (ADCY1) protein activity. The Fmr1 knockout neurons have increased Adcy1 mRNA translation and, consequently, excessive ADCY1 protein. The administration of NB001 in Fmr1 KO mice attenuated the ASD-like behaviors, such as social interaction deficits and stereotyped behaviors (Sethna et al., 2017).

7.3.2. Diterpenoid Forskolin

The diterpenoid forskolin (FSK EC 266-410-9) is an adenylate cyclase activator that increases intracellular cAMP. The administration of FSK during 15 days rescued the memory dysfunction, decreased muscle coordination and gait imbalance, improved the changes in enzyme activity in neuronal mitochondrial electron transport chain complexes, as well as decreased pro-inflammatory cytokines, oxidative stress, and lipid biomarkers in a dose-dependent manner in an ASD-animal model induced by intracerebroventricular injection of propionic acid (Mehan et al., 2020).

7.4. Other types of therapeutic molecules

7.4.1. 5-aminolevulinic acid

The 5-aminolevulinic acid is an endogenous non-protein amino acid precursor of the heme group. This treatment improved the ASD-like behaviors such as learning and memory, ameliorated oxidative stress and mitochondrial dysfunction in the hippocampus, and rescue the reduced number of PV interneurons of valproic acid-exposed rats (Matsuo et al., 2020).

7.4.2. Exogenous ATP and UTP

In primary astrocyte cultures derived from the Fmr1 KO mouse model, the presence of exogenous ATP and UTP triggered an elevation in intracellular calcium responses and in synaptogenic protein TSP-1 expression, which be modulated by P2Y receptors (Reynolds et al., 2021). The modulatory role of ATP is evidenced in a study using human primary skin fibroblasts from ASD patients, demonstrating that Ca^{2+} release in response to activation of exogenous ATP is decreased in ASD subjects and opening the way for new therapeutic targets (Schmunk et al., 2017).

7.4.2. Exogenous UMP and CMP

A group of four patients that presented developmental delay (especially language), seizure, ataxia, motor impairments, and other autistic-associated characteristics was treated with pyrimidines UMP and CMP, demonstrating improvement in several of these features. The treatment interruption resulted in the recurrence of the symptoms, indicating that the supplement with pyrimidines may counteract a continuous catabolic status in these patients (Page et al., 1997).

8. Concluding remarks

Neurodevelopmental disorders, including ASD, are a set of conditions that affect typical brain development. The embryonic period is especially susceptible to micro and macro-environmental changes. This includes, for example, immune changes via maternal immune activation, which can onset a plethora of dysregulations in the prenatal brain that persists through life: synaptic activity alterations, mitochondrial dysfunctions, aberrant behaviors, among others. Noteworthy, purinergic signaling plays a key regulatory role in all these processes. Beyond that, considering the purinergic system contribution in several outcomes, novel therapeutic approaches have been proposed mainly by the regulation of receptors, but also by enzymatic modulation and mitochondrial intermediates. Undoubtedly, more needs to be learned regarding how purinergic intermediates contribute both to the onset and the maintaining of neuropsychiatric disorders such as ASD; however, taken together, this overview of data highlights the purinergic signaling involvement both in typical and pathological brain development, as well as a target for novel therapeutic approaches.

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Legends of Figures

Figure 1. Main findings on purinergic system dysfunctions and autism-like features. Here we provide a clear overview of changes described in cell culture, in knockout animals for constituents of the purinergic system, and in animal models of ASD, as well as in humans diagnosed with ASD, emphasizing the important contribution of P2X4, P2X7, P2Y1, and A1 receptors in the pathophysiology of ASD. **1** Freitag et al., 2009; **2** Campbell et al., 2013; **3** Luo et al., 2018; **4** Takahashi et al., 2020; **5** Braun et al., 2007; **6** Stubbs et al., 1982; **7** Persico et al., 2000; **8** Bottini et al., 2001; **9** Avedaño et al., 2015; **10** Chávez et al., 2019; **11** Garré et al., 2020; **12** Horváth et al., 2019; **13** Trang et al., 2009; **14** Montilla et al., 2020; **15** Wyatt et al., 2013; **16** Mastrangelo et al., 2012; **17** Naviaux et al., 2013 ; **18** Hirsch et al., 2020; **19** Squillace et al., 2014; **20** Zimmermann et al., 2017; **21** López-Cruz et al., 2017

Figure 2. An integrative view of the changes described in ASD. Here, we show a compilation of alterations described both in patients with ASD and in animal models, evidencing the contribution of purinergic signaling in the most diverse biological processes. 1 Ahmad et al., 2017b; **2** Ahmad et al., 2018; **3** Ansari et al., 2017b **4** Ahmad et al., 2017a; **5** Ansari et al., 2017a; **6** Jimenez-Mateos et al., 2015; **7** García-Huerta et al., 2012; **8** Conte et al., 2020; **9** Reigada et al., 2019 **10** Giulivi et al., 2013 **11** Jyonouchi et al., 2019 **12** Abdel-Rahman et al., 2020**;** **13** Singh et al., 2020**; 14** Ahn et al., 2020**; 15** Vandenberg et al., 2021; **16** Rossi et al., 2019; **17** Eltokhi et al., 2020; **18** Smith et al., 2020; **19** Stathopoulos et al., 2020; **20** Masino et al., 2009; **21** Bertelli et al., 2006; **22** Bottini et al., 2001; **23** Hettinger et al., 2008

Figure 3. An overview of the therapeutic approaches. Considering the important role of purinergic signaling in ASD pathophysiology, we summarize the main therapeutic approaches described both in ASD and associate disorders, emphasizing purinergic signaling modulation as a novel approach both in studies about pathophysiological mechanisms and innovative therapies. **ADC**: adenylate cyclase; **ENT**: Equilibrative Nucleoside Transporter; **PDE**: phosphodiesterase.
1 Ahmad et al., 2017b; **2** Ahmad et al., 2017a **3** Ahmad et al., 2018; **4** Ansari et al., 2017a **5** Ansari et al., 2017b; **6** Amodeo et al., 2018; **7** Lewis et al., 2020; **8** Behmanesh et al., 2019; **9** Naviaux et al., 2017; **10** Hirsch et al., 2020; **11** Naviaux et al., 2014; **12** Naviaux et al., 2015; **13** Tsimis et al., 2017; **14** Jimenez-Pacheco et al., 2016; **15** Engel et al., 2012; **16** Kim et al., 2011; **17** Sethna et al., 2017; **18** Mehan et al., 2020; **19** Matsuo et al., 2020; **20** Schmunk et al., 2017; **21** Reynolds et al., 2021 **22** Page et al., 1997

Table 1: Summary of all findings described in the section "7. Therapeutic Approaches and Purinergic System in ASD". In this table, the therapeutic approaches are detailed, segregated by their functions in different biological tissues or structures, including descriptions of promising results in the context of ASD. Figure 3 illustrates these findings.

Therapeutic approach	Animal model	Outcome		Reference
Adenosine and P1 receptors modulators				
CGS 21680	BTBR mice	Splenic cells	↓ $CD4^+IL\text{-}21^+$, $CD4^+IL\text{-}22^+$, $CD4^+GATA3^+$, and $CD4^+T\text{-}bet^+$ ↓ $CD14^+TLR2^+$, $CD14^+TLR3^+$, $CD14^+TLR4^+$ ↑ $CD4^+CTLA\text{-}4$	(Ahmad et al., 2017a, 2017b)
		Splenic $CD8^+$ T cells	↓ $CCR3^+$, $CCR4^+$, $CCR5^+$, $CCR6^+$, $CCR7^+$, $CXCR3^+$, $CXCR4^+$, and $CXCR5^+$	(Ahmad et al., 2018)
		Total spleen and splenic $CD4^+$ T cells	↓ $IL\text{-}2^+$, $IL\text{-}6^+$, $IL\text{-}9^+$, $IFN\text{-}\gamma^+$, and $TNF\text{-}\alpha^+$ ↑ $TGF\text{-}\beta^+$	(Ansari et al., 2017a)
		$CD4^+$ cells	↓ IL-17A, RORγt, Stat3, and pStat3 ↑ $Foxp3^+$ and $IL\text{-}10^+$ (mRNA and protein expression)	(Ansari et al., 2017b)
		Brain tissue	↓ TLR2, TLR3, TLR4, and NF-κB mRNA and protein expression ↓ mRNA of C-C and C-X-C chemokine receptors ↓ IL-2, IL-6, IL-9, IFN-γ, and TNF-α ↑ TGF-β (mRNA and protein expression)	(Ahmad et al., 2017b, 2018; Ansari et al., 2017a)
		Behavior	Improvement in learning deficits, self-grooming behavior and response to the hot plate test	(Ansari et al., 2017a; Amodeo et al., 2018)
	C58 mice	Brain tissue	↑ BDNF mRNA expression (CGS, L-741,626, and CDPPB) ↑ Fos transcripts and Fos positive cells in dorsal striatum (CGS and CPA)	(Lewis et al., 2019; Muehlmann et al., 2020)
		Behavior	↓ repetitive motor behavior (CGS, L-741,626, and CDPPB) ↓ repetitive behavior (CGS and CPA)	(Lewis et al., 2019; Muehlmann et al., 2020)
	Deer mice	Behavior	↓ stereotypy behavior (CGS and CPA)	(Tanimura et al., 2010)
Propentofylline	Human	Behavior	↓ irritability (associated with risperidone)	(Behmanesh et al., 2019)
P2 receptors modulators				
Suramin	Human	Behavior	↓ repetitive behaviors Improvement in language and social interaction	(Naviaux et al., 2017)
	Rat ASD-model induced by VPA	Brain tissue	↓ IL-6 expression in medial prefrontal cortex	(Hirsch et al., 2020)
		Behavior	Improvement sociability behavior ↓ anxiety-like behavior	
	Rat ASD-model of MIA induced by poly(I:C)	Brain tissue	Normalization of metabolic pathways disturbed Improvement in the mitochondrial respiratory chain hyperactivity abnormalities Correction of phosphorylation of ERK1/2 and (CAMKII), Prevention of Purkinje cell loss Restoration of the diminished levels of P2X7 and P2Y2 immunocontent	(Naviaux et al., 2013, 2014)

		Behavior	Amelioration in social behavior and the novelty preference Prevention in sensorimotor coordination deficits	(Naviaux et al., 2013, 2014)
	Fmr1 KO mice	Brain tissue	Normalization of abnormal synaptosomes and metabolomics alteration	(Naviaux et al., 2015)
		Behavior	Improvement in the animal performance in novelty preference, marble burying, and social behavior	
Brilliant Blue G	Rat ASD-model of MIA induced by LPS	Behavior	↓ preterm birth rate Improvement in neuromotor tests of the offspring Restoration of the density of cortical neurons	(Tsimis et al., 2017)
JNJ-47965567	*Status epilepticus* animal model	Behavior	JNJ-47965567: ↓ spontaneous seizures ↓ microgliosis and astrogliosis	(Jimenez-Pacheco et al., 2016)
A438079, OxATP, and A740003	*Status epilepticus* animal model	Behavior	A438079: ↓ seizure duration and the neuronal death A438079 (associated with OxATP and A740003): ↑ pilocarpine-induced seizure susceptibility	(Kim and Kang, 2011; Engel et al., 2012)
Adenylate cyclase modulators				
NB001	*Fmr1* KO mice	Behavior	Attenuation of social interaction deficits and stereotyped behaviors	(Sethna et al., 2017)
Diterpenoid Forskolin	Rat ASD-model induced by propionic acid	Brain tissue	Improvement in neuronal mitochondrial electron transport chain complexes Reduction in pro-inflammatory cytokines, oxidative stress, and lipid biomarkers levels	(Mehan et al., 2020)
		Behavior	Amelioration in memory dysfunction Decrease in muscle coordination and gait imbalance	
Other types of therapeutic molecules				
5-aminolevulinic acid	Rat ASD-model induced by VPA	Brain tissue	Amelioration in oxidative stress and mitochondrial dysfunction in the hippocampus Normalization of reduced parvalbumin-positive interneurons	(Matsuo et al., 2020)
		Behavior	Improvement in learning and memory	
Exogenous ATP and UTP	Primary astrocyte cultures derived from the Fmr1 KO mouse model		Elevation in intracellular Ca^{2+} responses and in synaptogenic protein TSP-1 expression	(Reynolds et al., 2021)
	Human primary skin fibroblasts from ASD patients		Decrease in Ca^{2+} release in ASD subjects	(Schmunk et al., 2017)
Exogenous UMP and CMP			Improvement in developmental delay and other autistic-associated characteristics.	(Page et al., 1997)

Figure 1

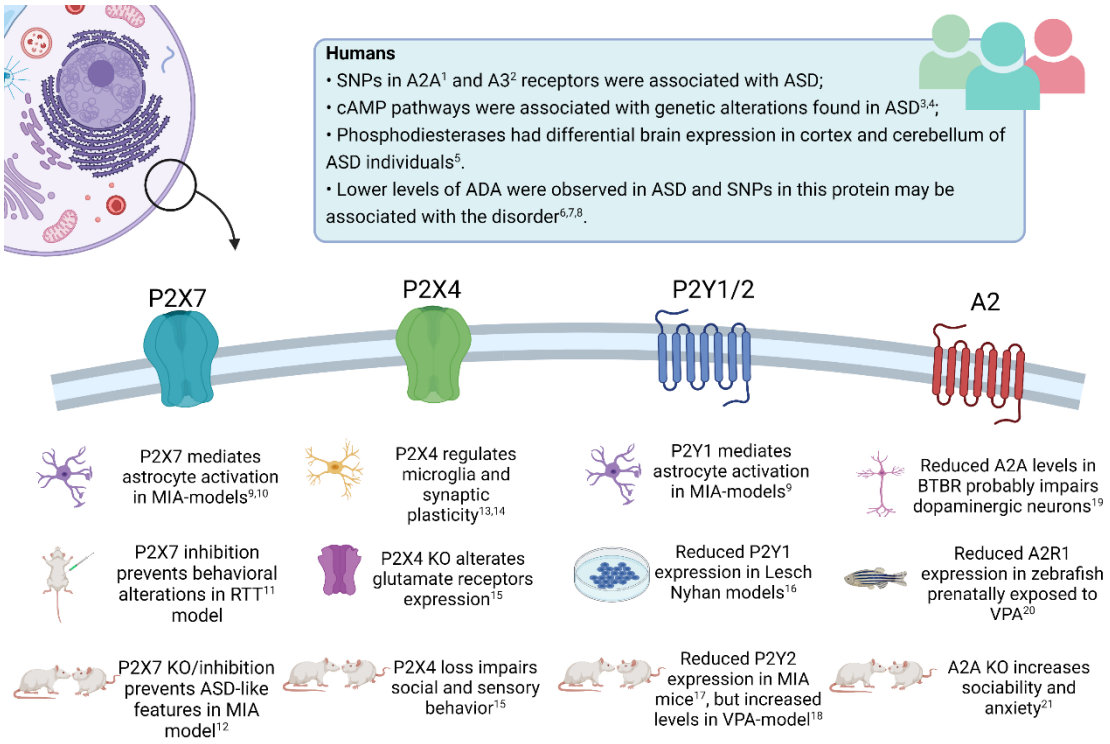

Humans
• SNPs in A2A[1] and A3[2] receptors were associated with ASD;
• cAMP pathways were associated with genetic alterations found in ASD[3,4];
• Phosphodiesterases had differential brain expression in cortex and cerebellum of ASD individuals[5].
• Lower levels of ADA were observed in ASD and SNPs in this protein may be associated with the disorder[6,7,8].
P2X7
P2X4
P2Y1/2
A2
P2X7 mediates astrocyte activation in MIA-models[9,10]
P2X4 regulates microglia and synaptic plasticity[13,14]
P2Y1 mediates astrocyte activation in MIA-models[9]
Reduced A2A levels in BTBR probably impairs dopaminergic neurons[19]
P2X7 inhibition prevents behavioral alterations in RTT[11] model
P2X4 KO alterates glutamate receptors expression[15]
Reduced P2Y1 expression in Lesch Nyhan models[16]
Reduced A2R1 expression in zebrafish prenatally exposed to VPA[20]
P2X7 KO/inhibition prevents ASD-like features in MIA model[12]
P2X4 loss impairs social and sensory behavior[15]
Reduced P2Y2 expression in MIA mice[17], but increased levels in VPA-model[18]
A2A KO increases sociability and anxiety[21]

Figure 2

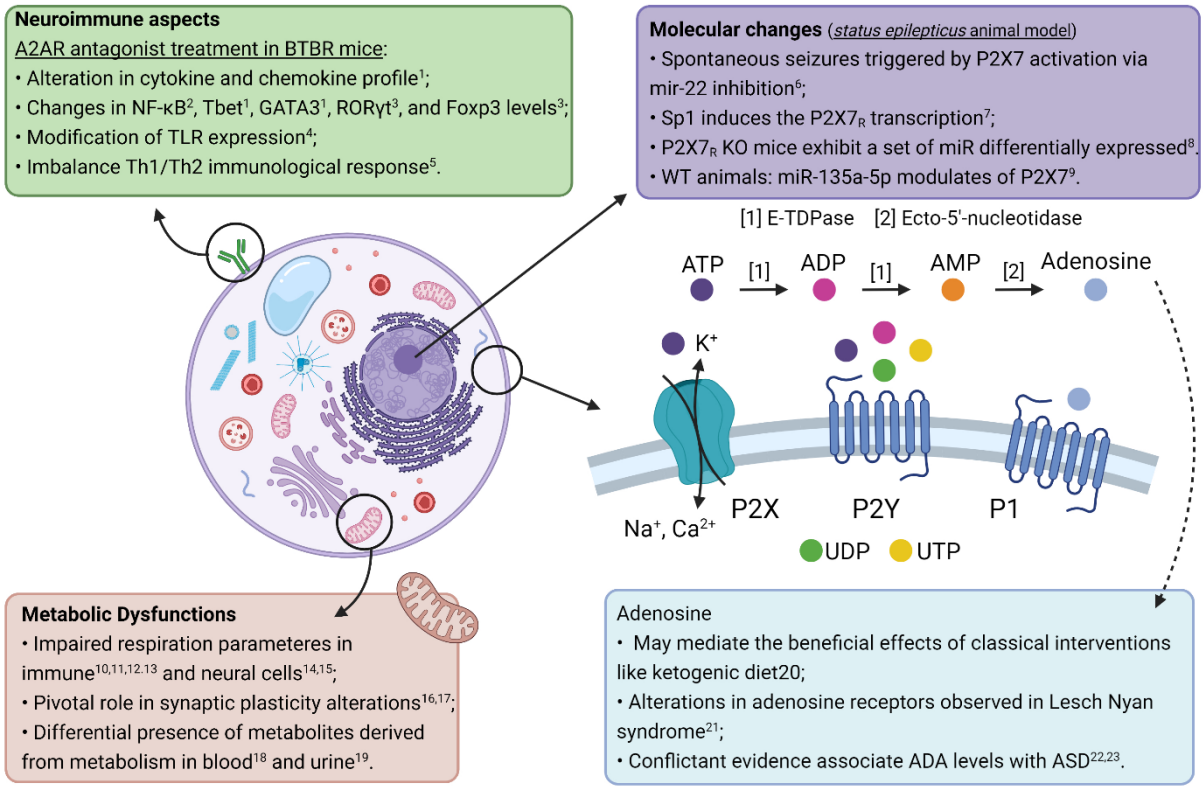
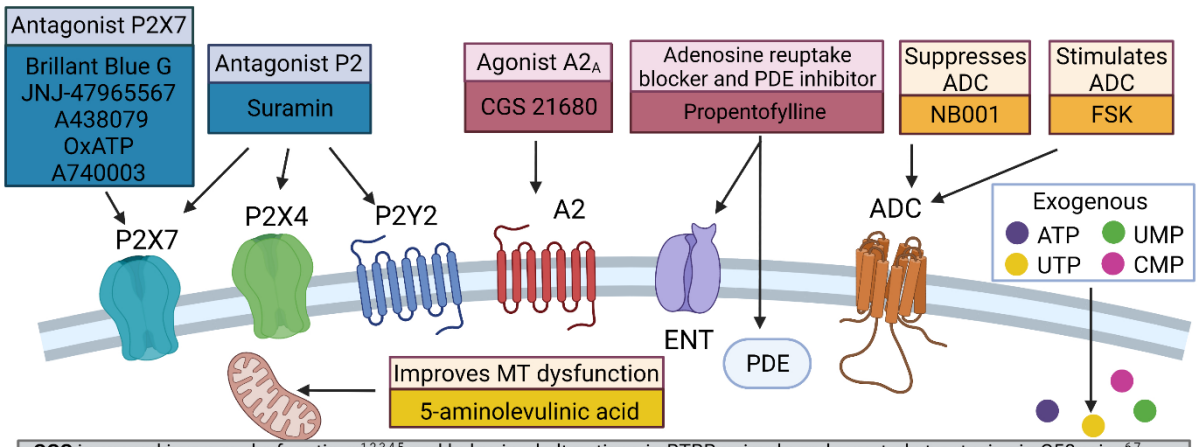

Neuroimmune aspects
A2AR antagonist treatment in BTBR mice:
• Alteration in cytokine and chemokine profile[1];
• Changes in NF-κB[2], Tbet[1], GATA3[1], RORγt[3], and Foxp3 levels[3];
• Modification of TLR expression[4];
• Imbalance Th1/Th2 immunological response[5].
Molecular changes (status epilepticus animal model)
• Spontaneous seizures triggered by P2X7 activation via mir-22 inhibition[6];
• Sp1 induces the P2X7R transcription[7];
• P2X7R KO mice exhibit a set of miR differentially expressed[8].
• WT animals: miR-135a-5p modulates of P2X7[9].
[1] E-TDPase [2] Ecto-5'-nucleotidase
ATP [1] ADP [1] AMP [2] Adenosine
K+
Na+, Ca2+
P2X
P2Y
P1
UDP
UTP
Metabolic Dysfunctions
• Impaired respiration parameteres in immune[10,11,12,13] and neural cells[14,15];
• Pivotal role in synaptic plasticity alterations[16,17];
• Differential presence of metabolites derived from metabolism in blood[18] and urine[19].
Adenosine
• May mediate the beneficial effects of classical interventions like ketogenic diet20;
• Alterations in adenosine receptors observed in Lesch Nyan syndrome[21];
• Conflictant evidence associate ADA levels with ASD[22,23].

Figure 3



- **CGS** improved immune dysfunctions[1,2,3,4,5] and behavioral alterations in BTBR animals and reverted sterotypies in C58 mice[6,7].
- **Propentofylline*** + risperidone improved irritability in ASD[8].
- **Suramin*** improved ASD-like behaviors in humans[9] and in VPA[10], Poly(I:C)[11] and Fmr KO models[12], as well as immune and metabolic features.
- **BBG** prevented behavioral and cortical cytoarchitecture impairments induced by prenatal exposure to LPS[13].
- **JNJ-47965567**[14] and **A438079**[15] reduced seizure events in epileptic mice, while **OxATP**[16], **A438079**[16], and **A740003**[16] increased seizures in other model.
- **NB001** improved ASD-like traits in *Fmr* KO mice[17].
- **FSK** improved motor deficits, mitochondrial disfunctions and immune imbalance in an ASD model[18].
- **5-aminolevulinic Acid** improved behavioral and mitochondrial dysfunctions in the VPA model[19].
- Exogenous **ATP** and **UTP** modulated astrocyte function in *Fmr* KO mice[20,21].
- Exogenous **UMP*** and **CMP*** improved several features in patients with developmental delay[22].

*** These treatments presented promising effects in ASD individuals**

PARTE III

3. DISCUSSÃO GERAL

No presente trabalho, utilizamos um dos modelos animais de autismo mais consolidados na literatura, o qual (como já ressaltado anteriormente) apresenta as validades de face (replica a sintomatologia encontrada em pacientes) e de construto (o mesmo agente etiológico reproduz a condição em humanos e modelos animais), além de possibilitar amplas investigações dentro da validade preditiva (possibilidade de desenvolvimento de estratégias terapêuticas) (MABUNGA et al., 2015).

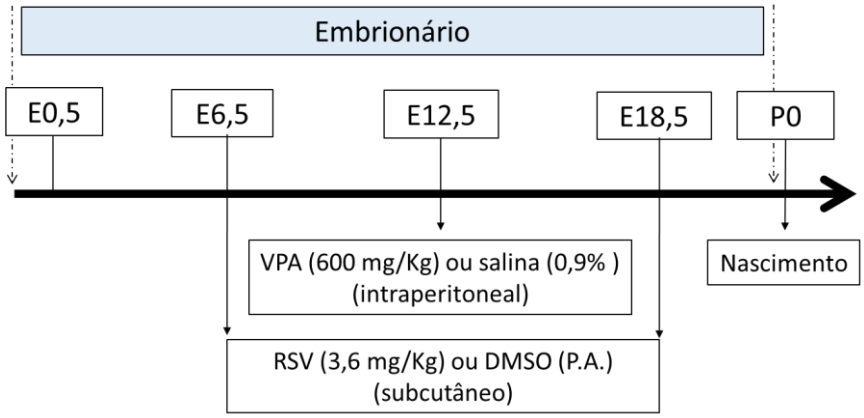
O nosso grupo de pesquisa (Grupo de Estudos Translacionais em Transtorno do Espectro Autista – GETTEA) atua em diferentes frentes para compreender a fisiopatologia do TEA sob diferentes óticas: análises em pacientes, análises *in silico*, análises *in vitro* e análises *in vivo* a partir do modelo animal VPA, sendo pioneiros na utilização dessa ferramenta de estudo no Brasil. Demonstramos previamente diversas alterações comportamentais (BAMBINI-JUNIOR et al., 2011, 2014; FONTES-DUTRA et al., 2018; HIRSCH et al., 2018), moleculares (HIRSCH et al., 2018) e celulares (FONTES-DUTRA et al., 2018; SANTOS-TERRA et al., 2021) induzidas pelo VPA bem como a prevenção dessas alterações pela administração intraútero do polifenol RSV. Sendo assim, os modelos animais se apresentam como ferramentas extremamente importantes e úteis no estudo de diversas condições, uma vez que permitem a aquisição de amostras biológicas, estudos comportamentais inviáveis em seres humanos, bem como a análise de vias biológicas e alterações metabólicas potencialmente associadas à fisiopatologia do transtorno – especialmente no TEA, que se mostra como uma desordem altamente heterogênea e de diagnóstico exclusivamente comportamental.

Nos propusemos a entender e investigar possíveis mecanismos subjacentes à macrocefalia, um relato clínico presente em, aproximadamente, 20% das crianças diagnosticadas com TEA (CHAWARSKA et al., 2011; HAZLETT et al., 2005) cuja causa ainda é desconhecida. Apesar de, na maioria dos casos, se apresentar como um evento transitório, o aumento do perímetro cefálico em idades pontuais pode estar relacionado às principais disfunções do TEA. Macrocefalia é a condição na qual o perímetro cefálico de uma criança está acima de 2 desvios-padrão, ou seja, que está acima do percentil 97. Embora usado como sinônimo de megalencefalia – designa o aumento da estrutura encefálica –, a macrocefalia é um termo amplo que abrange não só a megalencefalia, mas também outras causas de aumento do tamanho da cabeça

sem necessariamente o crescimento excessivo do cérebro, como por exemplo, acúmulo de fluido subdural (JONES; SAMANTA, 2021).

No capítulo I da presente tese são apresentados os experimentos realizados em ratos Wistar, bem como os 4 grupos experimentais que são utilizados na maioria das nossas pesquisas: 1) grupo controle (recebe apenas solução salina 0,9% e DMSO P.A. em volume equivalente – veículos para solubilidade do VPA e do RSV, respectivamente), 2) grupo RSV (recebe RSV na dose de 3,6 mg/Kg via subcutânea do dia E6,5 ao dia E18,5, e uma única injeção intraperitoneal de salina 0,9% no dia E12,5), 3) grupo VPA (recebe DMSO – veículo do RSV – via subcutânea entre E6,5 e E18,5, e uma única injeção intraperitoneal de VPA na dose de 600 mg/Kg no dia E12,5) e 4) grupo RSV+VPA (recebe RSV na dose de 3,6 mg/Kg via subcutânea do dia E6,5 ao dia E18,5, e uma única injeção intraperitoneal de VPA na dose de 600 mg/Kg no dia E12,5). Esse esquema está representado na Figura 3.

Figura 3 – Representação da linha do tempo para formação dos grupos experimentais



E: dia embrionário; P: dia pós-natal.

Primeiramente, avaliamos o conteúdo encefálico de água (também chamado de “medida de edema” na literatura) por meio da desidratação do encéfalo após retirada à fresco. A exposição pré-natal ao VPA alterou o conteúdo absoluto de água, aumentando a porcentagem de água livre no encéfalo de animais do grupo VPA, ao passo que esse fenômeno não se observa nos outros grupos. Além disso, considerando trabalhos prévios (SCHNEIDER; PRZEWŁOCKI, 2005), esperávamos encontrar um menor peso corporal dentre os animais do grupo VPA em relação aos outros grupos experimentais. Não só se confirmou, como também observamos uma

prevenção completa do RSV nesse parâmetro analisado. Por fim, ponderamos os resultados das porcentagens pelo peso corporal de cada animal, o que evidenciou ainda mais a maior proporção do volume de líquido encefálico no grupo VPA; isso significa dizer que esses animais apresentam um conteúdo encefálico maior que o grupo controle em uma estrutura corporal menor (o que se assemelha à condição clínica). Aqui, novamente, o tratamento pré-natal com RSV foi capaz de prevenir completamente o parâmetro analisado.

Um dos fatores envolvidos no desenvolvimento do edema encefálico é o dano à BHE; esse dano pode ser tanto iniciado quanto regulado por diversos mediadores pró-inflamatórios, como citocinas e quimiocinas, os quais regulam a magnitude do aporte de leucócitos no parênquima cerebral, bem como agem diretamente nas células endoteliais encefálicas, levando ao afrouxamento das junções oclusivas entre as células endoteliais e à formação de edema vasogênico (STAMATOVIC et al., 2006). A patogênese do edema encefálico o classificada como vasogênico ou citotóxico: o primeiro é definido como o acúmulo extracelular de líquido resultante da ruptura ou afrouxamento da BHE e consequente extravasamento de proteínas séricas (lesão vascular), enquanto que o segundo é caracterizado pelo inchaço celular causado pelo acúmulo intracelular de líquido (lesão de células parenquimatosas) (MICHINAGA; KOYAMA, 2015). No caso do TEA, ainda não é elucidado qual tipo é predominante, embora seja mais provável que envolva mecanismos de lesão vascular.

Sendo assim, demos sequência aos experimentos avaliando a permeabilidade da BHE a ao corante azul de Evans. A exposição pré-natal ao VPA causou nítido aumento na permeabilidade da BHE ao corante (evidenciado pela intensidade da fluorescência vermelha nas imagens representativas) principalmente nas regiões encefálicas pertencentes ao neocórtex (área somatossensorial – camadas 2/3 e 4/5 – e todas as sub-regiões do córtex pré-frontal medial – cingulado anterior, límbico e infralímbico, tanto em camadas profundas quanto em superficiais), mas também no plexo coroide (em contato com o neocórtex). Hipocampo e amígdala não apresentaram alterações de permeabilidade. Em todas as regiões onde houve alteração, o tratamento com o RSV foi capaz de prevenir completamente a permeabilidade da BHE ao corante, conforme observado no grupo RSV+VPA. Anteriormente, apenas Kumar e colegas (KUMAR; SHARMA; SHARMA, 2015; KUMAR; SHARMA, 2016a, 2016b) haviam demonstrado aumento de permeabilidade

a esse corante no modelo VPA, porém de forma macroscópica; aqui, refinamos a técnica demonstrando que as alterações são região-específica, bem como são passíveis de serem prevenidas pelo tratamento com o RSV. Apesar desse importante resultado, trata-se de uma técnica bastante inespecífica e com pouca precisão acerca de pormenores envolvidos no aumento da permeabilidade da BHE, uma vez que o corante azul de Evans não é metabolicamente inerte, é tóxico em altas dosagens e liga-se à albumina. Não existe um único marcador adequado para avaliação de BHE e todos os métodos conhecidos apresentam vantagens e desvantagens. No entanto, uma combinação de dextranos visualizáveis de diferentes tamanhos e moléculas radiomarcadas atualmente parece ser a abordagem mais apropriada para uma avaliação qualitativa e quantitativa mais robusta da integridade da BHE (SAUNDERS et al., 2015).

Seguindo nessa linha de raciocínio, investigamos a expressão e distribuição de AQP em diferentes regiões do SNC. Essas proteínas-canais desempenham diversos papéis, facilitando o movimento de água tanto para dentro quanto para fora do SNC (ROSU et al., 2019). A AQP1 apresentou um perfil diminuído em praticamente todas as regiões avaliadas no grupo VPA: plexo coroide, camadas profundas da área somatossensorial primária, região da amígdala e todas as sub-regiões do córtex pré-frontal medial. O plexo coroide é uma região particularmente importante na produção e liberação do LCR: animais nocaute para AQP1 apresentam redução de até 25% na taxa de secreção de LCR (OSHIO et al., 2003). Nessa região também observamos uma alteração morfológica bastante significativa em ambos os grupos expostos ao VPA prenatalmente. Uma lesão semelhante foi observada em um modelo de edema isquêmico cerebral (AKDEMIR et al., 2016), mas no presente estudo consideramos que não haja qualquer associação entre esta alteração morfológica e a fisiopatologia do TEA, o que pode ser simplesmente um efeito teratogênico *per se* do VPA.

Já a AQP4 apresentou perfis diversos de forma região-dependente: observou-se aumento no grupo VPA na área somatossensorial primária nas camadas mais profundas, com prevenção pelo RSV, além de uma diminuição em todas as sub-regiões do córtex pré-frontal medial, sem prevenção pelo RSV. Previamente, já havia sido demonstrado alterações no perfil de AQP4 em tecido encefálico *post mortem* de indivíduos com TEA. Um estudo de 2008 demonstra diminuição de 3% na área 9 de Brodmann (BA9 - equivalente ao córtex frontal), bem como uma expressão aumentada

de 39% no BA40 (córtex parietal, onde está localizada a área sensorial primária). Além disso, os autores observaram aumento da expressão da conexina 43 (CX43 - a principal proteína componente das *gap junctions* astrocíticas) no BA9, o que poderia significar aumento da sinalização neurônio-glia, indicando um aprimoramento da comunicação célula-célula no lobo frontal (uma área integrativa) (FATEMI et al., 2008). Ainda, a AQP4 tem sido relacionada ao sistema neuroimunológico (IKESHIMA-KATAOKA, 2016), o que representa um achado interessante no contexto de TEA, uma vez que o componente imunológico desse distúrbio está bem estabelecido (GOTTFRIED et al., 2015). Finalmente, estudos têm relacionado o nocaute de AQP4 tanto com redução no inchaço encefálico no edema citotóxico quanto com piora significativa de edema encefálico vasogênico (PAPADOPOULOS; VERKMAN, 2007).

A mudança de expressão região-dependente chama atenção por si só, mas também pelo fato de a AQP4 ser um canal colocalizado com os canais Kir4.1 (STROHSCHEIN et al., 2011) nos pés astrocíticos. Eventos como neuroinflamação aguda contribuem para a reorganização sináptica, resultando em alterações de longo prazo em relação à hiperexcitabilidade de todo o circuito neural (CLARKSON et al., 2017). Além disso, uma das principais teorias acerca da fisiopatologia do TEA envolve o desequilíbrio entre o delicado balanço excitatório-inibitório em algumas regiões específicas do SNC levando a alterações eletrofisiológicas. A presença de epilepsia ou episódios de convulsão em aproximadamente 30% dos indivíduos com TEA reforça o caráter excitatório predominante no TEA (SPENCE; SCHNEIDER, 2009). A atividade neuronal ocorre dentro da faixa fisiológica específica a partir de uma concentração extracelular de K^+ basal. O K^+ extracelular é crítico na definição do potencial de repouso da membrana de neurônios e astrócitos e, durante uma atividade excessiva, como em uma convulsão, seus níveis atingem um platô, o que influencia a transmissão sináptica e a plasticidade. Uma vez atingido esse platô, mesmo durante a atividade elétrica contínua, não ocorre aumento da concentração de K^+ devido ao mecanismo de remoção desse íon da fenda sináptica por meio da recaptação de K^+ pela glia via canais como o Kir4.1 (OLSEN et al., 2015), o que é vital para manter a homeostase cerebral (BELLOT-SAEZ et al., 2017). Provavelmente, a AQP4 é necessária para manter a depuração de K^+ eficiente, uma vez que alterações do fluxo de água são associadas a aumento da intensidade de convulsões epilépticas (AMIRY-

MOGHADDAM et al., 2003) e atraso no tamponamento de K^+ em camundongos nocaute para AQP4 (LU et al., 2008).

Infelizmente, por questões de pandemia, não foi possível analisar a expressão dos canais Kir4.1 no modelo VPA. Dessa forma, o nosso último experimento investigativo foi a avaliação tanto da imunomarcação quanto do número de astrócitos $GFAP^+$. Observamos aumento tanto da imunofluorescência de GFAP quanto do número de astrócitos $GFAP^+$ no córtex pré-frontal medial e na área somatossensorial primária pela exposição pré-natal ao VPA, o que corrobora com estudos anteriores que mostram ativação neuroglial em pacientes com TEA e modelos animais (BRISTOT SILVESTRIN et al., 2013; EDMONSON; ZIATS; RENNERT, 2014; VARGAS et al., 2005; ZHAO et al., 2019). Aqui, observamos um importante efeito preventivo do RSV nas camadas profundas das sub-regiões do córtex pré-frontal medial analisadas, retornando os níveis da imunofluorescência de GFAP para os níveis do grupo controle. Com base em dados anteriores, que indicam o efeito neuroprotetor de doses mais baixas de RSV em fatias de hipocampo (através da melhora da captação de glutamato pelos astrócitos e modulação da plasticidade sináptica) (BOBERMIN et al., 2012; DE ALMEIDA et al., 2008; QUINCOZES-SANTOS et al., 2013; QUINCOZES-SANTOS; GOTTFRIED, 2011) e melhora no quadro de neuroinflamação (uma marca registrada do TEA) em um modelo animal de TEA (AHMAD et al., 2018c; BHANDARI; KUHAD, 2017), seria possível que, também neste contexto, tenhamos um efeito benéfico sobre o metabolismo e função dos astrócitos, uma vez que este tratamento é eficaz na melhoria de diversos comprometimentos comportamentais em modelo animal de VPA (BAMBINI-JUNIOR et al., 2014; FONTES-DUTRA et al., 2018; HIRSCH et al., 2018).

Apesar de alguns estudos não terem demonstrado alterações nos parâmetros de astrócitos em tecidos *post mortem* de pessoas com TEA (LEE et al., 2017; MORGAN et al., 2014), os modelos animais da Síndrome do X Frágil (distúrbio com alta sobreposição ao TEA) apresentam uma perturbação específica na constituição das camadas corticais mais profundas, apresentando aumento do número de astrócitos (LEE et al., 2019). Essa dinâmica da desorganização cortical é amplamente descrita no TEA. Um dos achados mais relevantes em pacientes com TEA é a identificação de distúrbios na organização das minicolunas corticais (CASANOVA, 2007) e a presença de manchas com perda de delimitação das camadas corticais

(DENARDO et al., 2015; STONER et al., 2014), sendo as camadas mais profundas as mais afetadas.

Curiosamente, o hipocampo e a região da amígdala parecem ser mais vulneráveis aos efeitos do RSV quando se trata de astrócitos; especulamos que talvez a janela biológica de origem embrionária dessas estruturas possa ter participação no resultado obtido. Os núcleos amigdalares se originam em momentos diferentes entre os dias E10-E12 em ratos, antes da indução do modelo animal (em E12.5) e durante o tratamento pré-natal com RSV (entre E6.5 a E18.5) (SOMA et al., 2009). Sobre o hipocampo, embora sua origem embrionária seja em torno de E15 (HAYASHI et al., 2015), os efeitos da exposição pré-natal ao VPA parecem ser progressivos e de início tardio (SANTOS-TERRA et al., 2021). Mesmo o RSV apresentando importantes efeitos neuroprotetores, nesse caso, ele foi capaz de desempenhar um efeito *per se* no hipocampo no dia pós-natal (P) P30. Nós interpretamos esse resultado como algo positivo, considerando que o RSV talvez esteja desenvolvendo um *background* celular prévio para suportar melhor o dano progressivo induzido pelo VPA, uma vez que esse efeito progressivo se acentua na idade adulta.

Frequentemente, são descritas na literatura alterações na espessura cortical em indivíduos com TEA. Poderia ser uma das hipóteses relacionadas à macrocefalia transitória na primeira infância se esse dado não fosse tão controverso. Apesar de estudos demonstrarem que não há diferenças na espessura cortical entre indivíduos com TEA e indivíduos com neurodesenvolvimento típico entre 6 e 30 anos de idade (NUNES et al., 2020), há relatos de maior espessura cortical a partir da mesma faixa etária em crianças com esse transtorno, com diferenças diminuindo durante a idade adulta e a gravidade dos sintomas relacionados ao afeto social e à comunicação associada a essas anormalidades corticais (KHUNDRAKPAM et al., 2017). Já em adolescentes com alta funcionalidade, há relatos de diminuição da espessura cortical no lobo frontal inferior direito (correlacionado com maior prejuízo social), aumento da espessura cortical no lobo temporal direito e cíngulo posterior (associadas a piores escores no domínio de comunicação) e maiores áreas de superfície cortical em várias regiões do encéfalo, incluindo o cíngulo, lobos temporais e amígdala, bem como aumento da girificação em regiões associadas com a codificação de memórias visuais e áreas do componente sensório-motor (PEREIRA et al., 2018).

Especula-se também que o crescimento excessivo encefálico em crianças com TEA envolva aumento no número e no tamanho de neurônios no córtex pré-frontal. Numa avaliação *post mortem* do córtex pré-frontal de 7 indivíduos com TEA com idades entre 2 e 16, constatou-se um aumento de 67% no número de neurônios em comparação com indivíduos neurotípicos (COURCHESNE et al., 2011). A amígdala parece exibir o mesmo perfil de alteração (em amostra *post mortem* de indivíduos entre 2-48 anos), com aumento excessivo no número de neurônios maduros seguido por um declínio na idade adulta à medida que os neurônios imaturos são incorporados aos núcleos amigdalares, propondo um componente degenerativo com o passar do tempo (AVINO et al., 2018). Sabe-se que a AIM regula positivamente a expressão gênica do ciclo celular e a proliferação celular e pode causar crescimento excessivo do encéfalo (LOMBARDO et al., 2018; OSKVIG et al., 2012; SMITH; ELLIOTT; ANDERSON, 2012), podendo induzir a produção excessiva de neurônios (SMITH; ELLIOTT; ANDERSON, 2012), aumentar a espessura cortical (LE BELLE et al., 2014; SMITH; ELLIOTT; ANDERSON, 2012) e o tamanho do cérebro (LE BELLE et al., 2014). Além disso, as alterações de expressão gênica também envolvem processos de migração neuronal (OSKVIG et al., 2012), o que pode estar por trás da estratificação cortical anormal observada, com um aumento de 24% no número de neurônios nas camadas corticais 2/3 (SOUMIYA; FUKUMITSU; FURUKAWA, 2011). Curiosamente, em nossos estudos prévios, não encontramos alterações no número de neurônios totais nem no número de células não-neuronais em animais de 30 dias, ao passo que foi visto apenas intensa desorganização laminar entre as camadas com provável defeito de migração celular na área somatossensorial primária e região da amígdala (FONTES-DUTRA et al., 2018), o que nos leva a pensar que, pelo menos em animais, os mecanismos adjacentes à formação de edema não passam por aumento de celularidade (e consequentemente apoptose) e provavelmente envolvem mecanismos menos complexos, como aumento de líquido intracraniano como consequência do afrouxamento da BHE, que leva ao infiltrado inflamatório oriundo da periferia em direção ao SNC.

Considerando todos os dados, levantamos a hipótese de que as deficiências encefálicas induzidas pelo modelo VPA incluem um fundo de neuroinflamação desencadeado no encéfalo em desenvolvimento do embrião, o que contribui para o aumento da permeabilidade da BHE (e, consequentemente, edema devido à entrada

de água e infiltrado inflamatório). Em consequência, há uma diminuição nos níveis de AQP1 e AQP4 para manter a homeostase da água no cérebro. Paralelamente, a neuroinflamação desencadeia o processo de excitotoxicidade, levando a um fenótipo astrocítico reativo. O aumento da atividade astrocítica leva a um aumento da necessidade de tamponamento de K^+, que por sua vez aumenta os níveis de Kir4.1 e, consequentemente, de AQP4 de uma maneira específica da região (que, por sua vez, poderia ser o principal início da formação de edema cerebral). A impressão digital causada pelo VPA ocorre em várias regiões; uma vez que a área somatossensorial primária é uma área de processamento primário, tanto o impacto causado pelo VPA quanto os mecanismos de prevenção pelo RSV podem ser mais expressivos e menos complexos do que os que ocorrem no córtex pré-frontal medial, uma região associativa e mais complexa. Aqui, o RSV preveniu com sucesso as deficiências em relação à formação de edema, à permeabilidade da BHE, ao aumento de AQP4 na área somatossensorial primária, bem como possibilitou uma melhora funcional na reatividade glial no córtex pré-frontal medial. Desse modo, com base em evidências que apontam para o RSV como um estabilizador do ambiente neural, o RSV também poderia normalizar os níveis de K^+ e reestruturar as conexões sinápticas na área somatossensorial primária considerando a colocalização dos canais AQP4 e Kir4.1.

Um tema bastante recorrente nos estudos sobre TEA são as alterações neuroimunológicas, conforme abordado no Capítulo II da presente tese. Sabe-se que a AIM desencadeia diversas desordens neuropsiquiátricas, entre elas, TEA (ESTES; MCALLISTER, 2016); complementarmente, a literatura mais recente em modelos animais vem destacando a associação de processos inflamatórios maternos durante períodos críticos do desenvolvimento embrionário com crescimento excessivo do encéfalo e desencadeamento de comportamentos associados ao autismo na prole, possivelmente por meio de sinalização redox alterada em células-tronco e progenitores neurais (LE BELLE et al., 2014). Nesse sentido, para compreendermos melhor como as alterações imunológicos se apresentam dentro da fisiopatologia do TEA, foi necessário um extenso compilado da literatura, principalmente devido à carência de informações translacionais acerca do componente imunológico em modelos animais e em pacientes com TEA. Nesse sentido, escrevemos uma revisão contendo não só um levantamento das principais alterações reportadas como também comparando quais alterações são comuns entre humanos e o modelo animal VPA

separando por tipo de amostra biológica, com o objetivo de facilitar estudos posteriores do grupo e de outros pesquisadores sobre a neuroimunologia do TEA. Foi observado que o aumento de IL-6 e de TFN-α são alterações comuns tanto ao modelo animal quanto a estruturas encefálicas em humanos, bem como o aumento nos níveis de IL-6, IL-8, IL-10, TNF-α, IFNγ e TGFβ1 são achados tanto em amostras encefálicas quanto tecidos periféricos em humanos (representado na Figura 2 da revisão presente no capítulo II). Dessa forma, postulamos que a impressão digital causada pela exposição pré-natal ao VPA na prole poderia envolver mecanismos de AIM, uma vez que os animais do modelo animal VPA apresentam níveis aumentados de IL-1β, IL-6 e TFN-α no hipocampo e outras regiões do cérebro (DECKMANN et al., 2018), além de níveis aumentados de TFN-α e ativação microglial após a exposição pré-natal ao VPA (ZAMBERLETTI et al., 2019).

Apesar dos muitos mecanismos já elucidados, a fisiopatologia do TEA ainda permanece um quebra-cabeças longe de ser solucionado. Sabe-se que algumas vias biológicas estão intimamente relacionadas ou com o provável desencadeamento ou com a manutenção do transtorno. São alguns exemplos as vias:

- Da NOTCH, a qual já foi associada a regulação de processos inflamatórios (PARK et al., 2015), bem como se mostrou alterada tanto em modelo VPA (ZHANG et al., 2019) quanto em BTBR (AHMAD et al., 2021);
- Da PTEN, amplamente associada ao TEA (ABDELLI; SAMSAM; NASER, 2019; HOOSHMANDI; WONG; KHOUTORSKY, 2020; SHIN; SANTI; HUANG, 2021). Mais recentemente, a diminuição de seus níveis vem sendo correlacionada com o aumento nos níveis do coativador-1 'alfa' do receptor ativado por proliferador do peroxissoma (PGC-1α) e ciclo-oxigenase (COX) IV (e consequente disfunção mitocondrial) em hipocampo e córtex de camundongos do modelo animal VPA. O provável mecanismo é de que a PTEN pode desfosforilar AKT para inibir sua atividade, levando à diminuição da fosforilação da glicogênio sintase cinase 3 beta (GSK-3β, a qual fica ativada), aumentando a fosforilação de PGC-1α para promover sua degradação (FENG et al., 2021);
- Da GSK-3β, a qual parece ser crítica para a plasticidade sináptica nas sinapses glutamatérgicas na amígdala (WU et al., 2017) e está relacionada ao comportamento social, uma vez que seus níveis alterados encontrados na

amígdala (WU et al., 2017) e no córtex cingulado anterior (HOU et al., 2021; WANG et al., 2019) foram correlacionados com alterações de sociabilidade. Além disso, a nível molecular, a exposição pré-natal ao VPA aumenta a expressão de Wnt e ativa a via GSK-3β/β-catenina, o que foi correlacionado com macrocefalia e estruturas encefálicas alteradas observadas em encéfalos de animais do modelo VPA (GO et al., 2012);

- Da Wnt, sendo essa proteína crucial na sinalização de diversas outras proteínas das cascatas, como GSK-3β, β-catenina e mTOR. Quando expostos ao VPA, os animais apresentaram ativação da sinalização da Wnt por meio da regulação positiva da β-catenina e da fosfo-GSK-3β. Essa via, bem como a ativação da sinalização de mTOR, foram suprimidas após um tratamento com anti-inflamatório (QIN; DAI; YIN, 2016). O mesmo estudo também observou que a exposição ao VPA ativou a sinalização de mTOR e suprimiu a autofagia no córtex pré-frontal, hipocampo e cerebelo de ratos do modelo animal de TEA, caracterizada por fosfo-mTOR e fosfo-S6 aumentados, diminuição de Beclina-1, Atg5, Atg10, LC3-II e formação de autofagossomos;
- Além de outras sinalizações, como o caso do sistema purinérgico (conforme abordado no Capítulo III da presente tese).

Sabe-se que o desenvolvimento encefálico na vida embrionária é finamente regulado por uma variedade de processos biológicos; sinalizações como, por exemplo, a purinérgica, desempenham um papel essencial na organização tanto do desenvolvimento embrionário e fetal quanto na organogênese de forma tempo-dependente, controlando moléculas de sinalização purinérgica, como ATP, a liberação de Ca^{2+} da glia radial, expressão diferencial de subtipos de receptores, entre outros (FUMAGALLI et al., 2017; ULRICH; ABBRACCHIO; BURNSTOCK, 2012). No caso do TEA, já foram identificados polimorfismos de nucleotídeo único (SNP) nos genes dos receptores de adenosina A2A (ADORA2A) (FREITAG et al., 2009) e A3 (ADORA3), induzindo níveis aumentados de cGMP e resultando no aumento da atividade do transportador de serotonina (CAMPBELL et al., 2013). Também foi identificado, em tecido *post mortem*, enriquecimento da ontologia gênica para genes de sinalização purinérgica em um grupo de genes associados ao comportamento social prejudicado (GINSBERG et al., 2012), associação entre a atividade da adenilil ciclase e concentração de AMP cíclico (AMPc) com traços de TEA (TAKAHASHI et al., 2020),

além de presença diferencial de metabólitos derivados do metabolismo da purina na urina de indivíduos com TEA, indicando o impacto geral do metabolismo purinérgico nesse transtorno (GEVI et al., 2016). Por fim, a expressão de fosfodiesterases (PDE) demonstrou resultados diferenciais, estando alguns subtipos reduzidos em cerebelo e outros aumentados em BA9, o que é importante uma vez que essas enzimas regulam os níveis de AMPc, um segundo mensageiro na sinalização purinérgica (BRAUN et al., 2007).

Em modelos animais de TEA, a sinalização purinérgica também já se demonstrou alterada. Em modelo de AIM, o aumento da abertura dos canais Cx43 e Panx1 nos astrócitos do hipocampo foi associado à liberação de ATP e glutamato, os quais aumentaram os níveis de Ca^{2+}, levando a um perfil ativado em astrócitos e morte celular em neurônios (AVENDAÑO et al., 2015; CHÁVEZ et al., 2019). Quando ATP exógeno foi administrado, os animais exibiram um amplo espectro de alterações, incluindo características semelhantes às do TEA (ZOLKIPLI-CUNNINGHAM et al., 2021). Na cepa de camundongos BTBR, um modelo genético de TEA, observou-se diminuição da função do receptor A2A (SQUILLACE et al., 2014). Outro modelo genético de TEA, por deleção de um regulador de PDE4, induziu déficits de memória espacial e níveis reduzidos de AMPc no hipocampo (ZAMARBIDE et al., 2019). Por fim, no modelo VPA, foi observada aumento da hidrólise de AMP, da expressão de RNAm de A2R1 e do catabolismo de ATP/ADP no encéfalo de peixe-zebra (ZIMMERMANN et al., 2017), enquanto que em roedores foi observado aumento da razão AMP/ATP no mesencéfalo e redução da mesma no córtex – induzindo um desequilíbrio de sinalização purinérgica (HEGAZY; ALI; ELGOLY, 2015) –, bem como aumento da expressão dos receptores P2X4 e P2Y2 em hipocampo e P2X4 no córtex pré-frontal medial (HIRSCH et al., 2020).

Ainda sobre a influência da sinalização purinérgica em mecanismos subjacentes à fisiopatologia do TEA, quando há liberação de ATP extracelular de origem mitocondrial, ocorre uma sinalização indicando dano liberada pelas células sob estresse, uma vez que esse ATP pode desencadear processos inflamatórios (CHAN; GOLD; VON AHSEN, 2011; FAAS; SÁEZ; DE VOS, 2017) e induzir reações autoimunes (THEOHARIDES; ASADI; PATEL, 2013; ZHANG et al., 2012), o que é bem característico do TEA. De forma complementar, crianças com TEA apresentam níveis elevados de DNA mitocondrial circulante (PICARD et al., 2014); dessa forma, o

metabolismo energético mitocondrial comprometido (presente no TEA) tem sido proposto como um dos fatores desencadeadores desse transtorno (PASTURAL et al., 2009; PATOWARY et al., 2017). Além disso, os receptores purinérgicos do tipo P2X7 regulados por ATP estão emergindo como importantes reguladores da neuroinflamação e como novos alvos terapêuticos (DI VIRGILIO, 2015). Como os P2X7 possuem baixa afinidade por ATP, são considerados importantes sensores de danos e inflamação teciduais – condições nas quais os níveis de ATP extracelular aumentam consideravelmente – e sua ativação desencadeia respostas imunológicas que medeiam a maturação e a secreção de interleucinas, como a IL-1β (DENLINGER et al., 2001; SURPRENANT et al., 1996).

Em um estudo *in vitro* a partir da cocultura de astrócitos e células endoteliais (simulando as condições da BHE) foi observado que a ativação do receptor P2X7 por ATP induziu a liberação de IL-1β, a qual aumentou a atividade da metaloproteinase de matriz 9 (MMP-9) que por sua vez promoveu a ruptura da BHE pela degradação das proteínas de junção oclusiva ZO-1 e ocludina (YANG et al., 2016). A MMP-9 é uma enzima com atividade proteolítica zinco-dependente com habilidade em degradar o colágeno do tipo IV que compõe a lâmina basal cujos níveis aumentados já foram correlacionados com distúrbios do neurodesenvolvimento, incluindo TEA (REINHARD; RAZAK; ETHELL, 2015).

O receptor P2X7 parece estar intimamente relacionado com a funcionalidade da BHE. Substâncias como o 3,4-metilenodioximetamfetamina (*ecstasy*) são capazes de alterar a permeabilidade da BHE por meio do receptor P2X7, que por sua vez leva ao aumento da atividade de MMP-9 e MMP-3 e degradação da matriz extracelular (RUBIO-ARAIZ et al., 2014). Também foi relatado que a supressão do receptor P2X7 foi capaz de preservar a integridade da BHE após hemorragia intracerebral por meio da inibição da ativação do membro da família Ras homólogo A (RhoA, do inglês *Ras homolog family member A*) (ZHAO et al., 2016). Além disso, o receptor P2X7 localizado nos pericitos (co-expresso com PDGFβR, um marcador de pericitos) contribuiu para os mecanismos patológicos subjacentes à encefalomielite autoimune experimental em microvasos encefálicos influenciando a integridade da BHE por meio da diminuição da expressão de claudina-5 (GRYGOROWICZ; DĄBROWSKA-BOUTA; STRUŻYŃSKA, 2018).

Interessantemente, parece que os receptores purinérgicos são necessários para o desencadeamento do fenótipo do tipo autista, uma vez que tanto a prole originada de mães heterozigotas ou nocaute para P2X7 quanto fêmeas tratadas com um antagonista P2X7 específico não apresentaram quaisquer características associadas ao TEA, enquanto os tipos selvagens (*wild-type*) oriundos do modelo de AIM apresentaram alterações como déficits de sociabilidade, estereotipias, aumento do conteúdo encefálico de IL-6, CXCL1 e TNF-α, malformação de sinapse, interrupção da laminação cortical, entre outros (HORVÁTH et al., 2019). Tomadas em conjunto, essas evidências demonstram o envolvimento da desregulação purinérgica tanto no desencadeamento quanto na manutenção do TEA.

Ao longo dos anos, diversas estratégias terapêuticas têm sido propostas como ferramentas de estudo com o intuito de estudar os prováveis mecanismos envolvidos na fisiopatologia do TEA. No cenário nutracêutico, os polifenóis têm recebido grande atenção da comunidade científica devido ao seu potencial farmacológico; especialmente o RSV tem emergido como potencial estratégia devido às suas excelentes propriedades terapêuticas antioxidante e anti-inflamatória (VANG et al., 2011), agindo como neuroprotetor provavelmente contrapondo os efeitos pró-oxidantes e pró-inflamatórios do VPA. Juntamente a isso, é capaz de atenuar disfunções de BHE e reverter o acúmulo encefálico de água em modelo animal de isquemia por meio da regulação da MMP-9 (WEI et al., 2015). Outros mecanismos pelos quais o RSV provavelmente age como antioxidante e anti-inflamatório já foram propostos, como a via do ácido araquidônico (LI et al., 2018), fator nuclear kappa B (NF-κb) (FENG et al., 2016), proteína quinase ativada por mitogênio (MAPK) (YE; MENG, 2021) e proteína ativadora-1 (AP-1) (THIEL; RÖSSLER, 2014). Além disso, previne o dano oxidativo celular por meio da via do fator nuclear 2 relacionado ao eritroide-2 (Nrf2, do inglês, *nuclear factor erythroid 2–related factor 2*) (GAO et al., 2018; YANG et al., 2018), bem como tem ação na via SIRT1/AMPK e Nrf2 (MENG et al., 2016).

Por ser um nutracêutico com amplos efeitos benéficos já demonstrados, recentemente propusemos um estudo-piloto (MARCHEZAN *et al*., *manuscrito submetido*, Anexo 3 da presente tese) para avaliação da eficácia e segurança da suplementação de 200 mg de RSV por dia em crianças diagnosticadas com TEA de acordo com o DSM-V e recrutadas no Hospital de Clínicas de Porto Alegre (HCPA).

Cinco meninos com idades entre 10 e 13 anos compuseram o *n* amostral após assinatura do Termo de Consentimento Livre e Esclarecido pelos pais/responsáveis (FIPE-HCPA 170132/ CAAE 65862917100005327/ ReBEC número 6356). Os indivíduos receberam 200 mg de RSV diariamente durante 90 dias e foram avaliados antes, 45 dias e 90 dias após o início do tratamento. Foram aplicadas as escalas CGI (Impressão Clínica Global, do inglês *Clinical Global Impression*) e ABC (Lista de Verificação de Comportamento Aberrante, do inglês *Aberrant Behavior Checklist*). Além disso, também foi realizada a coleta de sangue periférico para avaliação de um perfil de microRNAs em células mononucleares do sangue periférico.

Nesse estudo, fomos os primeiros a mostrar que o tratamento de 90 dias com RSV foi capaz de melhorar substancialmente a pontuação de 3 dos 5 indivíduos na escala CGI. Já na escala ABC, houve uma diminuição significativa na pontuação total após o tratamento com RSV, sendo a subescala de irritabilidade a com maior diminuição significativa. A irritabilidade pode se manifestar de diversas formas, incluindo agressão, comportamento autolesivo e raiva, o que leva a dificuldades na interação social desses indivíduos. Curiosamente, todos os pacientes apresentaram um aumento estatisticamente significativo na expressão relativa do miR-195-5p após 90 dias de tratamento com RSV, o qual é envolvido em rotas relacionadas ao sistema imunológico, transdução de sinal, expressão gênica (transcrição), ciclo celular, morte celular programada, transporte de pequenas moléculas, doenças e respostas celulares a estímulos externos. Assim, um tratamento seguro, eficaz e sem efeitos colaterais capaz de modular o comportamento de irritabilidade (e, consequentemente, sociabilidade) poderia, sem dúvida, contribuir para uma melhora geral na funcionalidade desses indivíduos.

Cabe ressaltar que ainda existem muitos questionamentos em relação a utilização de suplementos à base de antioxidantes na dieta, principalmente em relação à suplementação em gestantes. No nosso modelo animal investigativo, o tratamento com RSV em ratas prenhes configura uma ferramenta de estudo, não sendo proposta de fato uma translacionalidade para suplementação em gestantes. Se em modelos animais essa administração se mostra segura e eficaz, em humanos poderia se supor o mesmo. No entanto, as gestantes são consideradas “cientificamente complexas” de serem incluídas em estudos, refletindo uma combinação de complexidade fisiológica e ética (AMERICAN COLLEGE OF OBSTETRICIANS AND GYNECOLOGISTS,

2015). As questões éticas envolvem a necessidade de equilibrar os interesses da gestante e do bebê em desenvolvimento, o que, em geral, está alinhado entre si, pois a saúde de um reflete a saúde de outro; no entanto, os interesses científicos podem divergir, especialmente quando a pesquisa que não está focada em questões da gestação, parto ou saúde fetal (AMERICAN COLLEGE OF OBSTETRICIANS AND GYNECOLOGISTS, 2015). Devido a preocupações éticas sobre a exposição de mulheres grávidas e fetos aos riscos de pesquisas científicas, a gestação é considerada um motivo para exclusão de estudos de pesquisa, mesmo quando os riscos são insignificantes e o estudo aborda questões relevantes para a saúde materna ou fetal (LYERLY; FADEN, 2013).

Nos últimos anos, diversos estudos descrevem os potenciais benefícios à saúde da dieta com polifenóis, descrevendo principalmente suas propriedades antioxidantes (SPENCER et al., 2008). Embora haja um amplo debate sobre as doses eficazes de proteção, a ingestão de polifenóis tem sido associada à redução do risco de várias doenças crônicas (DEL BO et al., 2019). Realizamos um levantamento acerca dos benefícios da utilização de polifenóis durante a gestação e observamos que o RSV apresentou um efeito benéfico em diversos contextos devido ao seu efeito antioxidante (Anexo 4). Embora os mecanismos envolvidos na proteção fornecida pelo tratamento com RSV não sejam conhecidos, os dados em conjunto destacam o suco de uva, o extrato de *Vitis vinífera* (videira), o extrato de uva e o próprio RSV isolado como novas estratégias terapêuticas promissoras.

Os dados apresentados no estudo clínico (Anexo 3) e no levantamento (Anexo 4) demonstram que o tratamento com RSV teve elevada tolerabilidade, sem efeitos colaterais, capaz de modular tanto alterações comportamentais quanto moleculares, além de ter se mostrado relevante no contexto gestacional. Assim, essa molécula desponta como uma potencial estratégia terapêutica, tanto como monoterapia quanto como adjuvante a um baixo custo, reforçando o seu caráter translacional protetor.

Tomados em conjunto, os dados apresentados no presente trabalho evidenciam a possível contribuição da disfunção da BHE no maior conteúdo de água observado no grupo VPA (os quais, em conjunto, podem possuir um *background* neuroinflamatório envolvido), bem como um perfil diferenciado tanto das AQP 1 e 4 quanto de astrócitos. Também mostramos que o RSV foi capaz de prevenir diversas alterações nesse contexto, possivelmente devido à sua ação anti-inflamatória,

antioxidante e neuroprotetora. Demonstramos, por meio de uma revisão da literatura, a translacionalidade do modelo animal VPA e de humanos com TEA na questão neuroimunológica, bem como evidenciamos, através de um capítulo de livro, o papel do sistema purinérgico na fisiopatologia do TEA. Conforme trabalho clínico em anexo, também demonstramos a eficácia e segurança da suplementação de RSV em pacientes pediátricos com TEA, sendo pioneiros nesse tipo de abordagem. Por fim, ressaltamos que, apesar dos inúmeros efeitos benéficos do uso de polifenóis, deve-se ter cautela na utilização principalmente em momentos cruciais como a gestação. Dessa forma, a investigação de mecanismos envolvidos nas alterações neuroimunológicas (tanto em idades pós-natais quanto embrionárias), bem como a utilização do RSV como ferramenta de estudo de vias biológicas envolvidas são estratégias promissoras para a continuação desse trabalho.

4. CONSIDERAÇÕES FINAIS E CONCLUSÕES

No presente trabalho, investigamos, em modelo animal, possíveis mecanismos subjacentes à macrocefalia presente em uma parcela de crianças com TEA. Além disso, avaliamos o efeito preventivo do RSV em todos os parâmetros analisados.

Observamos que a exposição pré-natal ao VPA levou à maior conteúdo encefálico de água livre (análogo à edema), prejudicou o crescimento corporal dos animais e promoveu maior permeabilidade de BHE ao corante azul de Evans. Além disso, também diminuiu o imunoconteúdo e a imunomarcação da AQP1 em praticamente todas as regiões encefálicas analisadas (plexo coroide, região da amígdala, área somatossensorial e córtex pré-frontal medial) ao passo que levou a um perfil diferencial de AQP4 região-dependente: diminuído no córtex pré-frontal medial e aumentado na área somatossensorial primária. Finalmente, observamos um aumento no número de astrócitos bem como na imunomarcação da GFAP novamente no córtex pré-frontal medial e na área somatossensorial primária nos animais expostos ao VPA. Em conjunto, os dados demonstram um importante efeito preventivo da administração pré-natal do RSV ao promover uma melhora funcional significativa.

Além disso, propusemos investigações translacionais sobre as bases neuroimunológicas envolvidas no TEA – reforçando ainda mais as validades do modelo VPA e possibilitando a ampliação de estudos nesse sentido –, bem como sobre contribuição das alterações no metabolismo das purinas na fisiopatologia do TEA, destacando o sistema purinérgico como um alvo promissor de estudos na busca por estratégias terapêuticas no TEA.

Portanto, podemos reforçar o papel protetor do RSV tanto nas disfunções da BHE em modelo de TEA quanto nos possíveis mecanismos subjacentes à macrocefalia devido à prevenção observada não só na manutenção da integridade da BHE, mas também nas alterações na dinâmica do conteúdo de água, no perfil de aquaporinas e de expressão de GFAP e astrócitos. Além disso, propusemos investigações translacionais sobre as bases neuroimunológicas envolvidas no TEA, bem como sobre alterações na sinalização purinérgica envolvidas nesse transtorno, levantando algumas hipóteses sobre possíveis mecanismos envolvidos na fisiopatologia do TEA.

5. PERSPECTIVAS

Devido à pandemia, os experimentos previstos para compor a presente tese precisaram ser interrompidos e adiados. Assim, as principais perspectivas envolvem a complementação dos experimentos previstos, bem como novos experimentos para responder às perguntas que surgiram durante a análise dos dados obtidos.

Uma vez que a exposição pré-natal ao VPA modula o balanço neuroimunológico, altera o comportamento e causa alterações morfofuncionais, aumentando a permeabilidade das barreiras neurais, cogitamos um envolvimento da placenta no desencadeamento de tais alterações. Seria importante determinar se o tratamento pré-natal com RSV poderia modular células do sistema imunológico, tanto a nível circulatório quanto tecidual (encefálico) e se esse efeito benéfico já poderia ser visualizado a nível embrionário.

Considerando os resultados gerados, destacando as alterações imunológicas e purinérgicas como alvos em potencial para estudos sobre os mecanismos envolvidos na indução do fenótipo do tipo autista pelo VPA, bem como na prevenção conferida pelo RSV, surgem as seguintes perspectivas:

- Placenta (remoção no dia gestacional 19):
    - Analisar por imunofluorescência a quimiocina CCL2 e a infiltração de células sanguíneas, incluindo leucócito, linfócito T, macrófago, mastócito, linfócito B (por meio de marcadores CD45, CD3, CD13, carboxipeptidase A (CPA), CD19, respectivamente);
    - Avaliar expressão gênica e proteica de IL-6 e seu receptor (IL-6R).
- Encéfalo de embriões no dia 19 (E19):
    - Analisar possíveis alterações na microglia (pela marcação por CD11b);
    - Analisar infiltrado de células sanguíneas, incluindo leucócito, linfócito T, macrófago, mastócito, linfócito B (por meio dos marcadores CD45, CD3, CD13, CPA, CD19, respectivamente) e de quimiocina CCL2;
    - Analisar a expressão gênica e proteica de IL-6 e IL-6R;
    - Analisar parâmetros de barreira hematoencefálica (BHE), incluindo marcação de pericitos, endotélio, astrócitos e AQP4;
    - Analisar AQP1 e AQP4 no epêndima do terceiro ventrículo e no plexo coroide.
- Animais adultos (amostras já obtidas):

- Analisar a expressão do canal Kir4.1 no modelo VPA e os possíveis efeitos do RSV;
- Buscar na literatura e analisar proteínas com provável envolvimento tanto nas disfunções nas barreiras neurais quanto na proteção promovida pela RSV, como a MMP-9;
- Realizar experimentos para análise do metabolismo astrocítico no modelo VPA e o possível efeito protetor do RSV;
- Realizar o Método de Cavalieri em cortes histológicos a fim de determinar se no modelo animal também há alteração do volume encefálico, além de analisar a espessura cortical em diferentes regiões encefálicas, bem como o possível efeito protetor do RSV;
- Analisar o perfil de citocinas pró-inflamatórias tanto circulantes quanto em tecido encefálico e o possível efeito protetor do RSV;
- Analisar a presença de infiltrado inflamatório no tecido encefálico e o possível efeito protetor do RSV.

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ANEXOS

ANEXO 1 – Carta de aprovação da Comissão de Ética no Uso de Animais (CEUA)

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRO-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 36229
Título: AVALIAÇÃO MOLECULAR DE MARCADORES DE CÉLULAS DO SISTEMA IMUNOLÓGICO NO SISTEMA NERVOSO CENTRAL E ALTERAÇÕES NA PERMEABILIDADE DE BARREIRAS NEURAIS EM MODELO ANIMAL DE AUTISMO

Vigência: 01/12/2018 à 01/03/2022

Pesquisadores:

Equipe UFRGS:

CARMEM JURACY SILVEIRA GOTTFRIED - coordenador desde 01/12/2018
MELLANIE FONTES DUTRA DA SILVA - Aluno de Doutorado desde 01/12/2018
IOHANNA DECKMANN - Aluno de Doutorado desde 01/12/2018
JÚLIO SANTOS TERRA MACHADO - Aluno de Doutorado desde 01/12/2018
Gustavo Brum Schwingel - Aluno de Doutorado desde 01/12/2018

Comissão De Ética No Uso De Animais aprovou o mesmo, em reunião realizada em 11/02/2019 - Plenarinho - Andar Térreo do Prédio da Reitoria - Campus Centro da UFRGS- Bairro Farroupilha - Porto Alegre- RS, em seus aspectos éticos e metodológicos, para a utilização de um total de 261 ratos Wistar, sendo 65 machos (com aproximadamente 80 dias) e 196 fêmeas (com aproximadamente 80 dias), oriundos do Centro de Reprodução e Experimentação de Animais de Laboratório (CREAL/UFRGS), de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Quinta-Feira, 21 de Fevereiro de 2019

MARCELO MELLER ALIEVI
Coordenador da comissão de ética

ANEXO 2 – Parecer de projeto de doutorado encaminhado para avaliação pelo Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

PPG-Ciências Biológicas Bioquímica

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

Parecer de Projeto de Doutorado

O projeto da aluna **Iohanna Deckmann**, intitulado: **"Avaliação molecular de marcadores de células do sistema imunológico no sistema nervoso central e alterações na permeabilidade de barreiras neurais em modelo animal de autismo."**, orientado pela Profa. Dra. Carmem Juracy Silveira Gottfried, encaminhado para avaliação pelo PPG - Bioquímica teve a seguinte análise e parecer:

ANÁLISE DO PROJETO

1. **Mérito científico**

[X] Relevante

[ ] Sugestões de alterações:

2. **Fundamentação**

[X] Adequada

[ ] Sugestões de alterações: Revisar erros de digitação, presentes ao longo de todo o texto.

3. **Objetivos**

[X] Bem definidos

[ ] Sugestões de alterações:

4. **Metodologia**

[X] Adequada

[ ] Sugestões de alterações:

5. **Cálculo do número amostral**

[x] Realizado: porém explicar o que será feito com as fêmeas das ninhadas e os machos genitores.

Não aplicável

[ ] Não realizado:

6. Considerações Éticas

[X] Descritas

[ ] Não aplicável

[ ] Sugestões de alterações:

7. Descarte dos Resíduos Químicos e Biológicos

[X] Descritos

[ ] Não aplicável

[ ] Sugestões de alterações:

8. Cronograma de Execução

[X] Apresentado

[ ] Não apresentado

9. Referências Bibliográficas

[X] Pertinentes

[ ] Sugestões de alterações: referência para a dose de resveratrol administrada.

PARECER FINAL:

[x] Aprovado

[ ] Diligência

Porto Alegre, 23 de maio de 2018

Atenciosamente,

Prof. Dr. Diogo Onofre Gomes de Souza
Coordenador do PPG em C.B.:
Bioquímica-ICBS/UFRGS

LETICIA RODRIGUES
PÓS-DOUTORANDA DO PPG BIOQUÍMICA
C.P.F. 968.655.120-49

ANEXO 3 – Primeira autoria compartilhada em manuscrito submetido

"Resveratrol treatment of autism spectrum disorders – a pilot study"

RESVERATROL TREATMENT OF AUTISM SPECTRUM DISORDER – A PILOT STUDY

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Coordination for the Improvement of Higher Education Personnel (CAPES, coauthors scholarship). We would like to thank statistical support group of the HCPA, Brazil.

Conflicts of interest: The authors declare that there is no conflict of interest. Due to its exploratory nature, treatment with resveratrol was not maintained after the period of the study.

Abbreviations: **ABC**, aberrant behavior checklist; **ASD**, autism spectrum disorder; **CGI**, clinical global impression; **DSM-5**, diagnostic and statistical manual of mental disorders, fifth edition; **HCPA**, Clinical Hospital of Porto Alegre; **IQR**, interquartile range; **miRNA**, microRNA; **PBMC**, peripheral blood mononuclear cell; **RSV**, resveratrol; **RTq-PCR**, real-time reverse transcription-polymerase chain reaction.

Abstract

Objectives: Considering autism spectrum disorder (ASD) as a neurodevelopmental condition associated with immune system impairments, we aimed to evaluate the efficacy, tolerability, and safety of the anti-inflammatory, antioxidant and neuroprotective trans-resveratrol (RSV) in behavioral impairments and in a set of eight microRNAs (miR) related to the immune system in pediatric subjects with ASD. **Methods**: This is an open-label pilot trial over a 3-month (90 days) study follow-up period designed to assess the effect of 200 mg/day RSV on five boys aged 10 to 13 (11.8±1.1) years diagnosed with ASD according to DSM-V. **Results**: The RSV treatment significantly reduced the Aberrant Behavior Checklist (ABC) total score (P=0.042) and Irritability (P=0.041), with no alteration in Stereotypical Behavior (P=0.066), Hyperactivity (P=0.068), and Lethargy/Social Withdrawal (P=0.078) subscales. On the Clinical Global Impression (CGI) scale, three individuals showed marked improvement in behavior; one showed mild improvement and the other had no changes. RSV treatment increased the miR-195-5p (p=0.043), an important modulator of targets related to inflammatory and immunological pathways. RSV administration did not present adverse effects and did not alter clinical laboratory results. **Conclusion**: RSV is a safe molecule for administrating in the pediatric population, able to modulate behavior alterations and molecules associated with the immune system, becoming a promising therapeutic strategy for large-scale studies in ASD, to investigate both behavioral and molecular approaches.

Keywords: autism spectrum disorder, ASD, inflammation, resveratrol, neuroinflammation, neuroprotection.

1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by 1) difficulty with communication and social interactions, and 2) restrictive and repetitive behaviors, interests and activities [1]. Nowadays, ASD prevalence is 1:54, an increase of approximately 150% since the 2000s [2]. Some medications can improve symptoms like inability to focus, anxiety, self-injury, or seizures; however, usually present undesirable side effects [3]. In addition, non-drug therapeutic approaches, such as behavior analysis, social skills training, and occupational therapy, can help ASD children; but may be expensive to the family [3]. Currently, no chemical treatments are able to revert the main symptoms of ASD [4].

The etiology of ASD is still unclear [5]. Nonetheless, the growing number of publications leaves no doubt that ASD is a multifactorial and heterogeneous disorder with a complex interaction among genetic and environmental, with important immunological background [6]. Recent studies suggest the need to define and recognize clinical subtypes of ASD to ameliorate the outcome of treatment [7]. Thus, immune pathways may provide clues to new pharmacological strategies in ASD [5,6,8].

In the nutraceutical scenario, polyphenols have received significant attention from the scientific community due to their pharmacological potential. The antioxidant and anti-inflammatory *trans*-resveratrol (RSV, 3,4′,5- trihydroxystilbene), found in grapes, berries, peanuts, and pines [9], is emerged as a potent molecule due to its outstanding therapeutic properties in cancer, cardiovascular and neural diseases [10,11]. Previously, our group demonstrated the preventive effects of prenatal RSV treatment in a myriad of changes observed in an ASD animal model, preventing autistic-like behavior [12], molecular, cellular [13], and microRNA (miRNA) alterations [14]. The miRNA are short non-coding RNA of 19-25 nucleotides that mediate gene silencing, regulating cell differentiation, development and homeostasis [15].

In the present study, five ASD-diagnosed male children received RSV in order to evaluate the RSV possible therapeutic effects, tolerability, and safety. Effects of RSV on the expression of eight miRNA associated to immune modulation was also evaluated.

2. Methods

2.1. Study Design and Logistics

This is an open-label pilot clinical study designed to evaluate the efficacy and

tolerability of administering 200 mg/day RSV to pediatric ASD patients recruited in the Clinical Hospital of Porto Alegre (HCPA). The application of the scales and the collection of biological samples occurred at the Clinical Research Center of HCPA. The study was undertaken from October 2017 to January 2018.

This study was approved by the Research Ethics Committee of the HCPA (17-0132 - CAAE 65862917100005327), registered in the Brazilian Registry of Clinical Trials (ReBEC, number 6356) and in the International Clinical Trials Registry Platform - World Health Organization (universal number U1111-1213-6255). Each patient's parent or guardian signed an informed consent declaration.

This protocol is following ethical and methodological aspects of the Resolution 466/2012 - Regulatory Guidelines and Norms for Research Involving Humans, and Resolution 251/97 - Research Norms for New Drugs, Medicines, Vaccines and Diagnostic Tests Involving Humans (in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Subjects

The study sample consisted of five boys aged 10 to 13 (11.8±1.1) years diagnosed with ASD according to the criteria described in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) [1].

The psychotropic medication was allowed as long as all subjects were utilizing the same dose for at least four weeks before entering the study, and as long as no dose adjustments were necessary throughout the study period.

Patients were excluded from the study if presented syndromes with clinical presentation similar to ASD; dose adjustment or interruption of any psychotropic medication during the study; use of anti-seizure medication; other active systemic diseases such as cardiovascular, liver or kidney pathologies.

2.3. Intervention

Subjects received 200 mg RSV daily (two 100 mg capsules as a single morning dose) for 90 days. The RSV employed in this study presented adequate purity for use in clinical trials (99% *trans*-RSV, *Farmácia Reativa*, Porto Alegre, RS, Brazil). Noteworthy, even RSV being a nutraceutical with several benefit effects related, is still an experimental drug.

2.4. Behavioral outcome measures

The scales CGI (Clinical Global Impression, universally recognized and used in therapeutic studies with patients with mental disorders) [16,17] and ABC (Aberrant Behavior Checklist, designed to assess behavioral symptoms in a population with psychiatric disorders) [18,19], were applied before and after 90 days of RSV treatment (detailed in Table S1 and S2, respectively).

2.5. Blood sample for biochemistry and microRNA quantification

Laboratory adverse effects follow-up

Peripheral blood was collected in EDTA at 0, 45, and 90 days of RSV treatment to perform the clinical laboratory exams.

Peripheral Blood Mononuclear Cell (PBMC) isolation

PBMC were harvested from 5 mL peripheral blood obtained via phlebotomy in EDTA (Histopaque 1.077 g/mL Sigma Aldrich, St. Louis, Missouri). Part of the cells were then prepared with Trypan Blue (1:1) for manual counting in a hemocytometer, while the remaining cells were stored in QIAzol® until the RNA extraction.

RNA Extraction, Reverse Transcription and Polimerase Chain Reaction (RT-qPCR)

After total RNA extraction and cDNA synthesis [20], the expression levels of eight different miRNAs (hsa-mir-21-5p, hsa-mir-25-3p, hsa-mir-30c-5p, hsa-mir-124-3p, hsa-mir-125a-5p, hsa-mir-146a-5p, hsa-mir-191-5p, hsa-mir-195-5p –Table S3) with proven roles in neural plasticity and the immune system were evaluated before and after RSV treatment. The RTq-PCR protocol and reagents used are detailed in the Supplementary Material. To calculate relative miRNA expression, the $2^{-\Delta\Delta Ct}$ [21] method was employed.

2.6. Signaling pathways and protein targets of altered miRNA.

The spidermiR package [22] was used to search for experimentally validated targets for miRNAs with altered relative expression. These targets were plotted in the Reactome diagram viewer to create a graph with hierarchical visualization of pathways related with each miRNA target [23]. The tool performs an enrichment analysis based on a statistical test that calculates the enrichment of biological pathways predefined in databases.

2.7. Statistical Analysis

Since this is a pilot study designed for proof of concept and there were no safety data regarding the use of RSV in children, a limited number of subjects were included, without calculating sample size. Mean values obtained from the ABC scale and miRNA relative expression were compared amongst each other using the Wilcoxon t test (nonparametric test to paired samples) formulated using a Monte Carlo approach. Finally, Cohen's d formula was employed for effect size, corrected for $n < 50$. For all analysis, was used Statistical Package for the Social Sciences (SPSS) program v. 21.0 for data processing and analyzing. P values < 0.05 were considered statistically significant.

3. Results

3.1. Clinical evaluation and biochemical measure

Regarding to patients' medication profile (Table S4), two subjects (4 and 5) used psychotropic treatment (sertraline and aripiprazole). All patients used at least one non-drug treatment.

At the end of RSV treatment, the intellectual score by CGI scale (Table 1) showed substantial improvement in subjects 3, 4 and 5, moderate improvement in subject 2 and no improvement in subject 1.

Observing the mean ABC score (Table 2), there was a significantly decrease in total score after RSV treatment (pre-RSV, median 67; IQR: 62.5-79 and post-RSV, median 49, IQR: 42.5-73.5, p=0.042). Interestingly, RSV treatment significantly decreased the irritability score (pre-RSV, median 19, IQR: 11.5-21.5 and post-RSV, median 12, IQR: 9-16, p=0.041). No difference was observed in the other subscales. Table S5 shows total and subscale scores for each patient, before and after RSV treatment. Patients had no biochemical alterations at 45 and 90-days of RSV treatment (Table S6).

3.2. MicroRNA analysis

GeNorm algorithm identified miR-21-5p, miR-30-5p and miR-191-5p as the most stable ones, which were used as reference genes for normalization and evaluation of the relative expression of the remaining miRNAs. Interestingly, all patients presented a statistically significant increase in relative expression of miR-195-5p after 90 days of RSV treatment (pre-RSV, median 0.93, IQR 0.82-1.34 and post-

RSV, median 1.83, IQR 1.73-2.24; p = 0.043) (Table 3). No statistical difference was found for the other four miRNA evaluated.

In Figure 1, all graphs highlight the Subject 1 miRNA profile, which had an opposite profile to the other patients in the miR-124-3p and miR-125a-5p relative expression (1B and 1C, respectively), showing decreased levels of these molecules when compared to the values before starting RSV treatment.

3.3. Relevant genes and pathways related to miR-195-5p

In order to identify the biological pathways involved in the miR-195-5p signaling, the experimentally validated targets of miR-195-5p ranked by spidermiR package (Table S7) were evaluated by the open-source database Reactome. The enrichment analysis showed in Figure 2, with the representative p-value of that enriched route (highlighted in darker colors), demonstrate the miR195-5p involvement in immune system, signal transduction, gene expression (transcription), cell cycle, programmed cell death, transport of small molecules, disease, and cellular responses to external stimuli (Table S8).

4. Discussion

Nowadays, only two medications, risperidone and aripiprazole, are approved by the *Food and Drug Administration* (FDA) to treat of disruptive symptoms in ASD patients [4,24]. However, these are associated with adverse metabolic effects, such as weight gain, dyslipidemia and hyperglycemia [25]. The present study comes up with RSV (200 mg/day) as a possible therapeutic strategy to improve social skills presenting no side effects by ameliorating the irritability condition. The absence of side effects corroborates with the literature, that demonstrates no side effects in doses below 500 mg/day [26–29].

Individual impairment, which affects many aspects of everyday functions [30], the direct and indirect economic burden of treatments and the impact on family suffering strengthens the need for effective interventions in ASD [31–33]. Thus, RSV emerges as an important strategy for a large-scale study in ASD. To our knowledge, this is the first study using RSV *per se* (not as a coadjutant) in patients diagnosed with ASD. There is a single work in the literature (a double-blind and placebo-controlled randomized trial) that used RSV in the ASD context as a risperidone adjunctive therapy in the treatment of irritability [34] without improvement in this parameter.

Here, we demonstrated a statistically significant reduction in the irritability subscale score for the study population. Irritability can manifest itself in different ways, including aggression, tantrums, self-injurious behavior and anger, and leads to difficulties in the social interaction of these individuals. Thus, a safe and effective treatment capable of modulating this behavior could undoubtedly contribute to a general improvement in the functioning of these individuals. The best responders were those 3 patients with normal or mild intelligence and also with normal speech (subjects 3, 4 and 5), which suggests that RSV may be more effective in patients with preserved cognitive abilities, or those with less severe impairments.

Considering the beneficial profile of RSV observed in the ASD animal model [12–14], and the decreased irritability of ASD patients observed in the present work, associated to the immune alterations already described in these patients, we searched the literature for the effects of RSV on immune cells (Table S9). This data compilation on the wide range of RSV effects in different immune cells reinforce its anti-inflammatory profile and the ability to decrease autoantibody production [35,36], allowing us suggest the RSV may improve behavioral skills in autistic patients throughout different cellular levels (extra-intracellular and nuclear) as well as over blood circulation.

In addition, our group demonstrated in the animal model that the prenatal administration of RSV successfully prevented behavioral sensory alterations, altered parvalbumin neuron localization and altered cortical organization [13]. Besides, both ASD animal models and ASD subjects demonstrated alterations in the central and peripheral immune system, including immune activation, self-antibody formation, cytokine/chemokine imbalance and an increase in the permeability of the blood-brain barrier [6,37].

The present analysis of miRNA demonstrated that after RSV treatment we observed a significant increase miR195-5p in all 5 studied patients. The opposite profile of miR-124-3p and miR-125a-5p relative expression in the subject 1 after RSV treatment emphasizes the possibility of different RSV response based on the ASD severity levels. Interestingly this patient is the same one with the highest ABC severity score (total score 85). Maybe ASD levels 1-2 have a better response to RSV treatment, highlighting a promising strategy to facilitate inclusion in education, tolerability in adverse environments and socialization.

The present data indicate that RSV may be triggering specific cellular mechanisms able to attenuate inflammation, oxidative stress and immunological

imbalances. In fact, miR-195 is associated with inhibition of the pro-inflammatory activation of macrophages, significantly reducing the concentration of the inflammatory cytokines IL-1β, IL-4, IL-6, and TNF-α [38]. It also inhibits the inflammatory process via inhibition of the TNF-α/NF-κB and VEGF/PI3K/Akt pathways [39]. Furthermore, miR-195 is known to target the brain-derived neurotrophic factor (BDNF) [40] with possible impact on synaptic connectivity and maturation (which are known to be perturbed in ASD). Considering that miR-195 may impact macrophage polarization status, favoring an anti-inflammatory phenotype even in pro-inflammatory conditions [38], miR-195 emerges as a potential tool to potentially treat ASD symptoms.

In summary, the achievements and critical analysis of the present case study (aiming to determine the best conditions for a large-scale clinical trial) were:

- As a nutraceutical, RSV can be administrated as mono therapy or as adjuvant to pharmacotherapies at a reasonable cost ($100 per individual);

- The interview guide produces the type and depth of data needed to answer the study question and is appropriate (validity and reliability) for use in the target population;

- The intervention was safe and without side effects;

- Here, we also demonstrated a promising effect of RSV at 1-2 autistic levels, could represent an alternative to mitigate irritability in challenging situations such as the school or work environment;

- Finally, the study had 100% adherence, indicating that in a larger study, even considering losses, it is possible to expect a good percentage of adherence in a 90-days treatment time.

Concluding, this study demonstrate that the RSV was well tolerated, without side effects, being beneficial in controlling 1-2 levels of ASD symptoms related to irritability. In addition, RSV treatment was associated with an increase in the expression of miR-195-5p, an important molecule that can mediate anti-inflammatory profile.

Building upon present findings, we highlight for further studies (a) the search for molecular roles of miR-195-5p, as well as other miRNA, in the etiology/pathophysiology of ASD and (b) the conduction of a placebo-controlled clinical trial, to fully evaluate the safety in a larger population and expand the screening of beneficial effects of RSV in different ASD levels.

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Author contributions

JM, ID, RR and CG: Conceptualization, data curation, formal analysis, project administration, visualization. RR and CG: Funding acquisition, supervision and resources. JM and ID: investigation, roles/Writing - original draft. JM, ID and GCF: Validation, methodology. CG, RR and RM: Writing - review & editing.

Data availability

The datasets generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

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Legends of Figures

Figure 1. Relative expression of microRNA in PBMC of autistic subjects. The differential expression of A) miR-25-3p, B) miR-124-3p, C) miR-125a-5p, D) miR-146a-5p and E) miR-195-5p was assessed before the treatment and after 90 days. In this graph, were plotted the values of the relative expression of each miRNA evaluated for each patient. Statistical analysis is described in Table 5, *p < 0.05 after Wilcoxon t test formulated using a Monte Carlo adjustment procedure.

Figure 2. Hierarchical visualization of pathways related to miR-195-5p targets. The pathways related to hsa-miR-195-5p targets are plotted by using the Reactome diagram viewer based on experimentally validated targets identified using SpidermiR (described in the Table S7). Biological pathways enriched to these microRNA targets are highlighted in darker colors and described in the Table S8 ($p<0.05$). Hsa: *homo sapiens*.

List of Tables

Table 1. Clinical Global Impression scale before and after treatment with Resveratrol.

	Pre-RSV (CGI-S)	Post-RSV (CGI-I)
Subject 1	Moderately Ill (4 in CGI-S)	No change (4 in CGI-I)
Subject 2	Severely Ill (6 in CGI-S)	Minimally improved (3 in CGI-I)
Subject 3	Moderately Ill (4 in CGI-S)	Much improved (2 in CGI-I)
Subject 4	Moderately Ill (4 in CGI-S)	Much improved (2 in CGI-I)
Subject 5	Moderately Ill (4 in CGI-S)	Much improved (2 in CGI-I)

CGI-S: Clinical Global Impression - Severity Scale; **CGI-I**: Clinical Global Impression - Improvement Scale.

Table 2. Mean Aberrant Behavior Checklist scores before and after treatment with resveratrol.

Subscale / Domain	Pre-RSV		Post-RSV		P	Effect Size
	mean±SD	Median, IQR (25%-75%)	mean±SD	Median, IQR (25%-75%)		
Total score	70.0± 9.487	67 (62.5-79)	56.2 ±17.138	49 (42.5-73.5)	0.042*	0.567
Irritability	17.0± 5.523	19 (11.5-21.5)	12.4± 3.578	12 (9-16)	0.041*	0.656
Lethargy/Social Withdrawal	20.4±7.369	23 (14-25.5)	16.8±8.289	16 (9.5-24.5)	0.078	0.447
Stereotypic Behavior	7.00±2.449	6 (5-9.5)	5.20±3.564	3 (2.5-9)	0.066	0.315
Hyperactivity	19.40±5.177	18 (15.5-24)	17.20±6.458	18 (11.5-22.5)	0.068	0.285
Inappropriate Speech	6.20±4.266	6 (3-9.5)	4.60±4.159	3 (1.5-8.5)	0.109	0.380

IQR: interquartile range; **RSV**: Resveratrol; **SD**: standard deviation. P values <0.05 were considered statistically significant.

Table 3. miRNA relative expression before and after treatment with resveratrol.

miRNA	Pre-RSV		Post-RSV		P
	mean±SD	Median, IQR (25%-75%)	mean±SD	Median, IQR (25%-75%)	
miR-25-3p	1.03±0.135	0.96 (0.95-1.14)	1.08±0.178	1.08 (0.93-1.24)	0.686
miR-124-3p	1.25±1.048	0.74 (0.62-2.14)	1.94±0.278	2.07 (1.65-2.17)	0.138
miR-125a-5p	1.10±0.541	0.75 (0.70-1.69)	1.51±0.493	1.33 (1.16-1.95)	0.225
miR-146a-5p	1.02±0.228	0.90 (0.86-1.25)	1.10±0.270	1.10 (0.88-1.32)	0.893
miR-195-5p	1.04±0.297	0.93 (0.82-1.34)	1.96±0.319	1.83 (1.73-2.24)	0.043*

IQR: interquartile range; **RSV**: Resveratrol; **SD**: standard deviation. P values <0.05 were considered statistically significant.

Supplementary Protocol. RTq-PCR detailed protocol.

Briefly, 1.25 mM stem-loop primers for each miRNA were added to a mix containing RNA, oligo(dT)25V (5 mM), 250 mM dNTPs (Ludwig, RS, Porto Alegre, Brazil) and RNAse-free water in a final volume of 17 µL. This was submitted to an incubation step at 65°C for 5 minutes, and then placed on ice. The MML-V RT enzyme (New England Biolabs, MA, USA) was used for cDNA synthesis according to the manufacturer's instructions. Each reaction mix was incubated at 16ºC for 30 minutes followed by another 30 minutes at 42ºC. All cDNA samples were diluted 50 X in RNAse-free water. Stem loop primers, initiation primers and the universal reverse primer were designed according to Chen and collaborators [20]. Real-time PCR was performed in a Bio-Rad CFX384 system (Bio-Rad, Hercules, CA, USA) utilizing SYBR Green I (Invitrogen, Carlsbad, CA, USA). The PCR reaction took place in a total volume of 10 µL containing 5 µL of diluted cDNA (1:50), 1 X SYBR Green I (Invitrogen, Carlsbad, CA, USA), 0.1 mM dNTPs, 1 X Buffer, 3 mM MgCl2, 0.25 U Taq DNA polymerase (Quatro G, Porto Alegre, RS, Brazil) and 200 nM of each forward and reverse primer. Samples were analyzed in triplicates in a 384-well plate, in which a control was also included. Reference genes were chosen according to the GeNorm® software, in order to determine the necessary miRNAs for normalization and to identify the most stable miRNAs to be used as normalizers. In order to determine miRNA amplification, PCR was set up as follows: an initial 5-minute step at 95ºC, followed by 40 cycles of 15 seconds at 95ºC for denaturation, 10 seconds at 60ºC for annealing and 10 seconds at 72ºC for elongation. Melting curve analysis was programmed at the end of the PCR reaction at the 65ºC and 95ºC interval, with readings at every 0.4ºC increase in temperature. Baselines and limits were determined manually using the Bio-Rad CFX software.

Table S1. *Clinical Global Impression* (CGI) scale.

Severity of illness (CGI-S) (How mentally ill is the patient at this time?)	
0 = Not assessed	**4 = Moderately Ill** *Noticeable, but modest, functional impairment or distress; symptom level may warrant medication*
1 = Normal, not at all Ill *Symptoms of disorder not present past seven days*	**5 = Markedly Ill** *Intrusive symptoms that distinctly impair social/occupational function or cause intrusive levels of distress*
2 = Borderline mentally Ill *Subtle or suspected pathology*	**6 = Severely Ill** *Disruptive pathology, behavior and function are frequently influenced by symptoms, may require assistance from others*
3 = Mildly Ill *Minimal distress or difficulty in social and occupational function*	**7 = Among the most extremely Ill patients** *Pathology drastically interferes in many life functions; may be hospitalized*
Global improvement (CGI-I) (How much has the patient changed?)	
0 = Not assessed	**4 = No change** *Symptoms remain essentially unchanged*
1 = Very much improved *Nearly all better; good level of functioning; minimal symptoms;*	**5 = Minimally worse** *Slightly worse but may not be clinically meaningful;*
2 = Much improved *Notably better with significant reduction of symptoms; increase in the level of functioning, but some symptoms remain*	**6 = Much worse** *Clinically significant increase in symptoms and diminished functioning*
3 = Minimally improved *Slightly better with little or no clinically meaningful reduction of symptoms.*	**7 = Very much worse** *Severe exacerbation of symptoms and loss of functioning*

Two components: CGI-S (Severity, evaluates the diseases' severity and is used only at the first visit in order to characterize the sample) and CGI-I (Improvement, evaluates the global improvement since the beginning of the treatment). Simplified and adapted to Brazilian Portuguese by Guy W and colleagues (GUY W, 1976) and Burner and Targum 2007 (BUSNER; TARGUM, 2007).

Table S2. *Aberrant Behavior Checklist* (ABC) scale.

0 - 29					30 - 58				
1. Excessively active at home, school, work, or elsewhere	0	1	2	3	30. Isolates himself/herself from other children or adults	0	1	2	3
2. Injures self on purpose	0	1	2	3	31. Hampers group activities	0	1	2	3
3. Indifferent, slow, inactive	0	1	2	3	32. Remains seated or standing in the same position for long periods of time	0	1	2	3
4. Aggressive to other children or adults (verbally or physically)	0	1	2	3	33. Talks out loud to him/herself	0	1	2	3
5. Tries to isolate himself from others	0	1	2	3	34. Cries over minor annoyances and hurts	0	1	2	3
6. Repetitive, meaningless body movements	0	1	2	3	35. Repetitive hand, body, or head movements	0	1	2	3
7. Noisy (rude and inappropriate noises)	0	1	2	3	36. Mood changes quickly	0	1	2	3
8. Screams inappropriately	0	1	2	3	37. Unresponsive to structured activities (does not react)	0	1	2	3
9. Talks excessively	0	1	2	3	38. Does not stay in seat (e.g., during lesson or training periods, meals, etc.)	0	1	2	3
10. Bad-tempered, fits of rage	0	1	2	3	39. Will not remain seated even for a short time	0	1	2	3
11. Stereotyped behavior; abnormal, repetitive movements	0	1	2	3	40. Is difficult to reach, contact or get through to	0	1	2	3
12. Worried, stares into space, head in the clouds	0	1	2	3	41. Cries and screams inappropriately	0	1	2	3
13. Impulsive (acts without thinking)	0	1	2	3	42. Prefers to be alone	0	1	2	3
14. Irritable and whiny	0	1	2	3	43. Does not try to communicate by words or gestures	0	1	2	3
15. Restless, incapable of staying still	0	1	2	3	44. Easily distractible	0	1	2	3
16. Withdrawn; prefers solitary activities	0	1	2	3	45. Shakes and swings extremities repeatedly	0	1	2	3
17. Eccentric, bizarre behavior	0	1	2	3	46. Repeats a word or frase over and over	0	1	2	3
18. Disobedient; difficult to control	0	1	2	3	47. Stamps feet, bangs objects or hits doors hard	0	1	2	3
19. Yells at inappropriate times	0	1	2	3	48. Constantly runs or jumps around the room	0	1	2	3

20. Fixed facial expression; lacks emotional responsiveness	0	1	2	3	49. Rocks his/her body backwards and forwards repeatedly	0	1	2	3
21. Annoys other people	0	1	2	3	50. Deliberately hurts himself/herself	0	1	2	3
22. Repetitive speech	0	1	2	3	51. Does not pay attention when someone speaks to him/her	0	1	2	3
23. Does nothing but sit and watch others	0	1	2	3	52. Does physical violence to self	0	1	2	3
24. Uncooperative	0	1	2	3	53. Inactive, never moves spontaneously	0	1	2	3
25. Depressive	0	1	2	3	54. Tends to be excessively active	0	1	2	3
26. Resists any kind of physical contact	0	1	2	3	55. Responds negatively to affection	0	1	2	3
27. Moves or rolls head back and forth repetitively	0	1	2	3	56. Ignores instructions deliberately	0	1	2	3
28. Does not pay attention to instructions	0	1	2	3	57. Has temper outbursts or tantrums when he/she does not get own way	0	1	2	3
29. Demands must be met immediately	0	1	2	3	58. Shows few social reactions to others	0	1	2	3

ABC questionnaire consist of 58 items subdivided into 5 subscales: I - irritability, agitation and crying (15 items); II - lethargy and social avoidance (16 items); III - stereotyped behavior (7 items); IV - hyperactivity (16 items); and V - inappropriate speech (4 items). **0**: no problem; **1**: behavior is a problem, but in a mild degree; **2**: the problem is of moderate severity; **3**: the problem is serious. Simplified and adapted to Brazilian Portuguese by Losapio and colleagues (LOSAPIO et al., 2011).

Table S3. Sequences of primers.

microRNA ID		Primer sequence (5'→ 3')
hsa-mir-21-5p	Forward	CCGGCGCTAGCTTATCAGACTGAT
	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA
hsa-mir-25-3p	Forward	TCAGCACATTGCACTTGTCTCGG
	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGAC
hsa-mir-30c-5p	Forward	GCGTCGCTGTAAACATCCTACACTC
	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCTGAG
hsa-mir-124-3p	Forward	CTAGCTTAAGGCACGCGGTGA
	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCATT
hsa-mir-125a-5p	Forward	GTCGCGATCCCTGAGACCCTTTA
	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAG
hsa-mir-146a-5p	Forward	CGTGGCGTGAGAACTGAATTCCA
	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACCCA
hsa-mir-191-5p	Forward	GGAGCGTCAACGGAATCCCAAAAG
	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGCTG
hsa-mir-195-5p	Forward	GGGCGCTAGCAGCACAGAAATA
	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCCAAT
U6	Forward	ATGAGTGCTCGCTTCGGCA
	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATA
RNU6B	Forward	ACGTACGCAAGGATGACACGC
	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAAT
Reverse universal		CCAGTGCAGGGTCCGAGGTA

Table S4. Clinical evaluation of ASD patients.

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5
Age (years)	12	12	13	10	12
IQ	ID moderate	ID moderate	133	ID mild	120
Normal exams	AEP, EEG, Karyotype, Fragile X and NMR	Audiometry, EEG, Karyotype, Fragile X and NMR	Karyotype, Fragile X and EEG	EEG, Karyotype, Fragile X and NMR	Focal paroxysmal EEG
Therapies	Psych, Phono, OT, School	Phono, School	Psychoped, Musical therapy, School	Psych, Phono, OT, School	Psych, Psychoped, School
Medications	None	None	None	Sertraline Aripiprazole	Sertraline

AEP: auditory evoked potential; **ASD**: Autism Spectrum Disorder; **EEG**: electroencephalography; **ID**: intellectual deficits; **NMR**: nuclear magnetic resonance; **OT**: occupational therapy; **Phono**: phonoaudiology; **Psych**: psychology; **Psychoped**: psychopedagogy.

Table S5. Individual *Aberrant Behavior Checklist* scores before and after treatment with resveratrol.

ABC Scale	Subject 1			Subject 2			Subject 3			Subject 4			Subject 5		
	Pre	Post	%	Pre	Post	%	Pre	Post	%	Pre	Post	%	Pre	Post	%
Total score	85	82	-4	73	65	-11	67	44	-34	61	49	-20	64	41	-36
Irritability	9	8	-11	20	16	-20	19	12	-37	23	16	-30	14	10	-29
Lethargy	27	28	4	23	21	-9	20	13	-35	8	6	-25	24	16	-33
Stereotypic behavior	9	8	-11	10	10	0	6	3	-50	6	3	-50	4	2	-50
Hyperactivity	28	27	-4	20	18	-10	16	13	-19	18	18	0	15	10	-33
Inappropriate speech	12	11	-8	0	0	0	6	3	-50	6	6	0	7	3	-57

%: Variation in the percentage of the score between evaluations after and before RSV treatment.

Table S6. Clinical laboratory exams.

	Subject 1			Subject 2			Subject 3			Subject 4			Subject 5		
	Pre	45 days	90 days	Pre	45 days	90 days	Pre	45 days	90 days	Pre	45 days	90 days	Pre	45 days	90 days
Hemoglobin	13.5	13.2	13.1	15.3	15.1	14.5	15.4	15.2	15.3	11.6	11.7	11.4	14.6	14.5	14.3
Hematocrit	38.3	37.5	36.9	44.8	44	43.1	43.8	43	42.8	35	35.4	35.1	42.8	42.6	42.1
Leukocyte	5710	6320	4620	5490	6700	5690	9810	8120	7620	9510	8910	8890	9000	11240	7290
Segmented (%)	44.4	56.5	48.7	42.8	48.5	38.7	46.1	44.3	32.2	49.4	59.6	49.5	44.9	68.5	39
Lymphocyte (%)	37.7	27.7	33.1	40.8	35.1	45.5	36.7	37.7	43.6	39	28.1	39	42.3	20.1	48.4
Platelets	340000	304000	287000	237000	235000	216000	355000	303000	283000	302000	304000	324000	313000	295000	327000
LDH	250	190	180	234	195	178	224	169	158	171	181	183	180	155	151
Triglycerides	57	59	50	45	39	46	60	80	74	189	111	112	135	87	79
Cholesterol (Total)	167	159	173	109	110	109	172	164	175	177	174	186	138	128	130
HDL	53	49	53	47	49	50	57	61	57	39	40	42	31	34	25
Glucose	83	85	81	92	94	90	93	87	85	89	86	87	85	92	83
Creatinine	0.49	0.45	0.46	0.8	0.72	0.71	0.69	0.69	0.6	0.51	0.54	0.55	0.68	0.62	0.64
TB	0.5	0.3	0.5	0.4	0.4	0.7	0.3	0.4	0.5	0.3	0.3	0.3	0.3	0.3	0.3
IB	0.4	0.2	0.3	0.3	0.2	0.4	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2
DB	0.1	0.1	0.2	0.1	0.2	0.3	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1
AST	26	20	21	21	20	19	18	14	16	16	18	17	17	15	17
ALT	21	20	19	16	18	16	11	12	12	13	16	17	17	15	18
Amylase	55	59	56	59	55	54	94	92	87	46	38	42	26	26	25

ALT: alanine aminotransferase; **AST**: aspartate aminotransferase; **DB**: Direct bilirubin; **HDL**: high-density lipoprotein cholesterol; **IB**: Indirect bilirubin; **LDH**: lactate dehydrogenase; **TB**: Total bilirubin.

Table S7. Experimentally validated targets for miR-195-5p ranked by spidermiR package.

miRNA ID	Experimentally validated targets						
hsa-mir-195-5p	ABCB7 AGER AGO1 ARL2 ATG14 Bace1 BCL2 BCL2L1 BCL2L2	BIRC5 BTRC CAB39 CAMKV CAND1 CBX4 CCL4 CCND1 CCND3	CCNE1 CDC25A CDC42 CDK4 CDK6 CDK8 CHEK1 CHUK COPB1	DICER1 E2F3 ELN FASN FGF2 HMGA1 INSR JAK2 KDR	KIAA0100 KRT7 LSM11 MBD1 MECP2 MYB NKD1 NOLC1 RAF1	RET RPL10 RPS6KB1 RUNX2 SH3BGRL2 SKI SLC2A3 SMAD7 SPTBN1	TAB3 TBCCD1 TMC6 TPI1 VEGFA WEE1 WNT7A YAP1 ZNF280C

ABCB7: ATP Binding Cassette Subfamily B Member 7; **AGER**: Advanced Glycosylation End-Product Specific Receptor; **AGO1**: Argonaute RISC Component 1; **ARL2**: ADP Ribosylation Factor Like GTPase 2; **ATG14**: Autophagy Related 14; **Bace1**: Beta-Secretase 1; **BCL2**: (B-cell lymphoma2): L2 and L11 (Apoptosis Regulator); **BIRC5**: Baculoviral IAP Repeat Containing 5 (member of apoptosis inhibition family); **BTRC**: Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase (modulates ubiquitination and subsequent proteasomal degradation); **CAB39**: Calcium Binding Protein 3; **CAMKV**: CaM Kinase Like Vesicle Associated; **CAND1**: Cullin Associated And Neddylation Dissociated 1 (Modulates ubiquitination); **CBX4**: Chromobox 4 (Involved in the sumoylation of transcriptional coactivators); **CCL4**: C-C Motif Chemokine Ligand 4 (Monokine with inflammatory and chemokinetic properties); **CCN**: (Cyclin) D1: D3: and E1 (regulates the cell-cycle during G(1)/S transition); **CDC:** (Cell Division Cycle) 25A and 42 (Inducer of mitotic progression); **CDK**: Cyclin Dependent Kinase 4 and 6 (regulate the cell-cycle during G(1)/S transition); **CDk8**: transcriptional coactivator; **CHEK1**: Checkpoint Kinase 1 (required for checkpoint-mediated cell cycle arrest and activation of DNA repair); **CHUK**: Component Of Inhibitor Of Nuclear Factor Kappa B Kinase Complex (plays a key role in the negative feedback of NF-kappa-B canonical signaling to limit inflammatory gene activation); **COPB1**: Coat Complex Subunit Beta 1 (mediate biosynthetic protein transport from the ER: via the Golgi up to the trans Golgi network); **DICER1**: Dicer 1: Dcr-1 Homolog (endoribonuclease playing a central role in short dsRNA-mediated post-transcriptional gene silencing.); **E2F3**: Transcription Factor 3 (modulates transcription of molecules Involved in cell cycle regulation or in DNA replication); **ELN**: Elastin (modulates arterial structure); **FASN**: Fatty Acid Synthase (catalyzes the formation of long-chain fatty acids from acetyl-CoA); **FGF2**: Fibroblast Growth Factor 2 (Plays an important role in the regulation of cell survival: cell division: cell differentiation and cell migration); **HMGA1**: High Mobility Group AT-Hook 1 (involved in the transcription regulation of genes containing: or in close proximity to A+T-rich regions); **INSR**: KH RNA Binding Domain Containing: Signal Transduction Associated 1 (putative regulator of mRNA stability and/or translation rates and mediates mRNA nuclear export.): **JAK2**: Janus Kinase 2 (Mediates essential signaling events in both innate and adaptive immunity); **KDR**: Kinase Insert Domain Receptor (Plays an essential role in the regulation of angiogenesis: vascular development: vascular permeability: and embryonic hematopoiesis); **KIAA0100** (involved in membrane trafficking); **KRT**: Keratin 7 (Blocks interferon-dependent

interfase); **LSM11**: LSM11, U7 Small Nuclear RNA Associated (Required for cell cycle progression from G1 to S phase); **MBD1**: Methyl-CpG Binding Domain Protein 1 (Transcriptional repressor); **MECP2**: Methyl-CpG Binding Protein 2 (Mediates transcriptional repression); **MYB**: MYB Proto-Oncogene: Transcription Factor (Transcriptional activator); **NKD1**: NKD Inhibitor Of WNT Signaling Pathway (antagonist of the canonical Wnt signaling pathway); **NOLC1**: Nucleolar And Coiled-Body Phosphoprotein 1 (Regulator of RNA polymerase I); **RAF1**: Raf-1 Proto-Oncogene: Serine/Threonine Kinase (Regulates Rho signaling and cell migration); **RET**: Ret Proto-Oncogene (Receptor tyrosine-protein kinase involved in numerous cellular mechanisms including neuronal navigation and cell proliferation: migration: and differentiation); **RPL10**: Ribosomal Protein L10 (Plays a role in the formation of actively translating ribosomes); **RPS6KB1**: Ribosomal Protein S6 Kinase B1 (Promote protein synthesis: cell growth: and cell proliferation); **RUNX2**: RUNX Family Transcription Factor 2 (Essential for osteoblastic differentiation and skeletal morphogenesis); **SH3BGRL2**: SH3 Domain Binding Glutamate Rich Protein Like 2 (Modulator of the thiotransferase glutaredoxin activity); **SKI**: SKI Proto-Oncogene (Repressor of TGF-beta signaling : and may play a role in neural tube development); **SLC2A3**: Solute Carrier Family 2 Member 3: or GLUT3 (Mediates neuronal glucose uptake); **SMAD7**: Mothers against decapentaplegic homolog 1 (transcription factor for TGFβ/BMP-dependent genes); **SPTBN1**: Spectrin Beta: Non-Erythrocytic 1 (actin crosslinking protein: modulating cell shape: arrangement of transmembrane proteins: and organization of organelles); **TAB3**: TGF-Beta Activated Kinase 1 (MAP3K7) Binding Protein 3 (Moduates NF-kappaB signal transduction pathway); **TBCCD1**: TBCC Domain Containing 1 (Plays a role in the regulation of centrosome and Golgi apparatus positioning: with consequences on cell shape and cell migration); **TMC6**: Transmembrane Channel Like 6 (ion channe); **TPI1**: Triosephosphate Isomerase 1 (atalyzes the isomerization of glyceraldehydes 3-phosphate (G3P) and dihydroxy-acetone phosphate (DHAP) in glycolysis and gluconeogenesis); **VEGFA**: Vascular Endothelial Growth Factor A (promotes angiogenesis); **WEE1**: “Wee (for reduced cell size)” nuclear tyrosine kinase member 1 (key regulator of cell cycle progression); **WNT7A**: Wnt (Wingless + Int-1): family member 7A (Regulates cell fate and patterning during embryogenesis); **YAP1**: Yes1 Associated Transcriptional Regulator (Involved in development: growth: repair: and homeostasis); **ZNF280C**: Zinc Finger Protein 280C (Part of transcription factors conferring DNA sequence specificity as the DNA-binding domain).

Table S8. Biological pathways enriched to miR-195-5p targets for *Homo sapiens* ranked by Reactome tool.

	miR-195-5p											
Pathway name	**Curated found**	**Curated Total**	**Interactors found**	**Interactors Total**	**Entities found**	**Entities Total**	**Entities ratio**	**Entities p-Value**	**Entities FDR**	**Reactions found**	**Reactions total**	**Reactions ratio**
Defective binding of RB1 mutants to E2F1,(E2F2, E2F3)	6	17	0	0	6	17	0.001	7.74E-9	4.72E-6	*1*	*1*	*0*
Aberrant regulation of mitotic G1/S transition in cancer due to RB1 defects	6	17	0	0	6	17	0.001	7.74E-9	4.72E-6	*1*	*2*	*0*
Small interfering RNA (siRNA) biogenesis	4	9	0	0	4	9	0	1.07E-6	4.36E-4	*5*	*5*	*0*
Post-transcriptional silencing by small RNAs	3	7	0	0	3	7	0	2.86E-5	7.97E-3	*3*	*3*	*0*
Cyclin D associated events in G1	7	51	4	72	9	111	0.005	4E-5	7.97E-3	*11*	*15*	*0.001*
G1 Phase	7	51	4	72	9	111	0.005	4E-5	7.97E-3	*11*	*15*	*0.001*
Regulation of MECP2 expression and activity	7	39	4	94	11	132	0.006	1.15E-4	1.9E-2	*13*	*14*	*0.001*
Loss of MECP2 binding ability to 5hmC-DNA	2	2	0	0	2	2	0	1.3E-4	1.9E-2	*1*	*1*	*0*
Interleukin-4 and Interleukin-13 signaling	11	216	1	143	11	347	0.017	1.41E-4	1.9E-2	*19*	*47*	*0.004*
MicroRNA (miRNA) biogenesis	4	31	0	4	4	35	0.002	2.08E-4	2.18E-2	*3*	*8*	*0.001*
Diseases of mitotic cell cycle	6	36	4	78	8	103	0.005	2.16E-4	2.18E-2	*2*	*3*	*0*
Aberrant regulation of mitotic cell cycle due to RB1 defects	6	36	4	78	8	103	0.005	2.16E-4	2.18E-2	*2*	*3*	*0*
Evasion of Oncogene Induced Senescence Due to Defective p16INK4A binding to CDK4 and CDK6	2	3	0	0	2	3	0	2.89E-4	2.51E-2	*1*	*1*	*0*
Evasion of Oxidative Stress Induced Senescence Due to Defective p16INK4A binding to CDK4 and CDK6	2	3	0	0	2	3	0	2.89E-4	2.51E-2	*1*	*1*	*0*
Competing endogenous RNAs (ceRNAs) regulate PTEN translation	3	19	0	0	3	19	0.001	5.33E-4	4.32E-2	*11*	*11*	*0.001*
Loss of MECP2 binding ability to 5mC-DNA	2	5	0	0	2	5	0	7.94E-4	6.04E-2	*2*	*2*	*0*
Loss of phosphorylation of MECP2 at T308	2	7	0	0	2	7	0	1.54E-3	1.04E-1	*1*	*1*	*0*
VEGF ligand-receptor interactions	3	8	4	25	5	28	0.001	1.62E-3	1.04E-1	*4*	*4*	*0*
VEGF binds to VEGFR leading to receptor dimerization	3	8	4	25	5	28	0.001	1.62E-3	1.04E-1	*3*	*3*	*0*

Obtained from the miR195-5p targets (supplementary Table S5), at the Reactome pathway database (Reacome 2020). **5hmC-DNA**: 5-Hydroxymethylcytosine (5hmC) is a DNA pyrimidine nitrogen base derived from cytosine; **CDK**: cyclin-dependent

kinases; **ceRNA**: competing endogenous RNA; **E2F**: transcription factor family; **G1**: cell cycle stage; **MECP2**: Methyl-CpG-binding protein 2; **miRNA**: microRNA; **NCoR/SMRT complex**: NCoR1 (nuclear receptor corepressor) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor; **p16INK4A**: tumor-suppressing protein that specifically binds to cyclin-dependent kinases CDK4 and CDK6; **PTEN**: Phosphatase and tensin homolog; **RB1**: retinoblastoma-associated protein; **S**: cell cycle stage (synthesis); **siRNA**: small interfering RNA; **T308**: threonine at position 308 of the amino acid sequence of the MECP2 protein; **VEGF**: vascular endothelial growth factor; **VEGFR**: vascular endothelial growth factor receptor.

Table S9. Effects resveratrol on immune cells

Macrophage	Anti-inflammatory profile Regulates the expression of TLR4 Decreases COX-2 expression and NF-κB activation Inhibits TBK1, RIP1, TRIF Inhibits the accumulation of acetylated α-tubulin Inhibits NLRP3-inflammasome activation Prevents LPS effect by decreasing CD14 and IRAK1 expression Exert antioxidant properties Reduces GM-CSF Modulates PGE2 levels
M2-like macrophage	Counteracts the immunosuppressive and tumor progressive influences of TAMs Decreases the activity of STAT3 Inhibits the IL-10, MCP-1 and VEGF Promotes TGFβ1 production
T cell	Inhibits T cell activation Regulates the function of Sirt1 Reduces TGFβ production Suppresses c-Jun Increases IL-10 production Activates Nfr2 signaling pathway
Regulatory T cell (Treg)	Suppress the $CD4^+CD25^+$ #
Th17	Reduces Th17 cells Reduces IL-17
NK	Dose-dependent effect on NK cell killing activity Facilitates NK cell to trigger signaling-dependent apoptosis
Regulatory B cell (tBreg)	Inactivates STAT3 Inhibits TGFβ expression Inhibits $FoxP3^+$ Tregs
B cell	Upregulates FcγRIIB Decreases antibody production Induces Sirt1 Decreases immunocomplex deposition Induces apoptosis of plasma cells

COX2: cyclooxygenase; **FcγRIIB**: a receptor for IgG; **FOXP3**: forkhead box P-3; **GM-CSF**: granulocyte–macrophage colony-stimulating factor; **IL-10**: interleukin-10; **IL-17**: interleukin 17; **IRAK**: interleukin-1 receptor-associated kinase; **LPS**: lipopolysaccharide; **MCP1**: monocyte chemoattractant protein-1; **NF-κB**: nuclear factor-Kappa B; **NLRP3**: nod-like receptor family: pyrin domain containing 3; **Nrf2**: nuclear factor erythroid 2-related factor 2; **PGE2**: prostaglandin E2; **RIP**: receptor-interacting protein; **Sirt1**: sirtuin 1; **STAT3**: signal transducer and activator of transcription; **TAMs**: tumor-associated macrophages; **TBK1**: TANK-binding kinase1; **TGF-β1**: transforming growth factor-β1; **Th17**: T helper 17; **TRIF**: toll-interleukin-1 receptor domain-containing adaptor inducing interferon; **TLR4**: toll-like receptor-4; **VEGF**: vascular endothelial growth factor. Compiled data from Gao et al., 2001; Malaguarnera, 2019; Shabani et al., 2020; and Zhou et al., 2020.

Figure 1

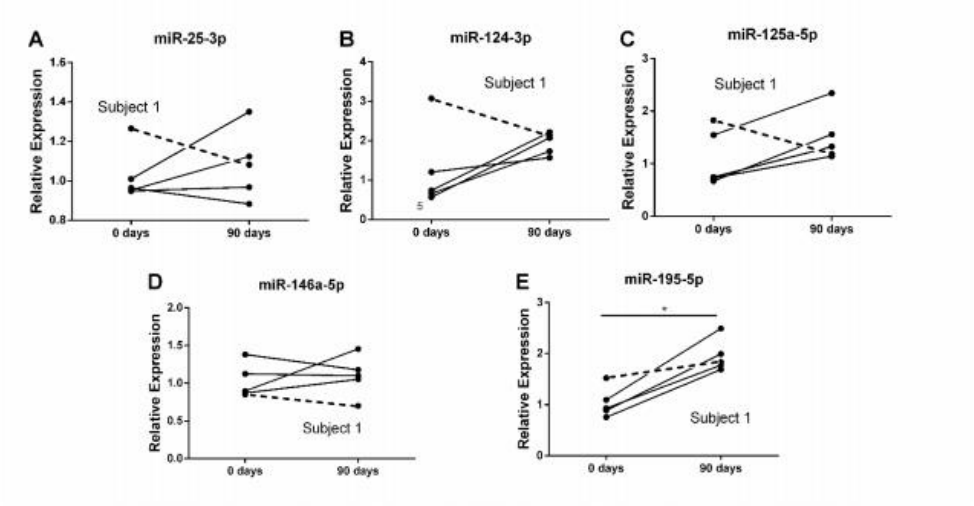
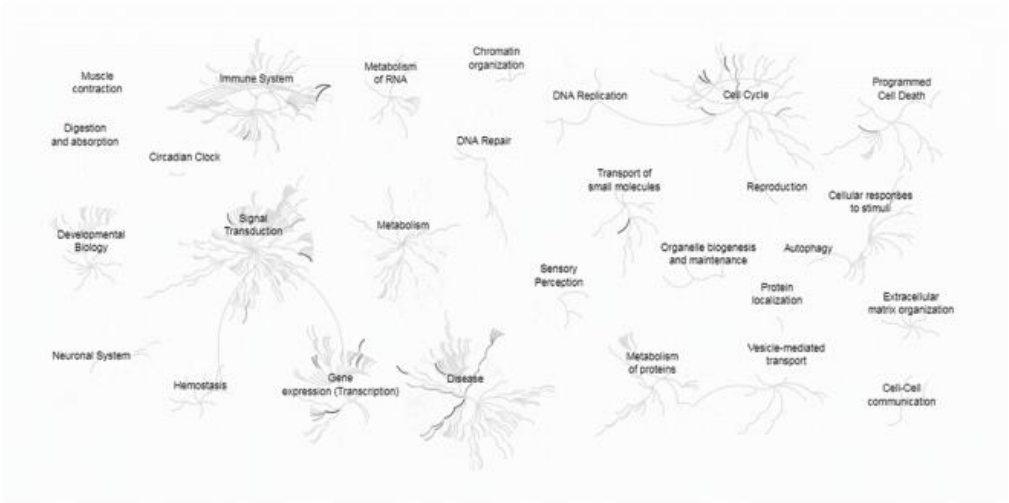
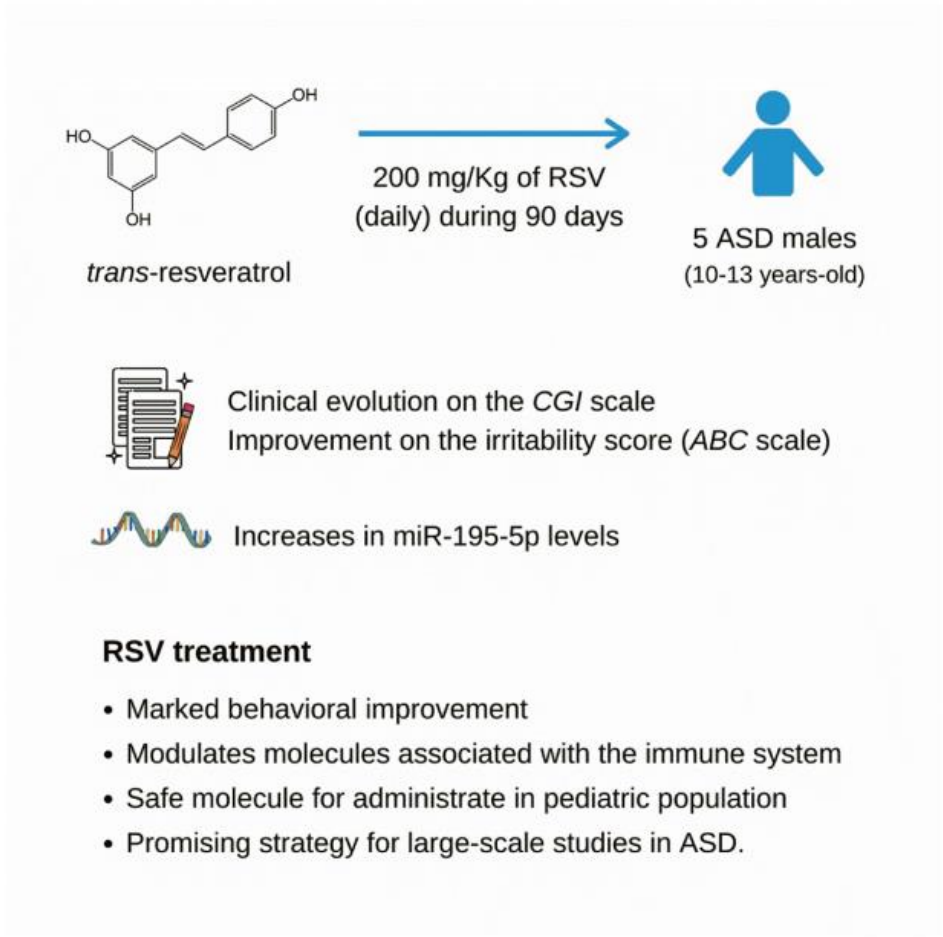


Figure 2



Graphical abstract



ANEXO 4 – Levantamento acerca da suplementação com RSV no contexto gestacional

No manuscrito a ser submetido (de autoria de Iohanna Deckmann, Júlio Santos-Terra, Fátima Martel e Jaqueline Vieira Carletti), revisamos o efeito da suplementação com diferentes polifenóis e os principais desfechos envolvendo as doenças mais comuns associadas à gravidez (pré-eclâmpsia, diabetes gestacional, restrição de crescimento fetal e constrição do ducto arterial fetal) uma vez que a exposição à pré-eclâmpsia materna e complicações perinatais foi associada a deficiência intelectual, transtornos específicos do desenvolvimento, TDAH e transtornos de conduta e outros transtornos comportamentais e emocionais na prole (KONG et al., 2022). Nessa seção, serão apresentados somente os resultados referentes ao RSV.

1. PRÉ-ECLÂMPSIA

1.1. Cultivo celular

1.1.1. Cultura de células endoteliais da veia umbilical humana (HUVECs) cultivadas com plasma de gestantes com pré-eclâmpsia

- Aumento da resposta antioxidante, diminuição nos níveis de espécies reativas de oxigênio (Caldeira-Dias et al., 2021);
- Prevenção das alterações de heme oxigenase-1 (HO-1) e NO, melhora dos níveis de GSH (Caldeira-Dias et al., 2019);
- Inibição da liberação induzida por citocinas do receptor-1 do fator de crescimento endotelial vascular solúvel (sVEGFR-1 ou sFlt-1) (Cudmore et al., 2012);
- Diminuição da produção de TNFα induzida por sFlt1 (Gurusinghe et al., 2017).

1.1.2. Linhagem celular de placenta humana HTR-8/SVneo cultivadas com plasma de gestantes com pré-eclâmpsia

- Prevenção de danos morfológicos e apoptose, proteção das células contra o estresse oxidativo induzido por H2O2, provavelmente pela ativação da autofagia via de SIRT1 (Wang et al., 2020);
- Prevenção de apoptose dependente de espécies reativas de oxigênio induzida por hipóxia (Zou et al., 2014);

- Estimulação da migração e invasão de trofoblastos, e ativação da transição epitelial-mesenquimal (Zou et al., 2019).

1.2. Modelos animais

1.2.1. Nocaute para catecol-O-metiltransferase ($COMT^{-/-}$)

- Aumento na velocidade do fluxo sanguíneo da artéria uterina e aumento peso fetal (Poudel et al., 2013);

1.2.2. Exposição a éster metílico de Nω-nitro-L-arginina (L-NAME)

- Prevenção dos efeitos do estresse oxidativo (Zou et al., 2014);
- Melhora da hipertensão e da proteinúria (Zou et al., 2019).

1.2.3. Exposição a acetato de desoxicorticosterona

- RSV não teve efeito no fluxo sanguíneo da placenta, no peso da placenta, no fluxo sanguíneo renal e no fluxo cerebral (Moraloglu et al., 2012).

1.3. Humanos (RSV como adjuvante à nifedipina (um dos tratamentos anti-hipertensivos de primeira linha)

- Diminuição do tempo necessário para controlar a pressão arterial e do número de doses necessárias para esse controle, sem apresentar efeito adverso à mãe e ao recém-nascido (Ding et al., 2017).

2. DIABETES MELLITUS GESTACIONAL

2.1. Cultivo celular

2.1.1. Células derivadas de mulheres grávidas

- Redução nos níveis de citocinas e quimiocinas pró-inflamatórias, e normalização da sinalização da insulina no tecido placentário, adiposo e muscular de gestantes saudáveis e expostos a agentes pró-inflamatórios (simulando o diabetes gestacional em cultura) (Tran et al., 2017).

2.1.2. Cultura de células endoteliais da veia umbilical humana (HUVECs) derivadas de gestações com diabetes

- Aumento da atividade e dos níveis de SIRT 1, 3 e 4 (Gui et al., 2015).

2.2. Modelos animais

2.2.1. Exposição precoce a elevados níveis de glicose

- Prevenção de malformações e lesões vasculares, diminuição do estresse oxidativo, redução da expressão do fator de transcrição PAX3 (Tan et al., 2015);
- Redução do peso corporal, dos níveis de glicose, dos níveis de insulina e da expressão de miR-23a-3p em adipócitos (Zheng and Chen, 2021);
- Atenuação dos níveis elevados de glicose em mães e filhotes, da resistência à insulina e do tamanho reduzido da ninhada (Yao et al., 2015).
- Prevenção do aumento dos níveis de glicose, normalização da secreção de insulina, regulação da expressão alterada de genes metabólicos no fígado e obesidade (Brawerman et al., 2019);

2.2.2. Embriopatia diabética

- Quando administrado nos primeiros dias da prenhez (E3-E12): melhora os níveis de glicose e lipídios das mães com diabetes, reduz estresse oxidativo, apoptose e previne malformações nos embriões (Singh et al., 2011);

2.2.3. Exposição a estreptozocina

- Redução da atividade de α-glucosidase e α-amilase, normalização dos níveis de glicose e redução dos parâmetros de estresse (Du et al., 2020);
- Tratamento pré-natal e pós-natal: redução dos comportamentos semelhantes ao TEA observados na prole de ratos do modelo de diabetes gestacional, associados a uma expressão reduzida de SOD2 na amígdala (o que provavelmente levou a um aumento do estresse oxidativo nesta região do encéfalo) (Wang et al., 2019b).

2.3. Humanos (gestantes com sobrepeso e alto risco de desenvolver diabetes gestacional)

- Após 30 e 60 dias, uma combinação de RSV com mioinositol e D-quiro-inositol melhorou os níveis de glicose no sangue e o perfil lipídico (Malvasi et al., 2017).

3. RESTRIÇÃO DO CRESCIMENTO FETAL

3.1. Modelos animais

3.1.1. Hipóxia

- Recuperação da disfunção cardíaca na prole, aumento dos níveis cardíacos de pAMPK e SOD2 apenas na prole do sexo feminino (Shah et al., 2017).

3.1.2. Nocaute para catecol-O-metiltransferase ($COMT^{-/-}$)

- Restauração do peso fetal e aumento da velocidade do fluxo sanguíneo da artéria uterina (Poudel et al., 2013).

3.1.3. Dieta rica em gordura

- Melhora do perfil metabólico da prole, do tamanho dos adipócitos, hiperlipidemia, eliminação de glicose e resistência à insulina (Dolinsky et al., 2011);
- Melhora da função cardíaca na prole do sexo feminino (Shah et al., 2017);
- Melhora da tolerância cardíaca à isquemia na prole (Rueda-Clausen et al., 2012).

3.1.4. Leitões com restrição de crescimento naturalmente

- Estímulo da biogênese mitocondrial, melhora a homeostase energética (Zhang et al., 2017);
- Redução da relação NAD+/NADH hepática (que inibe a oxidação de ácidos graxos e regenera o lactato, priorizando a glicose como principal fonte de energia), redução do alto teor de malondialdeído no fígado e aumento da atividade hepática de SIRT1 (Zhang et al., 2017);
- Atenuação do acúmulo de lipídios hepáticos ao melhorar a função mitocondrial e a oxidação de ácidos graxos, supressão da inflamação e estímulo à glutationa redutase e atividade da SOD (Cheng et al., 2021);
- Aumento da atividade da alanina aminotransferase, da atividade da lipoproteína lipase e da expressão do gene da proteína 1 quimiotática de monócitos (Cheng et al., 2020).

3.1.5. Suplementação crônica em ovelhas prenhes

- Aumento do fluxo sanguíneo da artéria uterina e o peso fetal, apesar de não haver diferenças nos níveis de SIRT1, CAMKII ou AKT/mTOR (Darby et al., 2019).

3.1.6. Ratos espontaneamente hipertensos

- A ingestão materna de RSV em ratos espontaneamente hipertensos não mostrou efeito no crescimento fetal, levou à alteração persistente na sinalização do NO e à restrição de crescimento em comparação ao grupo sem intervenção após o 21º dia pós-natal (Care et al., 2016).

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