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Marília Körbes Rockenbach

EFEITO DO RESVERATROL SOBRE PARÂMETROS ASTROCÍTICOS E PERMEABILIDADE DE BARREIRA HEMATOENCEFÁLICA NO MODELO ANIMAL DE AUTISMO INDUZIDO POR EXPOSIÇÃO PRÉ-NATAL AO ÁCIDO VALPROICO

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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharela em Biomedicina.

Orientadora: Profª. Dra. Carmem Gottfried
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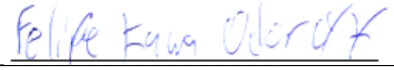

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RESUMO

O Transtorno do Espectro Autista (TEA) é uma desordem do neurodesenvolvimento de elevada prevalência, afetando 1:59 crianças de até 8 anos de idade nos Estados Unidos. De acordo com a 5ª edição do Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-5), o diagnóstico do TEA é dado pela díade comportamental composta por prejuízos na comunicação e interação social, bem como por interesses restritos, comportamentos repetitivos e estereotipados. Além das alterações comportamentais, o TEA pode apresentar muitas comorbidades associadas e um importante achado clínico é a presença de maior volume encefálico nos primeiros anos de vida. Embora a etiologia do TEA permaneça desconhecida, sabe-se da importância de fatores genéticos e ambientais, como a exposição pré-natal ao ácido valproico (VPA). O resveratrol (RSV) é uma molécula anti-inflamatória, anti-oxidante e neuroprotetora conhecida por prevenir comportamentos sociais no modelo animal de autismo induzido por exposição pré-natal ao VPA. O objetivo desse trabalho foi avaliar os efeitos do VPA na permeabilidade da barreira hematoencefálica (BHE), nas células $GFAP^+$ e na aquaporina 4 (AQP4) no córtex pré-frontal medial (CPFm) de ratos machos de 30 dias e o possível efeito protetivo do RSV. Ratas *Wistar* prenhes receberam doses diárias de 3.6 mg/kg de RSV ou DMSO (veículo) via subcutânea do dia embrionário 6.5 (E6.5) até E18.5 e no dia E12.5 foi administrada uma dose intraperitoneal de 600 mg/kg de VPA ou solução salina (0,9%). No dia pós-natal 30 (P30) os animais foram eutanasiados por sobredose anestésica de cetamina (300 mg/kg) e xilazina (40 mg/kg) e destinados a diferentes técnicas experimentais. Os animais avaliados para a permeabilidade da BHE ao corante azul de Evans foram injetados via intraperitoneal com uma solução de azul de Evans 2% diluído em salina (4 mg/kg), anestesiados e submetidos a perfusão transcardíaca com salina e paraformaldeído para posterior remoção e secção do encéfalo e análise de fluorescência. Para a imunofluorescência, os animais foram anestesiados, perfundidos e o encéfalo foi removido e seccionado. Animais do grupo VPA apresentaram maior permeabilidade da BHE ao corante azul de Evans e o RSV preveniu essa alteração. Os grupos que receberam VPA apresentaram aumento no número absoluto de células $GFAP^+$ nas camadas profundas do pré-límbico (PrL), além de um efeito sinérgico do RSV e do VPA no CC (superficial), no PrL (superficial) e no infralímbico (IL – profundo). Na razão de células $GFAP^+$ foi visto um aumento no número relativo nas camadas profundas e uma diminuição nas camadas superficiais do CC e do PrL no grupo VPA e o RSV foi capaz de prevenir essa alteração no PrL. Também foi observado uma diminuição no conteúdo de fluorescência de AQP4 em todas as regiões analisadas do CPFm no grupo VPA. Dessa forma, esse trabalho demonstrou alterações significativas na permeabilidade da BHE, no número e localização de células $GFAP^+$ e no conteúdo de fluorescência de AQP4 no CPFm do modelo VPA, bem como o uso do RSV como uma ferramenta para a investigação de mecanismos envolvidos na fisiopatologia do TEA.

Palavras-chave: Transtorno do Espectro Autista. Ácido valproico. Resveratrol. Barreira hematoencefálica. Astrócitos. Aquaporina 4.

ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental disorder of high prevalence, affecting 1:59 children up to 8 years of age in the United States. According to the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), the ASD diagnosis is given by the behavioral dyad composed by impairments in communication and social interaction, as well as restricted interests, repetitive and stereotyped behaviors. In addition to behavior alterations, ASD may present many associated comorbidities and an important clinical finding in this context is the presence of increased encephalic volume in the first years of life. Although the etiology of ASD remains unclear, the importance of genetic and environmental factors such as prenatal exposure to valproic acid (VPA) are known. Resveratrol (RSV) is an anti-inflammatory, antioxidant and neuroprotective molecule known to prevent social behaviors in the animal model of autism induced by prenatal exposure to VPA. The aim of this study was to evaluate the effects of VPA in blood-brain barrier permeability (BBB), GFAP$^+$ cells and aquaporin-4 (AQP4) in the medial prefrontal cortex (mPFC) of 30-day-old male rats and the possible protective effect of RSV. Pregnant Wistar rats received from embryonic day 6.5 (E6.5) up to E18.5 daily doses of 3.6 mg/kg RSV or DMSO (vehicle) via subcutaneous and on day E12.5 an intraperitoneal dose of 600 mg/kg VPA or saline solution (0.9%). On the postnatal day 30 (P30) the animals were euthanized by anesthetic overdose of ketamine (300 mg/kg) and xylazine (40 mg/kg) and destined to different experimental techniques. The animals evaluated for BBB permeability to the Evans blue dye were injected intraperitoneally with a solution of Evans blue 2% diluted in saline (4 mg/kg), anesthetized and submitted to transcardiac perfusion with saline and paraformaldehyde for posterior removal and section of the brain and fluorescence analysis. For immunofluorescence, the animals were anesthetized, perfused and the brain was removed and sectioned. VPA group animals presented greater BBB permeability to the Evans blue dye and RSV prevented this alteration. The groups that received VPA presented an increase in the absolute number of GFAP$^+$ cells in the deep layers of the prelimbic (PrL), in addition to a synergistic effect of RSV and VPA in CC (upper), PrL (upper) and infralimbic (IL – deeper). In the GFAP$^+$ cell ratio was seen an increase in the relative number in deep layers and a decrease in the upper layers of CC and PrL in the VPA group and RSV was able to prevent this chance in PrL. A decrease in the AQP4 fluorescence content was also observed in all mPFC regions analyzed in the VPA group. Thus, this work demonstrated significant changes in BBB permeability, number and location of GFAP$^+$ cells and in the fluorescent content of AQP4 in the mPFC of the VPA model, as well as the use of RSV as a tool for the investigation of mechanisms involved in ASD pathophysiology.

Keywords: Autism spectrum disorder. Valproic acid. Resveratrol. Blood brain barrier. Astrocytes. Aquaporin-4.

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LISTA DE ABREVIATURAS

AAT: aspartato aminotransferase

AMPA: alfa-amino-3-hidroxi-metil-5-4-isoxazolpropionico

AQP: aquaporina

ASD: *Autism spectrum disorder*

BA40: *Brodmann's area 40*

BA9: *Brodmann's area 9*

BBB: *Blood brain barrier*

BDNF: *brain derived neurotrophic factor*

BHE: Barreira hematoencefálica

CC: córtex cingulado

CDC: Centro de Controle e Prevenção de Doenças

CMM-UFRGS: *Microscopy and Microanalysis Center of Federal University of Rio Grande do Sul*

CNS: *Central Nervous System*

CPF: córtex pré-frontal

CPFl: córtex pré-frontal lateral

CPFm: córtex pré-frontal medial

CREB: *cAMP responsive element-binding protein*

Cx43: *connexin 43*

DMSO: *dimethyl sulfoxide*

DSM-5: 5ª edição do Manual Diagnóstico e Estatístico de Transtornos Mentais

E: *embryonic day*

ERK1/2: *extracelular signal-regulated kinase 1/2*

ESAM: moléculas de adesão seletivas de endotélio

GABA: ácido γ-aminobutírico

GABA-T: GABA-transaminase

GAD: glutamato descarboxilase

GAT: transportadores de GABA

GDH: glutamato desidrogenase

GDNF: *glial cell line-derived neurotrophic factor*

GFAP: *glial fibrilar acidic protein*

GLAST: transportador de glutamato-aspartato

Glt-1: transportador de glutamato 1

GS: glutamina sintetase

GSH: glutationa

HCPA: *Clinical Hospital of Porto Alegre*

HDAC: desacetilases de histonas

IL: infralímbico

IL-1: interleucina 1

IL-6: interleucina-6

JAMs: moléculas juncionas de adesão

Kir channels: *inwardly rectifying K^+ channels*

LPS: lipopolissacarídeo

MMP-9: *metallopeptidase 9*

mPFC: *medial prefrontal cortex*

NMDA: n-metil-D-aspartato

NO: óxido nítrico

NPA: asparagina-prolina-alanina

NVU: *neurovascular unit*

P: *postnatal day*

PAG: glutaminase ativada por fosforilação

PrL: pré-límbico

RSV: resveratrol

SIRT: sirtuína

SNAT: *sodium-coupled neutral amino acid transporters*

SNC: Sistema Nervoso Central

SOCS: *supressor of cytokine signaling*

SSADH: semialdeído succinato desidrogenase

TEA: Transtorno do espectro autista

TNF-α: fator de necrose tumoral

UEA: *Animal Experimentation Unit*

VPA: ácido valproico

ZOs: proteínas da zônula de oclusão

SUMÁRIO

1 INTRODUÇÃO

O Transtorno do Espectro Autista (TEA) é uma desordem complexa de etiologia desconhecida (GOTTFRIED et al., 2015), cuja principal característica são os prejuízos nos contextos sociais e a presença de estereotipia (AMERICAN PSYCHIATRIC ASSOCIATION, 2013). Uma importante ferramenta para o estudo desse transtorno são os modelos animais, como por exemplo, o modelo animal de autismo por exposição pré-natal ao ácido valproico (VPA) (NICOLINI; FAHNESTOCK, 2018). Dessa forma, muitos avanços na área foram feitos, no entanto ainda há muito para ser esclarecido.

1.1 TRANSTORNO DO ESPECTRO AUTISTA

O termo "autismo" foi usado pela primeira vez por Paul Eugen Bleuer, em 1911, para caracterizar o retraimento social observado em crianças com esquizofrenia (ASHOK; BAUGH; YERAGANI, 2012). Em 1924, a psiquiatra russa Grunya Efimovna Sukhareva descreveu um paciente como "do tipo introvertido, com uma tendência autista dentro de si" (ZELDOVICH, 2018).

Até pouco tempo, acreditava-se que o autismo havia sido descrito pela primeira vez na década de 40 por Leo Kanner e Hans Asperger. No entanto, duas décadas antes, Grunya publicou seu artigo descrevendo o caso de 6 crianças com características autistas (ZELDOVICH, 2018), cujos sintomas incluíam "falta de expressividade facial e de movimentos expressivos, afastamento de seus semelhantes, com tendência à solidão, fala de maneira estereotipada e interesses exclusivos" (SSUCHAREWA, 1926).

Em 1930, Georg Frankl e Anni Weiss relataram o caso de crianças retraídas socialmente, descrevendo características clássicas do autismo (ZELDOVICH, 2018). Apenas em 1943, Kanner descreveu 11 casos de crianças com autismo (KANNER, 1943) e, semelhantemente, Asperger em 1944 (ASPERGER, 1944).

Atualmente, a 5ª edição do Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-5) classifica o TEA como uma desordem do neurodesenvolvimento, caracterizada por prejuízos na comunicação e interação social, bem como por comportamentos repetitivos e estereotipados (compondo a chamada "díade comportamental"), e unifica transtornos que anteriormente eram separados: autismo clássico, síndrome de Asperger e transtorno global do desenvolvimento (AMERICAN PSYCHIATRIC ASSOCIATION, 2013). De acordo com o Centro de Controle e Prevenção de Doenças (CDC) dos Estados Unidos, a prevalência do TEA vem crescendo ao longo dos anos; atualmente, uma a cada 59 crianças até 8 anos de idade é

diagnosticada com o transtorno, sendo quatro vezes mais comum em homens do que em mulheres (BAIO et al., 2018). O TEA gera muitos impactos socioeconômicos, uma vez que influencia na capacidade de o indivíduo ser inserido no mercado de trabalho e de viver independentemente; sendo assim, uma gama de serviços médicos são necessários para as crianças afetadas, gerando impactos sobre a família e o orçamento público. Além disso, devido aos cuidados diários necessários, são despendidos muito tempo e atenção dos familiares (KAŁUŻNA-CZAPLIŃSKA; ŻURAWICZ; JÓŹWIK-PRUSKA, 2018). Dessa forma, mostra-se importante a busca pela compreensão da fisiopatologia do TEA a fim de auxiliar tanto no diagnóstico quanto na intervenção precoce contribuindo, assim, para uma melhora na qualidade de vida dos pacientes e familiares.

1.1.1 Diagnóstico e tratamento

O diagnóstico do TEA é baseado na observação clínica da díade comportamental (AMERICAN PSYCHIATRIC ASSOCIATION, 2013), ou seja, se dá exclusivamente por observação comportamental, visto que não há biomarcadores para a identificação desse transtorno (RUGGERI et al., 2014). Consequentemente, o diagnóstico ocorre de forma tardia, em torno dos 2-3 anos de idade, quando a criança não apresenta comportamentos “típicos” da sua idade, como por exemplo, a fala (PAUL et al., 2008).

Uma característica marcante do TEA é a elevada complexidade e heterogeneidade; além dos prejuízos comportamentais, os indivíduos apresentam um leque de sintomas e comorbidades, sendo que dificilmente duas pessoas com TEA manifestarão o mesmo conjunto de sintomas na mesma intensidade (MASI et al., 2017). Dentre as comorbidades, destacam-se alterações gastrointestinais, distúrbios do sono, agressividade, epilepsia e ansiedade (MANNION; LEADER, 2013). Ademais, um achado clínico de grande interesse no TEA é a presença de maior volume encefálico nos primeiros anos de vida (AYLWARD et al., 2002).

Além disso, segundo o CDC, não há tratamento farmacológico que atue especificamente nos sintomas centrais do TEA. No entanto, existem intervenções para outros sintomas ou comorbidades associadas ao transtorno, tais como prejuízo no sono, autoagressão e epilepsia, melhorando assim a qualidade de vida do paciente. Ainda, é importante considerar que, devido à heterogeneidade do TEA, as intervenções podem não contemplar todos os indivíduos da mesma maneira; dessa forma, cada tratamento deve ser individualizado e específico para as necessidades de cada paciente, podendo envolver por exemplo musicoterapia ou equinoterapia.

1.1.2 Etiologia e fatores de risco

Apesar dos vastos estudos acerca do TEA e dos avanços alcançados nos últimos anos, a sua etiologia permanece desconhecida. No entanto, há evidências de que fatores genéticos e ambientais, bem como as suas interações, estejam envolvidas no desencadeamento do transtorno. Além disso, acredita-se que as diferentes combinações entre esses fatores de risco estejam relacionadas com os diferentes graus de comprometimento presentes no espectro (GOTTFRIED et al., 2015).

Estudos envolvendo gêmeos mostram uma elevada herdabilidade do transtorno, com graus de concordância entre 70-90% para gêmeos monozigóticos e 10% para gêmeos dizigóticos (BAILEY et al., 1995; EL-FISHAWY; STATE, 2010), evidenciando a importância dos fatores genéticos. Além disso, muitos fatores ambientais já mostraram correlação com o TEA, como a ativação imune materna devido a infecções por diversos agentes durante a gestação, como bactérias e vírus, bem como a exposição a pesticidas e o uso de talidomida, de álcool e de ácido valproico durante a gestação (DIETERT; DIETERT; DEWITT, 2011; LYALL; SCHMIDT; HERTZ-PICCIOTTO, 2014; RODIER et al., 1997, 1996; SCHNEIDER et al., 2008; SCHNEIDER; PRZEWŁOCKI, 2005).

1.1.3 Modelo animal de autismo induzido por exposição pré-natal ao ácido valproico

O VPA (ácido 2-propilpentanoico) é um ácido carboxílico de cadeia ramificada, apresentando elevada lipossolubilidade e permeabilidade à barreira hematoencefálica (BHE) (BRUNI; WILDER, 1979). O VPA é utilizado para o tratamento da epilepsia, do transtorno bipolar e profilático da enxaqueca (REYNOLDS; SISK; RASGON, 2007), sendo rapidamente absorvido no trato gastrointestinal e se ligando extensivamente ($\geq 90\%$) a proteínas plasmáticas, além de apresentar tempo de meia vida curto (7-10 horas) e ser rapidamente eliminado do organismo, sendo metabolizado no fígado e excretado principalmente na urina (BRUNI; WILDER, 1979; SILVA et al., 2008).

Embora não esteja completamente elucidado, sabe-se que o VPA atua aumentando os níveis do neurotransmissor ácido γ-aminobutírico (GABA), e suprimindo a excitação glutamatérgica via receptores n-metil-D-aspartato (NMDA), alfa-amino-3-hidroxi-metil-5-4-isoxazolpropionico (AMPA) e de cainato (REYNOLDS; SISK; RASGON, 2007). Favorece também a transcrição gênica, inibindo as desacetilases de histonas (HDAC) (PHIEL et al., 2001), além de já ter sido demonstrada sua ação na ativação das sirtuínas, que são desacetilases dependentes de NAD (REID et al., 2005).

Como todos os medicamentos psicotrópicos atravessam a placenta, o primeiro trimestre gestacional é de especial atenção nesse contexto, pois nesse momento ocorre a organogênese, e isso representa uma janela biológica com reconhecido risco de teratogenicidade (MACFARLANE; GREENHALGH, 2018). O uso de medicação psicotrópica na gravidez é associado a complicações obstétricas (como baixo peso ao nascer) e complicações perinatais (ocorrendo logo após o nascimento) (MACFARLANE; GREENHALGH, 2018) e, especialmente o uso do VPA durante a gestação, está associado a um maior risco de desenvolvimento do TEA (CHRISTENSEN et al., 2013).

Embora existam hipóteses sobre como o VPA age para desencadear as alterações presentes no TEA, ainda não se sabe exatamente como ele atua nesse contexto (NICOLINI; FAHNESTOCK, 2018), sendo necessários mais estudos para compreender o mecanismo e as vias envolvidos na indução das mudanças associadas ao TEA pela exposição ao VPA.

O uso de modelos animais na pesquisa é de extrema importância no estudo de patologias e desordens, pois possibilita tanto a análise de vias quanto a obtenção de amostras biológicas para o estudo de possíveis mecanismos envolvidos na fisiopatologia desses transtornos, permitindo a aquisição de conhecimentos que possam auxiliar na clínica. Atualmente, existem diversos modelos animais de autismo e, embora existam limitações na interface entre humanos e animais (TORDJMAN et al., 2007), eles são amplamente utilizados na pesquisa, possibilitando diversas análises, além de serem mais acessíveis e replicáveis. Didaticamente, os modelos de autismo são divididos em: genéticos (mutações nos genes Fmr1, Mecp2, Shank2 e Shank3, por exemplo) (SCHROEDER et al., 2015), por lesão (como nos casos por ablação da amígdala, do hipocampo e do lobo temporal medial) (AMARAL; BAUMAN; MILLS SCHUMANN, 2003; BAUMAN; KEMPER, 1985), ativação imune materna (causada por infecções como rubéola e influenza, além de infusão de interleucina-6 (IL-6) e de lipopolissacarídeo - LPS) (DIETERT; DIETERT; DEWITT, 2011; MEYER, 2014) e farmacológicos (pela ação de agonistas β-2 adrenergicos, misoprostol e VPA, por exemplo) (DIETERT; DIETERT; DEWITT, 2011). Nesse último contexto, um modelo animal de autismo amplamente utilizado atualmente é o da exposição pré-natal ao VPA (BAMBINI-JUNIOR et al., 2011; NICOLINI; FAHNESTOCK, 2018; SCHNEIDER; PRZEWŁOCKI, 2005).

Diversos estudos em que foi utilizada a exposição pré-natal ao VPA mostraram que a prole de ratas expostas ao teratógeno apresentou alterações morfológicas no tronco encefálico, nos nervos cranianos e no cerebelo semelhantes às descritas em indivíduos com o transtorno (RODIER et al., 1996; RODIER et al., 1997; INGRAM et al., 2000), possibilitando uma primeira evidência do uso de VPA na indução de características do tipo autista em roedores.

Posteriormente, em 2005, um estudo demonstrou que ratos expostos no período pré-natal ao VPA apresentaram características comportamentais semelhantes às encontradas em indivíduos com TEA (SCHNEIDER; PRZEWŁOCKI, 2005), validando o modelo animal. Além disso, um estudo epidemiológico realizado na Dinamarca mostrou que gestantes que utilizaram VPA, principalmente durante o primeiro trimestre gestacional, apresentaram um risco maior de ter filhos com autismo (CHRISTENSEN et al., 2013).

1.2 BARREIRA HEMATOENCEFÁLICA

A BHE é uma barreira seletiva que limita a passagem de substâncias do sangue para o tecido encefálico, sendo composta por células endoteliais unidas por junções de oclusão, lâmina basal, pericitos que compartilham da mesma lâmina basal que as células endoteliais e pés astrocíticos (os quais serão discutidos separadamente no tópico "1.2.1 Astrócitos") (ABBOTT, 2013), compondo a unidade neurovascular. Neurônios e microglia também são observados na região perivascular, mas não contribuem diretamente para a manutenção da BHE (figura 1) (CHOI; KIM, 2008).

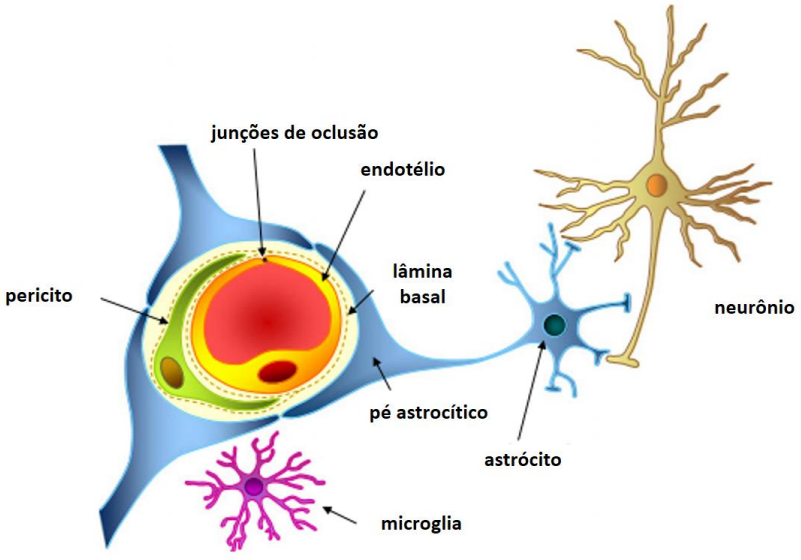

Figura 1 – Elementos que compõe a barreira hematoencefálica. A BHE é composta por células endoteliais unidas por junções de oclusão, lâmina basal, pericito e pés astrocíticos; neurônios e microglia também podem ser observados. Adaptado de: ABBOTT, 2013.

As junções de oclusão são responsáveis por unir fortemente as células endoteliais, sendo compostas pelas proteínas claudinas, ocludinas, moléculas juncionas de adesão (JAMs), moléculas de adesão seletivas de endotélio (ESAM) e proteínas da zônula de oclusão (ZOs).

Esse arranjo dificulta a passagem paracelular de moléculas e permite a livre passagem apenas de moléculas lipossolúveis e de baixo peso molecular (menores que 0,4 kDa) através da BHE, evitando a entrada de substâncias que poderiam causar danos encefálicos (ABBOTT et al., 2010; ABBOTT; RÖNNBÄCK; HANSSON, 2006). Outro componente que exerce importante função na manutenção da estrutura e da função da BHE é a lâmina basal; formada por proteínas da matriz extracelular como colágeno, laminina e perlecan, fornece suporte tanto para as células endoteliais quanto para os pericitos (ZHAO et al., 2015), os quais apresentam contratilidade devido à presença de actina de músculo liso em sua estrutura, sugerindo que exerçam importante papel no controle do fluxo sanguíneo, bem como na regulação da permeabilidade juncional (CARDOSO; BRITES; BRITO, 2010; LAI; KUO, 2005; LIU et al., 2012).

A disfunção da BHE permite que o encéfalo fique vulnerável a substâncias danosas que podem perturbar a homeostase desse tecido (OBERMEIER; DANEMAN; RANSOHOFF, 2013; ZHAO et al., 2015). Dessa forma, a integridade da BHE é diretamente relacionada à funcionalidade dos componentes da unidade neurovascular, bem como de suas comunicações.

1.2.1 Astrócitos

Os astrócitos são células gliais que expressam a proteína glial fibrilar ácida (GFAP) em seu citoesqueleto, um importante alvo para a marcação de astrócitos na pesquisa, podendo ser chamados de células $GFAP^+$ (PEKNY, 2001). Os prolongamentos astrocíticos formam “pés” em contato com a superfície externa do endotélio da BHE (CARDOSO; BRITES; BRITO, 2010), sendo os astrócitos caracterizados 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). Os astrócitos exercem importante função na manutenção da BHE, pois fornecem suporte estrutural, promovendo a formação da matriz extracelular (síntese de proteoglicanos). Também são importantes na neurogênese, no tamponamento de potássio, no suporte metabólico, em funções imunológicas e na modulação da plasticidade sináptica e da transmissão, além de auxiliarem na homeostase de neurotransmissores. (BLANCHETTE; DANEMAN, 2015; PALMER; OUSMAN, 2018).

Essa homeostase se dá pela recaptura e pelo metabolismo de glutamato e de GABA pelos astrócitos, os principais neurotransmissores excitatórios e inibitórios do encéfalo, respectivamente. A glutamina é o aminoácido precursor desses neurotransmissores, convertido a partir de glutamato por meio da enzima glutamina sintetase (GS), a qual está presente apenas nos astrócitos em condições normais (SCHOUSBOE; BAK; WAAGEPETERSEN, 2013), mas

em determinadas doenças como Alzheimer pode também ter uma produção residual em neurônios (FERNANDES et al., 2010; ROBINSON, 2000). Enquanto a maior parte do glutamato é recaptada pelos astrócitos por transportadores de glutamato (GLAST e Glt-1), grande parte do GABA é recaptado pelos neurônios GABAérgicos pré-sinápticos, por meio de transportadores de GABA (GAT) (SCHOUSBOE; BAK; WAAGEPETERSEN, 2013). O glutamato nos astrócitos pode ser convertido em glutamina pela GS, pode ser utilizado como substrato para o metabolismo oxidativo (havendo a atuação das enzimas glutamato desidrogenase (GDH) e de aminotransferases, como a aspartato aminotransferase (AAT)) e para a síntese de glutationa (GSH), um importante antioxidante biológico não-enzimático (ZEIDÁN-CHULIÁ et al., 2014). O GABA nos astrócitos pode ser convertido à glutamato a partir das enzimas GABA-transaminase (GABA-T) e desidrogenase succínico semialdeído (SSADH), que por sua vez pode ser transformado em glutamina pela GS. Para que essa glutamina seja enviada dos astrócitos para os neurônios GABAérgicos e glutamatérgicos existem transportadores de glutamina (*sodium-coupled neutral amino acid transporters* – SNAT) expressos em ambas as células. Nos neurônios glutamatérgicos a glutamina é convertida em glutamato pela glutaminase ativada por fosfato (PAG), enquanto que em neurônios GABAérgicos, o GABA é sintetizado por meio da descarboxilação do glutamato pela enzima glutamato descarboxilase (GAD) (SCHOUSBOE; BAK; WAAGEPETERSEN, 2013) (Figura 2), sendo a GAD65 e a GAD67 as isoformas mais importantes no Sistema Nervoso Central (SNC) (WATANABE et al., 2002).

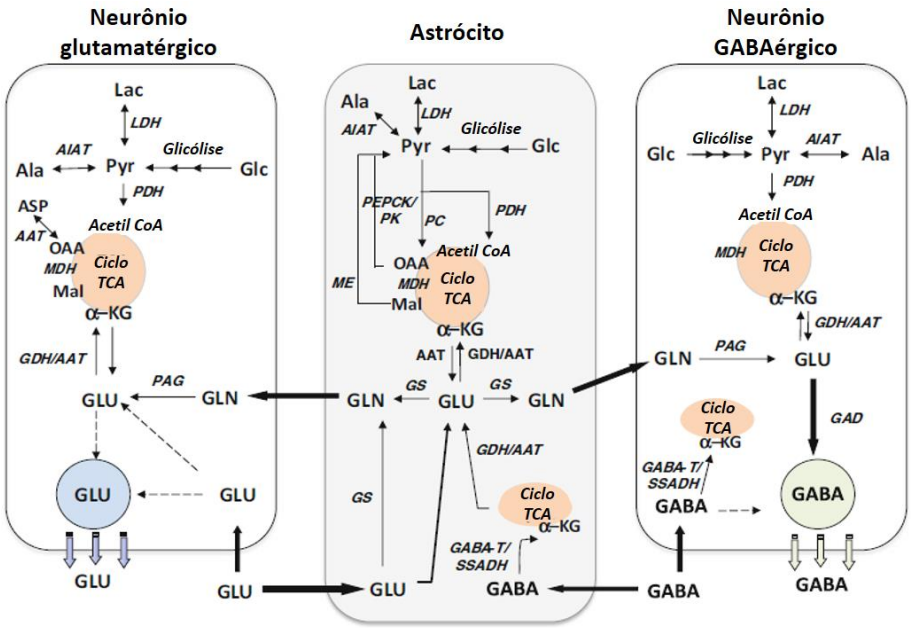

Figura 2 – Ciclo glutamato – glutamina. O glutamato (GLU) liberado na fenda sináptica é rapidamente captado pelos astrócitos por meio de transportadores. Nos astrócitos, o glutamato pode ser convertido em glutamina pela glutamina-sintetase (GS), no intermediário α-cetoglutarato do ciclo do ácido tricarboxílico (TCA) pelas enzimas glutamato desidrogenase (GDH) e aspartato aminotransferase (AAT) e ser destinado para a síntese de glutationa (não representado na figura). A glutamina é liberada pelos astrócitos e captada pelos neurônios glutamatérgicos, onde é convertida a glutamato pela glutaminase ativada por fosfato (PAG). O glutamato pode ser convertido no intermediário α-cetoglutarato ou ser encapsulado em vesículas e liberado na fenda sináptica. Nos neurônios GABAérgicos, a glutamina vinda dos astrócitos é convertida em glutamato pela PAG, o qual pode ser convertido a GABA pela glutamato descarboxilase (GAD) ou no intermediário α-cetoglutarato. *Ala* alanina, *ASP* aspartato, *Glc* glicose, *GLN* glutamina, *GLU* glutamato, *α-KG* α-cetoglutarato, *Lac* lactato, *Mal* malato, *OAA* oxaloacetato, *Pyr* piruvato, *ALAT* alanina aminotransferase, *AAT* aspartato aminotransferase, *GDH* glutamato desidrogenase, *GAD* glutamato descarboxilase, *GABA-T* GABA transaminase, *LDH* lactato desidrogenase, *MDH* malato desidrogenase, *ME* enzima málica, *PC* piruvato carboxilase, *PDH* piruvato desidrogenase, *SSADH* semialdeído succinato desidrogenase. Adaptado de: MCKENNA; FERREIRA, 2016.

Em condições normais, o processo inflamatório no encéfalo em desenvolvimento é controlado por diversos mecanismos, principalmente pelos astrócitos e pela microglia, sendo essas células cruciais para o reconhecimento de fatores pró-inflamatórios, os quais desempenham um papel importante na etiologia de vários distúrbios neurológicos e neuropsiquiátricos, incluindo o TEA (PETRELLI; PUCCI; BEZZI, 2016). Já em condições de perturbação da homeostase encefálica os astrócitos adquirem uma forma reativa, caracterizada por hipertrofia, presença de numerosos prolongamentos, aumento na produção de GFAP e proliferação astrocítica. Os astrócitos reativos podem exacerbar o dano tecidual, uma vez que eles liberam citocinas pró-inflamatórias (como o fator de necrose tumoral (TNF-α), a interleucina 1β (IL-1) e a IL-6), produzem e liberam óxido nítrico (NO) e espécies reativas de oxigênio (LIBERTO et al., 2004; LIDDELOW; BARRES, 2017), além de levar à redução na captação de glutamato, dificultando a ação dos astrócitos na homeostase de neurotransmissores (JAIN; KUMAR WADHWA; RAMANLAL JADHAV, 2015).

Diversos estudos relacionam disfunções astrocíticas a transtornos psiquiátricos (MONY et al., 2016; ZEIDÁN-CHULIÁ et al., 2014), já sendo demonstrada a associação entre o TEA e genes relacionados à ativação de células da glia e do sistema imune (VOINEAGU et al., 2011), além de aumento de gliose reativa e da proliferação de células da glia no encéfalo de indivíduos com o transtorno (PETRELLI; PUCCI; BEZZI, 2016). Ademais, sabe-se que há uma alteração nos níveis de citocinas no encéfalo e no sangue de pessoas com TEA, além de alterações encefálicas no modelo animal induzido por exposição ao VPA (DECKMANN et al., 2018). Semelhantemente, um estudo prévio do nosso grupo de pesquisa utilizando o mesmo

modelo demonstrou 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 - dados não publicados).

1.2.2 Aquaporina 4

As aquaporinas (AQP) são uma família de proteínas canais, cuja principal função é facilitar o movimento de água através das membranas celulares. Os monômeros de AQP apresentam, em geral, seis domínios transmembrana, embora eventualmente se descreva a aquaporina 4 (AQP4) com oito domínios, uma vez que dois de seus domínios não atravessam completamente a membrana (NAGELHUS; OTTERSEN, 2013; PAPADOPOULOS; VERKMAN, 2013). Além disso, as AQP apresentam um motivo conservado ao longo da sequência de aminoácidos asparagina-prolina-alanina (NPA), importante para a formação do poro pelo qual haverá a passagem de água e, em alguns casos, de outras moléculas (KOSINSKA ERIKSSON et al., 2013; NAGELHUS; OTTERSEN, 2013; VERKMAN; MITRA, 2000).

A AQP4 é o principal canal de água presente no SNC, localizada principalmente nos astrócitos presentes nas interfaces encéfalo-sangue e encéfalo-líquor, além de estar presente na membrana basolateral das células ependimárias. Essa proteína é importante no fluxo de água do tecido cerebral, apresentando papel crucial na migração astrocítica e na homeostase do potássio e do cálcio, além de participar da formação do edema encefálico (PAPADOPOULOS; VERKMAN, 2007, 2013). Complementarmente, alguns estudos em modelos *knockout* para AQP4 já 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).

No contexto do TEA, foi demonstrado uma diminuição na expressão de AQP4 no cerebelo de indivíduos com esse transtorno (FATEMI et al., 2008). Assim, considerando esse estudo prévio e a presença de edema em uma porcentagem significativa de indivíduos com TEA, fica evidente a importância do estudo das possíveis diferenças na expressão e/ou localização de AQP4 no TEA.

1.2.3 Alterações da permeabilidade e da dinâmica do volume encefálico no TEA

Uma observação clínica de grande interesse no TEA é o aumento do volume encefálico nos primeiros anos de vida, o qual regride ao longo dos anos, normalizando na idade adulta

(AYLWARD et al., 2002; HAZLETT et al., 2011). Além disso, estima-se que 20% das crianças com TEA apresentem macrocefalia (CHAWARSKA et al., 2011; HAZLETT et al., 2005).

O crescimento encefálico acelerado nos primeiros anos de vida, momento em que há aquisição de habilidades sociais e linguísticas, sugere que a aceleração prematura interfere na formação das regiões que dão suporte a essas habilidades (AYLWARD et al., 2002). Além disso, fisiologicamente há uma aceleração do crescimento encefálico na adolescência, período em que ocorre a maturação de habilidades relacionadas ao lobo frontal; em indivíduos com TEA ocorre uma desaceleração do crescimento encefálico neste período (AYLWARD et al., 2002).

Recentemente, um estudo prospectivo utilizando ressonância magnética funcional avaliou o volume encefálico em bebês com 6 meses de idade e foi capaz de predizer com 80% de precisão o diagnóstico de TEA que foi confirmado aos 24 meses de idade desses pacientes (EMERSON et al., 2017). Sendo assim, a dinâmica alterada do volume encefálico em indivíduos com TEA pode estar relacionada com as alterações na aquisição de habilidade sociais; no entanto, pouco se sabe sobre os mecanismos envolvidos no aumento do volume encefálico no TEA.

Em estudos avaliando a permeabilidade da BHE por meio da técnica do Azul de Evans, mostrou-se macroscopicamente a presença do corante no cerebelo de animais do modelo de autismo por exposição pré-natal ao VPA, evidenciando uma possível disfunção da BHE no modelo (KUMAR; SHARMA; SHARMA, 2015; KUMAR; SHARMA, 2016a, 2016b), uma vez que esse corante normalmente não entra no SNC por ter afinidade pela albumina e esta ser uma proteína de considerável peso molecular (PETRONILHO; GOLDMAN; BARICHELLO, 2019).

Além disso, foi observada uma maior permeabilidade da BHE ao corante Azul de Evans no plexo coroide e na área somatossensorial primária no grupo VPA em relação ao controle, evidenciando um possível afrouxamento da BHE, o que poderia estar relacionado ao quadro de edema encefálico (dados não publicados). Dessa forma, o estudo de elementos que compõe a BHE, como os astrócitos, nos modelos animais de autismo pode contribuir na compreensão dos mecanismos envolvidos no aumento do volume encefálico observado em crianças com TEA.

1.3 CÓRTEX PRÉ-FRONTAL MEDIAL E O TEA

O córtex pré-frontal (CPF) pode ser dividido em córtex pré-frontal lateral (CPFl) e em córtex pré-frontal medial (CPFm) (WOOD; GRAFMAN, 2003). O CPFm é uma das regiões

encefálicas responsáveis pela cognição e pelo comportamento social, além do processamento da linguagem, do planejamento e da tomada de decisões (GROSSMANN, 2013). Além disso, o CPFm faz conexões com regiões encefálicas envolvidas no processamento emocional (amígdala), na memória de trabalho e de longa duração (hipocampo) e em regiões sensoriais superiores (córtex temporal), sendo responsável pelo processamento e pela integração dessas informações (GROSSMANN, 2013). Essas conexões, bem como os *inputs* recebidos do ambiente, permitem que o indivíduo perceba o contexto e a situação na qual está inserido, moldando a sua personalidade e as suas ações. Acredita-se que o *feedback* de uma ação leva à plasticidade sináptica do CPFm, assegurando que determinado comportamento é apropriado em um contexto específico e que as decisões são tomadas de acordo com experiências prévias antecipando o resultado emocional e o estado corporal de experiências futuras (EUSTON; GRUBER; MCNAUGHTON, 2012).

Em roedores, o CPFm pode ser subdividido em córtex cingulado (CC), pré-límbico (PrL) e infralímbico (IL) (PAXINOS; WATSON, 2013). Enquanto as regiões ventrais (PrL ventral e IL) estão mais associadas ao controle emocional e autônomo, as regiões dorsais (CC e PrL dorsal) parecem ter mais relação com o controle do planejamento das ações (figura 3) (EUSTON; GRUBER; MCNAUGHTON, 2012). Essas características se devem às diferenças na conectividade: regiões dorsais apresentam uma baixa conexão com centros emocionais e autônomos, mas uma forte conectividade com áreas motoras e pré-motoras, enquanto nas regiões ventrais ocorre o oposto (EUSTON; GRUBER; MCNAUGHTON, 2012).

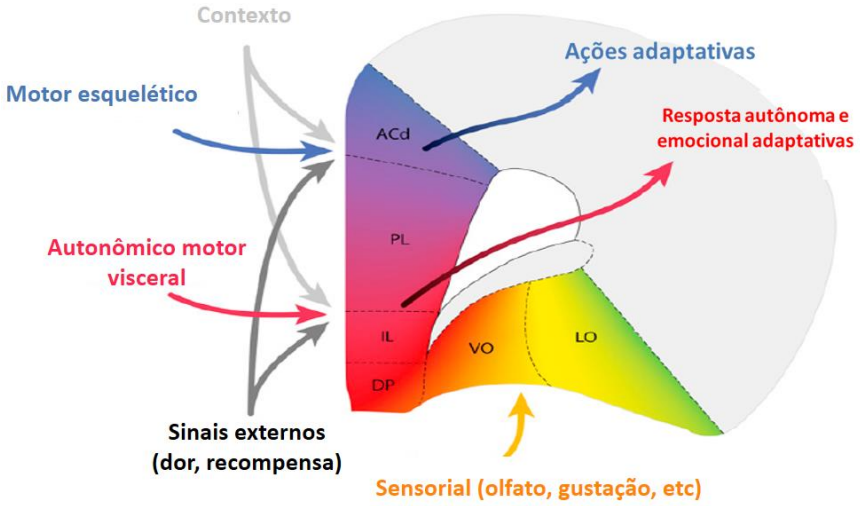

Figura 3 - Subdivisões do córtex pré-frontal de roedores. As áreas dorsais do CPFm têm mais relação com o controle das ações, enquanto as áreas ventrais estão mais associadas ao controle emocional e autônomo. ACd, cingulado; PL, pré-límbico; IL, infralímbico. Adaptado de: EUSTON; GRUBER; MCNAUGHTON, 2012.

Os córtices em geral (incluindo o CPFm) apresentam uma organização laminar, que compreende 6 camadas horizontais, as quais diferem entre si de acordo com a morfologia e densidade neuronal, conectividade, aferências e eferências. A camada mais externa é a molecular (I), que contém poucos corpos celulares neuronais; a camada granular externa (II) e a camada piramidal externa (III) apresentam elevada densidade de neurônios excitatórios piramidais; a camada granular interna (IV) compreende os neurônios estrelados espinhosos excitatórios; a camada de células piramidais interna (V) é formada por grandes neurônios piramidais corticofugais excitatórios; a camada fusiforme ou multiforme (VI) apresenta neurônios piramidais corticotalâmicos excitatórios (SWENSON; GULLEDGE, 2017).

As camadas II e III recebem projeções dos neurônios estrelados espinhosos, dos neurônios piramidais próximos e dos neurônios piramidais de áreas corticais de baixa e alta ordem. Além disso, enviam projeções para o neocórtex, estriado e amígdala. Dessa forma, essas camadas apresentam elevada conectividade, exercendo importante função na plasticidade associativa e participando das redes reverberatórias da memória de trabalho. A camada IV recebe *inputs* do núcleo talâmico e os axônios dos neurônios estrelados se projetam para as camadas II e III para inervar os neurônios piramidais, sendo de extrema importância na integração e na amplificação do *input* recebido, bem como na retransmissão dessa informação para os neurônios piramidais das camadas superiores. A camada V exerce importante papel, visto que seus neurônios são mais excitáveis e disparam em altas frequências em resposta ao estímulo sensorial, enviando projeções para alvos subcorticais profundos, incluindo a medula espinhal, o tronco cerebral e o *tectum*. Por fim, a camada VI apresenta *input* e *output* da mesma região, o núcleo talâmico. (SWENSON; GULLEDGE, 2017). Além disso, a superfície externa do córtex é composta pela glia limitante, uma camada de astrócitos que separam as artérias penetrantes do parênquima cerebral (ZHAO et al., 2015). Também há a divisão em camadas verticais (colunas), formadas por uma organização orientada de neurônios compartilhando de responsividade sensorial e/ou motora que, em conjunto com as diferentes aferências das camadas laminares, formam um "circuito cortical", que inclui vários subtipos de neurônios excitatórios e inibitórios (SWENSON; GULLEDGE, 2017).

Acredita-se que anormalidades a nível de integração nas subregiões do CPFm são responsáveis por algumas alterações encontradas em indivíduos com TEA, tanto a nível emocional, quanto de memória, de percepção sensorial e de habilidades motoras (SHALOM, 2009). Além disso, já foi demonstrada desorganização cortical de neurônios e citoarquitetura laminar anormal, contendo "fragmentos" desintegrados no CPF e no córtex temporal de pessoas com o transtorno (CASANOVA et al., 2002; STONER et al., 2014). Dessa forma, essas

anormalidades podem estar relacionadas às alterações de integração do CPFm e, em algum nível, a alterações comportamentais encontradas no TEA.

Outras evidências apontam que as conexões na camada V de células piramidais do CPFm são mais fracas, porém mais numerosas (maior conectividade) no modelo animal de autismo induzido pelo VPA, além de haver uma maior plasticidade sináptica nessa região no modelo (RINALDI; PERRODIN; MARKRAM, 2008). Disfunções nesse contexto (como a hiperfuncionalidade do CPFm) poderiam implicar em alterações comportamentais observadas em indivíduos com TEA, uma vez que o CPF é fundamental no controle executivo de outras regiões encefálicas (RINALDI; PERRODIN; MARKRAM, 2008).

Por fim, ainda falando de CPF e TEA, foi demonstrado um aumento da expressão de astrócitos e de microglia em indivíduos com o transtorno (EDMONSON; ZIATS; RENNERT, 2014) e, considerando o papel dessas células em questões imunológicas e, principalmente dos astrócitos na modulação da transmissão, na plasticidade sináptica e na manutenção da integridade da BHE (BLANCHETTE; DANEMAN, 2015; CARDOSO; BRITES; BRITO, 2010), alterações na expressão de células gliais poderiam estar associadas a anormalidades nas funções citadas.

Essas evidências, em conjunto, apontam para uma disfunção tanto a nível estrutural e celular, quanto de conectividade e de integração do CPFm no TEA, que podem estar associadas às alterações comportamentais relacionados ao transtorno, uma vez que muitos dos comportamentos alterados em indivíduos com TEA estão associados com essa região.

1.4 RESVERATROL

O resveratrol (3,4',5 – trihidroxiestilbeno - RSV) é um polifenol encontrado naturalmente em plantas, como em uvas e em amendoins, além de estar presente no vinho tinto. Já foi demonstrado seu efeito protetor e terapêutico devido às suas propriedades anti-inflamatória e antioxidante em diversas doenças, como no câncer, em doenças cardiovasculares, na diabetes e em doenças neurológicas, como o Alzheimer (BERMAN et al., 2017; PANGENI et al., 2014; SMOLIGA; BAUR; HAUSENBLAS, 2011). Além disso, o RSV vem atraindo a atenção de diversos pesquisadores, uma vez que já foi demonstrado também seu efeito neuroprotetor (QUINCOZES-SANTOS; GOTTFRIED, 2011). No entanto, tem sido proposto que o RSV pode apresentar propriedades citotóxicas e pró-oxidantes dependendo da sua concentração e tempo de exposição, já tendo sido demonstrado seu efeito pró-oxidante dose-dependente em células estreladas hepáticas (MARTINS et al., 2014).

Embora não se saiba completamente como o RSV age, muitos mecanismos têm sido propostos. Sabe-se que o RSV é um ativador da SIRT1, um membro da família das sirtuínas capazes de causar *downregulation* da proteína p53, ação relacionada com a sobrevivência celular (BAUR; SINCLAIR, 2006; MALHOTRA; BATH; ELBARBRY, 2015). Além disso, sua ação anti-inflamatória está associada com a inibição da ciclo-oxigenase, uma enzima crucial na produção de moléculas pró-inflamatórias (BAUR; SINCLAIR, 2006), além de já ter sido demonstrado o aumento da expressão de SOCS-1, uma proteína supressora de citocinas, pelo RSV em modelo animal de doença de Parkinson (MALHOTRA; BATH; ELBARBRY, 2015). O RSV também aumenta a produção de fatores neurotróficos, como GDNF (*glial cell line-derived neurotrophic factor*) e BDNF (*brain derived neurotrophic factor*), sendo proposto que essa ação se deva a modulação de ERK1/2 (*extracelular signal-regulated kinase 1/2*) e CREB (*cAMP responsive element-binding protein*) nos astrócitos (MALHOTRA; BATH; ELBARBRY, 2015).

Estudos do nosso grupo já demonstraram que o tratamento pré-natal com RSV foi capaz de prevenir alterações sociais, celulares, sensoriais e moleculares no modelo VPA. Por meio do teste de três câmaras foi demonstrado que os animais do grupo VPA não apresentam preferência entre interagir com um objeto ou com um semelhante, além de não demonstrarem interesse em novidade social, importantes prejuízos sociais que foram prevenidos pelo RSV (BAMBINI-JUNIOR et al., 2014). Além disso, o RSV preveniu alterações na organização cortical da área somatossensorial primária, bem como a hiperresponsividade sensorial dos animais do grupo VPA demonstrada pelo *Whisker Nuisance Task* (FONTES-DUTRA et al., 2018). Ademais, alterações na expressão de microRNA induzidas pelo VPA também foram prevenidas pelo RSV (HIRSCH et al., 2018). Dessa forma, esse composto se mostra uma ferramenta promissora no estudo de vias biológicas envolvidas na fisiopatologia do TEA.

1.5 JUSTIFICATIVA

Em virtude da elevada prevalência do TEA, dos gastos socioeconômicos envolvidos e da etiologia desconhecida, relacionada com o diagnóstico e o tratamento tardio, o estudo desse transtorno é de grande importância. Tendo em vista a característica clínica de aumento do volume encefálico nos primeiros anos de vida de crianças com TEA e a sua possível associação com as alterações sociais presentes no transtorno, o estudo de mecanismos que possam estar envolvidos com esse achado clínico, como a permeabilidade da BHE e a análise de astrócitos e de AQP4, podem contribuir para a melhor compreensão do TEA. Além disso, em virtude dos

efeitos do RSV e das evidências observadas no modelo animal de autismo por exposição ao VPA, esse composto se mostra uma ferramenta promissora de estudo no contexto do TEA. Assim, estratégias envolvendo 4 grupos experimentais (Controle, VPA, RSV e VPA + RSV) permitem o estudo de mecanismos associados com a fisiopatologia do TEA, tais como aumento do volume encefálico observado em pacientes e alterações celulares, bem como traçar paralelos com vias que possam estar envolvidas na etiologia desse transtorno.

1.6 OBJETIVOS

Compreender os mecanismos envolvidos no aumento do volume encefálico no TEA e traçar paralelos com vias que possam estar envolvidas na etiologia desse transtorno.

1.6.1 Objetivo geral

Avaliar permeabilidade da barreira hematoencefálica, níveis de aquaporina 4 e número de células $GFAP^+$ no córtex pré-frontal medial de animais de 30 dias no modelo animal de autismo induzido por exposição pré-natal ao ácido valproico, verificando um possível efeito preventivo do resveratrol administrado intra-útero.

1.6.2 Objetivos específicos

a) avaliar a permeabilidade da barreira hematoencefálica;

b) avaliar o conteúdo fluorescente de aquaporina 4 e de GFAP;

c) quantificar o número de células $GFAP^+$.

2 ARTIGO CIENTÍFICO

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Effects of resveratrol on astrocytic parameters and blood brain barrier permeability in animal model of autism induced by prenatal exposure to valproic acid

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Abstract

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by impairments in communication and social interaction, as well as by repetitive and stereotyped behaviors. An interesting clinical finding in ASD is the increase in brain volume in first years of life. Although its etiology remains unclear, an important known environmental risk factor is prenatal exposure to valproic acid (VPA). Resveratrol (RSV) is an anti-inflammatory, antioxidant and neuroprotective molecule known by preventing impairments in social behaviors in VPA animal model. Here, we analyze the VPA effect in blood-brain barrier (BBB) permeability, in $GFAP^+$-cells and in aquaporin-4 (AQP4) in medial prefrontal cortex (mPFC) of 30 days-old rats, and the protective effect of RSV. From embryonic day 6.5 (E6.5) to E18.5, pregnant Wistar rats received subcutaneously RSV (3.6 mg/kg) or dimethyl sulfoxide (DMSO - vehicle) and in E12.5 received intraperitoneally VPA (600 mg/kg) or saline (0.9%). Only male pups were used, euthanized at 30 days-old under deep anesthesia with ketamine (300 mg/Kg) and xylazine (40 mg/Kg). The animals evaluated for Evans blue dye permeability were injected with Evans blue 2% solution (4 mg/kg), anesthetized, submitted to transcardiac perfusion, the brain was removed, sectioned and analyzed by fluorescence. For immunofluorescence

technique, the animals were submitted to transcardiac perfusion, the brain was removed, fixed and sectioned. Animals from VPA group showed increased BBB permeability to Evans blue dye, prevented by RSV. There was an effect of VPA prenatal exposure in deeper layers of prelimbic (PrL) increasing the number of $GFAP^+$-cells in all group receiving VPA, and a synergistic effect of RSV and VPA in CC (upper) PrL (upper) and infralimbic (IL - deeper). Analyzing the ratio of $GFAP^+$-cells was observed that RSV prevents only in PrL the disorganization in GFAP distribution caused by VPA exposition through the layers in CC and PrL: decreases in upper and increases in deeper layers. Besides, there was a decrease in AQP4 fluorescent content in VPA group. Taken together, these data showed important alterations in BBB permeability, $GFAP^+$-cells and AQP4 in mPFC of VPA group and highlights the use of RSV as a valuable tool to explore ASD pathophysiology.

1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder of high prevalence, affecting 1:59 children aged 8 years in the USA (Baio et al., 2018). According to the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), the diagnosis consists of a behavioral dyad, composed by impairments in communication and social interaction, as well by repetitive behaviors, stereotypies and restricted interests (American Psychiatric Association, 2013). Although its etiology remains unclear, the importance of genetic and environmental factors to ASD is known (Gottfried et al., 2015). Studies involving twins show a high heritability of the disorder, with concordance between 70-90% for monozygotic twins and 10% for dizygotic twins (Bailey et al., 1995; El-Fishawy and State, 2010). Furthermore, the exposure to several environmental risk factors during pregnancy is associated with ASD onset, such as maternal infections, thalidomide, alcohol and valproic acid (VPA) (Christensen et al., 2013; Dietert et al., 2011; Rodier et al., 1996, 1997; Schneider and Przewłocki, 2005). Thus, a widely used animal model of autism is by prenatal exposure to VPA, presenting many similarities with autistic subjects (Bambini-Junior et al., 2011; Mabunga et al., 2015; Schneider and Przewłocki, 2005).

A brain region of interest in ASD is the medial prefrontal cortex (mPFC), which is important for cognition and social behavior, as well as language processing, planning and decision making (Grossmann, 2013). In addition to behavior impairments, were described various comorbidities in ASD, such as epilepsy, gastrointestinal disturbs and anxiety (Mannion and Leader, 2013). Moreover, an important clinical finding is the presence of increased brain volume in first years of life, normalized in adulthood (Aylward et al., 2002; Hazlett et al., 2011), being estimated that 20% of children with ASD present macrocephaly (Chawarska et al., 2011; Hazlett et al., 2005). Changes in neural growth dynamics may be related to alterations in social skill acquisition, since they occur in specific periods of the development: the acceleration of brain volume in early life, when social and linguistic skills are acquired, and the deceleration in adolescence, when occurs the maturation of abilities related to the frontal lobe (Aylward et al., 2002).

In this context, the blood brain barrier (BBB) take an important role limiting the crossing of substances from periphery to the brain (Abbott, 2013). The BBB is a selective barrier composed by endothelial cells joined by occlusion junctions, basal lamina, pericytes and astrocytic feet (Abbott, 2013), forming the neurovascular unit (NVU). Astrocytes are glial cells that provide structural support, contribute to neurotransmitters homeostasis and play a crucial role in the maintenance of BBB, as well as acting in synaptic plasticity and in immunological functions (Blanchette and Daneman, 2015; Cardoso et al., 2010; Palmer and Ousman, 2018).

Astrocytic changes have already been demonstrated in ASD (Edmonson et al., 2014; Rinaldi et al., 2008; Shalom, 2009; Stoner et al., 2014), indicating the possible contribution of this NVU component to the mechanisms involved in the increased brain volume in ASD individuals. Besides, studies investigating the BBB permeability through the Evans blue dye in VPA animal model showed the presence of this dye in cerebellum (Kumar et al., 2015; Kumar and Sharma, 2016a, 2016b). Similarly, recent data from our group demonstrated increased BBB permeability to the same dye in somatosensory area and in choroid plexus in the VPA model (unpublished data). Taking together, the present data indicate increased BBB permeability in the VPA group, which could be leading to brain edema.

Likewise, the study of aquaporins (AQP), a protein channels family that facilitates the intracellular transport of water, can also contribute to the understanding of the mechanisms involved in the brain volume increase (Nagelhus and Ottersen, 2013; Papadopoulos and Verkman, 2013). The aquaporin-4 (AQP4) is the major of the Central Nervous System (CNS), localized in astrocytes and in ependymal cells, playing an important role in water flow in brain tissue, in astrocytic migration and in potassium and calcium homeostasis, as well as in the formation of brain edema (Papadopoulos and Verkman, 2007, 2013).

Resveratrol (3,4`,5-trihydroxystilbene - RSV) is a naturally occurring polyphenol in plants, with anti-inflammatory, antioxidant (Berman et al., 2017) and neuroprotective (Quincozes-Santos and Gottfried, 2011) effects. Moreover, the administration of RSV during pregnancy in VPA animal model was able to prevent the VPA-induced alterations in behavior (Bambini-Junior et al., 2014), sensory system (Fontes-Dutra et al., 2018) and microRNA levels (Hirsch et al., 2018). Thus, this molecule is an excellent tool in the search for altered pathways in ASD. Taking together, alterations in parameters of BBB and in AQP4 could be associated with the increased brain volume observed in ASD subjects, and the prenatal administration of RSV is a potential tool to investigate the mechanisms involved in ASD pathophysiology.

2. Materials and Methods

2.1 Animals

Wistar rats were obtained from Animal Experimentation Unit (UEA) of Clinical Hospital of Porto Alegre (HCPA) and maintained under a standard 12/12h light/dark cycle at a constant temperature of 22 ± 2ºC and *ad libitum* access to food and water. The animals were handled in accordance with the guidelines established (Arouca Law 11.794/2008). This project was approved by the ethics committee of HCPA (CEUA/HCPA 130047).

Animals were mated overnight and in the next morning, the pregnancy was verified by spermatozoa presence in the vaginal fluid; once confirmed the pregnancy was considered the embryonic day 0.5 (E0.5). From E6.5 to E18.5, the pregnant rats received subcutaneously injection of RSV (3.6 mg/kg) or dimethyl sulfoxide (DMSO - vehicle). At E12.5, rats received a single injection intraperitoneally of 600 mg/kg VPA or saline solution 0,9%, as described previously (Bambini-Junior et al., 2014). Thus, were formed the experimental groups: Control (vehicles), RSV, VPA and RSV + VPA. At postnatal day 21 (P21) the litter was weaned and at P30 the male rats were euthanized under deep anesthesia with ketamine (300 mg/Kg) and xilazine (40 mg/Kg) for the experiment procedures (Panel 1).

2.2 Tissue preparation

The animals evaluated for permeability to Evans blue dye were injected intraperitoneally with Evans blue 2% solution diluted in saline (4 mg/kg) (Kumar et al., 2015; Kumar and Sharma, 2016b) and, after 2 hours, were anesthetized and submitted to transcardiac perfusion with saline solution 0.9% and paraformaldehyde 4%. The brain was removed and post-fixed during 6 hours in paraformaldehyde 4% and subsequently cryopreserved in increscent concentrations of sucrose (15% and 30%) until complete submersion. The tissues were kept in -80ºC ultra-freezer until realization of coronal slices of 25 μm in cryostat (Leica Microsystems GmbH) using a rat brain atlas (Paxinos and Watson, 2013) to identify the sections containing mPFC (bregma +3.24, interaural 12.24 mm).

For immunofluorescence technique, animals were anesthetized, euthanized by transcardiac perfusion with saline solution 0.9% and paraformaldehyde 4%, the brain was removed, preserved and sliced as described above.

2.3 Permeability to Evans blue dye

Considering that Evans blue dye emits fluorescence, the slices of 25 μm obtained in cryostat were incubated only with DAPI solution (1:10000 – Invitrogen MP01306) during 10 minutes, followed by 5 washes with PBS 0,1 M buffer (3 minutes each) and the mounting medium fluorshield (Sigma F6132) and coverslip were added. The images were obtained in the confocal microscope (Olympus FluoView 4.0) of Microscopy and Microanalysis Center (CMM-UFRGS), and three regions of the mPFC were captured: cingulate cortex (CC - upper and deeper), prelimbic (PrL - upper and deeper) and infralimbic (IL - upper and deeper), as demonstrated in Figure 1. The fluorescence was analyzed using the ImageJ software.

2.4 Immunofluorescence

Coronal brain slices (25 μm) of mPFC were obtained as described above. Slices were exposed to the following steps: 1) 3 washes with PBS 0.1 M buffer (3 minutes each); 2) permeabilization with PBS-Triton 0.3% (10 minutes); 3) 5 washes with PBS 0.1 M buffer (3 minutes each); 4) blockage with PBS-Triton 0.3% BSA 5% (1 hour); 5) incubation with primary antibodies in blocking solution for 48h at 4ºC; 6) 3 washes with PBS 0.1 M buffer (5 minutes each); 7) incubation with secondary antibodies for 2h at room temperature; 8) 3 washes with PBS 0.1 M buffer (3 minutes each); 9) incubation with DAPI solution (10 minutes); 10) 3 washes with PBS 0.1 M buffer (5 minutes each); 11) addition of mounting medium and coverslip.

Primary antibodies used were anti-AQP4 (produced in rabbit, diluted to 1:500 in blocking solution – PBS-Triton 0.3% BSA 1% - Santa Cruz Biotechnologies sc-20812) and anti-GFAP (produced in mouse, diluted to 1:500 in blocking solution – Cell Signaling mAb #3670); secondary antibodies used were anti-IgG of rabbit (Alexa 546) and mouse (Alexa 488) diluted to 1:2000 in blocking solution; DAPI solution was diluted to 1:10000 in blocking solution. Images were obtained in the confocal microscope of CMM-UFRGS, and the same three regions were captured. The fluorescence was analyzed using ImageJ software and the number of cells were analyzed using the same software with the Cell Counter plug-in and Olympus Fluoview software. For each animal, a blade containing three slices of the mPFC was analyzed.

2.5 Statistical analysis

Quantitative analyses were performed using GraphPad Prism 6 software, applying one-way ANOVA followed by Bonferroni`s posttest. Data is reported as mean ± standard deviation and $p<0.05$ was considered statistically significant.

3. Results

3.1 Prenatal administration of RSV prevents VPA-induced BBB permeability to Evans blue dye

An increased permeability to Evans blue was observed in mPFC regions analyzed in VPA group compared to control group and RSV prenatal treatment was capable to prevent this alteration (Figure 2): CC – upper (Control: 96.52 ± 17.62, RSV: 86.58 ± 22.16, VPA: 355.30 ± 74.43, RSV+VPA 78.36 ± 16.27, $F = 56.80$, $p<0.0001$); CC – deeper (Control: 89.58 ± 11.29, RSV: 91.55 ± 25.14, VPA: 402.30 ± 65.86, RSV+VPA 83.94 ± 8.458, $F = 95.56$, $p<0.0001$); PrL – upper (Control: 84.66 ± 20.02, RSV: 89.79 ± 24.82, VPA: 343.60 ± 110.00, RSV+VPA 75.93 ± 13.74, $F = 27.18$, $p<0.0001$); PrL – deeper (Control: 87.42 ± 15.02, RSV: 90.89 ± 24.00, VPA: 305.00 ± 129.5, RSV+VPA 74.09 ± 14.88, $F = 14.91$, $p<0.0001$); IL – upper (Control: 85.79 ± 9.783, RSV: 83.76 ± 18.57, VPA: 241.9 ± 113.3, RSV+VPA 84.74 ± 20.57, $F = 9.994$, $p = 0.0006$); IL – deeper (Control: 93.97 ± 14.61, RSV: 86.84 ± 18.33, VPA: 254.8 ± 97.16, RSV+VPA 83.97 ± 17.49, $F = 14.77$, $p<0.0001$).

3.2 The VPA group does not present alterations in the GFAP fluorescence content.

No differences was observed in GFAP fluorescence content from mPFC regions: CC – upper (Control: 381.6 ± 66.04, RSV: 316.1 ± 73.02, VPA: 406.4 ± 132.5, RSV+VPA 425.6 ± 144.1, $F = 0,5950$, $p = 0.6325$); CC – deeper (Control: 359.3 ± 56.14, RSV: 313.3 ± 59.27, VPA: 357.9 ± 106, RSV+VPA 331.4 ± 114.2, $F = 0.2189$, $p = 0.8811$); PrL – upper (Control: 385.3 ± 116.4, RSV: 282.9 ± 64.23, VPA: 335.2 ± 33.48, RSV+VPA 383.2 ± 90.62, $F = 1.1$, $p = 0.3939$); PrL – deeper (Control: 379.1 ± 70.08, RSV: 267.2 ± 24.25, VPA: 348.2 ± 73.79, RSV+VPA 314.8 ± 60.98, $F = 1.967$, $p = 0.1830$); IL – upper (Control: 389.7 ± 91.47, RSV: 275.6 ± 66.91, VPA: 314.3 ± 47.62, RSV+VPA 361.8 ± 44.79, $F = 1.954$, $p = 0.1850$); IL – deeper (Control: 365.1 ± 60.33, RSV: 260.7 ± 46.25, VPA: 319.8 ± 62.65, RSV+VPA 306.9 ± 71.96, $F = 1.706$, $p = 0.2285$).

3.3 The number of $GFAP^+$-cells per group was altered among groups depending of mPFC region.

These results are showed in Figure 3A,B. There was an effect of VPA prenatal exposure in deeper layers of PrL increasing the number of $GFAP^+$ cells in all groups receiving VPA (VPA and RSV+VPA groups) (Control: 92.25 ± 19.44, RSV: 97.50 ± 30.65, VPA: 195.9 ± 19.09, RSV+VPA 222.8 ± 29.73, $F = 26.07$, $p< 0.0001$). The RSV+VPA group increased $GFAP^+$-cells in CC (upper - Control: 122 ± 18.48, RSV: 122 ± 48.33, VPA: 172.5 ± 24.40, RSV+VPA 215 ± 36.77, $F = 6.422$, $p = 0.0107$), PrL (upper - Control: 118.3 ± 37.96, RSV: 95.17 ± 47.22, VPA: 156.6 ± 36.88, RSV+VPA 237.7 ± 19.21, $F = 8.892$, $p = 0.0036$) and IL

(deeper - Control: 80 ± 35.22, RSV: 85.83 ± 38.71, VPA: 161.1 ± 60.03, RSV+VPA 205.7 ± 16.75, F = 6.795, p = 0.0089), demonstrating a synergistic effect of RSV and VPA.

3.4 Effect of VPA on the ratio of GFAP$^+$ cells: increased relative number in deeper layers and decreased in upper layers in CC and PrL

These results are showed in Figure 3A,C. The VPA group decreased the number of GFAP$^+$-cells in upper layers of CC (Control: 0,609 ± 0.047, RSV: 0.558 ± 0.016, VPA: 0.497 ± 0.029, RSV+VPA 0.552 ± 0.057, F = 4.77, p = 0.0295) and PrL (Control: 0,556 ± 0,044, RSV: 0.547 ± 0.015, VPA: 0.42 ± 0.023, RSV+VPA 0.517 ± 0.016, F = 11.74, p = 0.0018). On the other hand, the VPA group increased the number of GFAP+-cells in deeper layers of CC (Control: 0,39 ± 0.047, RSV: 0.441 ± 0.016, VPA: 0,5030 ± 0,029, RSV+VPA 0.447 ± 0.057, F = 4.77, p = 0.0295) and PrL (Control: 0.443 ± 0.049, RSV: 0.453 ± 0.015, VPA: 0.579 ± 0.023, RSV+VPA 0.482 ± 0.016, F = 11.74, p = 0.0018). The RSV was able to prevent the VPA-induced alterations in PrL. No differences in this parameter was observed in IL (upper: Control: 0,578 ± 0.089, RSV: 0.525 ± 0.108, VPA: 0.499 ± 0.137, RSV+VPA 0.52 ± 0.039, F = 1.371, p = 0.3129 and deeper: Control: 0.421 ± 0.083, RSV: 0.474 ± 0.108, VPA: 0.5 ± 0.137, RSV+VPA 0.479 ± 0.039, F = 1.371, p = 0.3129).

3.5 The VPA group presented decreased AQP4 in mPFC regions

These results are showed in Figure 3A,D. The VPA group presented a significant decrease in the fluorescence content of AQP4 in all mPFC regions and the prenatal treatment with RSV was not able to prevent this effect: CC - upper (Control: 559 ± 68.74, RSV: 378.3 ± 92.29, VPA: 261.8 ± 89.43, RSV+VPA 508.5 ± 185.3, F = 4.835, p = 0.0249). CC – deeper (Control: 614.7 ± 155.4, RSV: 358.2 ± 87.94 (*per se* effect of RSV), VPA: 260.1 ± 48.64, RSV+VPA 432.7 ± 127, F = 7.316, p = 0.0057). PrL – upper (Control: 671.7 ± 308.7, RSV: 371.5 ± 94.17, VPA: 222.5 ± 46.70, RSV+VPA 378.1 ± 84.02, F = 4.661, p = 0.0245). PrL – deeper (Control: 672.2 ± 283, RSV: 343 ± 106.6, VPA: 228.4 ± 57.37, RSV+VPA 409.9 ± 81.86, F = 5.230, p = 0.0174). IL – upper (Control: 654.5 ± 299.9, RSV: 362.8 ± 120.2, VPA: 197.6 ± 29.85, RSV+VPA 314.9 ± 73.06, F = 5.028, p = 0.0196). IL – deeper (Control: 698.1 ± 269.3, RSV: 349.1 ± 125.5, VPA: 236.2 ± 60.24, RSV+VPA 364.6 ± 53.71, F = 6.168, p = 0.0103).

4. Discussion

In the present study, an increase in BBB permeability to Evans blue dye was observed in the VPA group and RSV prevented this alteration. In addition, was demonstrated an increase in the absolute number of GFAP+ cells in PrL (deeper) and a synergistic effect of RSV and VPA in upper layers of CC and PrL and in the deeper layers of IL. Regarding the GFAP+ cells ratio, an increase in the deeper layers and a decrease in the upper layers of CC and PrL was observed, and RSV prevented this change only in PrL. Moreover, a decrease in the AQP4 fluorescence content was demonstrated in the VPA group. These findings contribute in the understanding of mechanisms that could be involved in altered dynamics of brain volume in ASD and in the pathophysiology of this disorder.

We observed that the VPA induced a significant increase in BBB permeability to Evans blue dye in mPFC. Interestingly, this effect was prevented by prenatal administration of RSV. This dye binds to plasma albumin, a protein of 68 kDa poorly transported through the BBB (Nagaraja et al., 2008). So, a permeability to this dye in brain tissue is an important indicative of BBB impairment (Kaya and Ahishali, 2011). In agreement, previous studies demonstrated

the presence of Evans blue in the cerebellum of VPA group (Kumar et al., 2015; Kumar and Sharma, 2016b, 2016a). Similarly, recent data from our group demonstrated increased BBB permeability to the same dye in primary somatosensory area and in choroid plexus in the VPA model (unpublished data). These findings could be related to brain edema (Keep et al., 2017), which likewise was already demonstrated in VPA model through water content percentage technique, also prevented by RSV (Iohanna Deckmann, personal communication). Hence, BBB impairments could affect members of neurovascular unit (NVU), such as astrocytes, and aggravate impairments of BBB functions (Obermeier et al., 2013). Astrocytes play an important role in the maintenance of BBB, providing structural support, promoting the formation of the extracellular matrix and maintaining communication with other elements of NVU (Blanchette and Daneman, 2015). The elements of NVU secrete important molecules to the formation and maintenance of BBB (Zhao et al., 2015). Astrocytes can also interact with pericytes and regulate activation of matrix metallopeptidase 9 (MMP-9), that leads to degradation of endothelial tight junction and extracellular matrix proteins causing BBB disruption (Sweeney et al., 2016). Besides, studies have correlated neurodevelopmental disorders, including ASD, with increased expression of MMP-9 (Reinhard et al., 2015).

The neuroinflammation and immune alterations in autism VPA model have been demonstrated, suggesting a pro-inflammatory (Deckmann et al., 2018; Lucchina and Depino, 2014) and pro-oxidant effects by the exposition of this molecule (Chaudhary and Parvez, 2012; Tung and Winn, 2011). Furthermore, inflammation and oxidative stress are known to be an important cause of BBB damage, allowing brain vulnerability to toxic and pathogenic substances, as well as disrupting the brain homeostasis necessary for properly neural function (Obermeier et al., 2013). Therefore, considering the known anti-inflammatory and antioxidant properties of RSV, which was able to prevent the BBB impairments, it is possible that RSV is modulating pro-inflammatory and pro-oxidant effects caused by prenatal administration of VPA.

In this work, we observed that VPA group presented higher levels of $GFAP^+$-cells in deeper layers of PrL. This finding agrees to ASD context, since studies in animal models by LPS infusion showed a higher semi-quantitative scoring of $GFAP^+$-cells (O'Loughlin et al., 2017). The PrL is an important region related to social behaviors (Minami et al., 2017), an impairment characteristic in ASD individuals. Furthermore, deep layers (IV, V and VI) play an important role in cortical circuit, since IV layer is responsible for integration and amplification of thalamic nucleus input; V layer send important projections to subcortical areas, as spinal cord; and VI layer is important for feedback projection to the thalamus (Adesnik and Naka, 2018; Swenson and Gulledge, 2017). Thus, the effect of VPA in the absolute number of $GFAP^+$-cells in these regions could be related to impairments in cortical circuits, since altered neural connectivity in excitatory and inhibitory cortical circuits was already demonstrated in ASD (Zikopoulos and Barbas, 2013). Curiously, in upper layers of CC and PrL, as well as in deeper layers of IL was observed a synergistic effect of RSV and VPA. A common mechanism of RSV and VPA is the action in chromatin; both activate the NAD-dependent deacetylases (sirtuins), important enzymes responsible for histones deacetylation with consequent lower gene expression (Malhotra et al., 2015; Reid et al., 2005). Besides, VPA is also a histone deacetylase inhibitor, favoring gene transcription (Phiel et al., 2001). Therefore, the observed synergistic effect could be related to the acting of these molecules in the expression of genes associated with astrocytic proliferation.

Beyond that, analyzing the ratio between the number of $GFAP^+$-cells in different layers (upper and deeper) relative to absolute number of $GFAP^+$-cells in the region as a whole, are possible to observe alterations in CC and PrL in VPA group compared to the control: a higher relative number of $GFAP^+$-cells in deeper layers in relation to upper layers. Cortical laminar organization is important for the processing and integration of the information that arrives the

brain, being essential for normal cortical circuits and to the perceptions of the environmental and the context (Adesnik and Naka, 2018). Besides, abnormalities in cortical disorganization have been already demonstrated in CC of patients with ASD (Simms et al., 2009) and in primary somatosensory area in the VPA model, which was prevented by RSV (Fontes-Dutra et al., 2018). Thus, owing to the importance of astrocytes in neurogenesis, neuronal metabolic support, synaptic plasticity and neurotransmitters homeostasis (Palmer and Ousman, 2018), the alteration in the distribution of $GFAP^+$-cells in cortical layers could be related to cortical disorganization seen in previous studies, however more studies are needed to understand this relationship. In addition, apparently RSV was able to prevent the organization of $GFAP^+$-cells in PrL, a region related to social and affective behavior and associated with amygdala, an important structure for the expression of emotion (Minami et al., 2017), an interesting found in the context of ASD.

Moreover, although no differences were observed in the GFAP fluorescence content, there was an alteration in the morphology of $GFAP^+$-cells in groups exposed to VPA, with apparently longer cell processes, an indicative of reactive cells (Liberto et al., 2004; Liddelow and Barres, 2017). Thus, the increased BBB permeability could be associated to changes in brain homeostasis and astrocytic alterations. However, the Sholl technique is crucial to understanding astrocytic extensions and ramifications (Mavroudis et al., 2017).

Furthermore, other finding in the present study is the decreased expression of AQP4 in all mPFC regions evaluated in VPA group. A previous study described alterations in AQP4 profile in *post-mortem* brain from ASD subjects, including a 53% decrease in cerebellum and despite no statistically significant the authors also showed a decrease of 3% in Brodmann's Area 9 (BA9 – equivalent to the frontal cortex), as well as increased expression of 39% in BA40 (parietal cortex, where is located the primary sensory area). Besides, this study showed increased expression of connexin 43 (CX43 - the major component protein in astrocytic gap junctions) in BA9, which could signify increased glial-neuronal signaling, indicating an enhancement of cell-cell communication in frontal lobe, an integrative area, which may help explain impairments observed in ASD subjects (Fatemi et al., 2008). Beyond that, studies have related the role of AQP4 in neuroimmune system (Ikeshima-Kataoka, 2016); thus, alterations in this type of channel is an interesting finding in the ASD context, since the immune component of this disorder is well established (Deckmann et al., 2018). Moreover, studies showed that AQP4 *knockout* reduced brain swelling in cytotoxic edema, whereas it significantly worsened outcome in vasogenic brain edema (Papadopoulos and Verkman, 2007). In context of autism VPA model it is not elucidated what type of brain edema is present.

In addition, BBB permeability has been associated to albumin inflow into astrocyte, leading to downregulation of the Kir.4.1 potassium channel in epilepsy (Ivens et al., 2007), a common comorbidity in patients with ASD (Mannion and Leader, 2013). The "inwardly rectifying K^+ channels" (Kir channels), especially Kir4.1, is predominantly localized at end feet astrocytic processes; the co-localization of Kir4.1 with AQP4 was demonstrated, which are required for the astrocytes to maintain K^+ and water homeostasis in the CNS. The syncytial action of Kir4.1 and AQP4 in astrocyte contact capillaries, suggests that buffering of K^+ through Kir channels is dependent on concomitant transmembrane flux of water (Seifert et al., 2006). In retina, suppression of AQP4 attenuates the edema development, but the dysregulation of both AQP4 and Kir4.1 enhances the sensitivity to ischemic insults, indicating the coordinated role of these channels (Da and Verkman, 2004; Dalloz et al., 2003). So, the decrease in AQP4 could be, in one hand, avoid the development of brain edema; on the other hand, when the edema is already settled, it can: a) impair the elimination of excess water or b) contribute to avoid further water entrance. Nevertheless, more studies are needed to comprehend the role of AQP4 in edema formation in ASD.

In summary, we demonstrated significant effects of VPA on BBB permeability, in $GFAP^+$-cell profile, and in the fluorescence content of AQP4, with important effects of RSV on these parameters in mPFC, an important brain region related to social behavior, cognition and planning and decision making. These data contributes to a better understanding of the brain volume alterations observed in ASD.

4 Author contributions

ID, CG: experimental design and intellectual contribution. MK-R, ID, JS-T, MF-D: animal handling, obtaining samples analyses. MK-R, ID, JS-T, MF-D, CG: data discussion and manuscript preparation.

5 Conflict of interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Legends of panel and figures

Panel 1. Schematic timeline representation of experimental rat VPA model. Animals were mated overnight and in the next morning, the pregnancy was verified; once confirmed the pregnancy was considered the embryonic day 0.5 (E0.5). From E6.5 to E18.5, the pregnant rats received subcutaneously injection of RSV (3.6 mg/kg) or dimethyl sulfoxide (DMSO - vehicle). At E12.5, pregnant rats received a single injection intraperitoneally of 600 mg/kg VPA or saline solution 0,9%. At postnatal day 21 (P21) the litter was weaning and at P30 the male rats were euthanized for the experiment procedures. i.p., intraperitoneal; s.b., subcutaneous.

Figure 1. Scheme of the regions of the medial prefrontal cortex (mPFC) analyzed. Three regions of mPFC were analyzed: cingulate cortex (CC), pre-limbic (PrL) and infralimbic (IL); in the right, the representation of upper and deeper layers. Adapted from Paxinos Atlas (Paxinos and Watson, 2013).

Figure 2. Blood-brain barrier function by Evans blue dye permeability. A) Representative images of cingulate cortex upper region, DAPI nuclear dye is stained in blue and Evans blue dye fluorescence in red. **B)** Fluorescence quantification of Evans blue dye in cingulate cortex, prelimbic cortex and infralimbic cortex (upper and deeper). Scale bar: 50 μm. n sample = 4-7. One-Way ANOVA followed by Bonferroni post-test. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Figure 3. Representative immunofluorescence of glial fibrilar acidic protein (GFAP) and aquaporin-4 (AQP4) in medial prefrontal cortex. **A)** Representative images of cingulate cortex upper and deeper region, DAPI nuclear dye is stained in blue, $GFAP^+$-cells fluorescence in green and AQP4 fluorescence in red. **B)** Number of $GFAP^+$-cells in cingulate cortex, prelimbic cortex and infralimbic cortex (upper and deeper). **C)** Ratio of $GFAP^+$-cells in upper and deeper layers to total number $GFAP^+$-cells in region. **D)** Fluorescence quantification of AQP4 in same regions. Scale bar: 50 µm. n sample = 3-4. One-Way ANOVA followed by Bonferroni post-test. * $p < 0.05$.

8 List of figures

Panel 1

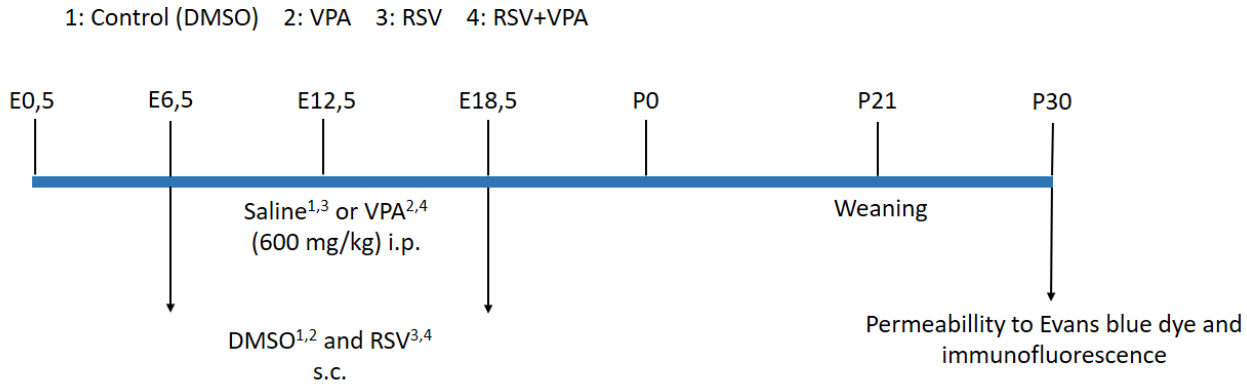


Figure 1

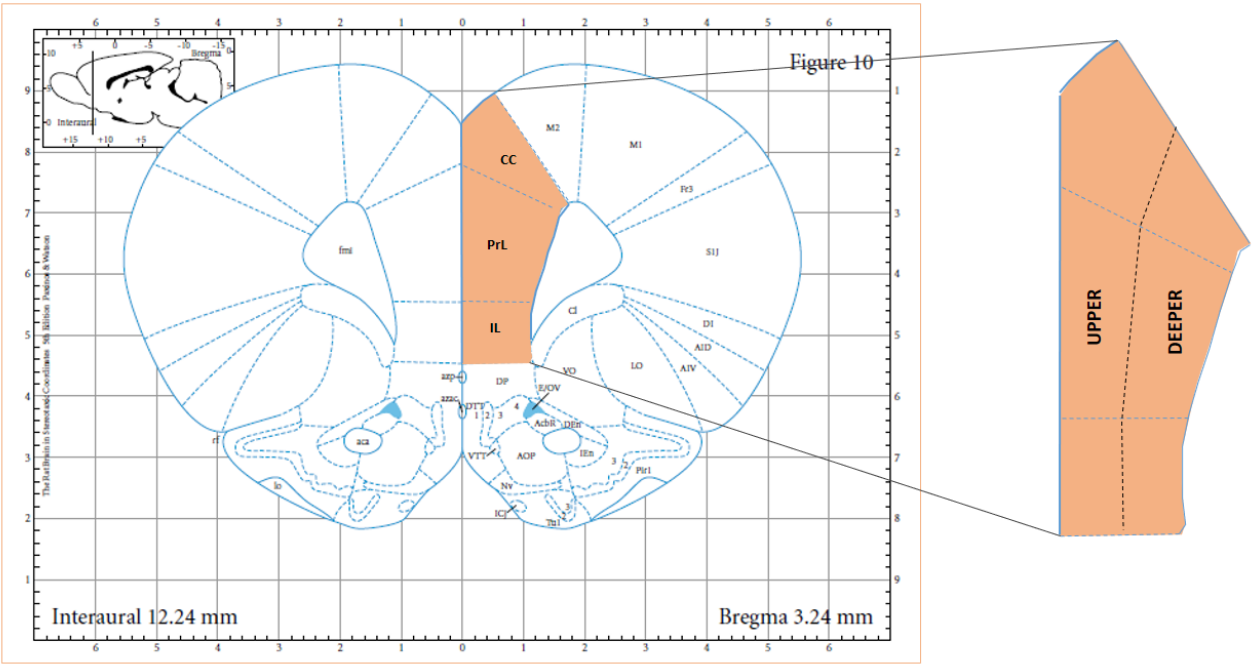
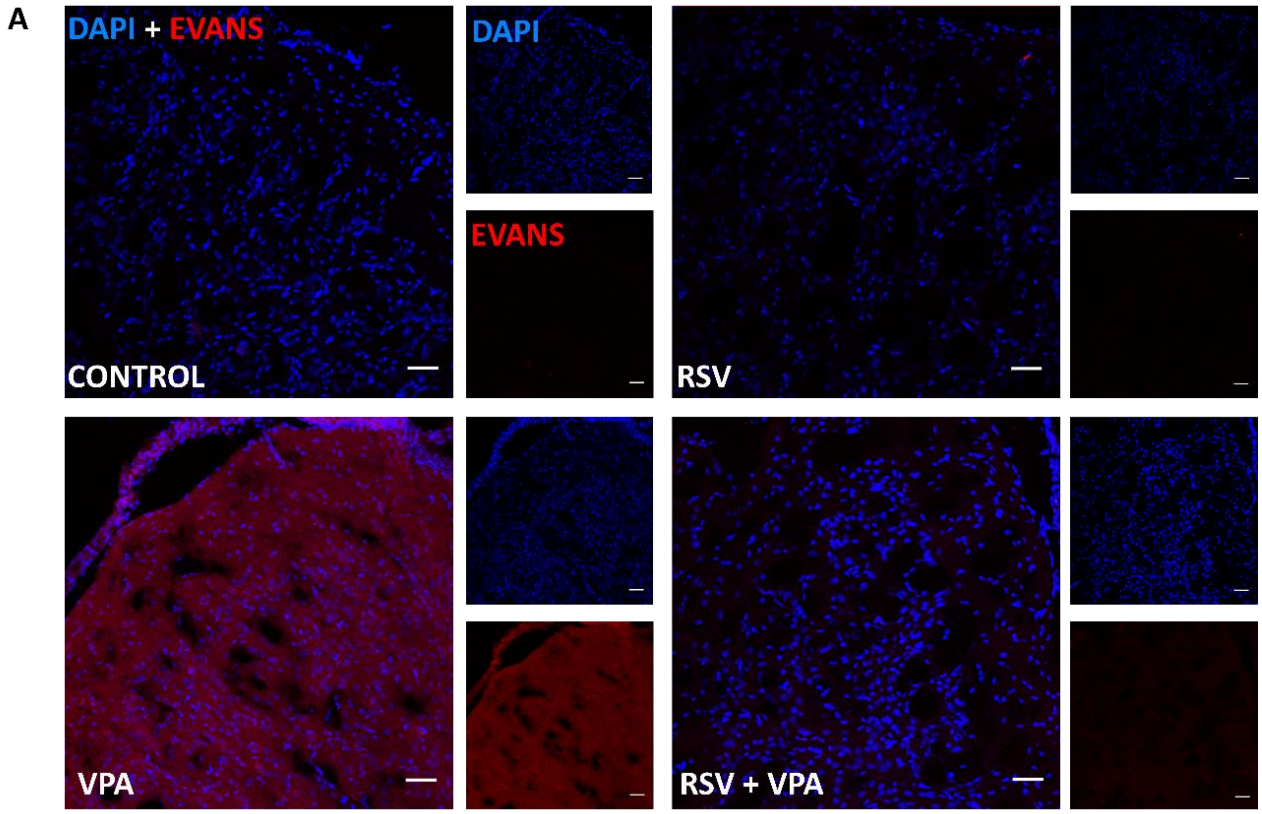


Figure 2


A
DAPI + EVANS
DAPI
EVANS
CONTROL
RSV
VPA
RSV + VPA

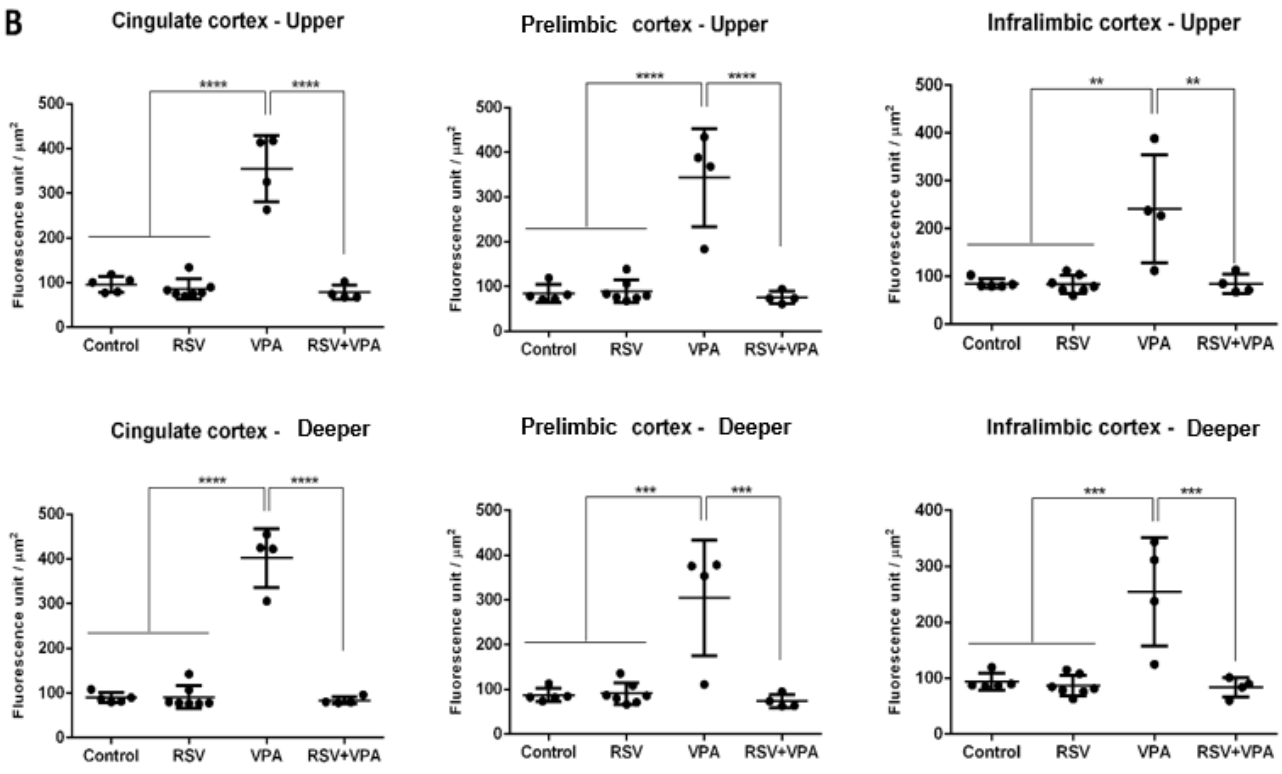
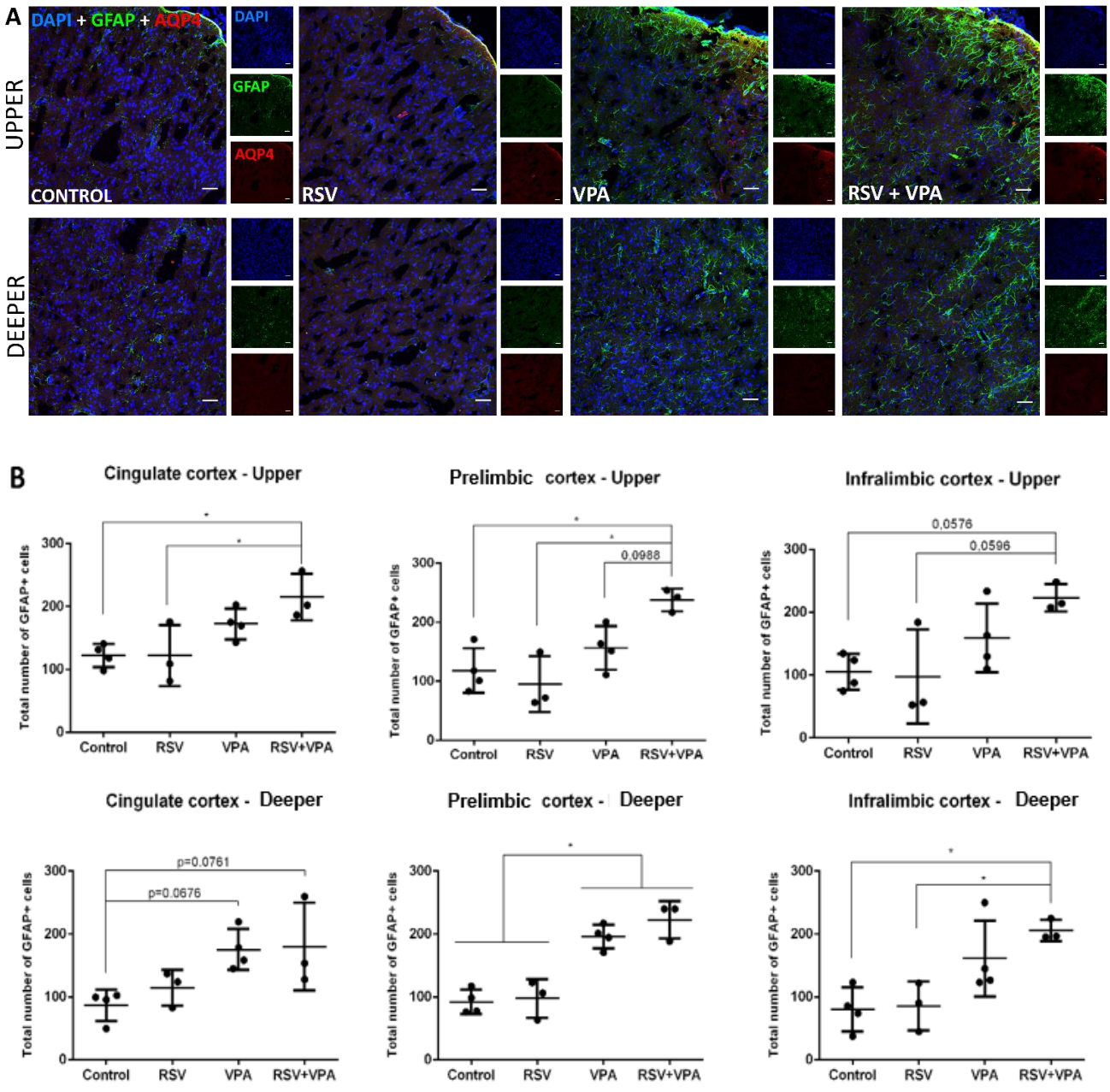
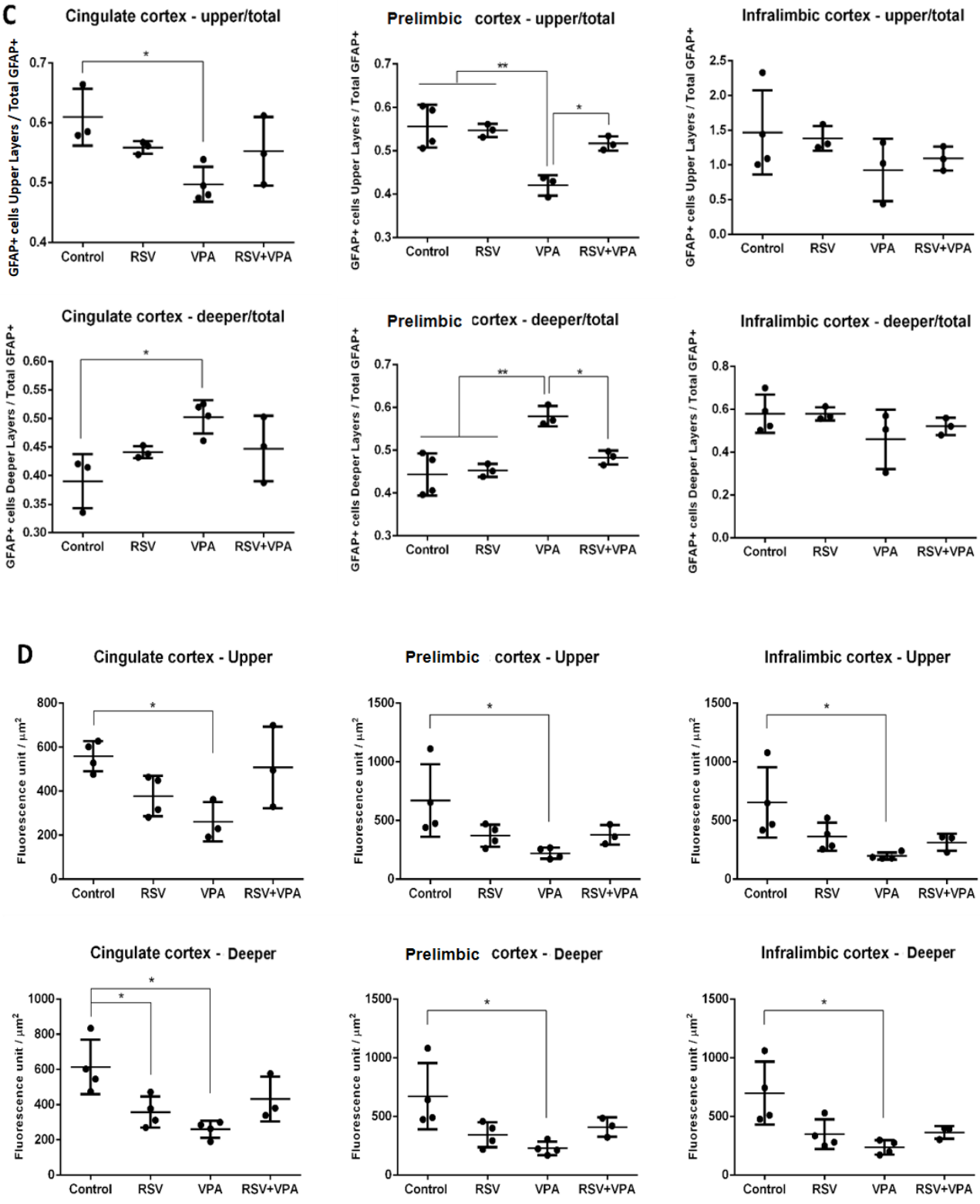

B
Cingulate cortex - Upper
Prelimbic cortex - Upper
Infralimbic cortex - Upper
Cingulate cortex - Deeper
Prelimbic cortex - Deeper
Infralimbic cortex - Deeper
Fluorescence unit / µm²
Control
RSV
VPA
RSV+VPA

Figure 3


A
DAPI + GFAP + AQP4
DAPI
GFAP
AQP4
UPPER
DEEPER
CONTROL
RSV
VPA
RSV + VPA
B
Cingulate cortex - Upper
Prelimbic cortex - Upper
Infralimbic cortex - Upper
Cingulate cortex - Deeper
Prelimbic cortex - Deeper
Infralimbic cortex - Deeper
Total number of GFAP+ cells
Control
RSV
VPA
RSV+VPA
p=0.0761
p=0.0676
0,0988
0,0576
0,0596


C
Cingulate cortex - upper/total
Prelimbic cortex - upper/total
Infralimbic cortex - upper/total
GFAP+ cells Upper Layers / Total GFAP+
Cingulate cortex - deeper/total
Prelimbic cortex - deeper/total
Infralimbic cortex - deeper/total
GFAP+ cells Deeper Layers / Total GFAP+
D
Cingulate cortex - Upper
Prelimbic cortex - Upper
Infralimbic cortex - Upper
Fluorescence unit / µm²
Cingulate cortex - Deeper
Prelimbic cortex - Deeper
Infralimbic cortex - Deeper
Control
RSV
VPA
RSV+VPA

3 CONCLUSÕES E PERSPECTIVAS

O presente trabalho demonstrou aumento da permeabilidade da BHE ao corante azul de Evans no CPFm do grupo VPA em relação ao controle, um importante indicativo de aumento da permeabilidade da BHE; o RSV foi capaz de prevenir essa alteração.

Observou-se um aumento no número absoluto de células $GFAP^+$ nas camadas profundas do PrL nos grupos expostos ao VPA em relação ao controle, além de um efeito sinérgico do VPA e do RSV nas camadas superficiais de CC e PrL e nas camadas profundas de IL. Em relação ao número relativo de células $GFAP^+$ nas camadas corticais, foi demonstrado uma razão aumentada nas camadas profundas e diminuída nas camadas superficiais do CC e do PrL no grupo VPA em comparação ao controle, sendo esse efeito prevenido pelo RSV apenas em PrL. Também foi observado alterações na morfologia astrocítica nos grupos que receberam VPA, semelhante às encontradas na astrocitose reativa, embora esse dado precise ser confirmado.

Além disso, foi observada uma diminuição na imunofluorescência de AQP4 no grupo VPA em relação ao controle. Dessa forma, importantes alterações na permeabilidade da BHE, no perfil astrocítico e na expressão de AQP4 foram observadas no grupo VPA, bem como o efeito do RSV em alterações morfológicas e funcionais presentes no modelo VPA, evidenciando o uso dessa molécula na compreensão de mecanismos envolvidos na fisiopatologia do TEA.

No entanto, mais estudos são necessários para compreender a relação desses achados no contexto do TEA e os mecanismos envolvidos no aumento da permeabilidade da BHE observado no modelo VPA. Portanto, com o intuito de esclarecer algumas dessas questões, são perspectivas desse trabalho:

a) Aumentar o número amostral;
b) Analisar a expressão de GFAP e de AQP4 por *Western Blotting*, a fim de confirmar os resultados encontrados;
c) Realizar a técnica de Sholl para mensurar prolongamentos e ramificações astrocíticas;
d) Analisar outros componentes da barreira hematoencefálica, como pericitos e células endoteliais, com o intuito de compreender os mecanismos envolvidos no aumento da permeabilidade da BHE no grupo VPA;
e) Analisar por microscopia eletrônica de transmissão as junções de oclusão que unem as células endoteliais na barreira hematoencefálica.

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ANEXO A – CARTA DE APROVAÇÃO CEUA/HCPA

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

A Comissão de Ética no Uso de Animais (CEUA/HCPA) analisou o projeto:

Projeto: 130047
Data da Versão do Projeto:

Pesquisadores:
RUDIMAR DOS SANTOS RIESGO
CARMEM GOTTFRIED
DIEGO MOURA BARONIO
VICTORIO BAMBINI JUNIOR

Título: DOSAGEM DE AQUAPORINAS EM MODELO ANIMAL DE AUTISMO INDUZIDO POR ÁCIDO VALPRÓICO

Este projeto foi APROVADO em seus aspectos éticos e metodológicos de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08/10/2008, que estabelece procedimentos para o uso científico de animais.

- Os membros da CEUA/HCPA não participaram do processo de avaliação de projetos onde constam como pesquisadores.
- Toda e qualquer alteração do Projeto deverá ser comunicada à CEUA/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEUA/HCPA.

Porto Alegre, 30 de setembro de 2013.

Dr. Alessandro Osvaldt
Coordenador CEUA/HCPA

ANEXO B - NORMAS DE PUBLICAÇÃO DA REVISTA *FRONTIERS IN NEUROSCIENCE*

Author Guidelines

Author Guidelines

1. Summary Table

Please view the table below for a summary on currently accepted article types and general manuscript style guidelines. Article types may vary depending on journal.

	Abstract (max. length)	Running title (5 words)	Figures and/or tables (combined)	Manuscript (max. length)	Peer review	Author fees	Submitted to PubMed Central or other indexing databases
Original Research	350 words	✔	15	12'000 words	✔	✔	✔
Review	350 words	✔	15	12'000 words	✔	✔	✔
Book Review	✘	✘	1	1'000 words	✔	✘	✔
Brief Research Report	250 words	✔	4	4'000 words	✔	✔	✔
Classification	250 words	✔	10	2'000 words	✔	✔	✔
Case Report	350 words	✔	4	3'000 words	✔	✔	✔
Clinical Study Protocol	350 words	✔	15	12'000 words	✔	✔	✔
Clinical Trial	350 words	✔	15	12'000 words	✔	✔	✔
Code	250 words	✔	3	3'000 words	✔	✔	✔
Community Case Study	350 words	✔	5	5'000 words	✔	✔	✔
Conceptual Analysis	350 words	✔	10	8'000 words	✔	✔	✔
CPC	250 words	✔	6	2'500 words	✔	✔	✔
Curriculum, Instruction, and Pedagogy	350 words	✔	5	5'000 words	✔	✔	✔
Data Report	✘	✔	2	3'000 words	✔	✔	✔
Editorial	✘	✘	0	1'000 words*	✔	✘	✔
Empirical Study	350 words	✔	10	8'000 words	✔	✔	✔
Evaluation	350 words	✔	5	6'000 words	✔	✔	✔

Field Grand Challenge	✘	✔	1	2'000 words	✔	✘	✔
Focused Review (1)	350 words	✔	5	5'000 words	✔	✘	✔
Frontiers Commentary (1)	✘	✘	1	1'000 words	✔	✘	✔
General Commentary	✘	✘	1	1'000 words	✔	✔	✔
Hypothesis and Theory	350 words	✔	15	12'000 words	✔	✔	✔
Methods	350 words	✔	15	12'000 words	✔	✔	✔
Mini Review	250 words	✔	2	3'000 words	✔	✔	✔
Opinion	✘	✔	1	2'000 words	✔	✔	✔
Policy & Practice Reviews	350 words	✔	15	12'000 words	✔	✔	✔
Policy Briefs	125 words	✔	5	3'000 words	✔	✔	✔
Protocols	350 words	✔	15	12'000 words	✔	✔	✔
Perspective	250 words	✔	2	3'000 words	✔	✔	✔
Registered Report	350 words	✔	15	12,000 words	✔	✔	✔
Research Snapshot	50 words	✔	1	500 words	✔	✔	✔
Specialty Grand Challenge	✘	✔	1	2'000 words	✔	✘	✔
Systematic Reviews	350 words	✔	15	12'000 words	✔	✔	✔
Technology Report	350 words	✔	15	12'000 words	✔	✔	✔

(1) Tier 2 article - field level article reserved to authors of selected Tier 1 articles.

* Editorials for Research Topics with 5 to 10 published articles have a maximum of 1'000 words, for Research Topics with more than 10 published articles the following applies: 1'100 words for 11 articles, 1'200 for 12 articles, 1'300 for 13 articles etc. up to maximum 5'000 words, for 50 or more papers.

Appendices and footnotes will be considered in the total length and word count of the article.

2. Manuscript Guidelines

2.1. Open access and copyright

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2.3. Manuscript Requirements and Style Guide

2.3.1. General standards

Word Files

If working with Word please use Frontiers Word templates. (http://www.frontiersin.org/Design/zip/Frontiers_Word_Templates.zip)

LaTeX Files

If you wish to submit your article as LaTeX, we recommend our Frontiers LaTeX templates (http://www.frontiersin.org/design/zip/Frontiers_LaTeX_Templates.zip).

These templates are meant as a guide, you are of course welcome to use any style or formatting and Frontiers journal style will be applied during typesetting.

Experiments

Authors are required to specifically state in their legends how many times experiments were performed (in general we require n=3 as a minimum) and what specific statistical analysis was performed.

2.3.1.1. Article Type

Frontiers requires authors to carefully select the appropriate article type for their manuscript, and to comply with the article-type descriptions defined in the journal's "Article Types", which can be seen from the "For Authors" menu on any Frontiers journal page. Please note that not all articles types are available for all journals/specialties. Please contact us if you have any questions. **Please pay close attention to the word count limits.**

Focused Reviews, Frontiers Commentaries and Grand Challenge articles are invited by the chief editor and cannot be part of any Frontiers Research Topic. Unless you were contacted by the chief editor or the editorial o±ce regarding the submission of a paper selected for tier 2 promotion, do not submit a Focused Review or a Frontiers Commentary - instead, submit a Review or a General Commentary.

Please see Additional Requirements for specific article types including Focused Reviews, General Commentaries, Protocols and Data Reports.

2.3.1.2. Manuscript Length

Frontiers encourages its authors to closely follow the article word count lengths given in the Summary Table. The manuscript length includes only the main body of the text, footnotes and all citations within it, and excludes abstract, section titles, figure and table captions, funding statements, acknowledgments and references in the bibliography. Please indicate the number of words and the number of figures included in your manuscript on the first page.

2.3.1.3. Language Editing

Frontiers requires manuscripts submitted to meet international standards for English language to be considered for publication.

For authors who would like their manuscript to receive language editing or proofing to improve the clarity of the manuscript and help highlight their research, Frontiers recommends the language-editing services provided by the following external partners:

Editage

Frontiers is pleased to recommend language-editing service provided by our external partner Editage to authors who believe their manuscripts would benefit from professional editing. These services may be particularly useful for researchers for whom English is not the primary language. They can help to improve the grammar, syntax and flow of your manuscripts prior to submission. Frontiers authors will receive a 10% discount by visiting the following link: http://editage.com/frontiers/ (http://editage.com/frontiers/)

The Charlesworth Group

Frontiers recommends the Charlesworth Group Author Services, who has a long standing track record in language editing and proofing. This is a third-party service for which Frontiers authors will receive a discount by visiting the following link: http://www.charlesworthauthorservices.com/~Frontiers (http://www.charlesworthauthorservices.com/~Frontiers).

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2.3.1.4. Language Style

The default language style at Frontiers is American English. If you prefer your article to be formatted in British English, please specify this on your manuscript first page. For any questions regarding style Frontiers recommends authors to consult the Chicago Manual of Style.

2.3.1.5. Search Engine Optimization (SEO)

There are a few simple ways to maximize your article's discoverability. Follow the steps below to improve search results of your article:

- Include a few of your article's keywords in the title of the article;
- Do not use long article titles;
- Pick 5 to 8 keywords using a mix of generic and more specific terms on the article subject(s);
- Use the maximum amount of keywords in the first 2 sentences of the abstract;
- Use some of the keywords in level 1 headings.

2.3.1.6. Title

The title is written in title case, centred, and in 16 point bold Times New Roman font at the top of page.

The title should be concise, omitting terms that are implicit and, where possible, be a statement of the main result or conclusion presented in the manuscript. Abbreviations should be avoided within the title.

Witty or creative titles are welcome, but only if relevant and within measure. Consider if a title meant to be thought-provoking might be misinterpreted as ouensive or alarming. In extreme cases, the editorial o±ce may veto a title and propose an alternative.

Authors should try to avoid, if possible:

- Titles that are a mere question without giving the answer.
- Unambitious titles, for example starting with "Towards", "A description of", "A characterization of", "Preliminary study on".
- Vague titles, for example starting with "Role of...", "Link between...", "Euect of..." that do not specify the role, link, or euect.
- Include terms that are out of place, for example the taxonomic a±liation apart from species name.

For Corrigenda, Book Reviews, General Commentaries and Editorials, the title of your manuscript should have the following format:

- "Corrigendum: Title of original article"
- "Book Review: Title of book"
- General Commentaries

- "Commentary: Title of original article" (This does not apply to Frontiers Commentaries)
- "Response: Commentary: Title of original article"

- "Editorial: Title of Research Topic"

For article types requiring it, the running title should be a maximum of 5 words in length. (see Summary Table)

2.3.1.7. Authors and A?liations

All names are listed together and separated by commas. Provide exact and correct author names as these will be indexed in o±cial archives. A±liations should be keyed to the author's name with superscript numbers and be listed as follows: Laboratory, Institute, Department, Organization, City, State abbreviation (USA, Canada, Australia), and Country (without detailed address information such as city zip codes or street names).

Example: Max Maximus, Department of Excellence, International University of Science, New York, NY, USA.

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Correspondence:

Dr. Max Maximus
maximus@gmail.com

If any authors wish to include a change of address, list the present address(es) below the correspondence details using a unique superscript symbol keyed to the author(s) in the author list.

2.3.1.8. Consortium/Group and Collaborative Authors

Consortium/group authorship should be listed in the manuscript with the other author(s). In cases where authorship is retained by the consortium/group, the consortium/group should be listed as an author separated by “,” or “and”. Consortium/group members can be listed in a separate section at the end of the manuscript.

Example: John Smith, Barbara Smith and The Collaborative Working Group.

In cases where work is presented by the author(s) on behalf of a consortium/group, it should be included in the manuscript author list separated with the wording “for” or “on behalf of”. The consortium/group will not retain authorship.

Example: John Smith and Barbara Smith on behalf of The Collaborative Working Group.

2.3.1.9. Headings and Sub-headings

Except for special names (e.g. GABAergic), capitalize only the first letter of headings and subheadings. Headings and subheadings need to be defined in Times New Roman, 12, bold. You may insert up to 5 heading levels into your manuscript (not more than for example: 3.2.2.1.2 **Heading title**).

2.3.1.10. Abstract

As a primary goal, the abstract should render the general significance and conceptual advance of the work clearly accessible to a broad readership. In the abstract, minimize the use of abbreviations and do not cite references. The text of the abstract section should be in 12 point normal Times New Roman. See Summary Table for abstract requirement and length according to article type.

For Clinical Trial article types, please include the Unique Identifier and the URL of the publicly accessible website on which the trial is registered.

2.3.1.11. Keywords

All article types: you may provide up to 8 keywords; at least 5 are mandatory.

2.3.1.12. Text

The entire document should be single-spaced and must contain page and line numbers in order to facilitate the review process. Your manuscript should be written using either LaTeX or MS-Word.

Templates are available (see above)

2.3.1.13. Nomenclature

- The use of abbreviations should be kept to a minimum. Non-standard abbreviations should be avoided unless they appear at least four times, and defined upon first use in the main text. Consider also giving a list of

non-standard abbreviations at the end, immediately before the Acknowledgments.

- Equations should be inserted in editable format from the equation editor.
- Italicize Gene symbols and use the approved gene nomenclature where it is available. For human genes, please refer to the HUGO Gene Nomenclature Committee (HGNC (https://www.genenames.org/)). New gene symbols should be submitted here (https://www.genenames.org/cgi-bin/request). Common Alternative gene aliases may also be reported, but should not be used alone in place of the HGNC symbol. Nomenclature committees for other species are listed here (https://www.genenames.org/about/faq#otherspecies). Protein products are not italicized.
- We encourage the use of Standard International Units in all manuscripts.
- Chemical compounds and biomolecules should be referred to using systematic nomenclature, preferably using the recommendations by IUPAC.
- Astronomical objects should be referred to using the nomenclature given by the International Astronomical Union provided here (http://cdsweb.u-strasbg.fr/Dic/how.htx).
- Life Science Identifiers (LSIDs) for ZOOBANK registered names or nomenclatural acts should be listed in the manuscript before the keywords. An LSID is represented as a uniform resource name (URN) with the following format:
  urn:lsid:::[:]
  For more information on LSIDs please see Inclusion of Zoological Nomenclature section.

2.3.1.14. Sections

Your manuscript is organized by headings and subheadings. For Original Research Articles, Clinical Trial Articles, and Technology Reports the section headings should be those appropriate for your field and the research itself.

For Original Research Articles, it is recommended to organize your manuscript in the following sections or their equivalents for your field:

Introduction

Succinct, with no subheadings.

Materials and Methods

This section may be divided by subheadings. This section should contain su±cient detail so that when read in conjunction with cited references, all procedures can be repeated. For experiments reporting results on animal or human subject research, an ethics approval statement should be included in this section (for further information, see section Materials and Data Policies)

Results

This section may be divided by subheadings. Footnotes should not be used and have to be transferred into the main text.

Discussion

This section may be divided by subheadings. Discussions should cover the key findings of the study: discuss any prior art related to the subject so to place the novelty of the discovery in the appropriate context; discuss the potential short-comings and limitations on their interpretations; discuss their integration into the current understanding of the problem and how this advances the current views; speculate on the future direction of the research and freely postulate theories that could be tested in the future.

For further information, please see Additional Requirements for specific article types including Focused Reviews, General Commentaries, Case Reports and Data Reports amongst others or you can check the descriptions defined in the journal's "Article Types", which can be seen from the "For Authors" menu on any Frontiers journal page.

2.3.1.15. Acknowledgments

This is a short text to acknowledge the contributions of specific colleagues, institutions, or agencies that aided the euorts of the authors.

2.3.1.16. Author Contributions Statement

The Author Contributions Statement is mandatory and should represent all the authors. It can be up to several sentences long and should briefly describe the tasks of individual authors. Please list only 2 initials for each author, without full stops, but separated by commas (e.g. JC, JS). In the case of two authors with the same initials, please use their middle initial to diuerentiate between them (e.g. REW, RSW). The Author Contributions Statement should be included at the end of the manuscript before the References.

2.3.1.17. Confiict of Interest Statement

A Conflict of Interest Statement needs to be included at the end of the manuscript before the references. Here, the authors need to declare whether or not the submitted work was carried out in the presence of any personal, professional or financial relationships that could potentially be construed as a conflict of interest. For more information on conflicts of interest, see our Editorial Policies.

2.3.1.18. Contribution to the Field Statement

When you submit your manuscript, you will be required to briefly summarize in 200 words your manuscript's contribution to, and position in, the existing literature of your field. This should be written avoiding any technical language or non-standard acronyms. The aim should be to convey the meaning and importance of this research to a non-expert. While Frontiers evaluates articles using objective criteria, rather than impact or novelty, your statement should frame the question(s) you have addressed in your work in the context of the current body of knowledge, providing evidence that the findings - whether positive or negative - contribute to progress in your research discipline. This will assist the Chief Editors to determine whether your manuscript fits within the scope of a specialty as defined in its mission statement; a detailed statement will also facilitate the identification of the Editors and Reviewers most appropriate to evaluate your work, ultimately expediting your manuscript's initial consideration.

Example Statement on: Markram K and Markram H (2010) The Intense World Theory – a unifying theory of the neurobiology of autism. Front. Hum. Neurosci. 4:224. doi: 10.3389/fnhum.2010.00224

Autism spectrum disorders are a group of neurodevelopmental disorders that auect up to 1 in 100 individuals. People with autism display an array of symptoms encompassing emotional processing, sociability, perception and memory, and present as uniquely as the individual. No theory has suggested a single underlying neuropathology to account for these diverse symptoms. The Intense World Theory, proposed here, describes a unifying pathology producing the wide spectrum of manifestations observed in autists. This theory focuses on the neocortex, fundamental for higher cognitive functions, and the limbic system, key for processing emotions and social signals. Drawing on discoveries in animal models and neuroimaging studies

in individuals with autism, we propose how a combination of genetics, toxin exposure and/or environmental stress could produce hyper-reactivity and hyper-plasticity in the microcircuits involved with perception, attention, memory and emotionality. These hyper-functioning circuits will eventually come to dominate their neighbors, leading to hyper-sensitivity to incoming stimuli, over-specialization in tasks and a hyper-preference syndrome. We make the case that this theory of enhanced brain function in autism explains many of the varied past results and resolves conflicting findings and views and makes some testable experimental predictions.

2.3.2. References

All citations in the text, figures or tables must be in the reference list and vice-versa. The references should only include articles that are published or accepted. Data sets that have been deposited to an online repository should be included in the reference list, include the version and unique identifier when available. For accepted but unpublished works use "in press" instead of page numbers. Unpublished data, submitted manuscripts, or personal communications should be cited within the text only, for the article types that allow such inclusions. Personal communications should be documented by a letter of permission. Website urls should be included as footnotes. Any inclusion of verbatim text must be contained in quotation marks and clearly reference the original source. Preprints can be cited as long as a DOI or archive URL is available, and the citation clearly mentions that the contribution is a preprint. If a peer-reviewed journal publication for the same preprint exists, the o±cial journal publication is the preferred source.

The following formatting styles are meant as a guide, as long as the full citation is complete and clear, Frontiers referencing style will be applied during typesetting.

- **SCIENCE, ENGINEERING, and HUMANITIES: For articles submitted in the domains of SCIENCE, ENGINEERING and HUMANITIES please apply Author-Year system for in-text citations.**

Reference list: provide the names of the first six authors followed by et al. and doi (https://www.crossref.org/guestquery/#textsearch) when available.

In-text citations should be called according to the surname of the first author, followed by the year. For works by 2 authors include both surnames, followed by the year. For works by more than 2 authors include only the surname of the first author, followed by et al., followed by the year. For Humanities and Social Sciences articles please include page numbers in the in-text citations.

Article in a print journal:

Sondheimer, N., and Lindquist, S. (2000). Rnq1: an epigenetic modifier of protein function in yeast. Mol. Cell. 5, 163-172.

Article in an online journal:

Tahimic, C.G.T., Wang, Y., Bikle, D.D. (2013). Anabolic euects of IGF-1 signaling on the skeleton. Front. Endocrinol. 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book:

Sorenson, P. W., and Caprio, J. C. (1998). "Chemoreception," in The Physiology of Fishes, ed. D. H. Evans (Boca Raton, FL: CRC Press), 375-405.

Book:

Cowan, W. M., Jessell, T. M., and Zipursky, S. L. (1997). Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press.

Abstract:

Hendricks, J., Applebaum, R., and Kunkel, S. (2010). A world apart? Bridging the gap between theory and applied social gerontology. Gerontologist 50, 284-293. Abstract retrieved from Abstracts in Social Gerontology database. (Accession No. 50360869)

Patent:

Marshall, S. P. (2000). Method and apparatus for eye tracking and monitoring pupil dilation to evaluate cognitive activity. U.S. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark O±ce.

Data:

Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm

disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

Theses and Dissertations:

Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

Preprint:

Smith, J. (2008). Title of the document. Preprint repository name [Preprint]. Available at: https://persistent-url (Accessed March 15, 2018).

For examples of citing other documents and general questions regarding reference style, please refer to the Chicago Manual of Style. (http://www.chicagomanualofstyle.org/home.html)

Frontiers Science Endnote Style (http://www.frontiersin.org/Design/ens/Frontiers-Science.ens)

Frontiers Science, Engineering and Humanities Bibstyle (http://www.frontiersin.org/Design/bst/frontiersinSCNS_ENG_HUMS.bst)

- **HEALTH, PHYSICS AND MATHEMATICS: For articles submitted in the domain of HEALTH or the journal Frontiers in Physics and Frontiers in Applied Mathematics and Statistics please apply the Vancouver system for in-text citations.**

Reference list: provide the names of the first six authors followed by et al. and doi (https://www.crossref.org/guestquery/#textsearch) when available.

In-text citations should be numbered consecutively in order of appearance in the text – identified by Arabic numerals in the parenthesis for Health articles, and in square brackets for Physics and Mathematics articles.

Reference examples

Article in a print journal:

Sondheimer N, Lindquist S. Rnq1: an epigenetic modifier of protein function in yeast. Mol Cell (2000) 5:163-72.

Article in an online journal:

Tahimic CGT, Wang Y, Bikle DD. Anabolic euects of IGF-1 signaling on the skeleton. Front Endocrinol (2013) 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book:

Sorenson PW, Caprio JC. "Chemoreception,". In: Evans DH, editor. The Physiology of Fishes. Boca Raton, FL: CRC Press (1998). p. 375-405.

Book:

Cowan WM, Jessell TM, Zipursky SL. Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press (1997). 345 p.

Abstract:

Christensen S, Oppacher F. An analysis of Koza's computational euort statistic for genetic programming. In: Foster JA, editor. Genetic Programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3–5; Kinsdale, Ireland. Berlin: Springer (2002). p. 182–91.

Patent:

Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. Flexible Endoscopic Grasping and Cutting Device and Positioning Tool Assembly. United States patent US 20020103498 (2002).

Data:

Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837 (https://dx.doi.org/10.5061/dryad.ps837)

Theses and Dissertations:

Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master’s thesis]. [Chicago (IL)]: University of Chicago

Preprint:

Smith, J. Title of the document. Preprint repository name [Preprint] (2008). Available at: https://persistent-url (Accessed March 15, 2018).

For examples of citing other documents and general questions regarding reference style, please refer to Citing Medicine (https://www.ncbi.nlm.nih.gov/books/NBK7256/).

Frontiers Health Endnote Style (http://www.frontiersin.org/Design/ens/Frontiers-Health.ens)

Frontiers Health and Physics Bibstyle (http://www.frontiersin.org/Design/bst/frontiersinHLTH%26FPHY.bst)

2.3.3. Disclaimer

Any necessary disclaimers which must be included in the published article should be clearly indicated in the manuscript.

2.3.4. Supplementary Material

Frontiers journals do not support pushing important results and information into supplementary sections. However, data that are not of primary importance to the text, or which cannot be included in the article because it is too large or the current format does not permit it (such as movies, raw data traces, power point presentations, etc.) can be uploaded during the submission procedure and will be displayed along with the published article. All supplementary files are deposited to FigShare for permanent storage, during the publication stage of the article, and receive a DOI.

The Supplementary Material can be uploaded as Data Sheet (word, excel, csv, cdx, fasta, pdf or zip files), Presentation (power point, pdf or zip files), Supplementary Image (cdx, eps, jpeg, pdf, png or tif), Supplementary Table (word, excel, csv or pdf), Audio (mp3, wav or wma) or Video (avi, divx, flv, mov, mp4, mpeg, mpg or wmv).

Supplementary material is not typeset so please ensure that all information is clearly presented, the appropriate caption is included in the file and not in the manuscript, and that the style conforms to the rest of the article. To avoid discrepancies between the published article and the supplementary material, please do not add the title, author list, a±liations or correspondence in the

supplementary files. For Supplementary Material templates (LaTex and Word) see Supplementary Material for Frontiers. (http://www.frontiersin.org/design/zip/Frontiers_Supplementary_Material.zip)

Suggested Fonts

The title is written in title case, centred, and in 16 point bold Times New Roman font at the top of page.

Headings and subheadings need to be defined in Times New Roman, 12, bold.

The text of the abstract section should be in 12 point normal Times New Roman.

The body text is in 12 point normal Times New Roman.

2.3.5. File Requirements

For Latex Files, when submitting your article please ensure to upload all relevant manuscript files including:

- tex file
- PDF
- .bib file (if the bibliography is not already included in the .tex file)

Figures should be included in the provided pdf. In case of acceptance, our Production O±ce might require high resolution files of the figures included in the manuscript in eps, jpg or tif format. In order to be able to upload more than one figure at a time, save the figures (labeled in order of appearance in the manuscript) in a zip file, and upload them as 'Supplementary Material Presentation'.

To facilitate the review process, please include a Word Count at the beginning of your manuscript, one option is teXcount which also has an online interface.

During the Interactive Review, authors are encouraged to upload versions using 'Track Changes'. Editors and Reviewers can only download the PDF file of the submitted manuscript .

2.3.6. Additional Requirements per article types

2.3.6.1. CrossMark Policy

CrossMark (https://www.crossref.org/crossmark/index.html) is a multi-publisher initiative to provide a standard way for readers to locate the current version of a piece of content. By applying the CrossMark logo Frontiers is committing to maintaining the content it publishes and to alerting readers to changes if and when they occur. Clicking on the CrossMark logo will tell you the current status of a document and may also give you additional publication record information about the document.

2.3.6.2. Commentaries on Articles

For General Commentaries, the title of your manuscript must have the following format: "Commentary: Title of the original article". At the beginning of your Commentary, please provide the complete citation of the article commented on. Authors commenting on a Frontiers article must submit their commentary for consideration to the same Journal and Specialty as the original article.

Rebuttals may be submitted in response to Commentaries; our limit in place is one commentary and one response. Rebuttals should be submitted as General Commentary articles and the title should have the following format: "Response: Commentary: Title of original article".

2.3.6.3. Book Reviews

The title of a book review needs to follow the format "Book Review: Title of book". For book Reviews, you must also provide the full book details at the beginning of the article in this format: "Book Review: Full book reference"

2.3.6.4. Focused Reviews

For Tier 2 invited **Focused Reviews**, to shape the paper on the importance of the research to the field, we recommend structuring the Review to discuss the paper's Introduction, Materials and Methods, Results and Discussion. In addition the authors must submit a short biography of the corresponding author(s). This short biography has a maximum of 600 characters, including spaces

A picture (5 x 5 cm, in *.tif or *.jpg, min 300 dpi) must be submitted along with the biography in the manuscript and separately during figure upload.

Focused Reviews highlight and explain key concepts of your work. Please highlight a minimum of four and a maximum of ten key concepts in bold in your manuscript and provide the definitions/explanations at the end of your manuscript under “Key Concepts”. Each definition has a maximum of 400 characters, including spaces.

2.3.6.5 Systematic Reviews

For Systematic Reviews, the following article structure applies.

- Title: include systematic review/meta-synthesis/meta-analysis as appropriate in the title

Each of the sections should include specific sub-sections as follows

- Abstract
  - Background
  - Methods
  - Results
  - Conclusions
- Introduction
  - Rationale
  - Objectives
  - Research question
- Methods
  - Study design
  - Participants, interventions, comparators
  - Systematic review protocol
  - Search strategy
  - Data sources, studies sections and data extraction
  - Data analysis
- Results
  - Provide a flow diagram of the studies retrieved for the review
  - Study selection and characteristics
  - Synthesized findings
  - Risk of bias
- Discussion

- Summary of main findings
- Limitations
- Conclusions

2.3.6.6. Data Reports

For Data Reports, please make sure to follow these additional specific guidelines.

1. The data sets (defined as a collection of data that contains individual data units organized in a standardized reusable format, including pre-processed or raw data) must be deposited in a public repository for long-term data preservation prior to submission of the Data Report. The data set(s) is to be fixed and made publicly available upon publication of the Data Report.

2. Our data sharing policy also requires that the dataset be made available to the Frontiers editors and reviewers during the review process of the manuscript. Prior to submission of your Data Report manuscript, please ensure that the repository you have selected supports confidential peer-review. If it does not, we recommend that the authors deposit the datasets to figshare or Dryad Digital Repository for the peer-review process. The data set(s) can then be transferred to another relevant repository before final publication, should the article be accepted for publication at Frontiers.

Note that it is the authors' responsibility to maintain the data sets after publication of the Data Report. Any published Frontiers Data Report article will be considered for retraction should the data be removed from the final selected repository after publication or the access become restricted.

3. The submitted manuscript must include the following details:

- Detailed statement of contribution of the data report to the field
- Name of the data set
- Name of the database/repository where the data set has been submitted
- Link to the data set for confidential peer-review (which can be updated after acceptance, prior to publication once the data is made public)
- Description of how the data was acquired, data collection period
- Filters applied to the data
- Overview of the data files and their formats

- Reference to and/or description of the protocols or methods used to collect the data
- Information on how readers may interpret the data set and reuse the data

All these elements will be peer-reviewed and are required for the publication of the Data Report.

Any future updates to the data set(s) should be deposited as independent versions in a repository and the relevant information may be published as General Commentaries linked on the Frontiers website to the initial Data Report.

Any detailed analyses or new scientific insights relating to the Data Report can be submitted as independent research articles which can also be linked on the Frontiers website to the Data Report article. The protocols and methodology used to collect the data can also be submitted as Methods articles.

2.3.6.7. Case Reports

Case Reports should include the following:

- Background
- Case Presentation

  For human patients: age, sex and occupation of the patient, presenting symptoms, the patient's history and any relevant family or social history, and relevant clinical findings
- For animal patients: age, sex, and breed of the animal, presenting problems, the animal's history, and relevant clinical findings.

  Description of laboratory investigations and diagnostic tests.
- Discussion of the underlying pathophysiology and the novelty or significance of the case. Authors are required to obtain written informed consent from the patients (or their legal representatives) for the publication.

2.3.6.8. Policy & Practice Reviews

For Policy and Practice Reviews, the following article structure applies:

- Abstract
- Introduction
- Sections on assessment of policy/guidelines options and implications
- Actionable Recommendations and Conclusions

2.3.6.9. Policy Briefs

For Policy Briefs, the following article structure applies:

- Abstract (bullet point format)
- Introduction
- Sections on Policy Options and Implications
- Section on Actionable Recommendations
- Conclusions

2.3.6.10. Protocols

For Protocols articles, please make sure to follow these additional specific guidelines.

1. The submitted manuscript must include the following sections:
    - An Abstract.
    - An Introduction outlining the protocol and summarizing its possible applications.
    - A Materials and Equipment section providing a list of reagents or other materials and/or equipment required to carry out the protocol. For basic-science protocols, the formulation of any solutions, e.g. buuers, should be clearly indicated in the Materials and Equipment section.
    - A Stepwise Procedures section listing, stepwise, the stages of the protocol. The timing of each step or related series of steps should be indicated, as should points at which it is possible to pause or halt the procedure without adversely influencing the outcome. For steps requiring repeated measurements, details of precision and accuracy should be presented. Limits of detection or quantification should also be stipulated where appropriate.

- An Anticipated Results section describing, and illustrating with figures, where possible, the expected outcome of the protocol. Any analytical software or methods should be presented in detail in this section, as should possible pitfalls and artifacts of the procedure and any troubleshooting measures to counteract them. These last may also be described in an optional Notes section.

- Code or training data sets referenced by the protocol and useful in its execution should be hosted in an online repository; their accession numbers or other stable identifiers should be referenced in the Anticipated Results.

2. The significance of the protocol and any advance represented by the method compared with other, similar methods should be presented in the contribution to the field statement accompanying your manuscript.

2.3.6.11. Code

The code should be novel and presented in human-readable format, adhere to the standard conventions of the language used (variable names, indentation, style and grammar), be well documented (comments in source), be provided with an example data set to show e±cacy, be compilable or executable free of errors (stating configuration of system used).

The code should only call standard (freely accessible) libraries or include required libraries, and include a detailed description of the use-scenarios, expected outcomes from the code and known limitations of the code.

Please therefore make sure to provide access to the following upon submission:

1. Abstract explicitly including the language of code

2. Keywords including the language of the code in the following format:"code:language"” e.g.: "code:matlab"

3. Contribution to the field statement including the utility of the code and its language

4. Main Text including:

- code description
- application and utility of the code
- link to an accessible online code repository where the most recent source code version is stored and curated (with an associated DOI for retrieval after review)
- access to test data and readme files
- methods used
- example of use
- known issues
- licensing information (Open Source licenses recommended)

5. Compressed Archive (.zip) of the reviewed version of the code as supplementary material (.zip archives are currently available under the “Presentation” dropdown menu).

2.3.6.12. Registered Report

Registered Reports are empirical research articles outlining a proposed methodology and analyses which are peer-reviewed and pre-registered before data collection. Registered Reports should include an Introduction, Methods and preliminary results from any pilot experiments (if applicable). If the Registered Report is endorsed following peer-review and the research is conducted according to the approved methodology, the manuscript will be given In Principle Acceptance. Following data collection, the authors should submit a complete manuscript containing the peer-reviewed sections included in the Registered Report, as well as the Results and Discussion sections. If the Results include unregistered analysis, these should be indicated separately as ‘Exploratory Analysis’. Authors have 1 year after their registered report is accepted to submit a full manuscript. The format is appropriate for any hypothesis-driven research, including both original studies and replications.

Registered Reports have a maximum word count of 3,000 and may include 2 Figures/Tables. Following data collection, the completed version of the manuscript should follow the guidelines for an Original Research article with a maximum word count of 12,000. Registered Reports incur a A-type article fee, charged after the acceptance of the completed manuscript.

2.4. Figure and Table Guidelines

2.4.1. CC-BY Licence

All figures, tables, and images will be published under a Creative Commons CC-BY licence (https://creativecommons.org/licenses/by/4.0/) and permission must be obtained for use of copyrighted material from other sources (including re-published/adapted/modified/partial figures and images from the internet). It is the responsibility of the authors to acquire the licenses, to follow any citation instructions requested by third-party rights holders, and cover any supplementary charges.

2.4.2. General Style Guidelines for Figures

The maximum number of figures and tables for all article types are shown in the Summary Table. Frontiers requires figures to be submitted individually, in the same order as they are referred to in the manuscript, the figures will then be automatically embedded at the end of the submitted manuscript. Kindly ensure that each table and figure is mentioned in the text and in numerical order.

For graphs, there must be a self-explanatory label (including units) along each axis. For figures with more than one panel, panels should be clearly indicated using labels (A), (B), (C), (D), etc. However, do not embed the part labels over any part of the image, these labels will be added during typesetting according to Frontiers journal style. Please note that figures which are not according to the guidelines will cause substantial delay during the production process.

Permissions may be necessary in the following scenarios:

- Republishing
- Modifying/adapting
- Partial Figures

It is the responsibility of the authors to acquire the licenses, to follow any citation instructions requested by third-party rights holders, and cover any supplementary charges.

2.4.3. General Style Guidelines for Tables

Tables should be inserted at the end of the manuscript. If you use a word processor, build your table in word. If you use a LaTeX processor, build your table in LaTeX. An empty line should be left before and after the table.

Please note that large tables covering several pages cannot be included in the final PDF for formatting reasons. These tables will be published as supplementary material on the online article abstract page at the time of acceptance. The author will notified during the typesetting of the final article if this is the case. A link in the final PDF will direct to the online material.

For additional information, please see our Editorial Policies: 3.5 Image Manipulation.

2.4.4. Figure and Table Requirements

Legends

Legends should be preceded by the appropriate label, for example "Figure 1" or "Table 4". Figure legends should be placed at the end of the manuscript (for supplementary images you must include the caption with the figure, uploaded as a separate file). Table legends must be placed immediately before the table. Please use only a single paragraph for the legend. Figure panels are referred to by bold capital letters in brackets: (A), (B), (C), (D), etc.

Image Size

Figure images should be prepared with the PDF layout in mind, individual figures should not be longer than one page and with a width that corresponds to 1 column or 2 columns.

- **All articles are prepared using the 2 column layout:** 2 column articles can contain images 85 mm or 180 mm wide.

2.4.5. Format

The following formats are accepted:

TIFF (.tif) TIFF files should be saved using LZW compression or any other non-lossy compression method.

JPEG (.jpg)

EPS (.eps) EPS files can be uploaded upon acceptance

Color Image Mode

Images must be submitted in the color mode RGB.

Resolution Requirements

All images must be uploaded separately in the submission procedure and have a resolution of **300 dpi at final size**. Check the resolution of your figure by enlarging it to 150%. If the resolution is too low, the image will appear blurry, jagged or have a stair-stepped euect.

Please note saving a figure directly as an image file (JPEG, TIF) can greatly auect the resolution of your image. To avoid this, one option is to export the file as PDF, then convert into TIFF or EPS using a graphics software. EPS files can be uploaded upon acceptance.

Chemical Structures

Chemical structures should be prepared using ChemDraw or a similar program. If working with ChemDraw please use Frontiers ChemDraw Template (https://www.frontiersin.org/files/zip/FrontChemTemplate.zip), if working with another program please follow the guidelines given below:

Drawing settings: chain angle, 120° bond spacing, 18% of width; fixed length, 14.4 pt; bold width, 2.0 pt; line width, 0.6 pt; margin width 1.6 pt; hash spacing 2.5 pt. Scale 100% Atom Label settings: font, Arial; size, 8 pt.

Assign all chemical compounds a bold, Arabic numeral in the order in which the compounds are presented in the manuscript text. Figures containing chemical structures should be submitted in a size appropriate for incorporation into the manuscript.

Legibility

Figures must be legible. Check the following:

- The smallest visible text is no less than 8 points in height, when viewed at actual size.
- Solid lines are not broken up.
- Image areas are not pixilated or stair stepped.
- Text is legible and of high quality.
- Any lines in the graphic are no smaller than 2 points width.

2.5. Funding disclosure

Details of all funding sources must be provided in the funding section of the manuscript including grant numbers, if applicable. All Frontiers articles are published with open access under the CC-BY Creative Commons attribution license. Articles published with Frontiers automatically fulfil or exceed the requirements for open access mandated by many institutions and funding bodies, including the National Institutes of Health, the Medical Research Council, Research Councils UK, and the Wellcome Trust. Frontiers submits funding data to the Open Funder Registry which is a funder identification service from CrossRef resulting from collaboration between scholarly publishers and funding agencies.

2.6. Materials and Data Policies

Frontiers is committed to open science and open data, and we strongly encourage authors to maximize the availability of data included in their articles by making generated data publicly available where possible, and ensuring that published data sets are cited in accordance with our data citation guidelines. We aim to achieve the best community standards regarding data availability, ensuring increased levels of transparency and reproducibility in our published articles.

Our policies on data availability are informed by community-driven standards, which Frontiers endorses, such as the Transparency and Openness (https://cos.io/our-services/top-guidelines/) (TOP) guidelines, and the joint declaration of data citation principles produced by FORCE 11 (https://www.force11.org/group/joint-declaration-data-citation-principles-final).

2.6.1. Availability of Materials

Authors are strongly encouraged to make all materials used to conduct their research available to other researchers. Research materials necessary to enable the reproduction of an experiment should be clearly indicated in the Materials and Methods section. Relevant materials such as protocols, analytic methods, and study material should preferably be uploaded to an online repository providing a global persistent link/identifier. If this is not possible, authors are strongly encouraged to make this material available upon request to interested researchers, and this should be stated in the manuscript.

Resource Identification Initiative

Authors wishing to participate in the Resource Identification Initiative (https://www.force11.org/group/resource-identification-initiative) should cite antibodies, genetically modified organisms, software tools, data, databases, and services using the corresponding catalog number and RRID in your current manuscript. For more information about the project and for steps on how to search for an RRID, please click here (https://www.frontiersin.org/files/pdf/letter_to_author.pdf).

2.6.2. Availability of Data

Frontiers requires that authors make all data relevant to the conclusions of the manuscript available to editors and reviewers during peer-review to enable complete and objective evaluation of the work described.

We strongly encourage authors to make the raw data supporting the conclusions of the manuscript available in publicly accessible repositories. To comply with best practice in their field of research, authors are required to make certain types of data available to readers at time of publication in specific stable, community-supported repositories such as those listed below. Authors are encouraged to contact our data availability o±ce at datapolicy@frontiersin.org (mailto:datapolicy@frontiersin.org) prior to submission with any queries concerning data reporting.

2.6.3. Data Citation Guidelines

Authors are encouraged to cite all datasets generated or analyzed in the study. Where datasets are cited, they should be included in the references list to maximize future usability. The following format should be used:

[Dataset] Author names. (year) Data Title. Repository name. Version. Persistant identifier

2.6.4. Data Availability Statements

Data availability statements are required for all manuscripts published with Frontiers. During the submission process, authors will be asked to detail the location of the raw data underlying the conclusions made in the manuscript, and whether it will be made available to other researchers following

publication. Authors will also be asked for the details of any existing datasets that have been analysed in the manuscript. These datasets should be cited in accordance with our data citation guidelines.

A statement will be automatically generated using the information provided in the submission form; however, manuscripts containing incomplete or incorrect statements will be prevented from entering the review process.

Examples of acceptable statements

1. **Datasets are in a publicly accessible repository:**

   The datasets [GENERATED/ANALYZED] for this study can be found in the [NAME OF REPOSITORY] [LINK]

2. **Datasets are available on request:**

   The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

3. **All relevant data is contained within the manuscript:**

   All datasets [GENERATED/ANALYZED] for this study are included in the manuscript and the supplementary files.

4. **Restrictions apply to the datasets:**

   The datasets for this manuscript are not publicly available because: [VALID REASON]. Requests to access the datasets should be directed to [NAME, EMAIL].

5. **Data has been obtained from a third party:**

   The data analyzed in this study was obtained from [SOURCE], the following licenses/restrictions apply [RESTRICTIONS]. Requests to access these datasets should be directed to [NAME, EMAIL].

6. **No datasets were generated for this study**

2.6.5. Recommended and Required Repositories

Authors are required to deposit the following data-types in public, community-supported repositories, such as those listed below, prior to publication of an associated Frontiers manuscript:

Data-type	Recommended Repositories	Metadata Standard
Genetic and genomic sequence (DNA/ RNA)^	GenBank DNA Data Bank of Japan (DDBJ) European Nucleotide Archive (ENA)	MiXS
Metagenomic sequence	EBI Metagenomics	MiXS
DNA and RNA trace or short-read sequencing data	NCBI Trace Archive NCBI Sequence Read Archive	MiXS
Genetic polymorphism data, including SNP and CNV data	dbSNP dbVar European Variation Archive DGVa	MiXS
Gene expression data; chromatin immunoprecipitation data (deep-sequencing or microarray)	ArrayExpress Gene Expression Omnibus (GEO)	MIAME / MINSEQE
Data linking genotype to phenotype	dbGaP	
Protein sequence data	UniProt	
Proteome profiling data	PRIDE PeptideAtlas ProteomeXchange	MIAPE
Small molecule, protein, protein complex data structural data	Crystallography Open Database Cambridge Structural Database wwPDB (Protein DataBank) Electron Microscopy Databank	CIF
Taxonomy data	Zoobank	

^ Genetic sequence variants should be annotated according to the guidelines established by the Human Variome Project (http://www.humanvariomeproject.org/resources/genetics-and-genomics-journals.html).

Authors are encouraged to consider deposition in public, community-supported repositories of the data-types listed below:

Data-type	Recommended Repositories	Metadata Standard

Protein-protein interaction data	Database of Interacting Proteins (DIP)	MIMIx
Metabolite and metabolome profiling data	MetaboLights Human Metabolome Database	MSI
Small-molecule screening data, chemical compound data	PubChem	CIF
Flow cytometry data	Flow Repository	
Brain Imaging data / Neuroimaging data	OpenNeuro INDI NITRC NeuroVault [Statistical maps]	BIDS
Trait data	TRY database	
Phenology data	National Phenology Network	
Any data	FigShare Dryad Digital Repository	None

2.6.6. Inclusion of Zoological Nomenclature

The International Code of Zoological Nomenclature, in a recent 2012 amendment to the 1999 Zoological Code (http://iczn.org/content/electronic-publication-made-available-amendment-code), allows all electronic-only papers, such as those published by the Frontiers journals, to have valid new taxon names and nomenclatural acts. However, these new names or nomenclatural acts must be registered in ZOOBANK (http://zoobank.org/) and have associated Life Science Identifiers (LSIDs). Registration must be done by the authors before publication. Should your manuscript include any zoological new taxon names and/or nomenclatural acts, please ensure that they are registered prior to final publication.

2.6.7. Inclusion of RNAseq Data

Studies employing RNASeq for comparative transcriptomic analyses must contain at least 3 biological replicates (unless otherwise justified). Each biological replicate should be represented in an independent library, each with a unique barcode if libraries are multiplexed for sequencing. Validation on a number of key transcripts highlighted in the study is also highly recommended.

Full data accompanying these experiments must be made available to reviewers at the time of submission in a freely accessible resource e.g the sequence read archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) or European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena). Depending on the question addressed in a manuscript, de novo assemblies of transcriptomes may also require multiple replicates and assembled sequences together with sequence annotation must be made freely available e.g figshare (https://figshare.com/) or dryad (https://datadryad.org/).

2.6.8 Inclusion of Proteomics Data

Authors should provide relevant information relating to how peptide/protein matches were undertaken, including methods used to process and analyse data, false discovery rates (FDR) for large-scale studies and threshold or cut-ou rates for peptide and protein matches. Further information should include software used, mass spectrometer type, sequence database and version, number of sequences in database, processing methods, mass tolerances used for matching, variable/fixed modifications, allowable missed cleavages, etc.

Authors should provide as supplementary material information used to identify proteins and/or peptides. This should include information such as accession numbers, observed mass (m/z), charge, delta mass, matched mass, peptide/protein scores, peptide modification, miscleavages, peptide sequence, match rank, matched species (for cross-species matching), number of peptide matches, etc. Ambiguous protein/peptide matches should be indicated.

For quantitative proteomics analyses, authors should provide information to justify the statistical significance, including biological replicates, statistical methods, estimates of uncertainty, and the methods used for calculating error.

For peptide matches with biologically relevant post-translational modifications (PTMs) and for any protein match that has occurred using a single mass spectrum, authors should include this information as raw data or annotated spectra, or submit data to an online repository (recommended option; see table below).

Raw or matched data and 2-DE images should be submitted to public proteomics repositories such as those participating in ProteomeXchange. Submission codes and/or links to data should be provided within the manuscript.

2.7. Statistics

Frontiers requires that all statements concerning quantitative diuerences should be based on quantitative data and statistical testing. For example, if a quantitative statement is made regarding the abundance of a certain protein based on a western blot, we request that the blot be scanned and the abundance assessed quantitatively using the correct analytic software (e.g. ImageJ) and statistics in order to support that statement.

Statistics should/must be applied for independent experiments. The number of independent samples and the deviation parameters (e.g. Standard Error of the Mean, Standard Deviation, Confidence Intervals) should be clearly stated in the Methods or the Figure legends. In general, technical replicates within a single experiment are not considered to be independent samples. Where multiple comparisons are employed (e.g. microarray data or Genome-wide association studies), any analysis should correct for false positive results. Descriptions of statistical procedures should include the software and analysis used, and must be su±ciently detailed to be reproduced.

3. Editorial Policies and Publication Ethics

Frontiers' ethical policies are a fundamental element of our commitment to the scholarly community. These policies apply to all the Frontiers in journal series. Frontiers has been a member of the Committee of Publication Ethics since January 2015 and follows COPE guidelines where applicable.

3.1. Authorship and Author Responsibilities

Frontiers follows the International Committee of Medical Journal Editors (http://www.icmje.org/recommendations/browse/roles-and-responsibilities/defining-the-role-of-authors-and-contributors.html) guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis or interpretation of data for the work;
- Drafting the work or revising it critically for important intellectual content;
- Provide approval for publication of the content;
- Agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Contributors, who do not meet these criteria, but nonetheless provided important contributions to the final manuscript should be included in the acknowledgements section. It is the authors responsibility to get written approval by persons named in the acknowledgement section. In order to provide appropriate credit to all authors, as well as assigning responsibility and accountability for published work, individual contributions should be specified as an Author Contributions statement. This should be included at the end of the manuscript, before the References. The statement should specify the contributions of all authors. You may consult the Frontiers manuscript guidelines for formatting instructions. Please see an example here:

AB, CDE and FG contributed conception and design of the study; AB organized the database; CDE performed the statistical analysis; FG wrote the first draft of the manuscript; HIJ, KL, AB, CDE and FG wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

The corresponding author takes primary responsibility for communication with the journal and editorial o±ce during the submission process, throughout peer review and during publication. The corresponding author is also responsible for ensuring that the submission adheres to all journal requirements including, but not exclusive to, details of authorship, study ethics and ethics approval, clinical trial registration documents and conflict of interest declaration. The corresponding author should also be available post-publication to respond to any queries or critiques.

Requests to modify the authors list after submission should be made to the editorial o±ce using the authorship changes form (http://www.frontiersin.org/files/pdf/Authorship_change_form.pdf).

3.2. Research Integrity

Material submitted to Frontiers must comply with the following policies to ensure ethical publication of academic work:

i. *Original content and duplicate publication:* Frontiers only publishes original content. Authors confirm the submission of original content in the Terms & Conditions upon submission. Manuscripts submitted to Frontiers must not have been previously published or be under consideration for publication elsewhere, either in whole or in part. If an article has been previously submitted for publication elsewhere, Frontiers will only consider publication if the article has been definitively rejected by the other publisher(s) at the point of submission to Frontiers.
ii. *Redundant publication:* Frontiers considers the submission and publication of very similar articles based on the same experiment or study to be unethical.
iii. *Fabrication and falsification:* Frontiers opposes both the fabrication of data or images (i.e. fake or made up data) and the falsification of data or images (i.e. the intentional misrepresentation or deceptive manipulation of data).
iv. *Plagiarism:* Plagiarism occurs when an author attempts to present previously published work as original content. Every manuscript submitted to Frontiers is screened for textual overlap by the software CrossCheck, powered by iThenticate. Manuscripts found to contain textual overlap are not considered for publication by Frontiers. For more details on what constitutes plagiarism, please see here.

We reserve the right to contact the a±liated institutions of authors, who have not acted according to good research and publication practices.

3.3. Translations

Frontiers accepts manuscript submissions that are exact translations of previously published work. This should be clearly stated in the manuscript upon submission. Permission from the original publisher and authors needs to be sought and also stated in the manuscript, and the relevant documents

should be provided as supplementary data for verification by the Editor and the editorial o±ce. The original work from which the manuscript has been translated should be clearly referenced.

> *"This is a ('language') language translation/reprint of ('insert title here') originally published in ('insert name here'). ('Insert name here') prepared this translation with support from (insert name of funding source, if any). Permission was granted by ('Insert name here')."*

Please note that Frontiers may request copies of related publications if there are any concerns about overlap or possible redundancy.

3.4. Plagiarism and Duplication

Frontiers checks all submitted manuscripts for plagiarism and duplication, and publishes only original content. Those manuscripts where plagiarism or duplication is shown to have occurred will not be considered for publication in a Frontiers journal. It is required that all submissions must consist as far as possible of content that has not been published previously. In accordance with COPE guidelines (http://publicationethics.org/files/International standards_authors_for website_11_Nov_2011.pdf), we expect that "original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations." This condition also applies to an author's own work.

For submissions adapted from theses, dissertations, conference abstracts or proceedings papers, please see the following sections for more information.

Theses and Dissertations

Frontiers allows the inclusion of content which first appeared in an author's thesis so long as this is the only form in which it has appeared, is in line with the author's university policy, and can be accessed online. If the thesis is not archived online, it is considered as original unpublished data and thus is subject to the unpublished data restrictions of some of our article types. This inclusion should be noted in the Acknowledgements section of the manuscript and the thesis should be cited and referenced accordingly in the Reference list. For some examples, please check our in Manuscript Requirements and Style Guide at 2.3.1

Conferences, Proceedings and Abstracts

Manuscripts that first appeared as conference papers must be expanded upon if they are to be considered as original work. You are required to add a substantial amount of original content in the form of new raw material (experiments, data) or new treatment of old data sets which lead to original discussion and/or conclusions, providing value that significantly exceeds the original conference version. As a rule of thumb, at least 30% of content must be original. Authors submitting such work are required to:

– Seek permission for reuse of the published conference paper if the author does not hold the copyright (proof of permission should be submitted as supplementary material or sent to editorial.o±ce@frontiersin.org with the manuscript ID upon submission).

– Cite the conference in the Acknowledgements section, or the references section if applicable.

Blogs

Although permissible, extended manuscript content which previously appeared online in non-academic media, e.g. blogs, should be declared at the time of submission in the acknowledgements section of the manuscript.

3.5. Image Manipulation

Frontiers takes concerns regarding image manipulation seriously. We request that no individual features within an image are modified (eg. enhanced, obscured, moved, recycled, removed or added). Image processing methods (e.g. changes to the brightness, contrast or color balance) must be applied to every pixel in the image and the changes should not alter the information illustrated in the figure. Where cropped images of blots are shown in figures, a full scan of the entire original gel(s) must be submitted as part of the supplementary material. Where control images are re-used for illustrative purposes, this must be clearly declared in the figure legend. If any form of image processing is legitimately required for the interpretation of the data, the software and the enhancement technique must be declared in the methods section of the manuscript. Image grouping and splicing must be

clearly stated in the manuscript and the figure text. Any concerns raised over undeclared image modifications will be investigated and the authors will be asked to provide the original images.

3.6. Confiicts of Interest

A conflict of interest can be anything potentially interfering with, or that could reasonably be perceived as interfering with, full and objective peer review, decision-making or publication of articles submitted to Frontiers. Personal, financial and professional a±liations or relationships can be perceived as conflicts of interest.

All authors and members of Frontiers Editorial Boards are required to disclose any actual and potential conflicts of interest at submission or upon accepting an editorial or review assignment.

The Frontiers review system is designed to guarantee the most transparent and objective editorial and review process, and because handling editor and reviewers' names are made public upon the publication of articles, conflicts of interest will be widely apparent.

Failure to declare competing interests can result in the rejection of a manuscript. If an undisclosed competing interest comes to light after publication, Frontiers will take action in accordance with internal policies and Committee on Publication Ethics guidelines.

What Should I Disclose?

As an author, disclosure of any potential conflicts of interest should be done during the submission process. Consider the following questions and make sure you disclose any positive answers:

1. Did you or your institution at any time receive payment or services from a third party for any aspect of the submitted work?
2. Do you have financial relationships with entities that could be perceived to influence, or that give the appearance of potentially influencing, what you wrote in the submitted work?
3. Do you have any patents and copyrights, whether pending, issued, licensed and/or receiving royalties related to the research?

4. Do you have other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work?

If you failed to disclose any of the potential conflicts of interest above during submission, or in case of doubt, please contact as soon as possible the Frontiers Editorial Office at editorial.office@frontiersin.org with the details of the potential conflicts.

Example statement: “Author xxx was employed by company xxxx. All other authors declare no competing interests.”

The handling editors and reviewers will be asked to consider the following potential conflicts of interest before accepting any editing or review assignment:

FAMILY	1. Are any of the authors a spouse or significant other, a member of the same family or a very close personal friend? Review Editors should also not be a member of the same family as the handling editor.
COLLABORATIONS	2. Are you currently hosting or have hosted a Frontiers Research Topic with any of the authors within the past 2 years? Are you currently hosting a Frontiers Research Topic with the Editor? 3. Are you currently collaborating or have you collaborated on a research project or a publication with any of the authors within the past 2 years? 4. Are you currently collaborating or have you collaborated with any of the authors as an advisor or in any other direct supervisory capacity in the past five years? 5. Are you currently collaborating or have you collaborated with any of the authors as a student or in any other direct subordinate capacity in the past five years? Note: Review Editors should not accept assignments if they have a close professional relationship with the handling editor, which in their view could affect the objectivity of the review.
AFFILIATION	6. Are you affiliated with the same institution as the editor? Are you affiliated with the same institution as any of the authors? If so, has this resulted in interactions, collaborations, or mutual interests with the authors that would compromise your impartiality in conducting this review? 7. Are you a current member of a committee or department that coincides with an affiliation with the editor or any of the authors?
FINANCIAL	8. Do you have a business or professional partnership with any author? 9. Do you have financial interests or business relations with any organization involved in this research or in the preparation of the manuscript? 10. Do you have any financial interest or competing interests in the content of the manuscript that might affect your ability to perform an objective review?

3.7. Bioethics

All research submitted to Frontiers for consideration must have been conducted in accordance with Frontiers guidelines on study ethics. In accordance with COPE guidelines, Frontiers reserves the right to reject any manuscript that editors believe does not uphold high ethical standards, even if authors have obtained ethical approval or if ethical approval is not required.

3.7.1. Studies involving animal subjects

All research involving regulated animals (i.e. all live vertebrates and higher invertebrates) must be performed in accordance with relevant institutional and national guidelines and regulations. Frontiers follows International Association of Veterinary Editors guidelines (http://www.veteditors.org/consensus-author-guidelines-on-animal-ethics-and-welfare-for-editors/) for publication of studies including animal research. Approval of research involving regulated animals must be obtained from the relevant institutional review board or ethics committee prior to commencing the study. Confirmation of this approval is required upon submission of a manuscript to Frontiers; authors must provide a statement identifying the full name of the ethics committee that approved the study. For most article types, this statement should appear in the Materials and Methods section. An example ethics statement:

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of [name of guidelines], [name of committee]. The protocol was approved by the [name of committee].

Should the study be exempt from ethics approval, authors need to clearly state the reasons in the declaration statement and in the manuscript. Studies involving privately owned animals should demonstrate the best practice veterinary care and confirm that informed consent has been granted by the owner/s, or the legal representative of the owner/s. Frontiers supports and encourages authors to follow the ARRIVE guidelines for the design, analysis and reporting of scientific research.

Humane Endpoints

All manuscripts describing studies where death is an endpoint will be subject to additional ethical considerations. Frontiers reserves the right to reject any manuscripts lacking in appropriate justification.

3.7.2. Studies involving human subjects

Research involving human subjects is expected to have been conducted in accordance with the World Medical Association's Declaration of Helsinki (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/). Studies involving human participants must be performed in accordance with relevant institutional and national guidelines, with the appropriate institutional ethics committee's prior approval and informed written consent from all human subjects involved in the study including for publication of the results. Conformation of this approval is required upon submission of a manuscript to Frontiers; authors must provide a statement identifying the full name of the ethics committee that approved the work and confirm that study subjects (or when appropriate, parent or guardian) have given written informed consent. For most article types, this statement should appear in the Materials and Methods section. An example ethics statement:

This study was carried out in accordance with the recommendations of [name of guidelines], [name of committee]. The protocol was approved by the [name of committee]. All subjects gave ***written informed consent*** *in accordance with the Declaration of Helsinki.*

Should the study be exempt from ethics approval, authors need to clearly state the reasons in the declaration statement and in the manuscript. In order to protect subject anonymity, identifying information should not be included in the manuscript unless such information is absolutely necessary for scientific purposes AND explicit approval has been granted by the subjects.

3.7.3. Inclusion of identifiable human data

Frontiers follows the ICMJE recommendations (http://www.icmje.org/recommendations/browse/roles-and-responsibilities/protection-of-research-participants.html) on the protection of research participants, which state that patients have a right to privacy that should not be violated without informed consent. We require

non-essential identifiable details to be omitted from all manuscripts, and written informed consent will be required if there is any doubt that anonymity can be maintained.

It is the responsibility of the researchers and authors to ensure that these principles are complied with, including the obtaining of written, informed consent.

Written informed consent can be documented on a form provided by an institution or ethics committee, and it must clearly state how the identifiable data will be used. Frontiers also makes available its own form (https://www.frontiersin.org/files/pdf/FrontiersConsentForm.pdf) , which may be used for this purpose, but use of the Frontiers form is not required if a suitable alternative form of consent, meeting the ICMJE recommendations (http://www.icmje.org/recommendations/browse/roles-and-responsibilities/protection-of-research-participants.html), is used. We consider it to be the author' duty to encourage participants or patients whose consent for publication is required to read and understand the ICMJE guidelines, for their information prior to completing the consent form. Participants should also be encouraged to ask any questions and to ensure they are comfortable before they sign the consent form.

The completed consent forms should be stored by authors or their respective institutions, in accordance with institutional policies. Frontiers does not need to view the completed form, and this should not be included with the submission. The completed form should be made available on request from the editor or editorial o±ce, both during the review process and post-publication.

The determination of what constitutes identifiable data lies with our editors and editorial o±ce stau, and manuscripts may be rejected if the required consent documents cannot be provided. Please note that written informed consent for publication is required for all case report articles where the patient or subject is identified or identifiable.

3.7.4. Clinical Trials

The World Health Organization (http://www.who.int/ictrp/en) defines a clinical trial as "any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the euects on health outcomes." In accordance with the Clinical Trial Registration Statement from the International Committee of Medical Journal Editors (ICMEJ) (http://www.icmje.org/), all clinical trials must be registered in a public trials registry at or before the onset of participant enrolment. This requirement applies to all clinical trials that begin enrolment after July 1, 2005. To meet the requirements of the ICMJE, and Frontiers', clinical trials can be registered with any Primary Registry in the WHO Registry Network (http://www.who.int/ictrp/network/primary/en/index.html) or an ICMJE approved registry (http://www.icmje.org/about-icmje/faqs/clinical-trials-registration/).

Clinical trial reports should be compliant with the Consolidated Standards of Reporting Trials (CONSORT) (http://www.consort-statement.org/?o=1011) both in terms of including a flow diagram presenting the enrolment, intervention allocation, follow-up, and data analysis with number of subjects for each and taking into account the CONSORT Checklist of items to include when reporting a randomized clinical trial.

The information on the clinical trial registration (Unique Identifier and URL) must be included in the abstract.

3.8. Corrections

Frontiers recognizes our responsibility to correct errors in previously published articles. If it is necessary to communicate important, scientifically relevant errors or missing information, and compelling evidence can be shown that a major claim of the original article was incorrect, a Correction should be submitted detailing the reason(s) for and location(s) of the change(s) needed using the below template. Corrections can be submitted if a small portion of an otherwise reliable publication proves to be misleading, e.g. an error in a figure that does not alter conclusions OR an error in statistical data not altering conclusions OR mislabeled figures OR wrong slide of microscopy provided, or if the author / contributor list is incorrect when a deserving author has been omitted or somebody who does not meet

authorship criteria has been included. The contribution to the field statement should be used to clearly state the reason for the Correction. Please note, a correction is not intended to replace the original manuscript.

The title of the submission should have the following format: "Corrigendum: Title of original article". It is advised to use the corrigendum Word and LaTeX templates (https://www.frontiersin.org/design/zip/Frontiers_Corrigendum_Templates.zip)

If the error was introduced during the publishing process, the Frontiers Production O?ce (mailto:production.o?ce@frontiersin.org) should be contacted.

3.9. Retractions

As a member of the Committee on Publication Ethics (COPE) (http://publicationethics.org/), Frontiers abides by their guidelines and recommendations in cases of potential retraction.

Frontiers also abides by two other key principles, as recommended by COPE:

- Retractions are not about punishing authors.
- Retraction statements should be public and linked to the original, retracted article.

While all potential retractions are subject to an internal investigation and will be judged on their own merits, Frontiers considers the following reasons as giving cause for concern and potential retraction:

- Clear evidence that findings are unreliable, either as a result of misconduct (e.g. data fabrication) or honest error (e.g. miscalculation or experimental error)
- Findings have previously been published elsewhere without proper attribution, permission or justification (i.e. cases of redundant publication)
- Major plagiarism
- The reporting of unethical research, the publication of an article that did not have the required ethics committee approval
- Legal issues pertaining to the content of the article e.g. libellous content

- Major authorship issues i.e. proven or strongly suspected cases of ghostwriting or sold (‘gift’) authorship
- Politically-motivated articles where objectivity is a serious concern
- The singling out of individuals or organizations for attack
- Faith issues (e.g. intelligent design)
- Papers that have made extraordinary claims without concomitant scientific or statistical evidence (e.g. pseudoscience)

Readers who would like to draw the editors' attention to published work that might require retraction should contact the authors of the article and write to the journal, making sure to include copies of all correspondence with authors.

Please find more details on our comments and complaints policy here (https://www.frontiersin.org/about/publishing-model)

3.10. Support and Ethical concerns

In our commitment to continuously improve our website, we welcome your feedback, questions and suggestions. Please visit our Help Center to find guidance on our platform or contact us at support@frontiersin.org (mailto:support@frontiersin.org).

For any ethical concerns, please contact us at editorial.o?ce@frontiersin.org (mailto:editorial.o?ce@frontiersin.org).