UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
CURSO DE GRADUAÇÃO EM BIOMEDICINA

Giovanna Carello Collar

ANÁLISE DE INTERNEURÔNIOS GABAÉRGICOS PARVALBUMINA-POSITIVOS E CALBINDINA-POSITIVOS NO CÓRTEX PRÉ-FRONTAL MEDIAL EM MODELO ANIMAL DE AUTISMO INDUZIDO POR ÁCIDO VALPROICO

Porto Alegre
2019

Giovanna Carello Collar

ANÁLISE DE INTERNEURÔNIOS GABAÉRGICOS PARVALBUMINA-POSITIVOS E CALBINDINA-POSITIVOS NO CÓRTEX PRÉ-FRONTAL MEDIAL EM MODELO ANIMAL DE AUTISMO INDUZIDO POR ÁCIDO VALPROICO

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: Dra. Carmem Juracy Silveira Gottfried
Co-orientador: Bel. Júlio Santos Terra Machado

Porto Alegre

2019

CIP - Catalogação na Publicação

Carello-Collar, Giovanna
ANÁLISE DE INTERNEURÔNIOS GABAÉRGICOS PARVALBUMINA-POSITIVOS E CALBINDINA-POSITIVOS NO CÓRTEX PRÉ-FRONTAL MEDIAL EM MODELO ANIMAL DE AUTISMO INDUZIDO POR ÁCIDO VALPROICO / Giovanna Carello-Collar. -- 2019.
133 f.
Orientadora: Carmem Juracy Silveira Gottfried.

Coorientador: Júlio Santos Terra Machado.

Trabalho de conclusão de curso (Graduação) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Curso de Biomedicina, Porto Alegre, BR-RS, 2019.

1. Transtorno do Espectro Autista. 2. Desequilíbrio excitatório/inibitório. 3. Modelo VPA. 4. Interneurônios GABAérgicos. 5. Córtex pré-frontal medial. I. Gottfried, Carmem Juracy Silveira, orient. II. Machado, Júlio Santos Terra, coorient. III. Título.

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

Giovanna Carello Collar

ANÁLISE DE INTERNEURÔNIOS GABAÉRGICOS PARVALBUMINA-POSITIVOS E CALBINDINA-POSITIVOS NO CÓRTEX PRÉ-FRONTAL MEDIAL EM MODELO ANIMAL DE AUTISMO INDUZIDO POR ÁCIDO VALPROICO

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.

Aprovado em: 04 de julho de 2019.

BANCA EXAMINADORA

Dr. Leo Anderson Meira Martins - UFRGS

Ma. Giovana Bristot - UFRGS

Dra. Carmem Juracy Silveira Gottfried - UFRGS (orientadora)

AGRADECIMENTOS

Esses agradecimentos não são somente sobre o resultado do fim de um semestre de ideias, hipóteses, experimentos e discussões, como também sobre o encerramento do meu ciclo no laboratório e de uma das várias etapas de minha jornada acadêmica e englobam todos aqueles que participaram deste caminho e que me acompanham desde 2015:

À minha orientadora, professora Carmem Gottfried, meu enorme agradecimento por ter aberto as portas do Laboratório de Plasticidade Neuroglial quando eu estava recém no segundo semestre da faculdade e por toda a ajuda e ensinamentos durante esses anos no lab e com este TCC.

Ao meu co-orientador, Júlio (às vezes Jílio), por ter idealizado o esboço deste trabalho e ter me dado a oportunidade de realizá-lo; obrigada pelos ensinamentos sobre imuno, pelas discussões de artigos, disponibilidade 24 horas por dia para tirar dúvidas e, é claro, muito obrigada pelas contribuições de ideias e por sempre fazer *to do lists* para me ajudar a "ejetar" este TCC. Agradeço de coração tua dedicação e empenho nesta co-orientação.

Aos meus colegas de trabalho, os quais viraram meus amigos dentro do laboratório e me acolheram desde o início. Obrigada pelas risadas, desmaios, rolês no Alemão, Vilma e Limas, congressos e por todos os ensinamentos não somente durante o TCC como também em todos esses anos. Obrigada Mari, minha bixete, companheira de laboratório e grande amiga, sem ti tudo teria sido mil vezes mais difícil com certeza. Obrigada Mell, por ser essa inspiração de cientista e professora. Obrigada Iohanna, que não era oficialmente minha co-orientadora, mas da mesma forma estava sempre disposta a ajudar. Levarei comigo todos os ensinamentos que vocês me passaram e lembrarei sempre do ImunoTeam. Obrigada Gui por eu me sentir parte importante do teu trabalho, obrigada Brum, Bruna, Kalunga e também a todos aqueles amigos do laboratório que seguiram outros caminhos, mas que foram muito importantes para mim durante todos esses anos: Mauro, Wal e Victorio. Mauro, obrigada por sempre ter incentivado os alunos de iniciação científica a sermos mais independentes e confiantes e ter dado dicas e conselhos. Wal, obrigada por toda a ajuda e paciência com as minhas histórias.

À minha família (vô Chico, mãe, Fê, pai e Matheus e, é claro, Frajola e Pretinha), por terem conseguido me aguentar nesses meses (admiro a coragem) e por terem compreendido minha ausência em alguns momentos importantes em família. Obrigada vô, por ter sido um exemplo de ser humano humilde, honesto e trabalhador. Mãe, nesse parágrafo não cabe tudo o que eu precisaria te agradecer. Obrigada por sempre segurar as pontas para mim, me incentivar em TUDO e me mostrar a paixão pela pesquisa. Tu és minha cientista preferida. Fê, obrigada por estar sempre comigo, mesmo que eu às vezes não me faça tanto presente por estar sempre,

como tu diz, “contando bolinhas verdes” e “nem parece que tá em casa porque tá sempre trancada no quarto estudando”. Pai, obrigada por todo o apoio, mesmo que de longe, e por entender o quanto a pesquisa significa para mim. Obrigada Teteus por ouvir todos os meus desabafos e reclamações (ufa) e por compartilhar essa vida científica comigo. Obrigada família por terem sido meu ponto de paz, refúgio e psicólogos nas horas vagas. Amo vocês!

Aos meus amigos da faculdade Vi, Paula, Jef, Bruna, Laura e Tati, futuros biomédicos que a UFRGS me deu o prazer de conhecer: obrigada por todos os momentos durante a faculdade e por compartilharem esse último ano de graduação comigo com muita reclamação, xororô e momentos de pânico, mas também com alívio e alegria de estarmos chegando ao fim!

Aos meus amigos de longa data, Jô, Rose, Rique, Vit, Jair e Pedro, que mesmo não entendendo muito bem o que eu fazia no lab, sempre pararam para me ouvir contar com entusiasmo sobre o que fazia meus olhinhos brilharem.

Aos meus amigos que não são brasileiros só por capricho do destino, Filippo e Mariah, não tenho nem como agradecer em palavras. Vocês foram muito importantes em momentos mágicos da minha vida. Muito obrigada por tudo.

Aos órgãos de fomento CNPq, CAPES, INCT-NIM, A.W.A.R.D Institute, FIPE-HCPA, PROPESQ-UFRGS por possibilitarem a realização deste trabalho.

E, por fim, não poderia deixar de registrar meu enorme agradecimento aos animais de pesquisa, pois sem eles boa parte dos avanços da ciência não teriam acontecido, e a todos os cientistas e pesquisadores que nos dão o *background* para embasarmos nossas hipóteses e construirmos nossos projetos.

“If I have seen further, it is by standing on the shoulders of Giants.”

Isaac Newton.

RESUMO

O transtorno do espectro autista (TEA) é uma desordem do neurodesenvolvimento que possui etiologia desconhecida, caracterizada por déficits de comunicação e de interação social, bem como padrões de comportamento, atividades ou interesses restritos e/ou repetitivos. Um conhecido fator de risco para o desencadeamento do TEA é a exposição pré-natal ao ácido valproico (VPA), o qual é utilizado para gerar características do tipo-autista em modelos animais quando administrado durante a prenhez. O desequilíbrio excitatório/inibitório (E/I) está relacionado com os prejuízos cognitivos, sociais e sensoriais encontrados no TEA, sendo que há evidências importantes da contribuição de sinalização inibitória alterada. Devido ao papel-chave dos interneurônios GABAérgicos para a manutenção do balanço E/I e a importância do córtex pré-frontal medial (CPFm) para comportamentos sociais nos roedores, os objetivos deste trabalho foram analisar a quantidade e a proporção dos interneurônios GABAérgicos calbindina-positivos (CB^+) e parvalbumina-positivos (PV^+) em relação aos neurônios totais $NeuN^+$ nas camadas superficiais e profundas das três regiões do CPFm: córtex cingulado anterior (aCC) e áreas pré-límbica (PrL) e infralímbica (IL). Ratas Wistar prenhes foram aleatoriamente divididas em dois grupos experimentais: Controle e VPA. No dia embrionário 12,5, salina ou VPA (600 mg/kg, i.p.) foram administrados para os grupos Controle e VPA, respectivamente. No dia pós-natal 30, o encéfalo de ratos machos da prole (n= 3-5 em ninhadas) foi removido para realização da técnica de imunofluorescência e posterior análise em microscópio confocal (CEUA-UFRGS #140367). No CPFm como um todo, o grupo VPA apresentou um aumento no número de neurônios totais $NeuN^+$ (p=0,0086). Já na análise das sub-regiões, observou-se diminuição do número de CB^+ (p=0,0278) nas camadas profundas do aCC e na proporção destes interneurônios (p=0,0255) em relação aos neurônios totais nas camadas superficiais de PrL e de IL (p=0,0173). Já em relação aos PV^+, houve aumento do número (p=0,0369) e na proporção (p=0,0044) destas células nas camadas superficiais de PrL e aumento do número nas camadas profundas (p=0,0245) de PrL. Interessantemente, houve diminuição da proporção de PV^+ nas camadas superficiais (p=0,0003) e profundas (p=0,0460) do aCC e nas camadas superficiais de IL (p=0,0169). Os achados sugerem que a exposição pré-natal ao VPA possa estar relacionada com alterações no processo de migração dos interneurônios em direção a áreas corticais, uma vez que a composição de todos os resultados nos permitiu observar diferentes padrões de alterações específicas nas sub-regiões. Além disso, a alteração em neurônios totais $NeuN^+$ - os quais são majoritariamente representados por neurônios piramidais - poderia demonstrar o papel do componente excitatório nesse contexto. A exposição pré-natal ao VPA conduziu a alterações pós-natais importantes no CPFm, tanto no número quanto na distribuição de interneurônios, sugerindo uma possível relação com os prejuízos sociais já observados no modelo. Estes dados contribuem para elucidar elementos-chave relacionados com alterações neurais observadas no TEA, bem como na compreensão de vias envolvidas no desequilíbrio E/I.

Palavras-chave: Transtorno do espectro autista. Ácido valproico. Desequilíbrio excitatório/inibitório. Interneurônios GABAérgicos. Córtex pré-frontal medial.

ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with unknown etiology, characterized by deficits in social interaction and social communication, as well as patterns of restricted and/or repetitive behaviors, activities or interests. An established environmental risk factor for ASD is the prenatal exposure to valproic acid (VPA). Based on this observation, VPA in commonly used to generate autistic-like behaviors in animal models when administered during pregnancy. Excitatory/inhibitory (E/I) imbalance is related to social, cognitive and sensory impairments found in ASD and there are strong evidences of an altered inhibitory signaling contribution. Given the key role of GABAergic interneurons for the maintenance of E/I balance and, since medial prefrontal cortex (mPFC) is pivotal to social behaviors in rodents, we aimed to evaluate quantity and proportion of calbindin-positive (CB^+) and parvalbumin-positive (PV^+) interneurons (both GABAergic ones) on upper and deeper layers of anterior cingulate cortex (aCC) and prelimbic (PrL) and infralimbic (IL) areas, all three mPFC subregions. Pregnant Wistar rats were randomly divided into two experimental groups: Control and VPA. At embryonic day 12.5, saline or VPA (600 mg/kg, i.p.) were administered to Control and VPA group, respectively. At postnatal day 30, brain of offspring male rats (n=3-5 in litters) was removed in order to perform imunofluorescence technique and subsequent confocal microscopy analysis (CEUA-UFRGS #140367). When analyzing the whole mPFC, VPA group presented an increase in the number of total neurons $NeuN^+$ ($p= 0.0086$). In subregion analysis, it was observed a decrease in the number of CB^+ ($p= 0.0278$) at deeper layers of aCC and also in the proportion of these interneurons ($p=0.0255$) into total neurons scenario at upper layers of PrL and IL areas ($p= 0.0173$). PV^+ analysis revealed an increase in number ($p= 0.0369$) and proportion ($p= 0.0044$) of these cells at upper layers of PrL, as well as an increase in the number at deeper layers of PrL ($p= 0.0245$). Interestingly, there was a decrease in PV^+ proportion within total neurons scenario at upper ($p=0.0003$) and deeper layers ($p= 0.0460$) of aCC, as well as upper layers of IL ($p= 0.0169$). Taken together, these results suggest that prenatal exposure to VPA may be related to alterations of interneuron migratory process towards cortical areas, once the composition of all results allowed us to observe different patterns of specific alterations in the subregions. Besides, the alteration in the number of total neurons $NeuN^+$, which are mostly represented by pyramidal ones, could also demonstrate the role of excitatory component in this context. Prenatal exposure to VPA led to important postnatal changes in mPFC, in both neuronal number and distribution, suggesting possible correlation with the social impairments observed in the model. These data contribute to elucidate key elements related to neural changes observed in ASD, as well as to the understanding of pathways involved in E/I imbalance.

Keywords: Autism Spectrum Disorder. Valproic Acid. Excitatory/Inhibitory Imbalance. GABAergic interneurons. Medial Prefrontal Cortex.

LISTA DE FIGURAS

LISTA DE TABELAS E QUADROS

LISTA DE ABREVIATURAS E SIGLAS EM PORTUGUÊS

aCC – Córtex Cingulado Anterior (do inglês *Anterior Cingulate Cortex*)

CB – Calbindina

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

CEUA – Comissão de Ética no Uso de Animais

CGE – Eminência Gangliônica Caudal (do inglês *Caudal Ganglionic Eminence*)

CMM – Centro de Microscopia e Microanálise

CoA – Coenzima A

CONCEA – Conselho Nacional de Controle de Experimentação Animal

CPF – Córtex Pré-Frontal

CPFdl – Córtex Pré-Frontal Dorso Lateral

CPFm – Córtex Pré-Frontal Medial

CR – Calretinina

CREAL – Centro de Reprodução e Experimentação de Animais de Laboratório

DSM – Manual Diagnóstico e Estatístico de Transtornos Mentais (do inglês *Diagnostic and Statistical Manual of Mental Disorders*)

E – Dia Embrionário

E/I – Excitatório/Inibitório

EUA – Estados Unidos da América

GABA – Ácido Gama Aminobutírico (do inglês *Gamma Aminobutyric Acid*)

GAD – Ácido Glutâmico Descarboxilase (do inglês *Glutamic Acid Descarboxylase*)

GE – Eminência Gangliônica (do inglês *Ganglionic Eminence*)

GETTEA – Grupo de Estudos Translacionais em Transtorno do Espectro Autista

HCPA – Hospital de Clínicas de Porto Alegre

HDAC – Desacetilase de Histonas (do inglês *Histone Deacetylase*)

ICBS – Instituto de Ciências Básicas da Saúde

LGE – Eminência Gangliônica Lateral (do inglês *Lateral Ganglionic Eminence*)

MeCP2 – Proteína 2 Ligadora de Metil-CpG (do inglês *Methyl-CpG Binding Protein 2*)

MGE – Eminência Gangliônica Medial (do inglês *Medial Ganglionic Eminence*)

NLGN – Neuroliguina

PV – Parvalbumina

RELN - Relina

RNA – Ácido Ribonucleico (do inglês *Ribonucleic Acid*)

TDAH – Transtorno do Déficit de Atenção com Hiperatividade

TEA – Transtorno do Espectro Autista

UFRGS – Universidade Federal do Rio Grande do Sul

VPA – Ácido Valproico (do inglês *Valproic Acid*)

VIP – Peptídeo Intestinal Vasoativo (do inglês *Vasoactive Intestinal Peptide*)

LISTA DE ABREVIATURAS E SIGLAS EM INGLÊS

aCC – Anterior Cingulate Cortex

ASD – Autism Spectrum Disorder

BSA – Bovine Serum Albumin

CB – Calbindin

CGE – Caudal Ganglionic Eminence

DAPI - 4,6-diamidino-2-phenylindole

dlPFC – Dorsolateral Prefrontal Cortex

DMSO - Dimethyl Sulfoxide

E/I – Excitatory/Inhibitory

GABA – Gamma Aminobutyric Acid

GE – Ganglionic Eminence

IL – Infralimbic

KCC2 - Potassium-Chloride Cotransporter 2

LGE – Lateral Ganglionic Eminence

MeCP2 – Methyl-CpG Binding Protein 2

MGE – Medial Ganglionic Eminence

mPFC – Medial Prefrontal Cortex

mRNA – Messenger Ribonucleic Acid

NRG – Neuroregulin

P – Postnatal Day

PFA – Paraformaldehyde

PBS - Phosphate-Buffered Saline

PFC – Prefrontal Cortex

PrL – Prelimbic

PV – Parvalbumin

SD – Standard Deviation

Sox6 - Sex Determining Region Y-box 6

VPA – Valproic Acid

SUMÁRIO

1 INTRODUÇÃO

1.1 TRANSTORNO DO ESPECTRO AUTISTA

O transtorno do espectro autista (TEA) é uma desordem do neurodesenvolvimento heterogênea e multifatorial, sem etiologia conhecida. Ao longo das últimas décadas, diversos estudos têm contribuído para tentar elucidar a fisiopatologia do TEA; entretanto, apesar dos grandes esforços, muito pouco ainda é compreendido.

1.1.1 Histórico

O termo "autismo" foi primeiramente introduzido em 1908 pelo psiquiatra suíço Paul Eugen Bleuler para descrever o afastamento social e distanciamento da realidade frequentemente apresentados por crianças com diagnóstico de esquizofrenia na época (BLEULER, 1908, 1912; ISLER, 2018). Décadas mais tarde, em 1943, o psiquiatra austríaco Leo Kanner publicou a obra "Distúrbios Autísticos do Contato Afetivo", onde descreveu um grupo de crianças que apresentavam características em comum, tais como dificuldades severas de integração social, "extrema solidão", ecolalia e "desejo ansiosamente obsessivo pela manutenção da mesmice", formando uma síndrome única, até então não descrita; para esse transtorno, utilizou o termo "autismo infantil precoce" (KANNER, 1943). Um ano depois, quase ao mesmo tempo que Leo Kanner, o psiquiatra e pesquisador austríaco Hans Asperger escreveu o artigo "A Psicopatia Autista na Infância" (ASPERGER, 1944, 1991) onde descreve, assim como Kanner, crianças com déficits em habilidades sociais e de comunicação (PEARCE, 2005). Ele nomeou esse conjunto de sintomas como síndrome de Asperger, uma vez que essas crianças apresentavam alta inteligência não-verbal, diferente do observado por Kanner.

Essas descrições clínicas do autismo na década de 1940 por Leo Kanner e Hans Asperger foram, por muito tempo, consideradas as primeiras caracterizações do transtorno. Entretanto, cerca de duas décadas antes, em 1926, a psiquiatra russa Grunya Ssucharewa já havia definido o autismo de maneira semelhante aos critérios comportamentais utilizados atualmente para o diagnóstico, sendo, portanto, a verdadeira pioneira na caracterização do autismo (SSUCHAREWA, 1926a, 1926b).

Interessantemente, o autismo foi caracterizado por três pesquisadores distintos (Grunya, Kanner e Asperger), em três locais do mundo diferentes (Rússia, Estados Unidos da América (EUA) e Alemanha, respectivamente) de maneira independente, visto que Kanner e Asperger publicaram seus achados quase concomitantemente, possivelmente sem ter nenhum conhecimento das observações um do outro e, a princípio, sem saberem da descrição já

realizada anos antes por Grunya. De qualquer modo, todas essas descrições do autismo são relativamente muito recentes se formos compará-las com o histórico de descrição de outras condições, como a epilepsia - cujo relato mais antigo data de 2000 a.C (MOREIRA, 2004), doença de Parkinson - descrita em 1817 (GOETZ, 2011), doença de Alzheimer - descrita por Alois Alzheimer em 1906 (HIPPIUS; NEUNDÖRFER, 2003), dentre outras.

1.1.2 Critérios diagnósticos

A 5ª edição do Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-5) (AMERICAN PSYCHIATRIC ASSOCIATION, 2013) definiu que o autismo deveria ter um diagnóstico unificado, com o espectro variando de leve a severo. Assim, o autismo passou a ser nomeado TEA, nomenclatura utilizada atualmente. Os critérios diagnósticos – os quais devem apresentar-se no início da primeira infância - são agrupados em dois grandes domínios comportamentais:

a) déficits persistentes de comunicação e interação social através de múltiplos contextos, como, por exemplo: déficits de comunicação não-verbal e prejuízos no desenvolvimento, manutenção e entendimento de relacionamentos com outros indivíduos;
b) padrões de comportamento, interesses ou atividades restritos e repetitivos, como, por exemplo: movimentos ou fala estereotipados e inflexibilidade em aderir a novas rotinas.

Tipicamente, esta díade comportamental é identificada em torno dos três anos de idade (alguns estudos apontam idades mais precoces, como 6 a 18 meses), embora possa não se manifestar completamente até a idade escolar (SZATMARI *et al*., 2016).

O TEA é um transtorno do neurodesenvolvimento marcadamente caracterizado por alterações sociais. Além dos prejuízos centrais citados acima, uma observação comum em crianças com autismo – antes de serem diagnosticadas – é o processamento alterado de pistas sociais durante o primeiro ano de vida, o que as coloca em uma situação de habilidade social cada vez mais prejudicada (OSTERLING; DAWSON, 1994; WELSH; ESTES, 2018). Ademais, indiferença a vozes (SPERDIN; SCHAER, 2016) e a faces (GRELOTTI; GAUTHIER; SCHULTZ, 2002) no TEA conduzem a déficits no desenvolvimento de uma adaptação na interação social com os outros e dificuldades no entendimento de comportamentos. Talvez por este motivo crianças com TEA prefiram ocasiões que não exijam extenso contato social (KLIN *et al*., 2009). Não se sabe porque crianças com autismo

apresentam esta alteração em estágios precoces do desenvolvimento, mas esta aparente indiferença a pistas sociais em última análise dificulta o desenvolvimento normal da rede do chamado "encéfalo social" ou pelo menos partes dessa rede (GOTTS *et al*., 2012; PELPHREY *et al*., 2011). Além disso, sugere-se que indivíduos com TEA apresentem alterações em parâmetros associados à empatia, ainda que existam controvérsias nesse ponto (BARON-COHEN; WHEELWRIGHT, 2004; JONES *et al*., 2010).

Uma vez que ainda não há biomarcadores para o TEA, o diagnóstico é somente clínico e baseado apenas na detecção dos dois domínios comportamentais mencionados anteriormente. Desse modo, embora possa haver casos de diagnóstico em torno dos 2 anos de idade, a maior parte das crianças com autismo é diagnosticada tardiamente, após os 4 anos de vida (CHRISTENSEN *et al*., 2018) – quando há um contexto de acompanhamento multiprofissional adequado. Além disso, apesar da especificação dos critérios diagnósticos no DSM-5, ainda há certa dificuldade em sua determinação adequada por profissionais da saúde devido à grande heterogeneidade do transtorno, visto que o TEA se apresenta como um amplo espectro. Como consequência disso, as intervenções normalmente também são tardias, situação potencialmente crítica no contexto de um transtorno do neurodesenvolvimento como o TEA.

1.1.3 Impacto e custos

O TEA é uma desordem que tem grande impacto na qualidade de vida dos indivíduos afetados, seus familiares, cuidadores e comunidade, uma vez que é um transtorno de início precoce, e, como ainda não há cura, acompanha o indivíduo durante toda a vida. Os pacientes necessitam de cuidado médico e parental, educação especial, medicamentos para o tratamento dos sintomas, dentre outros; assim, o TEA apresenta tanto custo emocional quanto financeiro. Estima-se que os custos totais por ano com crianças com TEA nos EUA sejam de 11,5 a 60,9 bilhões de dólares (BUESCHER *et al*., 2014; LAVELLE *et al*., 2014).

1.1.4 Comorbidades

Evidências epidemiológicas indicam que algumas comorbidades são mais prevalentes em crianças com TEA quando comparadas às crianças da população em geral (KOHANE *et al*., 2012); apesar da alta prevalência dessas comorbidades, muitas não são acompanhadas rotineiramente (BUIE *et al*., 2010; MALOW *et al*., 2012).

O TEA apresenta diversas comorbidades; as mais frequentes estão apresentadas na tabela abaixo:

Tabela 1 – Algumas das comorbidades mais frequentes do TEA

Comorbidade	Prevalência em indivíduos com TEA	Referência
Epilepsia	~25-30%	(TUCHMAN; CUCCARO, 2011)
Distúrbios de sono	50-80%	(KRAKOWIAK *et al*., 2008; WIGGS; STORES, 2004)
Disfunções gastrointestinais	9-70%	(BUIE *et al*., 2010; COURY *et al*., 2012; HOLINGUE *et al*., 2018)
Transtorno do Déficit de Atenção com Hiperatividade (TDAH)	22-83%	(MATSON; RIESKE; WILLIAMS, 2013; SOKOLOVA *et al*., 2017)
Deficiência intelectual	~30%	(CHRISTENSEN *et al*., 2018)

Fonte: elaborada pela autora

Além disso, também pode-se citar outras condições associadas ao TEA, como disfunções imunes (alergias alimentares, rinite alérgica, eczema) (CHEN *et al*., 2013; MIYAZAKI *et al*., 2015), psoríase (ZERBO *et al*., 2015a), asma (GURNEY; MCPHEETERS; DAVIS, 2006) ansiedade e depressão (CROEN *et al*., 2015; MATSON; CERVANTES, 2014), além de reduzido contato visual (ZWAIGENBAUM *et al*., 2005), déficits motores (PAN; TSAI; CHU, 2009), desordens de humor e retardo mental (CASANOVA, 2007; GESCHWIND, 2009). Estudos indicam elevadas taxas de mortalidade em pacientes com TEA (HIRVIKOSKI *et al*., 2016; SCHENDEL *et al*., 2016), fato que pode estar associado às complicações que surgem das comorbidades e das condições associadas.

1.1.5 Epidemiologia

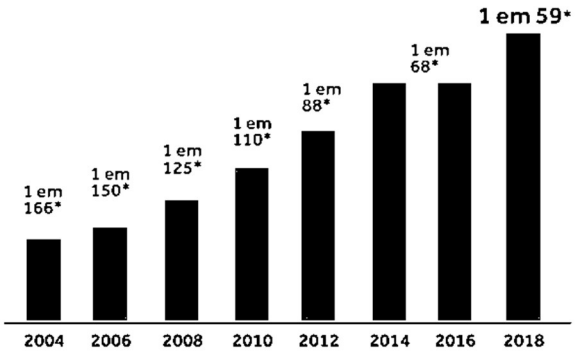
Um dos primeiros estudos epidemiológicos sobre o TEA foi realizado em 1966 na Inglaterra (LOTTER, 1966). Neste estudo, toda a população de crianças de 8 a 10 anos do Condado de Middlesex foi analisada e observou-se uma prevalência de 4,5 crianças com autismo a cada 10.000 (1:2.222). A partir de então, alguns outros estudos foram realizados no Canadá, no Brasil e nos EUA:

No Canadá, um estudo realizado em 2015 mostrou uma prevalência de 1:66 em crianças e adolescentes de 5 a 17 anos de idade, sendo o TEA identificado 4 vezes mais em meninos do que em meninas (OFNER; COLES; DECOU, 2018). No Brasil, existe apenas um estudo

epidemiológico, realizado em 2011 com crianças de Atibaia, São Paulo, no qual encontraram uma frequência de 0,3% (1:333) (PAULA *et al*., 2011).

Nos EUA, pesquisas epidemiológicas do Centro de Controle e Prevenção de Doenças (CDC) com crianças tendo em torno de 8 anos de idade mostraram que a incidência do TEA vem crescendo, visto que podemos observar um aumento da prevalência ao longo dos anos (CHRISTENSEN *et al*., 2018):

Figura 1 - Prevalência do TEA nos EUA ao longo dos anos



ADAPTADO DE (CHRISTENSEN *et al*., 2018). Arte: ADAPTADO DE Portal Tismoo.

Os dados mais atuais do CDC a respeito da prevalência (1:59) foram obtidos em 2014 e publicados em 2018, sendo, atualmente, os oficialmente utilizados (CHRISTENSEN *et al*., 2018). Este dado é semelhante aos resultados obtidos no Canadá (1:63 se consideradas somente as crianças com 8 anos de idade). Ainda, nota-se que esta prevalência é extremamente alta por si só e também maior do que as apresentadas por outras desordens de grande impacto social, emocional e econômico, como esquizofrenia (1:100) (WORLD HEALTH ORGANIZATION., 2000) e epilepsia (1:250 – 1:66) (WORLD HEALTH ORGANIZATION, 2019).

O TEA ocorre em todos os grupos sociais, raças e etnias e é em torno de 4 vezes mais prevalente em meninos do que em meninas (CHRISTENSEN *et al*., 2018), embora esta razão diminua com o aumento da severidade do transtorno (WERLING; GESCHWIND, 2013).

Apesar do aumento da incidência, é importante levar em consideração diversos fatores que podem influenciar nesse crescente número de novos casos. Cabe ressaltar que, ao longo dos diferentes estudos (desde 1966 até 2014), houve modificações nos critérios diagnósticos e inclusão e exclusão de outras desordens na categoria de TEA. Alguns outros fatores também devem ser considerados: grande complexidade e heterogeneidade do transtorno, melhoria no diagnóstico e conscientização da comunidade a respeito do TEA, além da exposição mais recorrente dos pais a fatores de risco para o TEA associados à vida moderna, como obesidade

materna (LI *et al*., 2016) e adiamento da maternidade (GUINCHAT *et al*., 2012; IDRING *et al*., 2014; SANDIN *et al*., 2012). Todos esses aspectos representam um desafio na investigação do aumento da incidência decorrente de uma verdadeira elevação no número de novos casos ou consequência dos diversos fatores citados acima (HERTZ-PICCIOTTO; DELWICHE, 2009; KING; BEARMAN, 2011; LIU; KING; BEARMAN, 2010). Além disso, deve-se ressaltar que as estimativas de prevalência após 2014 ainda não estão disponíveis e, portanto, o impacto das mudanças na classificação do TEA no DSM-5 em 2013 somente será melhor observado ao longo dos próximos anos (LYALL *et al*., 2017).

1.1.6 Desencadeamento

Apesar do progresso alcançado no entendimento da fisiopatologia do TEA e na identificação de fatores de risco implicados com o desencadeamento do transtorno ao longo das últimas décadas, sua etiologia permanece desconhecida. Ao invés de uma única causa, o consenso nos últimos anos é o de que o TEA é uma desordem de etiologia heterogênea e multifatorial (BEVERSDORF, 2016). Evidências epidemiológicas mostram o forte papel de fatores de risco genéticos (CAGLAYAN, 2010; EL-FISHAWY; STATE, 2010; GESCHWIND, 2011) e ambientais (RISCH *et al*., 2014; SEALEY *et al*., 2016) implicados no desencadeamento do TEA. Ademais, pode haver a interação entre fatores genéticos e ambientais, além de contribuições epigenéticas (LYALL *et al*., 2017).

A contribuição genética na etiologia do transtorno é fortemente sustentada por estudos históricos com irmãos gêmeos e famílias; estima-se que a herdabilidade do TEA nos EUA e na Europa seja de 50 a 95% (COLVERT *et al*., 2015; HALLMAYER *et al*., 2011; SANDIN *et al*., 2014). Ao longo da última década, diversos estudos têm identificado variações genéticas relacionadas ao transtorno, incluindo mutações herdadas e *de novo*, além de variações no número de cópias (BOURGERON, 2015). Diversos genes têm sido identificados (LI; ZOU; BROWN, 2012), como NLGN3, NLGN4 (JAMAIN *et al*., 2003) e SHANK3 (ZHOU *et al*., 2019). Além disso, até 15% dos casos de TEA podem ser relacionados a uma causa genética conhecida via síndromes monogênicas, tais como a síndrome do X frágil e esclerose tuberosa (DEVLIN; SCHERER, 2012).

Além dos fatores de risco genéticos, a exposição a fatores ambientais específicos pode estar associada com o desencadeamento de TEA. Os fatores de risco ambientais mais comumente identificados são:

Quadro 1 - Exemplos de fatores de risco ambientais

Fator de risco ambiental	Referências

Idade parental avançada	(GUINCHAT *et al*., 2012; IDRING *et al*., 2014; SANDIN *et al*., 2012)
Intervalo pequeno entre gestações	(GUNNES *et al.*, 2013; ZERBO *et al*., 2015b)
Fatores imunológicos, como infecções maternas durante a gestação e histórico familiar de doenças autoimunes	(ATLADOTTIR *et al*., 2009; KEIL *et al*., 2010; LEE *et al*., 2015; ZERBO *et al*., 2015c)
Diabetes gestacional	(GARDENER; SPIEGELMAN; BUKA, 2009)
Uso materno de determinados medicamentos durante a gestação	(BROMLEY *et al*., 2008; CHRISTENSEN *et al*., 2013; GIDAYA *et al*., 2016; MEZZACAPPA *et al*., 2017; STRÖMLAND *et al*., 1994; STRÖMLAND; MILLER, 1993)

Fonte: elaborado pela autora

A respeito do uso materno de determinados medicamentos durante a gestação, pode-se citar a talidomida (STRÖMLAND *et al*., 1994; STRÖMLAND; MILLER, 1993), alguns antidepressivos (MEZZACAPPA *et al*., 2017), antiasmáticos (GIDAYA *et al*., 2016) e antiepilépticos, como o ácido valproico (VPA) (BROMLEY *et al*., 2008; CHRISTENSEN *et al*., 2013).

1.2 ÁCIDO VALPROICO

O VPA é um ácido graxo sintetizado em 1882 (BURTON, 1882) como um solvente orgânico que atravessa a barreira hematoencefálica e a placenta (BRUNI; WILDER, 1979). Descoberto como fármaco da classe dos anticonvulsivantes na década de 60 (MEUNIER; H., 1963), passou a ser então utilizado para tratar certos tipos de crises epilépticas (crises de ausência e crises tônico-clônicas) (LÖSCHER, 2002). Ademais, é prescrito para o tratamento de mania em indivíduos com transtorno bipolar (FOUNTOULAKIS *et al*., 2005) e para prevenir enxaquecas (CHRONICLE; MULLENERS, 2004). Em alguns casos, é utilizado como estabilizador de humor para o tratamento de episódios de agressão em crianças com TDAH (REYNOLDS; SISK; RASGON, 2007).

Mesmo após mais de 50 anos de uso, os mecanismos pelos quais o VPA exerce seus efeitos ainda não são bem entendidos. Sabe-se que este fármaco é um inibidor de vários subtipos das desacetilases de histonas (HDAC) (Quadro 2) e pode atuar aumentando os níveis do ácido

gama-aminobutírico (GABA) no encéfalo (BALDINO; GELLER, 1981; GODIN *et al*., 1969; HARITON *et al*., 1984; KUKINO; DEGUCHI, 1978). Esse aumento nos níveis de GABA provavelmente acontece através da inibição da enzima succiníco-semialdeído desidrogenase, que oxida succinil semialdeído. A inibição dessa enzima consequentemente resulta em aumento dos níveis encefálicos de succinil semialdeído, o que por sua vez inibe a enzima GABA transaminase, o que impede o catabolismo de GABA (JOHANNESSEN, 2000). Ainda, o VPA afeta diversas enzimas que estão integradas à síntese e degradação do GABA. Assim, suas propriedades anticonvulsivantes podem estar relacionadas ao consequente aumento na concentração de GABA no encéfalo, justamente por inibir a enzima que cataboliza o GABA, ou por estimular a sua síntese, além de provavelmente bloquear a recaptação de GABA na fenda sináptica e na glia. Além disso, o VPA também pode estar atuando por suprimir disparos neuronais de alta-frequência e por alterar as propriedades de canais de sódio dependentes de voltagem, inibindo-os (MCLEAN; MACDONALD, 1986). Por fim, alguns estudos têm sugerido que o VPA causa uma diminuição na transmissão excitatória no encéfalo (ZEISE; KASPAROW; ZIEGLGÄNSBERGER, 1991): em estudos com roedores, foi observado que o VPA diminui os níveis de aspartato – aminoácido localizado em neurônios glutamatérgicos excitatórios – em todo o encéfalo, através de mecanismos não muito bem esclarecidos (CHAPMAN *et al*., 1982; LÖSCHER; HÖSTERMANN, 1994; SCHECHTER; TRANIER; GROVE, 1978).

Quadro 2 – Alguns alvos do VPA

Alvos	Mecanismo	Referências
HDAC 9	Inibição	(KANAI *et al*., 2004; ROSENBERG, 2007; STOCKHAUSEN *et al*., 2005)
HDAC 2	Inibição	(GOTTLICHER, 2004; KRÄMER *et al*., 2003)
4-aminobutirato aminotransferase	Inibição	(BRUNI; WILDER, 1979; LÖSCHER, 1982; ROSENBERG, 2007)
Desidrogenase acil-CoA mitocondrial (importante para a β-oxidação de ácidos graxos)	Inibição	(BAZINET *et al*., 2006; ITO *et al*., 1990)

α-cetoglutarato desidrogenase mitocondrial (converte α-cetoglutarato a succinil-CoA)	Inibição	(JOHANNESSEN; JOHANNESSEN, 2003)
Succinato-semialdeído desidrogenase mitocondrial	Inibição	(JOHANNESSEN; JOHANNESSEN, 2003)
Canal de sódio dependente de voltagem	Inibição	(FARBER *et al*., 2002)

Fonte: elaborado pela autora

Embora amplamente utilizado na clínica, diversos estudos na literatura descrevem os riscos associados à exposição ao VPA durante o período gestacional, principalmente durante o primeiro trimestre: malformações congênitas, atraso no desenvolvimento e função cognitiva reduzida. Além disso, há alertas de segurança do fármaco para pacientes mulheres a respeito do risco do tratamento com este medicamento, incluindo também defeitos no fechamento do tubo neural (US FOOD AND DRUG ADMINISTRATION, 2009), bem como risco de prejuízos no desenvolvimento cognitivo (US FOOD AND DRUG ADMINISTRATION, 2011). O VPA é um dos fármacos antiepilépticos administrados durante a gravidez, entretanto, ele já foi identificado como sendo um dos fármacos antiepilépticos mais teratogênicos (MEADOR, 2008; WERLER *et al*., 2011). Os efeitos teratogênicos do VPA podem estar relacionados à deficiência de ácido fólico, indução de estresse oxidativo e inibição da HDAC (ORNOY, 2009). Devido ao fato de o VPA poder causar defeitos de nascimento em humanos, ele é classificado como um fármaco categoria D para gestantes pelo *American Food and Drug Administration* (DUFOUR-RAINFRAY *et al*., 2011).

Por fim, a exposição pré-natal ao VPA durante o primeiro trimestre de gravidez está associada a um maior risco de desencadeamento de TEA nos filhos dessas gestantes. Essa associação foi originalmente baseada no aumento da frequência de sintomas de autismo em crianças diagnosticadas com a síndrome fetal do valproato (CHRISTIANSON; CHESTER; KROMBERG, 1994; WILLIAMS *et al*., 2001; WILLIAMS; HERSH, 1997). Em um estudo realizado na Dinamarca com todos os nascidos vivos do país entre os anos de 1996 e 2006, observou-se que o uso materno de valproato durante a gestação estava associado com um risco significativamente aumentado de desencadeamento de TEA nos filhos (CHRISTENSEN *et al*., 2013).

1.2.1 Modelo animal de autismo induzido por exposição pré-natal ao VPA

Na década de 90, estudos com a talidomida e TEA levantaram a hipótese de que o TEA poderia estar associado com alterações promovidas por esse fármaco, como prejuízos no fechamento do tubo neural. Porém, para replicar tal situação em roedores, a talidomida não seria adequada, pois já era sabido que seus efeitos teratogênicos observados em primatas não se replicavam em roedores (SCHUMACHER *et al*., 1972). Portanto, Rodier e colegas, em 1996, (RODIER *et al*., 1996) expuseram ratas prenhes ao VPA, teratógeno que, na época, surgia como um novo possível fator de risco ambiental para o desencadeamento de TEA. A dose de 350 mg/kg de VPA foi administrada no dia embrionário (E) 11,5 (E11,5) – o qual corresponde ao dia do fechamento do tubo neural em roedores - E12 ou E12,5 e observou-se alterações em alguns pares de nervos cranianos nos animais do grupo VPA quando comparados com o controle (RODIER *et al*., 1996). Este foi o primeiro estudo utilizando o VPA pré-natalmente para mimetizar defeitos encefálicos observados em pacientes com TEA; entretanto, nenhum aspecto comportamental foi avaliado. Somente em 2005, Schneider e Przewlocki (SCHNEIDER; PRZEWŁOCKI, 2005a) investigaram possíveis alterações comportamentais em animais do modelo VPA (600 mg/kg, E12,5). No estudo, viu-se que estes animais apresentavam comportamentos repetitivos e estereotipados e déficits de interação social, prejuízos comportamentais centrais utilizados para o diagnóstico de TEA. Ainda, os animais do modelo VPA apresentaram diminuição da sensibilidade a estímulos nociceptivos e aumento da sensibilidade a estímulos não dolorosos, outro sintoma importante do TEA (AMERICAN PSYCHIATRIC ASSOCIATION, 2013). Em outro estudo, a exposição pré-natal ao VPA resultou em alterações comportamentais e moleculares que diferem entre o sexo, sendo estas muito mais marcantes em machos do que em fêmeas do modelo VPA (SCHNEIDER *et al*., 2008). Este dado tem grande relevância clínica, uma vez que o TEA é 4 vezes mais prevalente em indivíduos do sexo masculino (CHRISTENSEN *et al*., 2018). A partir de então, diversos testes comportamentais que avaliaram não somente os sintomas centrais do TEA como também as comorbidades e condições associadas ao transtorno (CRAWLEY, 2007) foram realizados em vários trabalhos, evidenciando prejuízos como aumento no comportamento do tipo depressivo (NAKASATO *et al*., 2008), do tipo ansioso (MEHTA; GANDAL; SIEGEL, 2011), desregulação do ritmo circadiano (TSUJINO *et al*., 2007), aspectos observados também em pacientes com TEA (CROEN *et al*., 2015; KRAKOWIAK *et al*., 2008; MATSON; CERVANTES, 2014). Além de modificações a nível comportamental (BAMBINI-JUNIOR *et al*., 2014; HIRSCH *et al*., 2018a, 2018b; SCHNEIDER; PRZEWŁOCKI, 2005b), o modelo VPA também apresenta alterações a nível molecular (GOTTFRIED *et al*., 2013; ROULLET *et*

al., 2010), morfológico (DENDRINOS; HEMELT; KELLER, 2011; FAVRE *et al*., 2013; GOTTFRIED *et al*., 2013; RODIER *et al*., 1997) e eletrofisiológico (DAWSON *et al*., 2005; MARKRAM *et al*., 2008; RINALDI; SILBERBERG; MARKRAM, 2008) similares àquelas encontradas em indivíduos com TEA. Ressalta-se também que os animais do modelo VPA apresentam alterações no comportamento do tipo-empatia (FONTES-DUTRA *et al*., 2019), de modo similar ao observado em pacientes (BARON-COHEN; WHEELWRIGHT, 2004; JONES *et al*., 2010).

Atualmente, o modelo animal de autismo induzido por exposição pré-natal ao VPA é um dos modelos mais amplamente utilizados para o entendimento da fisiopatologia do TEA e já está bem consolidado e validado na literatura, pois encaixa-se em diversos critérios usualmente descritos como validades de construto, de face e preditiva. A validade de construto caracteriza-se pela similaridade de etiologia da doença apresentada pelos seres humanos e o modelo animal que mimetiza tal condição. Esta validade é encontrada no modelo VPA: a utilização de VPA durante a gestação é um fator de risco para o TEA e, em modelos animais, induz a prole a ter características do tipo-autista quando administrado durante a prenhez. A validade de face caracteriza-se pela presença de alterações comportamentais, bioquímicas e fisiológicas no modelo animal semelhantes àquelas encontradas em pacientes com a doença. Como já mencionado, o modelo VPA apresenta tais características, tendo, portanto, validade de face (ROULLET; LAI; FOSTER, 2013). E, por fim, a validade preditiva avalia a resposta do modelo animal a um tratamento, tanto para analisar a similaridade de resposta observada em pacientes ou para a identificação de fármacos benéficos para uso em seres humanos. Apesar desta última validade não ser tão bem caracterizada no modelo VPA, algumas moléculas promissoras e intervenções ambientais são utilizadas no modelo tanto para a busca de potenciais tratamentos como também como ferramenta de estudo dos mecanismos envolvidos no transtorno (BAMBINI-JUNIOR *et al*., 2014; MABUNGA *et al*., 2015; SCHNEIDER; TURCZAK; PRZEWŁOCKI, 2006).

Devido a todas essas questões, o modelo animal de autismo induzido por exposição pré-natal ao VPA apresenta translacionalidade para o fenótipo, fatores de risco e possíveis intervenções que são observadas no TEA, sendo, portanto, uma ótima ferramenta de estudo. Além disso, devido a dificuldade de obtenção de amostras encefálicas *post-mortem* de pacientes com TEA e a diversidade de análises comportamentais, bioquímicas e moleculares que podem

ser realizadas em modelos animais, o modelo VPA mostra-se fundamental e de extrema importância.

1.3 DESEQUILÍBRIO EXCITATÓRIO/INIBITÓRIO E TEA

Acredita-se que as redes neurais *in vivo* funcionem de maneira balanceada, onde neurônios excitatórios e inibitórios e outras células neurais mantêm níveis de atividade finamente regulados (CARLSON, 2012). Desse modo, o chamado balanço excitatório/inibitório (E/I) refere-se às contribuições relativas de *inputs* sinápticos excitatórios e inibitórios direcionadas a um circuito ou evento neural (MEGÍAS *et al*., 2001). O balanço E/I auxilia na manutenção da atividade neuronal dentro de uma faixa segura e estreita e é uma característica fundamental para a atividade das redes neurais, bem como sua computação (EICHLER, SABRINA A.; MEIER, 2008); é essencial para controlar a cognição, aprendizado, memória e comportamentos emocionais (TURRIGIANO; NELSON, 2004). Para que haja a manutenção do equilíbrio E/I, é preciso a regulação adequada do desenvolvimento de sinapses excitatórias e inibitórias e de transmissão sináptica, da plasticidade sináptica homeostática e excitabilidade intrínseca neuronal.

Existem diversas teorias acerca da fisiopatologia do TEA (YENKOYAN *et al*., 2017), sendo a hipótese de desequilíbrio E/I uma delas (GOGOLLA *et al*., 2009; NELSON; VALAKH, 2015; RUBENSTEIN, J.L.R & MERZENICH, 2003), ou seja, uma das possíveis explicações para a complexidade do TEA pode estar na desregulação do balanço entre excitação e inibição durante períodos críticos de desenvolvimento, e isto pode estar relacionado com os déficits cognitivos, sociais, comportamentais, sensoriais e motores observados no transtorno (MARKRAM; MARKRAM, 2010; RUBENSTEIN, J.L.R & MERZENICH, 2003; RUBENSTEIN, 2010). Além disso, muitos estudos apresentam a ideia de que o desequilíbrio E/I pode estar presente também em transtornos como síndrome de Down, neurofibromatose e esquizofrenia (RAMAMOORTHI; LIN, 2011), as quais compartilham alguns sintomas e características com o TEA (CHANNELL *et al*., 2015; EVANS *et al*., 2014).

Entretanto, ainda existem divergências se essas alterações são causadas devido a um aumento ou a uma diminuição da razão excitação/inibição (razão E/I).

1.3.1 Aumento na razão E/I

Em 2001, Hussman e colaboradores publicaram o primeiro estudo a respeito deste tema no TEA, sugerindo que houvesse uma diminuição na sinalização GABAérgica (CELLOT; CHERUBINI, 2014; HUSSMAN, 2001; NELSON; VALAKH, 2015; SÜDHOF, 2008). Dois

anos mais tarde, Rubenstein e Merzenich propuseram a ideia de aumento na razão E/I no TEA, o que conduziria a uma hiperexcitabilidade dos circuitos corticais (RUBENSTEIN; MERZENICH, 2003). Esta teoria reforçava a hipótese de Hussman e era uma possível explicação para a sua observação de sinalização GABAérgica reduzida no TEA; ainda, tal desequilíbrio poderia também explicar a propensão destes indivíduos a desenvolverem epilepsia, conhecida como resultado de hiperexcitabilidade neural. Indivíduos com TEA desenvolvem epilepsia a uma taxa 25x maior do que a população em geral (BOLTON *et al*., 2011) – interessantemente, a epilepsia é uma das comorbidades mais comumente associadas com o TEA, ocorrendo em até 1/3 destes indivíduos (R. MUHLE, S. V. TRENTACOSTE, 2004; TUCHMAN; RAPIN, 2002).

1.3.2 Diminuição na razão E/I

Alguns indivíduos com TEA apresentam uma redução na razão E/I, o que vai de encontro com a hipótese inicial. Estudos sugerem que o TEA possa ser um transtorno hipoglutamatérgico (CARLSSON, 1998) ou que haja um excesso de inibição. Essas observações também estão presentes na síndrome de Down (BELICHENKO *et al*., 2009), condição que compartilha vários sintomas com o TEA (CHANNELL *et al*., 2015), como interesses restritos e comportamentos repetitivos (EVANS *et al*., 2014). Ainda, tanto inibição aumentada como excitação reduzida têm sido observadas em modelo animal da síndrome de Rett com alelo de MeCP2 mutado (DANI *et al*., 2005) e em camundongos que expressam mutação no alelo humano da Neuroliguina 3, ambos genes cuja alteração está associada com alguns casos de TEA (FUKUDA *et al*., 2005; TABUCHI *et al*., 2007).

O desequilíbrio E/I também é frequentemente observado em modelos animais de autismo (LEE; LEE; KIM, 2017a), inclusive no modelo VPA, no qual há relatos de aumento na razão E/I (KANG; KIM, 2015; LIN *et al*., 2013; RINALDI *et al*., 2007).

Com base nestas duas grandes linhas de raciocínio, o consenso atual é que tanto a sinalização excitatória quanto a inibitória estão alteradas no TEA. Uma explicação para isso é que alterações primárias na excitação ou na inibição modificam a rede neuronal e esta mudança pode, por si só, levar a alterações secundárias em uma tentativa de ajustar a função sináptica e neuronal para retomar a homeostase da circuitaria. Por isso, é difícil separar os efeitos primários resultantes de alterações causadas pelo TEA dos mecanismos compensatórios (efeitos secundários) que o organismo apresenta em uma tentativa de manter a circuitaria funcional e normal. Ainda, à medida que o TEA se desenvolve, os mecanismos homeostáticos tornam-se

insuficientes e não conseguem mais restaurar a atividade normal, o que acaba prejudicando toda a circuitaria (NELSON; VALAKH, 2015).

1.4 INTERNEURÔNIOS GABAÉRGICOS CALBINDINA-POSITIVOS E PARVALBUMINA-POSITIVOS

Circuitos encefálicos complexos compreendem redes hierárquicas de neurônios excitatórios e inibitórios. Dentro do córtex cerebral, há dois tipos principais de neurônios: os piramidais glutamatérgicos, os quais são excitatórios, e os interneurônios, inibitórios. As células piramidais corticais transmitem informação entre diferentes camadas no córtex e para outras regiões encefálicas; já os interneurônios GABAérgicos corticais - compreendendo apenas 10-15% da população total de neurônios (MARKRAM *et al*., 2004) - contribuem fortemente para a inibição a nível local, exercendo papel fundamental. Ademais, estas células conferem flexibilidade à circuitaria neural, modulando de forma dinâmica as respostas excitatórias dos neurônios piramidais (ISAACSON; SCANZIANI, 2011; KLAUSBERGER *et al*., 2003). Através da inibição dos neurônios piramidais vizinhos, os interneurônios GABAérgicos atuam como importantes moduladores da sinalização excitatória, controlando a geração e o momento de disparo dos neurônios piramidais (POUILLE; SCANZIANI, 2001).

Os interneurônios GABAérgicos corticais são uma ampla família que pode ser classificada (ASCOLI *et al*., 2008) através de:

a) características morfológicas (MARKRAM *et al*., 2004; TAMÁS; SOMOGYI; BUHL, 1998);
b) propriedades eletrofisiológicas (CONNORS; GUTNICK, 1990; MARKRAM *et al*., 2004; MCCORMICK *et al*., 1985);
c) marcadores histológicos (DEFELIPE, 1993; KAWAGUCHI; KUBOTA, 1997; MARKRAM *et al*., 2004).

Essas diferentes classificações são feitas na tentativa de categorizar as subpopulações de interneurônios sem que haja sobreposição de uma classe em outra. Entretanto, cabe ressaltar que alguns subtipos de interneurônios podem fazer parte de mais de uma classificação.

Aqui, será dado enfoque para a classificação de acordo com os marcadores histológicos, a qual é baseada, dentre outros, na imunorreatividade dos interneurônios às proteínas ligadoras de cálcio calbindina (CB), calretinina (CR) e parvalbumina (PV). Essas proteínas tendem a ser expressas em três subpopulações de interneurônios separadamente; entretanto, já foi encontrado um pequeno grau de sobreposição (co-localização) na expressão de CB e CR em interneurônios

de algumas regiões encefálicas em modelos animais (ROGERS; RÉSIBOIS, 1992) e de CB e PV no núcleo geniculado lateral dorsal (DEMEULEMEESTER *et al*., 1989), na amígdala basolateral (MCDONALD; BETETTE, 2001) e no hipocampo (GULYÁS *et al*., 1991) de modelos animais, assim como no córtex temporal de seres humanos (DEL RÍO; DEFELIPE, 1997). Entretanto, esses achados não são um consenso, visto que muitos estudos também mostram que não há co-localização, principalmente em se tratando dos interneurônios positivos para parvalbumina (PV^+). Portanto, apesar de existirem algumas divergências e problemas em relação à classificação, a categorização com base nas proteínas ligadoras de cálcio é umas das mais utilizadas, pois há suporte na literatura de que estes três marcadores identificam três subpopulações distintas de interneurônios (DEFELIPE *et al*., 2013).

Neste trabalho, foram estudados os interneurônios GABAérgicos corticais positivos para calbindina (CB^+) e os PV^+.

1.4.1 Interneurônios CB^+

A maioria dos interneurônios CB^+ (Figura 2) é classificada morfologicamente como *double-bouquet cells*; as quais (1) inervam os espinhos dendríticos e os dendritos dos neurônios piramidais (DEFELIPE *et al*., 1990; DEFELIPE; HENDRY; JONES, 1989; DEL RÍO; DEFELIPE, 1995), (2) são encontradas principalmente na camada II/III do córtex cerebral (DEL RÍO; DEFELIPE, 1995), (3) caracterizam-se por apresentarem um padrão de arranjo vertical de seus axônios (SOMOGYI; COWEY, 1984), além de (4) fazerem parte da estrutura de minicolunas no córtex cerebral de macacos (DEFELIPE *et al*., 1990). Uma pequena parcela dos neurônios CB^+ é caracterizada morfologicamente como células neurogliaformes (um tipo de interneurônio GABAérgico) e células Martinotti (camada IV/V) (CONDÉ *et al*., 1994; DEFELIPE, 1997).

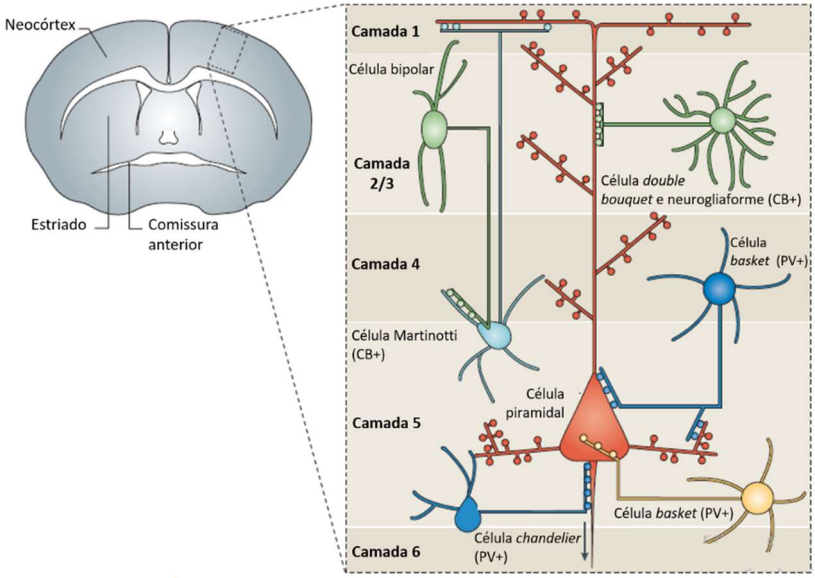
1.4.2 Interneurônios PV^+

Os interneurônios PV^+ representam aproximadamente 40% da população total de interneurônios GABAérgicos corticais em roedores (RUDY *et al*., 2011) e 25% em primatas (CONDÉ *et al*., 1994). Estas células são caracterizadas por (1) apresentarem um padrão de disparo característico, conhecido como "*fast-spiking*", (2) baixa resistência de *input* e (3) rápida alta amplitude após hiperpolarização (KAWAGUCHI *et al*., 1987; KAWAGUCHI; KUBOTA, 1997). Por apresentarem essas diferentes propriedades, têm a habilidade de disparar um rápido trem de potenciais de ação como nenhum outro neurônio no córtex cerebral. Há indícios de que um único interneurônio PV^+ faz sinapse com quase todos os neurônios piramidais adjacentes

(PACKER; YUSTE, 2011), o que permite a estas células um alto nível de inibição *feedback* e *feedforward* (HU; GAN; JONAS, 2014). A subpopulação de interneurônios PV^+ é subdividida, de acordo com as suas características morfológicas (Figura 2), em *chandelier cells* e *basket cells*:

As *chandelier cells* podem ser multipolares ou *bitufted cells* e têm sido encontradas na camada cortical IV/V (MARÍN, 2012), além de fazerem sinapse no segmento inicial do axônio de células piramidais (ASCOLI *et al*., 2008; KAWAGUCHI; KUBOTA, 1997). Já as *basket cells* fazem sinapse no soma e nos dendritos proximais dos neurônios alvo (ASCOLI *et al*., 2008; KAWAGUCHI; KUBOTA, 1997), têm dendritos grandes, multipolares e sem espinhos, além de grande arborização axonal, o que permite a estas células inibirem neurônios nas camadas superiores e mais profundas do córtex, tanto de neurônios vizinhos como os localizados em minicolunas distantes (MARKRAM *et al*., 2004).

Figura 2 - Exemplos de subtipos de interneurônios do neocórtex de roedores.



ADAPTADO DE (MARÍN, 2012).

1.4.3 Origem e migração dos interneurônios CB^+ e PV^+

A maioria dos interneurônios GABAérgicos origina-se primariamente na eminência gangliônica (GE) (CORBIN; BUTT, 2011), uma estrutura encefálica transitória localizada na área ventral do telencéfalo, presente somente durante o desenvolvimento embrionário (KELSOM; LU, 2013). A GE é dividida em medial (MGE), lateral (LGE) e caudal (CGE). A MGE é o local de origem de 50 a 60% da população de interneurônios corticais em camundongos (BUTT *et al*., 2005; PLEASURE *et al*., 2000; WONDERS; ANDERSON, 2006), principalmente os PV^+ (WONDERS; ANDERSON, 2006) e CB^+ (KELSOM; LU, 2013). O

segundo local é a CGE, produzindo 30 a 40% dos interneurônios corticais (ANDERSON *et al*., 2001; NERY; FISHELL; CORBIN, 2002), dando origem às *double bouquet cells* (principal tipo de CB^+) (BUTT *et al*., 2005; PLEASURE *et al*., 2000). Cabe ressaltar que existem diferenças entre roedores e primatas em relação às origens e vias de migração dos interneurônios GABAérgicos (KELSOM; LU, 2013): nos primatas, a maioria destes não se origina somente das GE (LETINIC; ZONCU; RAKIC, 2002), como também de zonas proliferativas do telencéfalo dorsal.

Nos roedores, após serem gerados e se especificarem em seus subtipos (CB^+, PV^+ e outros), os interneurônios GABAérgicos iniciam sua migração em direção às áreas corticais no E12,5 (KELSOM; LU, 2013), momento importante do desenvolvimento embrionário. A migração se completa, em sua maior parte, somente ao nascimento, e envolve a atividade de vários fatores quimiotáticos, de transcrição, neurotransmissores e motogenes (FAUX *et al*., 2009; MARSH *et al*., 2008).

1.4.4 Equilíbrio E/I e interneurônios CB^+ e PV^+

Para que o balanço E/I seja mantido, a sinalização inibitória precisa responder às flutuações que ocorrem neste equilíbrio devido aos *inputs* excitatórios que chegam até o córtex cerebral (ATALLAH; SCANZIANI, 2009; GALARRETA; HESTRIN, 1998; SHU; HASENSTAUB; MCCORMICK, 2003; XUE; ATALLAH; SCANZIANI, 2014). Uma vez que se acredita que a inibição contribua para aumentar a seletividade de respostas excitatórias em diversas áreas encefálicas, a perda de inibição pode conduzir a um aumento no ruído e imprecisão no aprendizado e cognição. Por isso, é de extrema importância que se mantenha um nível correto de inibição, visto que isso mantém de forma adequada a razão sinal/ruído, ou seja, faz com que o indivíduo consiga filtrar mais adequadamente uma informação, se concentrando melhor nos pontos importantes (FERGUSON; GAO, 2018). Muitos genes associados ao TEA são expressos nos interneurônios e já foram encontrados alterados (*GAD1*, *RELN*, *VIP*, entre outros) (WANG *et al*., 2018). Mutações nestes genes prejudicam o desenvolvimento dos interneurônios, bem como o *input*/*output*, além de excitabilidade e disparo, síntese e liberação de GABA e formação de sinapses inibitórias com os neurônios-alvo (LEE; LEE; KIM, 2017a).

Desordens neuropsiquiátricas, como a esquizofrenia, e do neurodesenvolvimento, como o TEA, apresentam alterações no balanço E/I. Devido à extrema importância dos interneurônios PV^+ para o balanço E/I, alterações nestas células podem perturbar toda a fina regulação que mantém o equilíbrio entre excitação e inibição (CLINE, 2005; POLLEUX; LAUDER, 2004;

RUBENSTEIN, J.L.R & MERZENICH, 2003). Já se observou diminuição no número de interneurônios PV^+ no córtex pré-frontal medial (CPFm) (HASHEMI *et al*., 2016) e no córtex pré-frontal dorsolateral (CPFdl) de pacientes com TEA (ZIKOPOULOS; BARBAS, 2013). Estas evidências podem ajudar a explicar a dessincronização de atividade oscilatória observada no transtorno (DEFELIPE, 1999) e estão de acordo com achados de neurotransmissão inibitória comprometida, refletida por reduzida oscilação gama (WILSON *et al*., 2007). Em modelos animais de autismo, também existe uma grande quantidade de estudos evidenciando déficits de interneurônios PV^+: no córtex de camundongos com deleção do gene *MeCP2*, cuja mutação está relacionada com comportamentos observados no TEA na síndrome de Rett e de Angelman (FUKUDA *et al*., 2005); no córtex cingulado anterior (aCC) e no córtex parietal de camundongos *u*$PAR^{-/-}$, um modelo animal de autismo já bem caracterizado (POWELL *et al*., 2003); no córtex parietal de animais do modelo VPA (GOGOLLA *et al*., 2009); no CPFm de animais do modelo de autismo por ativação imune materna (MEYER *et al*., 2008), dentre vários outros. Ainda, cabe ressaltar que camundongos *knockout* para PV (camundongos $PV^{-/-}$) apresentam diversas características do tipo-autista (prejuízos de comunicação e de interação social e padrões de comportamento repetitivos e estereotipados, bem como reduzida sensibilidade a estímulos dolorosos e maior susceptibilidade a desenvolver crises epilépticas). Neste mesmo estudo, camundongos heterozigotos ($PV^{+/-}$) também apresentaram prejuízos de comunicação e interação social, indicando que uma simples redução no número de interneurônios PV^+ possa ser suficiente para desencadear características do tipo-autista nesses animais (WÖHR *et al*., 2015). Além disso, alterações nas oscilações gama no CPFm foram observadas em camundongos com *knock in* de Neuroliguina 3 – um dos diversos genes associados ao TEA (JAMAIN *et al*., 2003) – e isto conduziu a déficits sociais nestes animais (CAO *et al*., 2018), o que também é observado em pacientes com TEA e em modelos animais de autismo (AMERICAN PSYCHIATRIC ASSOCIATION, 2013; SCHNEIDER; PRZEWŁOCKI, 2005). Também existem alterações em interneurônios PV^+ descritas na esquizofrenia; um dos achados mais comuns neste transtorno em relação a estas células é a redução do RNA mensageiro ou do conteúdo proteico de PV nos interneurônios PV^+. Estudos na literatura mostram esta alteração, por exemplo, no CPFdl de pacientes com esquizofrenia (FUNG *et al*., 2010; HASHIMOTO *et al*., 2003, 2008; MELLIOS *et al*., 2009; VOLK *et al*., 2012). Modelos animais de esquizofrenia também apresentam alterações em interneurônios PV^+ em algumas regiões encefálicas, como déficits no hipocampo (ABDUL-MONIM; NEILL; REYNOLDS, 2007; PENSCHUCK *et al*., 2006), além de aumento destes no córtex cingulado (ABDUL-MONIM; NEILL; REYNOLDS, 2007).

Como os interneurônios são uma peça chave para a manutenção correta do equilíbrio E/I, os interneurônios CB^+ surgem como uma possível fonte de respostas para o desequilíbrio E/I observado no TEA. Entretanto, a quantidade de estudos na literatura a respeito destas células em pacientes com TEA é escassa: sabe-se que há aumento na densidade de interneurônios CB^+ no giro denteado, uma importante região do hipocampo (LAWRENCE *et al*., 2010); já no córtex cingulado posterior e no giro fusiforme, (OBLAK *et al*., 2011), bem como no CPFdl (ZIKOPOULOS; BARBAS, 2013) não há diferenças significativas na densidade quando comparados com indivíduos controle. Além disso, há uma redução no número de células de Purkinje cerebelares imunorreativas para CB d28k em uma subpopulação de pacientes com TEA (WHITNEY *et al*., 2008). Em modelos animais, de nosso conhecimento, há um único estudo: camundongos *u*$PAR^{-/-}$, um modelo animal de autismo já bem caracterizado, têm 50% menos interneurônios CB^+ no córtex frontoparietal (LEVITT; EAGLESON; POWELL, 2004; POWELL *et al*., 2003). Já na esquizofrenia, há mais estudos sobre os interneurônios CB^+: em pacientes, há evidências de redução na densidade destas células no córtex pré-frontal (CPF) (BEASLEY *et al*., 2002; REYNOLDS *et al*., 2000), no *planum temporale* (área cortical posterior ao córtex auditivo, centro da área de Wernicke) (CHANCE; WALKER; CROW, 2005) e na região CA2 do hipocampo (IRITANI *et al*., 1999), além de aumento na densidade destas células no CPFdl (DAVISS; LEWIS, 1995) e no aCC (WOO *et al*., 2008).

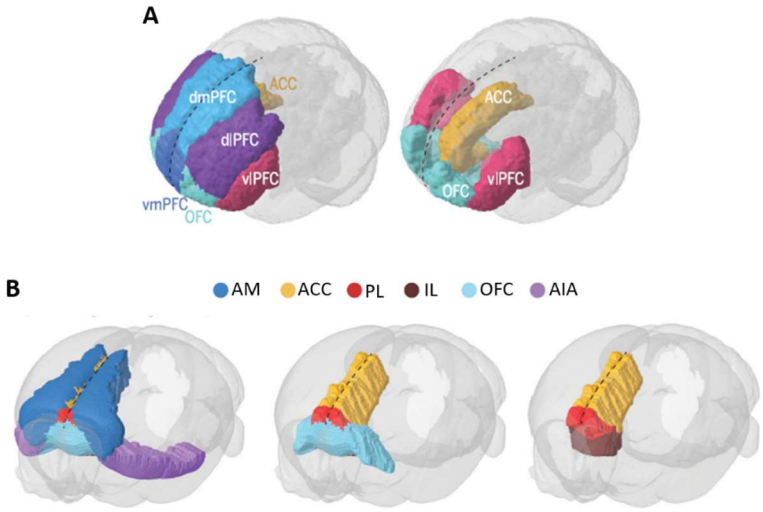
O funcionamento alterado de diversas áreas encefálicas é uma característica marcante do TEA. Além disso, as alterações sociais - tanto as presentes na díade comportamental de diagnóstico, quanto outras que se manifestam ainda no primeiro ano de vida ou mais tardiamente - resultam em prejuízos de comunicação social e de interação com os indivíduos. Como diversos comportamentos e funções sociais são dependentes do CPF, o funcionamento alterado desta região encefálica pode estar relacionado com o fenótipo social e cognitivo encontrado no TEA. Desse modo, o CPF surge como uma potencial área de investigação.

1.5 CÓRTEX PRÉ-FRONTAL E TEA

No encéfalo de mamíferos, o CPF corresponde ao córtex cerebral que recobre a parte frontal do lobo frontal (MURRAY; WISE; GRAHAM, 2017). O CPF localiza-se anteriormente ao córtex pré-motor e à área motora suplementar (STUSS; BENSON, 1984); em humanos, é dividido funcionalmente em córtex orbitofrontal, CPF lateral (subdividido em CPFdl e CPF ventrolateral) e CPFm (subdividido em aCC, CPF dorsomedial e CPF ventromedial) (Figura 3).

Já em roedores, existem algumas diferenças: o CPF é constituído pela área motora secundária, CPFm [subdividido em aCC, área pré-límbica (PrL) e área infralímbica (IL)], córtex orbitofrontal e área insular agranular (Figura 3).

Figura 3 - Divisão funcional do córtex pré-frontal (CPF) em humanos (A) e roedores (B)



(A) dmPFC - CPF dorsomedial; ACC - córtex cingulado anterior; dlPFC - CPF dorsolateral; vlPFC - CPF ventrolateral; vmPFC - CPF ventromedial; OFC - córtex orbitofrontal; (B) AM - área motora secundária; PL - área pré-límbica; IL - área infralímbica e AIA - área insular agranular. ADAPTADO DE (CARLÉN, 2017).

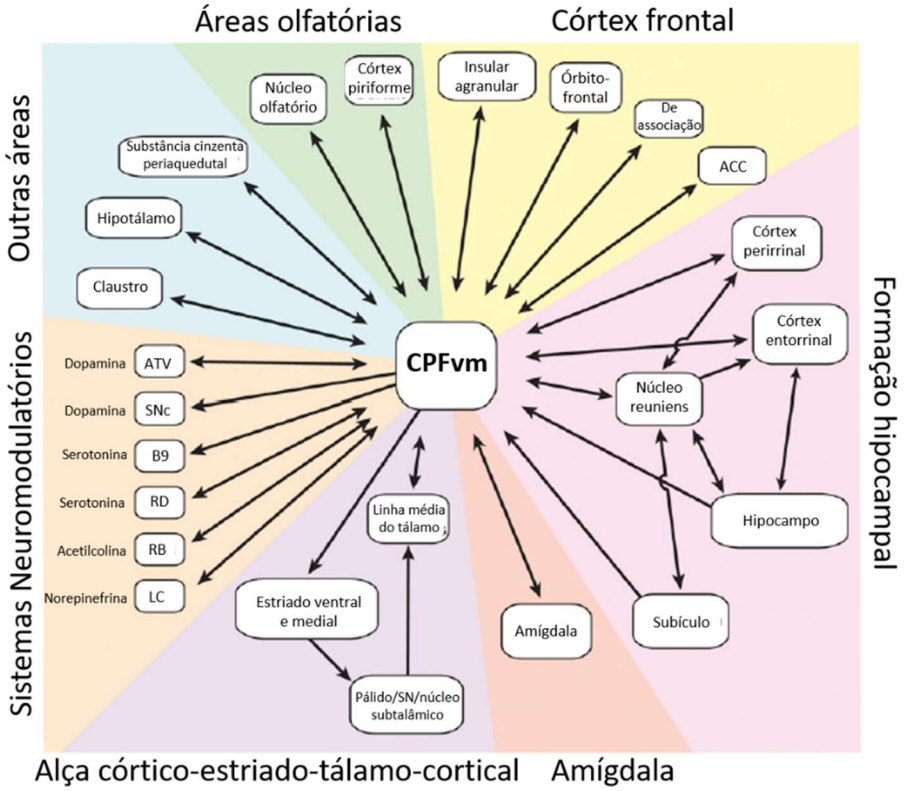
Há certa inconsistência a respeito da nomenclatura e limites anatômicos das sub-regiões do CPF nas diferentes espécies (LAUBACH *et al*., 2018), bem como dificuldade na obtenção de um consenso sobre a homologia das diferentes áreas deste córtex entre humanos e roedores. Muitos estudos chegaram à conclusão de que a área pré-límbica do CPFm dos roedores é a que mais tem homologia funcional ao CPFdl e ao aCC dos humanos e primatas não-humanos (SEAMANS; LAPISH; DURSTEWITZ, 2008; UYLINGS; GROENEWEGEN; KOLB, 2003); por isso, a partir daqui, quando CPFdl e CPFm forem citados, estes corresponderão, respectivamente, à área cortical homóloga em humanos e roedores.

O CPFdl está associado com funções cognitivas e executivas, como a memória de trabalho (QIN *et al*., 2009), *goal-directed action* (intenção de realizar uma ação já com um propósito determinado), *abstract reasoning* (capacidade de analisar a informação, detectar padrões e solucionar o problema) e concentração (MILLER; COHEN, 2001), bem como modulação da dor (LORENZ; MINOSHIMA; CASEY, 2003). Estudos de imagem já relacionaram o CPFdl e o aCC dos humanos com o controle cognitivo (MACDONALD, 2000). O aCC está relacionado com diversas funções: detecção de erros e problemas de lógica, tomada

de decisão e concentração, bem como capacidade de decisão e de ação de acordo com experiências anteriores (SEAMANS; LAPISH; DURSTEWITZ, 2008).

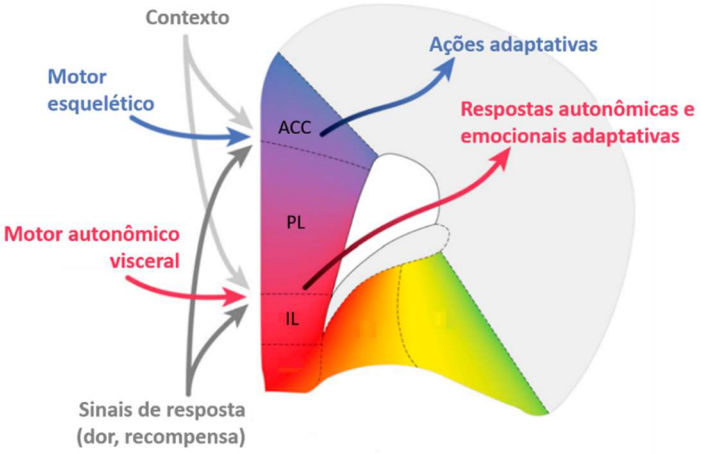
Em relação aos *inputs* e *outputs* do CPFdl e do CPFm, o CPFdl tem conexões recíprocas com regiões encefálicas que estão implicadas com o controle motor (gânglios basais, córtex pré-motor, área motora suplementar) (GROSSMANN, 2013). A maior fonte de aferências se origina do tálamo, mas também há *inputs* de estruturas límbicas, como a amígdala e hipocampo, os quais dão informação emocional. Também recebe *inputs* de regiões neuromodulatórias, como a área tegmental ventral, *locus coeruleus*, rafe dorsal e do prosencéfalo basal, o que pode influenciar no nível de atenção (HOOVER; VERTES, 2007; KURODA *et al*., 1996). Já o CPFm (Figuras 4 e 5) está fortemente interconectado com áreas insulares anteriores, conhecidas por estarem envolvidas na interocepção (ALLEN *et al*., 1991) e percepção de dor (JASMIN; GRANATO; OHARA, 2004). Outra conexão importante é com a substância cinzenta periaquedutal, região envolvida com a agressão, comportamento de defesa e modulação da dor (NELSON; TRAINOR, 2007) (SEWARDS; SEWARDS, 2002). O CPFm processa esses *inputs* tão heterogêneos para, finalmente, guiar a execução de um comportamento adequado para tal estímulo.

Figura 4 - Principais conexões anatômicas do córtex pré-frontal ventromedial (CPFvm)



ACC, córtex cingulado anterior; SN, substância nigra; LC, *locus coeruleus*; RB, região de Brocá; RD, rafe dorsal; B9, células serotoninérgicas B9; SNc, substância nigra *pars compacta;* ATV, área tegmental ventral. ADAPTADO DE (EUSTON, 2012).

Figura 5 - Visão esquemática dos *inputs* e *outputs* de diferentes regiões para o CPFm



ACC, córtex cingulado anterior; PL, área pré-límbica; IL, área infralímbica. ADAPTADO DE (EUSTON, 2012).

O CPF é subdividido em 6 camadas (I-VI), definidas assim pelo tipo morfológico celular que as compõem, além da conectividade, origens de desenvolvimento e padrões de expressão gênica das células em cada camada (GUY; STAIGER, 2017). Um padrão de organização neuronal típico dos córtices são as minicolunas, em humanos, e as colunas, em roedores. Uma minicoluna é uma unidade funcional básica do encéfalo que organiza os neurônios no espaço cortical (FAVOROV; KELLY, 1994a, 1994b; MOUNTCASTLE, 1997; TOMMERDAHL *et al*., 1993); essa coluna vertical atravessa as diversas camadas corticais (camada VI até a II). Neurônios dentro da minicoluna recebem *inputs* que são comuns para todos, têm *outputs* também comuns, estão interconectados e provavelmente constituem uma unidade computacional fundamental do córtex cerebral (CASANOVA *et al*., 2002). Nestas minicolunas estão os interneurônios GABAérgicos, os quais fazem a inibição lateral e acredita-se que isso é o que delineia uma minicoluna da outra (DEFELIPE *et al*., 1990; FAVOROV; KELLY, 1994a, 1994b). No encéfalo de primatas, vias cortico-corticais e cortico-subcorticais ocorrem através de neurônios excitatórios. Quando essas vias alcançam seus alvos no córtex, há a formação de sinapses excitatórias com neurônios excitatórios locais e inibitórios locais, participando de microcircuitos locais dentro de minicolunas ou minicolunas vizinhas no córtex (ZIKOPOULOS; BARBAS, 2013).

1.5.1 Equilíbrio E/I e CPF

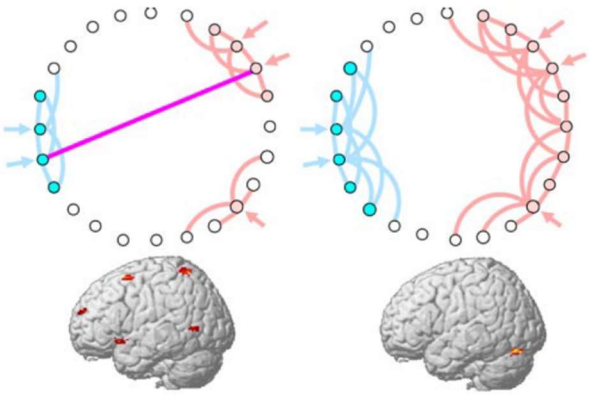
O balanço E/I é essencial para a execução apropriada de diversos comportamentos dependentes do CPF (FERGUSON; GAO, 2018). Estudos evidenciam que, ao aumentar de forma exagerada a excitabilidade dos interneurônios PV^+ no CPFm (diminuição da razão E/I) há um prejuízo significativo na memória de trabalho, flexibilidade cognitiva e interação social (FERGUSON; GAO, 2018), comportamentos dependentes do CPF. Ainda, estudos mostram que um aumento na razão E/I também é prejudicial: a manipulação optogenética de neurônios piramidais e de interneurônios PV^+ no CPFm de camundongos prejudicou o comportamento social (YIZHAR *et al*., 2011) e os déficits sociais puderam ser reduzidos ao aumentar o tom inibitório dos interneurônios. A manutenção do equilíbrio E/I no CPFm tem uma participação fundamental do tálamo: os *inputs* vindos desta região encefálica garantem forte ativação dos interneurônios PV^+ (SCHMITT *et al*., 2017), enquanto que uma atenuação da atividade do tálamo aumenta a razão E/I por reduzir a inibição dos interneurônios nas células piramidais corticais (FERGUSON; GAO, 2018).

Recentemente, descobriu-se que as *basket cells* são necessárias e suficientes para a geração e manutenção das oscilações gama (30-80 Hz) (BUZSAKI *et al*., 2004; CARDIN *et al*., 2009; SOHAL *et al*., 2009), uma faixa de oscilação relacionada com a cognição, memória de trabalho, processamento e integração da informação (GAETZ *et al*., 2011; HOWARD *et al*., 2003; LUNDQVIST *et al*., 2016) - todos comportamentos dependentes do CPF. Em indivíduos neurotípicos, essa oscilação é modulada por uma variedade de processos integrativos, incluindo *feature binding* (capacidade de integração de todas as características de um mesmo objeto) (TALLON-BAUDRY *et al*., 1998), *top-down feature selection* (capacidade de discernir as características de um objeto, como seu tamanho, por exemplo) (HERRMANN; MECKLINGER, 2001), atenção (MÜLLER; GRUBER; KEIL, 2000), processamento de face (KEIL *et al*., 2001b; RODRIGUEZ *et al*., 1999) e *emotional arousal* (capacidade de sentir emoções) (KEIL *et al*., 2001a). Prejuízos comportamentais tanto no TEA como na esquizofrenia têm sido associados com a elevação basal (ou seja, não evocada) de oscilações de alta frequência (na faixa dos 30-80Hz), faixa característica das oscilações gama (OREKHOVA *et al*., 2007; ROJAS *et al*., 2008; WILSON *et al*., 2007). De fato, já existem evidências de que oscilações gama anormais conduzem ao desequilíbrio E/I e déficits sociais em pacientes com TEA (ROJAS; WILSON, 2014; YIZHAR *et al*., 2011). A elevação na razão E/I é acompanhada por elevação nas oscilações gama (SOHAL *et al*., 2009). Esses resultados sugerem que uma razão E/I aumentada, causada por mau funcionamento dos interneurônios

PV^+, induz a oscilações gama excessivas e comportamentos do tipo-autista (LEE; LEE; KIM, 2017b).

Ainda, há alta conectividade local e baixa conectividade entre regiões distantes (Figura 6), o que é evidenciado principalmente por estudos de ressonância magnética funcional (DICHTER, 2012; MINSHEW; KELLER, 2010). Essa falta de conectividade entre regiões encefálicas no TEA pode estar refletida em uma falta de sincronia nas oscilações gama no eletroencefalograma (BROCK *et al*., 2002). Irmãos não autistas de pessoas com TEA parecem compartilhar a hipoativação dos córtices pré-frontal e medial temporal, mas não a hiperativação do córtex parietal, sugerindo que esta baixa atividade nessas duas regiões possa ser um endofenótipo, que reflete padrões familiares de organização encefálica que coloca os indivíduos em um alto risco para o TEA (BELMONTE; BARON-COHEN, 2004).

Figura 6 - Hiperconectividade local e hipoconectividade entre regiões distantes no TEA



À esquerda, observa-se uma representação da conectividade no encéfalo típico (estímulos são processados localmente e há integração entre diferentes regiões). À direita, observa-se a representação do encéfalo no TEA (estímulos são hiperprocessados localmente e há pouca integração entre diferentes regiões) ADAPTADO DE (BELMONTE, 2004).

Uma mudança frequentemente observada na estrutura cortical da substância cinzenta em crianças e adultos com TEA é a minicolunopatia (BUXHOEVEDEN *et al*., 2006; CASANOVA, 2006; CASANOVA *et al*., 2002, 2010; CASANOVA; BUXHOEVEDEN; GOMEZ, 2003; CASANOVA; BUXHOEVEDEN; BROWN, 2002). Casanova e colaboradores realizaram diversos estudos a respeito das minicolunas em indivíduos com TEA e a observação foi a de que o CPFdl tem mais minicolunas, porém estas são mais estreitas do que a de indivíduos neurotípicos. Entretanto, ainda não foram elucidados os mecanismos pelos quais este fenômeno ocorre. O que se sabe é que as *double bouquet cells* definem a organização minicolunar (MOUNTCASTLE, 1997). Alterações na densidade e morfologia neuronal, bem como na distribuição laminar e colunar, podem afetar as conexões excitatórias e inibitórias e os

circuitos. Por exemplo, isso pode afetar o neurópilo (ZIKOPOULOS; BARBAS, 2013), que é o espaço que circunda as minicolunas e é o canal para as projeções dos circuitos locais de excitação e inibição (PETERS; SETHARES, 1996) e isso pode prejudicar o balanço E/I no TEA. Já foi observado que o neurópilo no CPFdl de pacientes com TEA é mais estreito e há uma menor compactação - ou seja, as células são mais esparsas (CASANOVA *et al*., 2002).

Algumas outras alterações no CPF de indivíduos com TEA também já foram descritas na literatura, como aumento no volume da substância cinzenta em crianças, o que pode ser, em alguns casos, devido a um aumento no número de neurônios, pelo menos em alguns córtices pré-frontais (COURCHESNE *et al*., 2011); de fato, já foi observado que há 79% mais neurônios no CPFdl e 29% mais neurônios no córtex mesial pré-frontal (CPFm sem o aCC) de crianças com TEA - embora esta alteração já não seja mais observada no encéfalo de adultos (ZIKOPOULOS; BARBAS, 2010) - e aumento relativo de 67% no número total de neurônios no CPF (COURCHESNE *et al*., 2011). Embora não se saiba a causa disto, tal anormalidade parece ser de origem pré-natal e, consequentemente, estas alterações influenciam todo o equilíbrio E/I. Ainda, foi identificado em pacientes com TEA partes de córtex que estão desorganizadas, incluindo o CPF (STONER *et al*., 2014). Provavelmente isso resulta de desregulação da formação das camadas e diferenciação neuronal específica de cada camada em estágios pré-natais de desenvolvimento, o que também contribui de maneira crítica para as alterações E/I encontradas no transtorno. Por fim, dados sugerem forte ativação anormal no córtex parietal, ao mesmo tempo em que regiões integrativas nos córtices pré-frontal e medial temporal estão anormalmente quiescentes (BELMONTE; BARON-COHEN, 2004). Todos esses achados evidenciam o grande papel do CPF para o E/I e as alterações encontradas suportam o papel chave dos interneurônios e da correta organização cortical para a manutenção de estados adequados de excitação e inibição corticais.

1.6 JUSTIFICATIVA

Este presente trabalho justifica-se devido ao fato de o TEA:

a) ser um transtorno heterogêneo e multifatorial sem etiologia conhecida;
b) ter uma prevalência elevada;
c) causar déficits de comunicação e de interação social que prejudicam a inserção e o convívio dos indivíduos na comunidade;
d) apresentar diversas comorbidades, como, por exemplo, epilepsia, o que prejudica a qualidade de vida dos pacientes;

e) gerar um extenso custo econômico, social e emocional (tanto para os pacientes como para os familiares e a sociedade).

Devido a isso, o entendimento acerca da fisiopatologia do TEA mostra-se crucial e, por causa da limitação de estudos em seres humanos e dificuldade de obtenção de tecidos *post-mortem* de pacientes com TEA, a pesquisa básica utilizando modelos animais faz-se necessária e mostra-se de extrema importância e relevância.

Devido ao papel chave dos interneurônios CB^+ e PV^+ para a manutenção do equilíbrio E/I no CPFm, justifica-se a necessidade de investigação destas células nesta região encefálica crucial e bastante afetada no TEA. A partir da realização deste trabalho, espera-se obter resultados que auxiliem a elucidar alguns parâmetros no âmbito de desequilíbrio E/I, CPFm e TEA e contribuam para avanços no entendimento da fisiopatologia do TEA.

1.7 OBJETIVOS

1.7.1 Objetivo geral

Analisar a distribuição laminar cortical e entre sub-regiões dos interneurônios GABAérgicos CB^+ e PV^+ e neurônios totais $NeuN^+$ nas camadas superficiais (II/III) e profundas (IV/V) no CPFm em ratos Wistar de 30 dias do modelo animal de autismo induzido por exposição pré-natal ao VPA.

1.7.2 Objetivos específicos:

a) quantificar os interneurônios CB^+ e PV^+ e os neurônios totais $NeuN^+$ no CPFm, dividindo-o em suas sub-regiões aCC, PrL e IL e camadas superficiais (II/III) e profundas (IV/V);
b) calcular a proporção de interneurônios CB^+ e PV^+ em relação aos neurônios totais $NeuN^+$ no CPFm, dividindo-o em suas sub-regiões aCC, PrL e IL e camadas superficiais (II/III) e profundas (IV/V).

2 TRABALHO EXPERIMENTAL NA FORMA DE ARTIGO CIENTÍFICO

A ser submetido de acordo com as normas de publicação da revista *Frontiers in Neuroscience*

ANALYSIS OF PARVALBUMIN-POSITIVE AND CALBINDIN-POSITIVE GABAERGIC INTERNEURONS IN MEDIAL PREFRONTAL CORTEX OF AUTISM ANIMAL MODEL INDUCED BY VALPROIC ACID

Giovanna Carello Collar[a,b,c], Júlio Santos Terra Machado[a,b,c], Mellanie Fontes Dutra da Silva[a,b,c], Iohanna Deckmann[a,b,c], Gustavo Brum Schwingel[a,b,c] and Carmem Gottfried[a,b,c]*

[a]Laboratory of Neuroglial Plasticity, Institute of Health's Basics Science, Department of Biochemistry, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

[b]Department of Biochemistry, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

[c]Translational Research Group in Autism Spectrum Disorders (GETTEA), Institute of Health's Basics Science, Department of Biochemistry, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

*Correspondence address: Carmem Gottfried, Department of Biochemistry, Institute of Health's Basics Science, Federal University of Rio Grande do Sul, Ramiro Barcelos Street, 2600, building 21111, 90035-003, Porto Alegre – RS, Brazil. Fax: +55 51 33085551. E-mail address: cgottfried@ufrgs.br

ANALYSIS OF PARVALBUMIN-POSITIVE AND CALBINDIN-POSITIVE GABAERGIC INTERNEURONS IN MEDIAL PREFRONTAL CORTEX OF AUTISM ANIMAL MODEL INDUCED BY VALPROIC ACID

Giovanna Carello-Collar[1,2,3], Júlio Santos-Terra[1,2,3], Mellanie Fontes-Dutra[1,2,3], Iohanna Deckmann[1,2,3], Gustavo Brum Schwingel[1,2,3] and Carmem Gottfried[1,2,3*]

[1]Laboratory of Neuroglial Plasticity, Institute of Health's Basics Science, Department of Biochemistry, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

[2]Department of Biochemistry, UFRGS, Porto Alegre, Brazil.

[3]Translational Research Group in Autism Spectrum Disorders (GETTEA), Institute of Health's Basics Science, Department of Biochemistry, UFRGS, Porto Alegre, Brazil.

***Corresponding author:**
Carmem Gottfried
cgottfried@ufrgs.br

Keywords: Autism Spectrum Disorder, Valproic Acid, Excitatory/Inhibitory Imbalance, Parvalbumin interneurons, Calbindin interneurons, Medial Prefrontal Cortex.

Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with unknown etiology. Excitatory/inhibitory (E/I) imbalance is related to social, cognitive and sensory impairments found in ASD and may have a strong contribution of altered inhibitory signaling. Given the key role of GABAergic interneurons for the maintenance of E/I balance and, since medial prefrontal cortex (mPFC) is pivotal to social behaviors in rodents, we aimed to evaluate in valproic acid (VPA) autism model quantity and proportion of calbindin-positive (CB^+) and parvalbumin-positive (PV^+) interneurons (both GABAergic ones) on upper and deeper layers of anterior cingulate cortex (aCC) and prelimbic (PrL) and infralimbic (IL) areas, all three mPFC subregions. When analyzing the whole mPFC, VPA group displayed increased number of total neurons $NeuN^+$. In subregion analysis, the composition of all results allowed us to observe different patterns of specific alterations both in number and proportion of CB^+ and PV^+ interneurons within total neurons scenario, as well as altered pattern of cortical lamination. Taken together, the results suggest that prenatal exposure to VPA may be related to alterations of interneuron composition in mPFC possibly due to impairments in migratory process and influences in other neuronal subpopulations. Prenatal exposure to VPA led to alterations in mPFC neuronal scenario, which may be possibly related to the social impairments observed in VPA model.

The understanding about the mechanisms by which E/I imbalance occurs in ASD still remains with many questionings. Data showed here contribute to the attempt to elucidate possible key moments in which some of the alterations observed in ASD concerning E/I imbalance can be originated.

1 Introduction

Autism spectrum disorder (ASD) is characterized by deficits in social interaction and social communication, as well as patterns of restricted and repetitive behaviors, activities or interests (American Psychiatric Association, 2013). In addition to the core symptoms, ASD patients can exhibit a set of associated conditions, for example, epilepsy - one of the most prevalent comorbidities in ASD, which affects approximately one third of the patients (R. Muhle, S. V. Trentacoste, 2004; Tuchman and Cuccaro, 2011; Tuchman and Rapin, 2002). Furthermore, toddlers with ASD process social cues in an atypical way during their first year of life (Osterling and Dawson, 1994; Welsh and Estes, 2018), which impairs subsequent social behaviors. Moreover, they orient preferentially to non-social contingencies (Klin et al., 2009) and apparently have potential impairments on empathy-behaviors (Baron-Cohen and Wheelwright, 2004; Jones et al., 2010).

ASD has an elevated prevalence (1:59) (Christensen et al., 2018) and, despite the progress achieved on the understanding of ASD's pathophysiology, its etiology remains unknown. Besides that, ASD is a heterogeneous and multifactorial disorder (Beversdorf, 2016) with an intricate interaction among epigenetic (Lyall et al., 2017), genetic (Caglayan, 2010; El-Fishawy and State, 2010; Geschwind, 2011) and environmental (Risch et al., 2014; Sealey et al., 2016) risk factors strongly associated with its triggering. Regarding the environmental ones, here we highlight the prenatal exposure to valproic acid (VPA) (Christensen et al., 2013; Roullet et al., 2013) – which findings conducted to the well-stablished VPA animal model of autism (Bambini-Junior et al., 2011, 2014; Fontes-Dutra et al., 2018; Gottfried et al., 2013; Hirsch et al., 2018; Rodier et al., 1996; Schneider and Przewłocki, 2005).

The complexity of ASD's pathophysiology has been targeted for theories in an attempt to explain the basis of this disorder (Yenkoyan et al., 2017). The excitatory/inhibitory (E/I) imbalance hypothesis during critical periods of embryonic development (Gogolla et al., 2009; Nelson and Valakh, 2015; Rubenstein, J.L.R & Merzenich, 2003) addresses mechanisms by which impaired neuronal connectivity affects brain circuitry; however, it still remains with many questions. E/I imbalance may alters the functioning of several brain regions, such as prefrontal cortex (PFC). This region can be divided, among other areas, into dorsolateral PFC (dlPFC) in humans and medial PFC (mPFC) in

rodents. The rodent mPFC is divided into anterior cingulate cortex (aCC) and prelimbic (PrL) and infralimbic (IL) areas (Carlén, 2017) and is the functional homologous region to the human dlPFC (Seamans et al., 2008; Uylings et al., 2003). As the E/I balance is pivotal for appropriated achievement of PFC-dependent behaviors, alterations on this circuitry are related to impairments on PFC functions, including working memory, cognitive flexibility and social interaction (Ferguson and Gao, 2018; Yizhar et al., 2011) and also related to social, behavioral, sensorial, motor and cognitive phenotypes found in ASD (Markram and Markram, 2010; Rubenstein, J.L.R & Merzenich, 2003; Rubenstein, 2010).

GABAergic interneurons display a pivotal role in the maintenance of E/I balance in PFC. They strongly contribute to local inhibition within cortical circuitry and modulate excitatory responses in a dynamic way (Isaacson and Scanziani, 2011; Klausberger et al., 2003), controlling pyramidal cells generation and time of firing (Pouille and Scanziani, 2001) and preventing the excess of excitation (Dichter and Ayala, 1987). Calbindin-positive and parvalbumin-positive GABAergic interneurons (CB^+ and PV^+, respectively) represent a huge percentage of total GABAergic cortical interneuron population. Whereas not much is known about CB^+ interneurons, the PV^+ population is characterized by having a unique pattern of firing, known as fast-spiking (Kawaguchi et al., 1987; Kawaguchi and Kubota, 1997), generating a rapid train of action potentials like any other neuron. Also, they are necessary and sufficient to generate and maintain gamma oscillations (30-80Hz) (Buzsaki et al., 2004; Cardin et al., 2009; Sohal et al., 2009), a range of oscillation related to cognition, working memory, and processing and integration of information in PFC (Gaetz et al., 2011; Howard et al., 2003; Lundqvist et al., 2016). Alterations in these oscillations are present in ASD (McNally and McCarley, 2016; Rojas and Wilson, 2014), leading to E/I imbalance (Rojas and Wilson, 2014) and to PFC-related social deficits in animal models of autism (Cao et al., 2018) and patients (Rojas and Wilson, 2014). A minor dysregulation of GABAergic interneurons can affect thousands of excitatory cells and may imbalance the entire delicate regulation between excitation and inhibition (Cline, 2005; Polleux and Lauder, 2004; Rubenstein, J.L.R & Merzenich, 2003).

Given the key role of GABAergic interneurons for the maintenance of E/I balance in PFC and, since PFC is pivotal to social behaviors, the altered E/I balance and social impairments seen in ASD can have a strong contribution of altered inhibitory signaling. Due the fact that there is not much understanding about ASD's pathophysiology yet, here we asked how the general neuronal organization was structured and whether CB^+ and PV^+ interneurons had impaired pattern of distribution across layers in aCC and prelimbic and infralimbic areas in the VPA model.

2 Materials and Methods

2.1 VPA Model

Wistar rats were obtained from Center of Reproduction and Experimentation of Laboratory Animals (CREAL) of Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil, and maintained in Hospital de Clínicas de Porto Alegre (HCPA) and Department of Biochemistry from Institute of Health's Basics Science (ICBS) facilities under a standard 12/12h light/dark cycle (light cycle starting at 7am and ending at 7pm) at a constant temperature of 22 ± 1°C. The animals had *ad libitum* access to food and water and were handled in accordance with the guidelines established by the National Council for the Control of Animal Experimentation (CONCEA) of Brazil. This project was approved by UFRGS ethics committee (CEUA-UFRGS #140367).

As shown in Panel 1, animals were mated overnight and pregnancy was verified by next morning through presence of spermatozoa in the vaginal smear. This was considered the embryonic day 0.5 (E0.5). Pregnant females were then divided randomly into two experimental groups: Control and VPA. On E12.5, VPA model was induced, as first described by Schneider and Przewlocki (Schneider and Przewlocki, 2005): rats of VPA group received a single intraperitoneal injection of VPA at 600 mg/kg (Acros Organics, NJ, USA) while control group received saline solution 0.9%. At postnatal day 21 (P21), the offspring was weaned and only the male ones were kept.

2.2 Tissue Preparation and Immunofluorescence Technique

P30 male rats (n=3-5, in litters) were euthanized by anesthetic overdose and subjected to transcardiac perfusion with 0.9%-NaCl solution followed by 1.5%- and 4%-paraformaldehyde (PFA) solutions before brain removal. After dissection, the tissue was post-fixed for 4h in a 4%-PFA solution and subsequently cryoprotected by sequential immersions in 15%- and 30%-sucrose phosphate-buffered saline (PBS) solutions (the tissue was kept in each solution until complete submersion). Brains were embedded in Tissue-Tek® and kept in -80°C ultra-freezer until immunofluorescence procedures.

The 7th edition of Paxinos rat brain atlas (Paxinos and Watson, 2013) was utilized to identify mPFC region according to the following parameters: Bregma 3.24 mm; interaural: 12.24 mm (Figure 10 of Paxinos). Coronal brain slices (25 μm) were made utilizing cryostat (Leica Microsystems GmbH). The immunostaining procedure was performed as it follows: (1) 4%-PFA exposure (10 min); (2) three washes (5 min each) with PBS 0.1M; (3) permeabilization with PBS-Triton 0.1% (10 min); (4) three washes (5 min each) with PBS 0.1M; (5) citrate buffer antigen retrieval (60°C for 1h); (6) two washes (5 min each) with PBS-Triton 0.1%; (7) blockade with bovine serum albumin (BSA) 5% in

PBS-Triton 0.1% (1h); (8) incubation with primary antibodies Anti-Parvalbumin (Abcam ab64555), Anti-Calbindin (Abcam ab82812) and Anti-NeuN (Abcam ab17748) – each one diluted 1:500 in blocking solution (BSA 5% in PBS-Triton 0.1%) – for 48h at 4°C; (9) five washes (3 min each) with PBS 0.1M; (10) incubation with both secondary antibodies Alexa Fluor 488 Goat Anti-Mouse IgG (Molecular Probes, A11029) and Alexa Fluor 546 Goat Anti-Rabbit IgG (Molecular Probes, A11036) – both diluted 1:2,000 in blocking solution for 2h at room temperature; (11) five washes (3 min each) with PBS 0.1M; (12) incubation with DAPI Nucleic Acid Stain solution (Invitrogen, MP01306, 1:10000) (10 min); (13) five washes (3 min each) with PBS 0.1M; (14) addition of Mounting Medium Fluorshield (Sigma-Aldrich, F6182) followed by coverslip.

2.3 Confocal Microscopy and Image Analysis

The division of mPFC in three subregions (aCC, PrL and IL) was visually made in a confocal microscope (Olympus FluoView FV1000 confocal laser scanning) of UFRGS Microscopy and Microanalysis Center from one hemi-slice per glass slide. Each mPFC subregion was yet visually subdivided in upper (II/III) and deeper (IV/V) layers. As a result, each animal had six images. Each image was obtained at least eight times (eight tomes) (20x magnification) and the analysis was made from merged tomes. The neuronal quantification results are shown in absolute number of $NeuN^+$ labeled cells (here used as a marker for total neurons), PV^+ labeled cells (PV^+ neurons) and CB^+ labeled cells (CB^+ neurons) (Panel 2). $NeuN^+$ quantification was performed for each assay: 1) CB^+, $NeuN^+$ and DAPI and 2) PV^+, $NeuN^+$ and DAPI. Total neurons $NeuN^+$ quantification results had few differences between the assays – as we can see in Figure 1Ab and 1Bb. These small differences seem to be related to a higher background in CB^+, $NeuN^+$ and DAPI assay, which slightly impairs the visualization. Because of that, the results of total neurons $NeuN^+$ quantification shown in Table 1, 2 and 3 are only from PV+, $NeuN^+$ and DAPI assay. Analyses were performed using ImageJ software Cell Counter plug-in by two independent researchers. The ratio of interneurons was performed as it follows: *absolute number of PV^+ or CB^+ / absolute number of Total Neurons $NeuN^+$*.

2.4 Statistical Analysis

Student's T Test followed by Welch's correction was performed using Graph Pad Prism 6 software. Data are reported as mean ± standard deviation (SD). Statistically significant p values: *p<0.05, **<0.01, ***<0.001.

3 Results

3.1 VPA group displays increased number of total neurons NeuN$^+$ on mPFC

When analyzing the whole mPFC, VPA group presented a significant increase in the number of total neurons NeuN$^+$ when compared to control group (Control: 2598 ± 122.5, VPA: 3482 ± 183.4, p= 0.0086) – data shown from PV$^+$ experiments (Figure 1Ab). Neither the number of CB$^+$ and PV$^+$ neurons nor their proportions within mPFC total neurons scenario were affected by prenatal exposure to VPA: number of PV$^+$ neurons (Figure 1Aa): Control: 228.0 ± 15.45, VPA: 224.8 ± 35.90, p= 0.9377; number of CB$^+$ neurons (Figure 1Ba): Control: 136.2 ± 12.19, VPA: 122.0 ± 27.71, p=0.6626; PV$^+$ ratio (Figure 1Ac): Control: 0.08431 ± 0.005051, VPA: 0.06358 ± 0.007179, p= 0.0610; CB$^+$ ratio (Figure 1Bc): Control: 0.04949 ± 0.005505, VPA: 0.03848 ± 0.006202, p= 0.2331.

3.2 VPA group presents proportion and distribution impairments of PV$^+$ and CB$^+$ interneurons across aCC layers

Then, we went through the subregions of mPFC (aCC, PrL and IL) and their upper and deeper layers. As shown in Table 1, at upper layers of aCC, VPA group presented diminished PV$^+$ ratio (Control: 0.08400 ± 0.003180; VPA: 0.05513 ± 0.002124; p= 0.0003). At deeper layers of aCC, VPA group presented diminished PV$^+$ ratio (Control: 0.09694 ± 0.01337; VPA: 0.05653 ± 0.009855; p= 0.0460), as well as diminished number of CB$^+$ neurons (Control: 47.20 ± 7.172; VPA: 22.75 ± 5.006; p= 0.0278). When analyzing the whole aCC (upper layers + deeper layers), VPA group presented diminished PV$^+$ ratio when compared to control (Control: 0.09678 ± 0.005418; VPA: 0.06008 ± 0.005611; p= 0.0061). There were no differences statistically significant among the other analyses in the aCC: upper layers, number of total neurons NeuN$^+$ (Control: 350.8 ± 29.06; VPA: 435.5 ± 64.43; p= 0.2938), number of PV$^+$ neurons (Control: 29.20 ± 1.934; VPA: 26.00 ± 4.041; p= 0.5275), number of CB$^+$ neurons (Control: 15.20 ± 4.128; VPA: 16.00 ± 4.899; p= 0.9045), CB$^+$ ratio (Control: 0.05189 ± 0.005582; VPA: 0.04192 ± 0.008916; p= 0.7866); deeper layers, number of total neurons NeuN$^+$ (Control: 484.6 ± 31.66; VPA: 655.0 ± 57.99; p= 0.0521), number of PV$^+$ neurons (Control: 47.20 ± 7.172; VPA: 44.33 ± 8.452; p= 0.8069), CB$^+$ ratio (Control: 0.06139 ± 0.009970, VPA: 0.03932 ± 0.005643; p= 0.1152); whole aCC (upper layers + deeper layers), number of total neurons NeuN$^+$ (Control: 872.5 ± 51.32; VPA: 1161 ± 137.3; p= 0.1587), number of PV$^+$ neurons (Control: 84.25 ± 5.808; VPA: 70.33 ± 12.47; p= 0.3891), number of CB$^+$ neurons (Control: 37.20 ± 6.224, VPA: 38.75 ± 9.886; p= 0.8994), CB$^+$ ratio (Control: 0.04965 ± 0.007097, VPA: 0.04026 ± 0.006774; p= 0.3710).

3.3 VPA group presents abnormal proportion and distribution impairments of PV^+ and CB^+ interneurons across prelimbic layers

As shown in Table 2, at upper layers of prelimbic area, VPA group presented an increase in the number of PV^+ neurons (Control: 28.80 ± 3.625; VPA: 40.00 ± 2.000; p= 0.0369) and PV^+ ratio (Control: 0.07180 ± 0.0008816; VPA: 0.08789 ± 0.001814; p= 0.0044), a decrease in CB^+ ratio (Control: 0.08041 ± 0.01349; VPA: 0.03512 ± 0.007272; p= 0.0255), as well as an increase in the number of total neurons $NeuN^+$ (Control: 337.6 ± 21.97; VPA: 448.3 ± 19.92; p= 0.0074). At deeper layers, VPA group presented an increase in the number of PV^+ neurons (Control: 32.00 ± 1.225; VPA: 50.00 ± 3.464; p= 0.0245) and in the number of total neurons $NeuN^+$ (Control: 515.4 ± 24.84; VPA: 744.0 ± 48.38; p= 0.0104). When analyzing the whole prelimbic area (upper layers + deeper layers), there was an increase in the number of total neurons $NeuN^+$ (Control: 853.0 ± 35.50; VPA: 1192 ± 57.98; p= 0.0039). There were no differences statistically significant among the other analysis in prelimbic area: at upper layers, number of CB^+ neurons (Control: 25.20 ± 3.929; VPA: 16.00 ± 4.882; p= 0.1909); at deeper layers, PV^+ ratio (Control: 0.06239 ± 0.003016; VPA: 0.05584 ± 0.008117; p= 0.4933), number of CB^+ neurons (Control: 23.20 ± 1.934; VPA: 26.75 ± 7.040; p= 0.6560); whole prelimbic area (upper layers + deeper layers), number of PV^+ neurons (Control: 64.40 ± 6.765; VPA: 78.25 ± 12.09; p= 0.3648), PV^+ ratio (Control: 0.07533 ± 0.006955; VPA: 0.06472 ± 0.008034; p= 0.3538), number of CB^+ neurons (Control: 48.40 ± 3.614; VPA: 42.75 ± 11.76; p= 0.6725), CB^+ ratio (Control: 0.06348 ± 0.009881; VPA: 0.03856 ± 0.007843; p= 0.0891).

3.4 VPA group presents abnormal proportion and distribution impairments of PV^+ and CB^+ interneurons across infralimbic layers

As shown in Table 3, at upper layers of infralimbic area, VPA group presented a decrease in both PV^+ ratio (Control: 0.08257 ± 0.004630; VPA: 0.06520 ± 0.001922; p= 0.0169) and CB^+ ratio (Control: 0.06557 ± 0.009221; VPA: 0.03151 ± 0.003986; p= 0.0173), as well as an increase in the number of total neurons $NeuN^+$ (Control: 349.0 ± 25.03; VPA: 481.0 ± 9.443; p= 0.0042). When analyzing the whole infralimbic area (upper layers + deeper layers), we can observe an increase in the number of total neurons $NeuN^+$ (Control: 909.6 ± 59.50; VPA: 1199 ± 50.58; p= 0.0076). There were no differences statistically significant among the other analyses in infralimbic area: upper layers, number of PV^+ neurons (Control: 28.80 ± 2.634; VPA: 36.25 ± 4.990; p= 0.2481), number of CB^+ neurons (Control: 20.80 ± 2.458; VPA: 14.25 ± 3.326; p= 0.1655), at deeper layers, number of total neurons $NeuN^+$ (Control: 560.6 ± 38.84; VPA: 718.0 ± 56.31; p= 0.0642), number of PV^+ neurons

(Control: 44.40 ± 4.179; VPA: 44.00 ± 6.658; p=0.9621), PV^+ ratio (Control: 0.08066 ± 0.009038; VPA: 0.06124 ± 0.007621; p= 0.1530), number of CB^+ neurons (Control: 29.80 ± 4.893; VPA: 26.25 ± 3.092; p= 0.5606), CB^+ ratio (Control: 0.05739 ± 0.01566; VPA: 0.04003 ± 0.004933; p=0.3411), whole infralimbic area (upper layers + deeper layers): number of PV^+ neurons (Control: 73.20 ± 4.903; VPA: 75.33 ± 5.783; p= 0.7904), PV^+ ratio (Control: 0.08161 ± 0.006711; VPA: 0.06278 ± 0.004230; p= 0.0556), number of CB^+ neurons (Control: 50.60 ± 7.264; VPA: 40.50 ± 6.397; p= 0.3314), CB^+ ratio (Control: 0.05982 ± 0.01294; VPA: 0.03655 ± 0.004274; p= 0.1503).

4 Discussion

When analyzing mPFC, the number of total neurons $NeuN^+$ in VPA group was increased when compared to control (Figure 1Ab), while neither the number of CB^+ or PV^+ neurons nor their proportions were altered (Figures 1Aa, 1Ac, 1Ba and 1Bc). When analyzing the subregions of mPFC (aCC, PrL and IL), as well as their upper and deeper layers, the same pattern concerning the number of total neurons $NeuN^+$ repeated: in VPA group, there was an increase of total neurons $NeuN^+$ when comparing to control group, with the exception of aCC (whole region) and IL (only deeper layers). This subregion analysis enabled us to investigate specific changes in cortical laminar organization through analysis of number and proportion of CB^+ and PV^+ interneurons across the layers. The number of PV^+ neurons in VPA group was increased in PrL when compared to control group (both in upper and deeper layers – Table 2); interestingly, the number of CB^+ neurons was decreased on aCC (deeper layers – Table 1). Then, we decided to normalize the number of interneurons (CB^+ or PV^+) by the number of total neurons $NeuN^+$ in order to obtain a ratio. By utilizing this approach, we can have an interesting insight about the E/I balance in the different regions (alterations on the proportion of inhibitory neurons related to total neurons $NeuN^+$ – mostly pyramidal excitatory – may cause significant impairments in the local neural circuits). Indeed, then we could see that prenatal exposure to VPA impaired the proportion of PV^+ and CB^+ interneurons within the total neurons scenario in many regions and layers. PV^+ ratio was decreased in aCC (both in the upper and deeper layers – Table 1), PrL (upper layers – Table 2) and IL (upper layers – Table 3). VPA affected in a more discreet way CB^+ proportion, once the same pattern of decreased ratio was observed only in PrL (upper layers – Table 2) and IL (upper layers – Table 3), while other sites had the same ratio of control group.

The increased number of total neurons $NeuN^+$ exhibited by VPA group in our work corroborates with data from literature: there is 79% more neurons on dlPFC and 29% more neurons on mesial prefrontal cortex (mPFC without aCC) in children with ASD (Zikopoulos and Barbas, 2010), as well

as a relative increase of 67% of them in PFC (Courchesne et al., 2011). Regardless of PV^+ interneuron analysis, it was already seen a decrease on the number of PV^+ interneurons on mPFC (Hashemi et al., 2016) and dlPFC (Zikopoulos and Barbas, 2013) both from ASD *post mortem* analysis, besides many findings about this interneurons in animal models of autism (Gogolla et al., 2009; Meyer et al., 2008; Powell et al., 2003); in contrast, little is known about CB^+ ones: a study suggest that, in ASD patients, there are no significant differences in the density of CB^+ neurons on dlPFC when compared to neurotypical individuals (Zikopoulos and Barbas, 2013) and, in animal models, there is a decrease of 50% of CB^+ neurons on frontoparietal cortex of *u*$PAR^{-/-}$ mice (Levitt et al., 2004). Here we show, for the first time to our knowledge, data from CB^+ analysis on mPFC of VPA model, as well as a deeper and thinner analysis both of CB^+ and PV^+ interneurons, once our data are from three different regions of mPFC and also from their upper and deeper layers. In this way, we could observe different patterns of impaired number and proportion of interneurons across regions and impaired lamination across mPFC layers (Figure 2).

Once there are no statistically significant differences between both groups concerning the absolute number of interneurons (PV^+ or CB^+) when analyzing the whole mPFC, we started to hypothesize that the prenatal exposure to VPA may not be affecting interneuron quantity *per se*. It might implicate more in route interneuron alterations to reach the cortex and intracortical areas than in cell loss during cellular generation - nonetheless, more studies investigating cell loss in different embryonic stages are needed. Since interneuron migration starts at E12.5 (Kelsom and Lu, 2013) and VPA insult is made also at E12.5, our hypothesis was that the prenatal exposure to VPA could be affecting interneuron migration.

Interneurons generation and specification occur in a transitory brain structure in the telencephalon called ganglionic eminence (GE), which is divided in medial (MGE), lateral (LGE) and caudal (CGE). It only exists anatomically during embryonic development (Corbin and Butt, 2011) and becomes evident approximately at E11.5 in rodents (O'Rahilly and Gardner, 1979). After proliferation and specification end in GE, interneurons start a tangential route of migration at E12.5 towards corticoestriatal junction and into the cortical wall. Then, they start the intracortical migration – which is completed only by birth – into the laminated cortex that was generated by pyramidal neurons (Faux et al., 2012). Many molecules have a pivotal role toward interneuron migration to cortical areas and adequate lamination in the cortex (Guo and Anton, 2014), as motogens, transcription factors, chemotactic factors and neurotransmitters.

Transcription factors of homeobox family such Sox6 and Lhx6 – which are expressed by MGE-derived interneurons - are crucial for specification (Azim et al., 2009; Batista-Brito et al., 2009; Butt et al., 2008; Liodis et al., 2007), migration to cortex (Gong et al., 2003; Lavdas et al., 1999), properly integration of interneurons into cortical layers (Zhao et al., 2008) and have already presented significant alterations in disorders related to ASD. Loss of Sox6 in MGE-derived interneurons results in accumulation of them out cortical areas (Azim et al., 2009; Batista-Brito et al., 2009). The mechanism by which Lhx6 promotes migration may be through promoting the expression of ErbB4 - a receptor for neuregulin 1 that is also involved in interneuron migration (Zhao et al., 2008). Once Sox6 and Lhx6 are necessary for migration, alterations in their gene expression probably affect interneuron migration. On our work, once we observed decreased number of CB^+ interneurons in aCC of VPA group - which may be due to migratory deficits - we hypothesized that VPA could be affecting the gene expression of Sox6 and Lhx6. Analyses in progress at our research group of Sox6 and Lhx6 will clarify the expression pattern of these two transcription factors in VPA model.

Also, the neurotransmitter GABA plays a critical role (Faux et al., 2012): GABA elicits a depolarizing response in $GABA_A$ receptors – which are expressed by migrating interneurons (Cuzon et al., 2006; Cuzon Carlson and Yeh, 2011; Lopez-Bendito et al., 2003). Impairments in GABA activity result in an accumulation of interneurons at the corticoestriatal region and prevent their entry into the cortical wall. On the other hand, the addition of GABA increases migration (Cuzon et al., 2006). Here we showed that VPA group had an increase in absolute number of PV^+ interneurons at PrL. One mechanism of action of VPA is by increasing the level of GABA on the brain (Baldino and Geller, 1981; Godin et al., 1969; Johannessen, 2000; Kukino and Deguchi, 1978). Thus, we can hypothesize that our result may be related indirectly to VPA mechanisms of action.

The fact that only some subregions and layers presented impairments on the absolute number and/or proportion of interneurons, while others did not, is intriguing. Also, in some subregions both CB^+ and PV^+ neurons presented impairments, whereas in others only PV^+ were affected. So, what can possibly explain that in some subregions migration has worked and in others it did not? Also, why in some subregions migration of CB^+ and PV^+ was different? In order to explain, we must consider the site where each interneuron is generated and the embryonic period of migratory onset specific to each interneuron subtype. Regarding site of origin, it is known that MGE originates PV^+ (Butt et al., 2005; Wonders and Anderson, 2006; Xu et al., 2004) and CB^+ (Kelsom and Lu, 2013), whilst CGE originates double bouquet cells (the majority class of CB^+ interneurons) (Butth et al., 2005; Pleasure et al., 2000). Thus, PV^+ and double bouquet cells are generated in different sites, MGE and CGE, respectively. Also,

we have to consider that within CB^+ population, there are two different sites of origin (CGE for double bouquet cells and MGE for the non-double bouquet ones). Concerning time of migratory onset, neurogenesis of MGE-derived interneurons in rodents starts around E9 and finishes around E16, while of CGE-derived ones starts around E12 (Miyoshi et al., 2007, 2010). Based on this, a subset of MGE-derived interneurons has already started migration process at E9, before VPA insult, and thus maybe this population is not too much affected by prenatal exposure to VPA. Also, at E16, there are still interneurons migrating to cortex; by this time, days after VPA insult, maybe this set of interneurons can reach the cortex in a more adequate cellular and molecular environment than the ones that try to migrate around E12.5, the day of VPA insult. Altogether, this observations and statements may shed some light why some regions presented specific interneuron subtype impairments. Also intriguing is the fact that the same pattern of damage that occurred in interneurons was not observed in the number of total neurons. Once the majority of total neurons are excitatory, it may be explained due to interneuron generation and origin site be independent from the excitatory ones (Mione et al., 1994; Parnavelas et al., 1991). This way, other molecules and time of migratory onset are involved in pyramidal excitatory neuronal migration.

Also, the same subtype of interneuron presented different patterns of alterations across subregions. So we asked if VPA exposure could be affecting the correct interneuron subregion localization. Albeit the molecular mechanisms regulating intracortical migration are largely unknown, potassium-chloride cotransporter 2 (KCC2) – which is localized at interneuron membrane - has a crucial role (Bortone and Polleux, 2009). The stop signal that ends migration is the upregulation of KCC2, resulting in interneuron hyperpolarization – the called shift between excitatory to inhibitory $GABA_A$ receptor (Bortone and Polleux, 2009). Rat cortical neurons exposed to VPA in cell culture resulted in downregulation of KCC2 messenger RNA (mRNA) expression. Interestingly, in the same study, mRNA expression of α4 $GABA_A$ subunit was upregulated by VPA, whilst its Rγ2 subunit was downregulated (Fukuchi et al., 2009). Altogether, VPA impairments on KCC2 and in α4 and Rγ2 $GABA_A$ subunits mRNA expression, besides the action of VPA in GABA metabolism itself, may be related to the interneuron subtype region-specific impairments seen in mPFC.

Cortical lamination is the final step on migratory process. In rodents, it starts around E11 and completes approximately at P14 (Faux et al., 2012). In our work, some impairments were present only on upper or deeper layers, not on both, indicative of impaired interneuron lamination. Nevertheless, how interneurons determine their very final position in the cortex is not known, but at least MGE-derived ones follow cues generated by cortical pyramidal cells (Hevner et al., 2004; Lodato et al., 2011;

Pla et al., 2006), which influence interneuron adequate lamination (Faux et al., 2012). Thus, we hypothesized that impairments in laminar distribution of PV^+ and CB^+ interneurons seen in this work may have an influence of excitatory cells (probably increased as already discussed).

Finally, the altered number of interneurons and impaired proportion of them within cortical neurons scenario probably have deep consequences on E/I balance, which is essential to properly execution of a set of PFC-dependent behaviors (Ferguson and Gao, 2018), as cognition, learning, memory, emotional behaviors (Turrigiano and Nelson, 2004) and social parameters. Disruptions on PFC E/I balance, as seen in ASD, during critical periods of embryonic development may be related to cognitive, social, behavioral and sensorial deficits found in ASD patients (Markram and Markram, 2010; Rubenstein, J.L.R & Merzenich, 2003; Rubenstein, 2010).

5 Conclusion

Prenatal exposure to VPA led to significant postnatal changes in mPFC, in both neuronal number and proportion, suggesting possible correlation with the social impairments observed in VPA model. Prenatal exposure to VPA may be related to alterations in interneuron migratory process towards cortical areas, once the composition of all results allowed us to observe different patterns of specific alterations in mPFC subregions. Besides, the alteration in the number of total neurons $NeuN^+$, which are mostly represented by pyramidal neurons, could demonstrate also the role of the excitatory component in this context. Our results contribute to demonstrate a general pattern of cortical disorganization, which, in final view, may have impact in E/I balance and lead to several outcomes. Also, these data contribute to elucidate key elements related to neural changes observed in ASD, as well as to the understanding of pathways involved in E/I imbalance. The complexity of results demonstrates the necessity to expand studies focusing on neuronal ontogeny in ASD context in order to clarify possible breaking points during development.

6 Conflict of Interest

Authors declare that there are no conflicts of interest.

7 Author Contributions

GC-C, JS-T, CG, MF-D, ID, GBS: experimental design and intellectual contribution. GC-C, JS-T, MF-D, ID, GBS: experimental procedures. GC-C, JS-T, CG, MF-D, ID, GBS: data discussion. GC-C, JS-T, CG: manuscript preparation.

8 Acknowledgments

This work was supported by National Institute of Science and Technology on NeuroImmunomodulation (INCT-NIM) #465489/2014-1, National Council of Technologic and Scientific Development (CNPq), Coordination for the Improvement of Higher Education Personnel (CAPES), Hospital de Clínicas de Porto Alegre (FIPE-HCPA) and PROPESQ-UFRGS.

9 References

American Psychiatric Association *Manual Diagnóstico e Estatístico de Transtornos Mentais - DSM-5*. doi:10.5007/1807-1384.2014v11n2p96.

Azim, E., Jabaudon, D., Fame, R. M., and Macklis, J. D. (2009). SOX6 controls dorsal progenitor identity and interneuron diversity during neocortical development. *Nat. Neurosci.* 12, 1238–1247. doi:10.1038/nn.2387.

Baldino, F., and Geller, H. M. (1981). Effect of sodium valporate on hypothalamic neurons in vivo and in vitro. *Brain Res.* 219, 231–237. doi:10.1016/0006-8993(81)90288-2.

Bambini-Junior, V., Rodrigues, L., Behr, G. A., Moreira, J. C. F., Riesgo, R., and Gottfried, C. (2011). Animal model of autism induced by prenatal exposure to valproate: Behavioral changes and liver parameters. *Brain Res.* 1408, 8–16. doi:10.1016/J.BRAINRES.2011.06.015.

Bambini-Junior, V., Zanatta, G., Della Flora Nunes, G., Mueller de Melo, G., Michels, M., Fontes-Dutra, M., et al. (2014). Resveratrol prevents social deficts in animal model of autism induced by valproic acid. *Neurosci. Lett.* 583, 176–181. doi:10.1016/j.neulet.2014.09.039.

Baron-Cohen, S., and Wheelwright, S. (2004). The Empathy Quotient: An Investigation of Adults with Asperger Syndrome or High Functioning Autism, and Normal Sex Differences. *J. Autism Dev. Disord.* 34, 163–175. doi:10.1023/B:JADD.0000022607.19833.00.

Batista-Brito, R., Rossignol, E., Hjerling-Leffler, J., Denaxa, M., Wegner, M., Lefebvre, V., et al. (2009). The Cell-Intrinsic Requirement of Sox6 for Cortical Interneuron Development. *Neuron* 63, 466–481. doi:10.1016/J.NEURON.2009.08.005.

Beversdorf, D. Q. (2016). Phenotyping, etiological factors, and biomarkers: Toward precision medicine in autism spectrum disorders. *J. Dev. Behav. Pediatr.* 37, 659–673. doi:10.1097/DBP.0000000000000351.

Bortone, D., and Polleux, F. (2009). KCC2 Expression Promotes the Termination of Cortical Interneuron Migration in a Voltage-Sensitive Calcium-Dependent Manner. *Neuron* 62, 53–71. doi:10.1016/J.NEURON.2009.01.034.

Butt, S. J. B., Fuccillo, M., Nery, S., Noctor, S., Kriegstein, A., Corbin, J. G., et al. (2005). The Temporal and Spatial Origins of Cortical Interneurons Predict Their Physiological Subtype.

Neuron 48, 591–604. doi:10.1016/J.NEURON.2005.09.034.

Butt, S. J. B., Sousa, V. H., Fuccillo, M. V., Hjerling-Leffler, J., Miyoshi, G., Kimura, S., et al. (2008). The Requirement of Nkx2-1 in the Temporal Specification of Cortical Interneuron Subtypes. *Neuron* 59, 722–732. doi:10.1016/J.NEURON.2008.07.031.

Buzsaki, G., Draughn, A., Buzsáki, G., Draguhn, A., Buzsaki, G., and Draughn, A. (2004). Neuronal Oscillations in Cortical Networks. *Science (80-.).* 304, 1926–1930. doi:10.1126/science.1099745.

Caglayan, A. O. (2010). Genetic causes of syndromic and non-syndromic autism. *Dev. Med. Child Neurol.* 52, 130–138. doi:10.1111/j.1469-8749.2009.03523.x.

Cao, W., Lin, S., Xia, Q. qiang, Du, Y. lan, Yang, Q., Zhang, M. ying, et al. (2018). Gamma Oscillation Dysfunction in mPFC Leads to Social Deficits in Neuroligin 3 R451C Knockin Mice. *Neuron* 97, 1–8. doi:10.1016/j.neuron.2018.04.025.

Cardin, J. A., Carlén, M., Meletis, K., Knoblich, U., Zhang, F., Deisseroth, K., et al. (2009). Driving fast-spiking cells induces gamma rhythm and controls sensory responses. *Nature* 459, 663–667. doi:10.1038/nature08002.

Carlén, M. (2017). What constitutes the prefrontal cortex? *Sci.* 358, 478–482. doi:10.1126/science.aan8868.

Christensen, D. L., Braun, K. V. N., Baio, J., Bilder, D., Charles, J., Constantino, J. N., et al. (2018). Prevalence and Characteristics of Autism Spectrum Disorder Among Children Aged 8 Years - Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2012. *Morb. Mortal. Wkly. Report. Surveill. Summ. (Washington, D.C. 2002)* 65, 1–23. doi:10.15585/mmwr.ss6513a1.

Christensen, J., Grnøborg, T. K., Srøensen, M. J., Schendel, D., Parner, E. T., Pedersen, L. H., et al. (2013). Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. *JAMA - J. Am. Med. Assoc.* 309, 1696–1703. doi:10.1001/jama.2013.2270.

Cline, H. (2005). Synaptogenesis: A Balancing Act between Excitation and Inhibition. *Curr. Biol.* 15, R203–R205. doi:10.1016/J.CUB.2005.03.010.

Corbin, J. G., and Butt, S. J. B. (2011). Developmental mechanisms for the generation of telencephalic interneurons. *Dev. Neurobiol.* 71, 710–732. doi:10.1002/dneu.20890.

Courchesne, E., Mouton, P. R., Calhoun, M. E., Semendeferi, K., Ahrens-Barbeau, C., Hallet, M. J., et al. (2011). Neuron Number and Size in Prefrontal Cortex of Children With Autism. *JAMA* 306, 2001–2010. doi:10.1001/jama.2011.1638.

Cuzon Carlson, V. C., and Yeh, H. H. (2011). GABAA Receptor Subunit Profiles of Tangentially Migrating Neurons Derived From the Medial Ganglionic Eminence. *Cereb. Cortex* 21, 1792–1802. doi:10.1093/cercor/bhq247.

Cuzon, V. C., Yeh, P. W., Cheng, Q., and Yeh, H. H. (2006). Ambient GABA Promotes Cortical

Entry of Tangentially Migrating Cells Derived from the Medial Ganglionic Eminence. *Cereb. Cortex* 16, 1377–1388. doi:10.1093/cercor/bhj084.

DeFelipe, J. (1999). Chandelier cells and epilepsy. *Brain* 122, 1807–1822. doi:10.1093/brain/122.10.1807.

Dichter, M. A., and Ayala, G. F. (1987). Cellular mechanisms of epilepsy: A Status report. *Science (80-.).* 237, 157–164. doi:10.1126/science.3037700.

El-Fishawy, P., and State, M. W. (2010). The Genetics of Autism: Key Issues, Recent Findings, and Clinical Implications. *Psychiatr. Clin. North Am.* 33, 83–105. doi:10.1016/J.PSC.2009.12.002.

Faux, C., Rakic, S., Andrews, W., and Britto, J. M. (2012). Neurons on the Move: Migration and Lamination of Cortical Interneurons. *Neurosignals* 20, 168–189. doi:10.1159/000334489.

Ferguson, B. R., and Gao, W.-J. (2018). PV Interneurons: Critical Regulators of E/I Balance for Prefrontal Cortex-Dependent Behavior and Psychiatric Disorders. *Front. Neural Circuits* 12, 1–13. doi:10.3389/fncir.2018.00037.

Fontes-Dutra, M., Santos-Terra, J., Deckmann, I., Schwingel, G. B., Nunes, G. D. F., Hirsch, M. M., et al. (2018). Resveratrol prevents cellular and behavioral sensory alterations in the animal model of autism induced by valproic acid. *Front. Synaptic Neurosci.* 10, 1–12. doi:10.3389/fnsyn.2018.00009.

Fukuchi, M., Nii, T., Ishimaru, N., Minamino, A., Hara, D., Takasaki, I., et al. (2009). Valproic acid induces up- or down-regulation of gene expression responsible for the neuronal excitation and inhibition in rat cortical neurons through its epigenetic actions. *Neurosci. Res.* 65, 35–43. doi:10.1016/j.neures.2009.05.002.

Gaetz, W., Edgar, J. C., Wang, D. J., and Roberts, T. P. L. (2011). Relating MEG measured motor cortical oscillations to resting γ-Aminobutyric acid (GABA) concentration. *Neuroimage* 55, 616–621. doi:10.1016/J.NEUROIMAGE.2010.12.077.

Geschwind, D. H. (2011). Genetics of autism spectrum disorders. *Trends Cogn. Sci.* 15, 409–16. doi:10.1016/j.tics.2011.07.003.

Godin, Y., Heiner, L., Mark, J., and Mandel, P. (1969). Effects of di-n-propylacetate, and anticonvulsive compound, on GABA metabolism. *J. Neurochem.* 16, 869–873. doi:10.1111/j.1471-4159.1969.tb08975.x.

Gogolla, N., LeBlanc, J. J., Quast, K. B., Südhof, T. C., Fagiolini, M., and Hensch, T. K. (2009). Common circuit defect of excitatory-inhibitory balance in mouse models of autism. *J. Neurodev. Disord.* 1, 172–181. doi:10.1007/s11689-009-9023-x.

Gong, S., Zheng, C., Doughty, M. L., Losos, K., Didkovsky, N., Schambra, U. B., et al. (2003). A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature* 425, 917–925. doi:10.1038/nature02033.

Gottfried, C., Bambini-Junior, V., Baronio, D., Zanatta, G., Silvestrin, R. B., Vaccaro, T., et al.

(2013). Valproic Acid in Autism Spectrum Disorder: From an Environmental Risk Factor to a Reliable Animal Model. *Recent Adv. Autism Spectr. Disord.* 1, 64. doi:10.5772/32009.

Guo, J., and Anton, E. S. (2014). Decision making during interneuron migration in the developing cerebral cortex. *Trends Cell Biol.* 24, 342–351. doi:10.1016/J.TCB.2013.12.001.

Hashemi, E., Ariza, J., Rogers, H., Noctor, S. C., and Martínez-Cerdeño, V. (2016). The Number of Parvalbumin-Expressing Interneurons Is Decreased in the Medial Prefrontal Cortex in Autism. *Cereb. Cortex*, 1–13. doi:10.1093/cercor/bhw021.

Hevner, R. ., Daza, R. A. ., Englund, C., Kohtz, J., and Fink, A. (2004). Postnatal shifts of interneuron position in the neocortex of normal and reeler mice: evidence for inward radial migration. *Neuroscience* 124, 605–618. doi:10.1016/J.NEUROSCIENCE.2003.11.033.

Hirsch, M. M., Deckmann, I., Fontes-Dutra, M., Bauer-Negrini, G., Della-Flora Nunes, G., Nunes, W., et al. (2018). Behavioral alterations in autism model induced by valproic acid and translational analysis of circulating microRNA. *Food Chem. Toxicol.* 115, 336–343. doi:10.1016/J.FCT.2018.02.061.

Howard, M. W., Rizzuto, D. S., Caplan, J. B., Madsen, J. R., Lisman, J., Aschenbrenner-Scheibe, R., et al. (2003). Gamma Oscillations Correlate with Working Memory Load in Humans. *Cereb. Cortex* 13, 1369–1374. doi:10.1093/cercor/bhg084.

Isaacson, J. S., and Scanziani, M. (2011). How inhibition shapes cortical activity. *Neuron* 72, 231–243. doi:10.1016/j.neuron.2011.09.027.

Johannessen, C. U. (2000). Mechanisms of action of valproate: A commentatory. *Neurochem. Int.* 37, 103–110. doi:10.1016/S0197-0186(00)00013-9.

Jones, A. P., Happé, F. G. E., Gilbert, F., Burnett, S., and Viding, E. (2010). Feeling, caring, knowing: different types of empathy deficit in boys with psychopathic tendencies and autism spectrum disorder. *J. Child Psychol. Psychiatry* 51, 1188–1197. doi:10.1111/j.1469-7610.2010.02280.x.

Kawaguchi, Y., Katsumaru, H., Kosaka, T., Heizmann, C. W., and Hama, K. (1987). Fast spiking cells in rat hippocampus (CA1 region) contain the calcium-binding protein parvalbumin. *Brain Res.* doi:10.1016/0006-8993(87)90921-8.

Kawaguchi, Y., and Kubota, Y. (1997). GABAergic cell subtypes and their synaptic connections in rat frontal cortex. *Cereb. Cortex* 7, 476–486. doi:10.1093/cercor/7.6.476.

Kelsom, C., and Lu, W. (2013). Development and specification of GABAergic cortical interneurons. *Cell Biosci.* 3, 1–19. doi:10.1186/2045-3701-3-19.

Klausberger, T., Magill, P. J., Márton, L. F., Roberts, J. D. B., Cobden, P. M., Buzsáki, G., et al. (2003). Brain-state- and cell-type-specific firing of hippocampal interneurons in vivo. *Nature* 421, 844–848. doi:10.1038/nature01374.

Klin, A., Lin, D. J., Gorrindo, P., Ramsay, G., and Jones, W. (2009). Two-year-olds with autism

orient to non-social contingencies rather than biological motion. *Nature* 459, 257–261. doi:10.1038/nature07868.

KUKINO, K., and DEGUCHI, T. (1978). Anticonvulsant activity and effects of sodium dipropylacetate on cerebral 5-hydroxytryptamine and GABA in reserpinized mice. *Chem. Pharm. Bull. (Tokyo).* 26, 3551–3555. doi:10.1248/cpb.26.3551.

Lavdas, A. A., Grigoriou, M., Pachnis, V., and Parnavelas, J. G. (1999). The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. *J. Neurosci.* 19, 7881–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10479690 [Accessed June 2, 2019].

Levitt, P., Eagleson, K. L., and Powell, E. M. (2004). Regulation of neocortical interneuron development and the implications for neurodevelopmental disorders. *Trends Neurosci.* 27, 400–406. doi:10.1016/J.TINS.2004.05.008.

Liodis, P., Denaxa, M., Grigoriou, M., Akufo-Addo, C., Yanagawa, Y., and Pachnis, V. (2007). Lhx6 Activity Is Required for the Normal Migration and Specification of Cortical Interneuron Subtypes. *J. Neurosci.* 27, 3078–3089. doi:10.1523/JNEUROSCI.3055-06.2007.

Lodato, S., Rouaux, C., Quast, K. B., Jantrachotechatchawan, C., Studer, M., Hensch, T. K., et al. (2011). Excitatory Projection Neuron Subtypes Control the Distribution of Local Inhibitory Interneurons in the Cerebral Cortex. *Neuron* 69, 763–779. doi:10.1016/J.NEURON.2011.01.015.

Lopez-Bendito, G., Luján, R., Shigemoto, R., Ganter, P., Paulsen, O., and Molnár, Z. (2003). Blockade of GABAB Receptors Alters the Tangential Migration of Cortical Neurons. *Cereb. Cortex* 13, 932–942. doi:10.1093/cercor/13.9.932.

Lundqvist, M., Rose, J., Herman, P., Brincat, S. L. L., Buschman, T. J. J., and Miller, E. K. K. (2016). Gamma and Beta Bursts Underlie Working Memory. *Neuron* 90, 1–13. doi:10.1016/j.neuron.2016.02.028.

Lyall, K., Croen, L., Daniels, J., Fallin, M. D., Ladd-Acosta, C., Lee, B., et al. (2017). The Changing Epidemiology of Autism Spectrum Disorders. *Annu. Rev. Public Health* 38, 81–102. doi:10.1146/annurev-publhealth-031816-044318.

Markram, K., and Markram, H. (2010). The Intense World Theory – A Unifying Theory of the Neurobiology of Autism. *Front. Hum. Neurosci.* 4, 1–29. doi:10.3389/fnhum.2010.00224.

McNally, J. M., and McCarley, R. W. (2016). Gamma band oscillations: A key to understanding schizophrenia symptoms and neural circuit abnormalities. *Curr. Opin. Psychiatry* 29, 202–210. doi:10.1097/YCO.0000000000000244.

Meyer, U., Nyffeler, M., Yee, B. K., Knuesel, I., and Feldon, J. (2008). Adult brain and behavioral pathological markers of prenatal immune challenge during early/middle and late fetal development in mice. *Brain. Behav. Immun.* 22, 469–486. doi:10.1016/J.BBI.2007.09.012.

Mione, M. C., Danevic, C., Boardman, P., Harris, B., and Parnavelas, J. G. (1994). Lineage analysis

559 reveals neurotransmitter (GABA or glutamate) but not calcium-binding protein homogeneity in
560 clonally related cortical neurons. *J. Neurosci.* 14, 107–23. doi:10.1523/JNEUROSCI.14-01-
561 00107.1994.

562 Miyoshi, G., Butt, S. J. B., Takebayashi, H., and Fishell, G. (2007). Physiologically Distinct
563 Temporal Cohorts of Cortical Interneurons Arise from Telencephalic Olig2-Expressing
564 Precursors. *J. Neurosci.* 27, 7786–7798. doi:10.1523/JNEUROSCI.1807-07.2007.

565 Miyoshi, G., Hjerling-Leffler, J., Karayannis, T., Sousa, V. H., Butt, S. J. B., Battiste, J., et al.
566 (2010). Genetic fate mapping reveals that the caudal ganglionic eminence produces a large and
567 diverse population of superficial cortical interneurons. *J. Neurosci.* 30, 1582–94.
568 doi:10.1523/JNEUROSCI.4515-09.2010.

569 Nelson, S. B., and Valakh, V. (2015). Excitatory/Inhibitory Balance and Circuit Homeostasis in
570 Autism Spectrum Disorders. *Neuron* 87, 684–698. doi:10.1016/j.neuron.2015.07.033.

571 O'Rahilly, R., and Gardner, E. (1979). The initial development of the human brain. *Cells Tissues*
572 *Organs* 104, 123–133. doi:10.1159/000145061.

573 Osterling, J., and Dawson, G. (1994). Early recognition of children with autism: A study of first
574 birthday home videotapes. *J. Autism Dev. Disord.* 24, 247–257. doi:10.1007/BF02172225.

575 Parnavelas, J. G., Barfield, J. A., Franke, E., and Luskin, M. B. (1991). Separate Progenitor Cells
576 Give Rise to Pyramidal and Nonpyramidal Neurons in the Rat Telencephalon. *Cereb. Cortex* 1,
577 463–468. doi:10.1093/cercor/1.6.463.

578 Pla, R., Borrell, V., Flames, N., and Marín, O. (2006). Layer acquisition by cortical GABAergic
579 interneurons is independent of Reelin signaling. *J. Neurosci.* 26, 6924–34.
580 doi:10.1523/JNEUROSCI.0245-06.2006.

581 Pleasure, S. J., Anderson, S., Hevner, R., Bagri, A., Marin, O., Lowenstein, D. H., et al. (2000). Cell
582 Migration from the Ganglionic Eminences Is Required for the Development of Hippocampal
583 GABAergic Interneurons. *Neuron* 28, 727–740. doi:10.1016/S0896-6273(00)00149-5.

584 Polleux, F., and Lauder, J. M. (2004). Toward a developmental neurobiology of autism. *Ment.*
585 *Retard. Dev. Disabil. Res. Rev.* 10, 303–17. doi:10.1002/mrdd.20044.

586 Pouille, F., and Scanziani, M. (2001). Enforcement of temporal fidelity in pyramidal cells by somatic
587 feed-forward inhibition. *Science (80-.).* 293, 1159–1163. doi:10.1126/science.1060342.

588 Powell, E. M., Campbell, D. B., Stanwood, G. D., Davis, C., Noebels, J. L., and Levitt, P. (2003).
589 Genetic disruption of cortical interneuron development causes region- and GABA cell type-
590 specific deficits, epilepsy, and behavioral dysfunction. *J. Neurosci.* 23, 622–631.

591 R. Muhle, S. V. Trentacoste, I. R. (2004). The genetics of autism. *Pediatrics* 113, 472–486.
592 doi:10.1016/j.spen.2004.07.003.

593 Risch, N., Hoffmann, T. J., Anderson, M., Croen, L. A., Grether, J. K., and Windham, G. C. (2014).
594 Familial recurrence of autism spectrum disorder: Evaluating genetic and environmental

595 contributions. *Am. J. Psychiatry* 171, 1206–1213. doi:10.1176/appi.ajp.2014.13101359.

596 Rodier, P. M., Ingram, J. L., Tisdale, B., Nelson, S., and Romano, J. (1996). Embryological origin for
597 autism: Developmental anomalies of the cranial nerve motor nuclei. *J. Comp. Neurol.* 370, 247–
598 261. doi:10.1002/(SICI)1096-9861(19960624)370:2<247::AID-CNE8>3.0.CO;2-2.

599 Rojas, D. C., and Wilson, L. B. (2014). γ-band abnormalities as markers of autism spectrum
600 disorders. *Biomark. Med.* 8, 353–368. doi:10.2217/bmm.14.15.

601 Roullet, F. I., Lai, J. K. Y., and Foster, J. A. (2013). In utero exposure to valproic acid and autism - A
602 current review of clinical and animal studies. *Neurotoxicol. Teratol.* 36, 47–56.
603 doi:10.1016/j.ntt.2013.01.004.

604 Rubenstein, J.L.R & Merzenich, M. M. (2003). Model of autism: increased ratio of
605 excitation/inhibition in key neural systems. *Genes, Brain Behav.* 2, 255–267.
606 doi:10.1046/j.1601-183X.2003.00037.x.

607 Rubenstein, J. L. R. (2010). Three hypotheses for developmental defects that may underlie some
608 forms of autism spectrum disorder. *Curr. Opin. Neurol.* 23, 118–123.
609 doi:10.1097/WCO.0b013e328336eb13.

610 Schneider, T., and Przewłocki, R. (2005). Behavioral alterations in rats prenatally to valproic acid:
611 Animal model of autism. *Neuropsychopharmacology* 30, 80–89. doi:10.1038/sj.npp.1300518.

612 Sealey, L. A., Hughes, B. W., Sriskanda, A. N., Guest, J. R., Gibson, A. D., Johnson-Williams, L., et
613 al. (2016). Environmental factors in the development of autism spectrum disorders. *Environ. Int.*
614 88, 288–298. doi:10.1016/j.envint.2015.12.021.

615 Seamans, J. K., Lapish, C. C., and Durstewitz, D. (2008). Comparing the prefrontal cortex of rats and
616 primates: Insights from electrophysiology. *Neurotox. Res.* 14, 249–262.
617 doi:10.1007/BF03033814.

618 Sohal, V. S., Zhang, F., Yizhar, O., and Deisseroth, K. (2009). Parvalbumin neurons and gamma
619 rhythms enhance cortical circuit performance. *Nature* 459, 698–702. doi:10.1038/nature07991.

620 Tuchman, R., and Cuccaro, M. (2011). Epilepsy and autism: Neurodevelopmental perspective. *Curr.*
621 *Neurol. Neurosci. Rep.* 11, 428–434. doi:10.1007/s11910-011-0195-x.

622 Tuchman, R., and Rapin, I. (2002). Epilepsy in autism. *Lancet Neurol.* 1, 352–358.
623 doi:10.1016/S1474-4422(02)00160-6.

624 Turrigiano, G. G., and Nelson, S. B. (2004). Homeostatic plasticity in the developing nervous
625 system. *Nat. Rev. Neurosci.* 5, 97–107. doi:10.1038/nrn1327.

626 Uylings, H. B. M., Groenewegen, H. J., and Kolb, B. (2003). Do rats have a prefrontal cortex?
627 *Behav. Brain Res.* 146, 3–17. doi:10.1016/J.BBR.2003.09.028.

628 Welsh, J. P., and Estes, A. M. (2018). Exploring the social brain. *Elife* 7. doi:10.7554/eLife.35392.

629 Wilson, T. W., Rojas, D. C., Reite, M. L., Teale, P. D., and Rogers, S. J. (2007). Children and

Adolescents with Autism Exhibit Reduced MEG Steady-State Gamma Responses. *Biol. Psychiatry* 62, 192–197. doi:10.1016/J.BIOPSYCH.2006.07.002.

Wonders, C. P., and Anderson, S. A. (2006). The origin and specification of cortical interneurons. *Nat. Rev. Neurosci.* 7, 687–96. doi:10.1038/nrn1954.

Xu, Q., Cobos, I., De La Cruz, E., Rubenstein, J. L., and Anderson, S. A. (2004). Origins of Cortical Interneuron Subtypes. *J. Neurosci.* 24, 2612–2622. doi:10.1523/JNEUROSCI.5667-03.2004.

Yenkoyan, K., Grigoryan, A., Fereshetyan, K., and Yepremyan, D. (2017). Advances in understanding the pathophysiology of autism spectrum disorders. *Behav. Brain Res.* 331, 92–101. doi:10.1016/j.bbr.2017.04.038.

Yizhar, O., Fenno, L. E., Prigge, M., Schneider, F., Davidson, T. J., O'Shea, D. J., et al. (2011). Neocortical excitation/inhibition balance in information processing and social dysfunction. *Nature* 477, 171–178. doi:10.1038/nature10360.

Zhao, Y., Flandin, P., Long, J. E., Cuesta, M. Dela, Westphal, H., and Rubenstein, J. L. R. (2008). Distinct molecular pathways for development of telencephalic interneuron subtypes revealed through analysis of Lhx6 mutants. *J. Comp. Neurol.* 510, 79–99. doi:10.1002/CNE.21772.

Zikopoulos, B., and Barbas, H. (2010). Changes in prefrontal axons may disrupt the network in autism. *J. Neurosci.* 30, 14595–609. doi:10.1523/JNEUROSCI.2257-10.2010.

Zikopoulos, B., and Barbas, H. (2013). Altered neural connectivity in excitatory and inhibitory cortical circuits in autism. *Front. Hum. Neurosci.* 7, 609. doi:10.3389/fnhum.2013.00609.

Legends of Figures

Panel 1. **Timeline of experiments.** Single dose of VPA (600 mg/kg, i.p.). Only offspring male rats were utilized for histological analysis. E: embryonic day. P: postnatal day. (CEUA-UFRGS#140367). n=3-5 (in litters).

Panel 2. **Representative image of immunofluorescence assays.** (A) Merge image of PV^+ (green), $NeuN^+$ (red) and DAPI (blue) in Control and VPA groups. (B) Merge image of CB^+ (green), $NeuN^+$ (red) and DAPI (blue) in Control and VPA groups. These images, specifically, were chosen in an illustrative purpose.

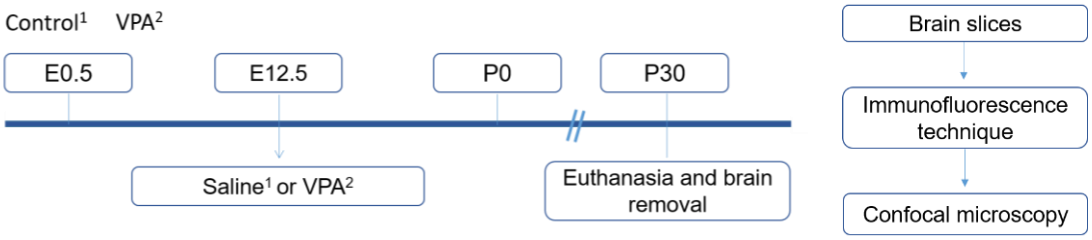
Figure 1. **Increased number of total neurons $NeuN^+$ on mPFC of VPA group when compared to control group.** Analysis of PV^+ neurons (A) and CB^+ neurons (B). Values are plotted as mean ± standard deviation. n= 4-5 (in litters). Statistical analysis: Student's *t* Test followed by Welch's correction. *p<0.05. **p<0,01.

Figure 2. **Schematic representation of the results obtained in this work.** Illustrative picture of a brain hemisphere, highlighting mPFC (gray area) and with ampliation of its subregions (aCC, PrL and IL).

678 **Figures**

679 Panel 1

680 681 682



683

684

685

686

687

688

689

690

691

692

693

694

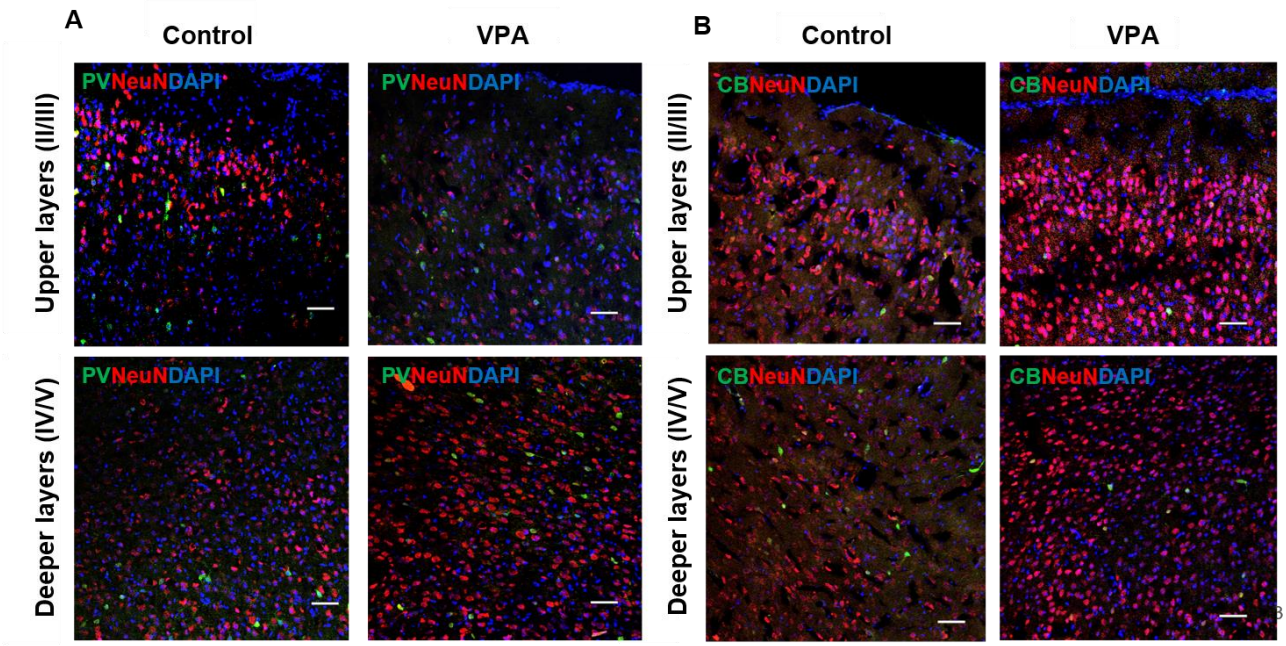
695

696

697

698

699 Panel 2



700

701

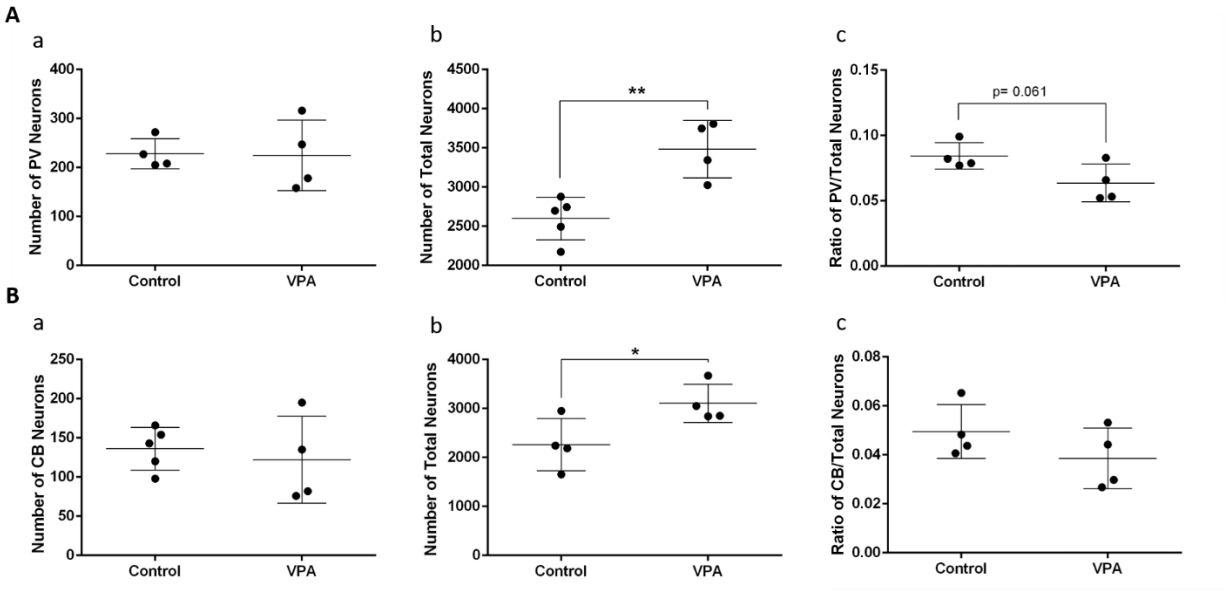
702

703

704

705

706 Figure 1



707

Figure 2

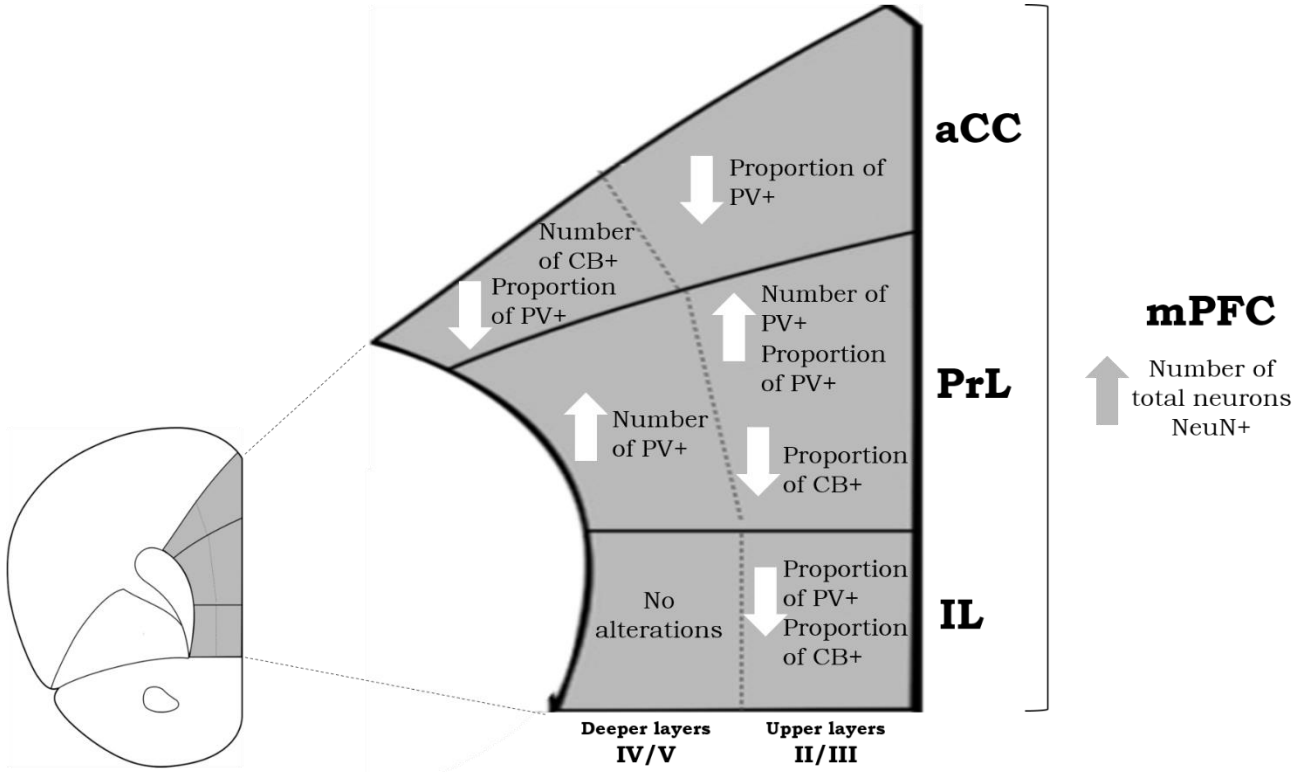

aCC
Proportion of PV+
Number of CB+
Proportion of PV+
Number of PV+
Proportion of PV+
PrL
Number of PV+
Proportion of CB+
No alterations
Proportion of PV+
Proportion of CB+
IL
Deeper layers IV/V
Upper layers II/III
mPFC
Number of total neurons NeuN+

Table 1. VPA group displays abnormal proportion and distribution impairments of PV^+ and CB^+ interneurons across aCC layers

Region	Parameter	Control	VPA	p
Upper Layers	total neurons	350.8 ± 29.06	435.5 ± 64.43	0.2938
	PV+ neurons	29.20 ± 1.934	26.00 ± 4.041	0.5275
	Ratio (PV+/total neurons)	0.08400 ± 0.003180	0.05513 ± 0.002124	**0.0003*****
	CB+ neurons	15.20 ± 4.128	16.00 ± 4.899	0.9045
	Ratio (CB+/total neurons)	0.05189 ± 0.005582	0.04192 ± 0.008916	0.7866
Deeper Layers	total neurons	484.6 ± 31.66	655.0 ± 57.99	0.0521
	PV+ neurons	47.20 ± 7.172	44.33 ± 8.452	0.8069
	Ratio (PV+/total neurons)	0.09694 ± 0.01337	0.05653 ± 0.009855	**0.0460***
	CB+ neurons	47.20 ± 7.172	22.75 ± 5.006	**0.0278***
	Ratio (CB+/total neurons)	0.06139 ± 0.009970	0.03932 ± 0.005643	0.1152
Whole aCC	total neurons	872.5 ± 51.32	1161 ± 137.3	0.1587
	PV+ neurons	84.25 ± 5.808	70.33 ± 12.47	0.3891
	Ratio (PV+/total neurons)	0.09678 ± 0.005418	0.06008 ± 0.005611	**0.0061****
	CB+ neurons	37.20 ± 6.224	38.75 ± 9.886	0.8994
	Ratio (CB+/total neurons)	0.04965 ± 0.007097	0.04026 ± 0.006774	0.3710

Values are plotted as mean ± standard deviation. n= 3-5 (in litters). Statistical analysis: Student's *t* Test followed by Welch's correction. *p<0.05. **p<0.01 ***p<0.001.

Table 2. VPA group presents abnormal proportion and distribution impairments of PV^+ and CB^+ interneurons across prelimbic layers

Region	Parameter	Control	VPA	p
Upper Layers	total neurons	337.6 ± 21.97	448.3 ± 19.92	**0.0074****
	PV+ neurons	28.80 ± 3.625	40.00 ± 2.000	**0.0369***
	Ratio (PV+/total neurons)	0.07180 ± 0.0008816	0.08789 ± 0.001814	**0.0044****
	CB+ neurons	25.20 ± 3.929	16.00 ± 4.882	0.1909
	Ratio (CB+/total neurons)	0.08041 ± 0.01349	0.03512 ± 0.007272	**0.0255***
Deeper Layers	total neurons	515.4 ± 24.84	744.0 ± 48.38	**0.0104***
	PV+ neurons	32.00 ± 1.225	50.00 ± 3.464	**0.0245***
	Ratio (PV+/total neurons)	0.06239 ± 0.003016	0.05584 ± 0.008117	0.4933
	CB+ neurons	23.20 ± 1.934	26.75 ± 7.040	0.6560
	Ratio (CB+/total neurons)	0.05155 ± 0.009063	0.04130 ± 0.008741	0.4430
Whole PrL	total neurons	853.0 ± 35.50	1192 ± 57.98	**0.0039****
	PV+ neurons	64.40 ± 6.765	78.25 ± 12.09	0.3648
	Ratio (PV+/total neurons)	0.07533 ± 0.006955	0.06472 ± 0.008034	0.3538
	CB+ neurons	48.40 ± 3.614	42.75 ± 11.76	0.6725
	Ratio (CB+/total neurons)	0.06348 ± 0.009881	0.03856 ± 0.007843	0.0891

Values are plotted as mean ± standard deviation. n= 3-5 (in litters). Statistical analysis: Student's *t* Test followed by Welch's correction. *p<0.05. **p<0.01.

Table 3. VPA group presents abnormal proportion and distribution impairments of PV^+ and CB^+ interneurons across infralimbic layers

Region	Parameter	Control	VPA	p
Upper Layers	total neurons	349.0 ± 25.03	481.0 ± 9.443	**0.0042****
	PV+ neurons	28.80 ± 2.634	36.25 ± 4.990	0.2481
	Ratio (PV+/total neurons)	0.08257 ± 0.004630	0.06520 ± 0.001922	**0.0169***
	CB+ neurons	20.80 ± 2.458	14.25 ± 3.326	0.1655
	Ratio (CB+/total neurons)	0.06557 ± 0.009221	0.03151 ± 0.003986	**0.0173***
Deeper Layers	total neurons	560.6 ± 38.84	718.0 ± 56.31	0.0642
	PV+ neurons	44.40 ± 4.179	44.00 ± 6.658	0.9621
	Ratio (PV+/total neurons)	0.08066 ± 0.009038	0.06124 ± 0.007621	0.1530
	CB+ neurons	29.80 ± 4.893	26.25 ± 3.092	0.5606
	Ratio (CB+/total neurons)	0.05739 ± 0.01566	0.04003 ± 0.004933	0.3411
Whole IL	total neurons	909.6 ± 59.50	1199 ± 50.58	**0.0076****
	PV+ neurons	73.20 ± 4.903	75.33 ± 5.783	0.7904
	Ratio (PV+/total neurons)	0.08161 ± 0.006711	0.06278 ± 0.004230	0.0556
	CB+ neurons	50.60 ± 7.264	40.50 ± 6.397	0.3314
	Ratio (CB+/total neurons)	0.05982 ± 0.01294	0.03655 ± 0.004274	0.1503

Values are plotted as mean ± standard deviation. n= 3-5 (in litters). Statistical analysis: Student's *t* Test followed by Welch's correction. *p<0.05. **p<0.01.

Supplementary Figures

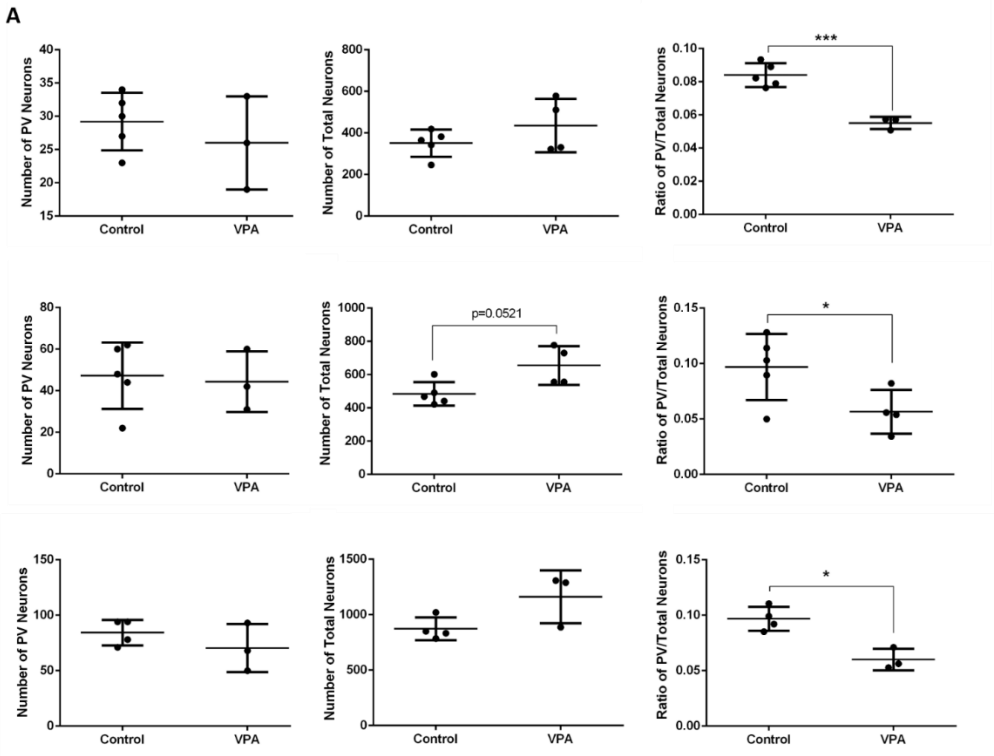
Legends of Figures

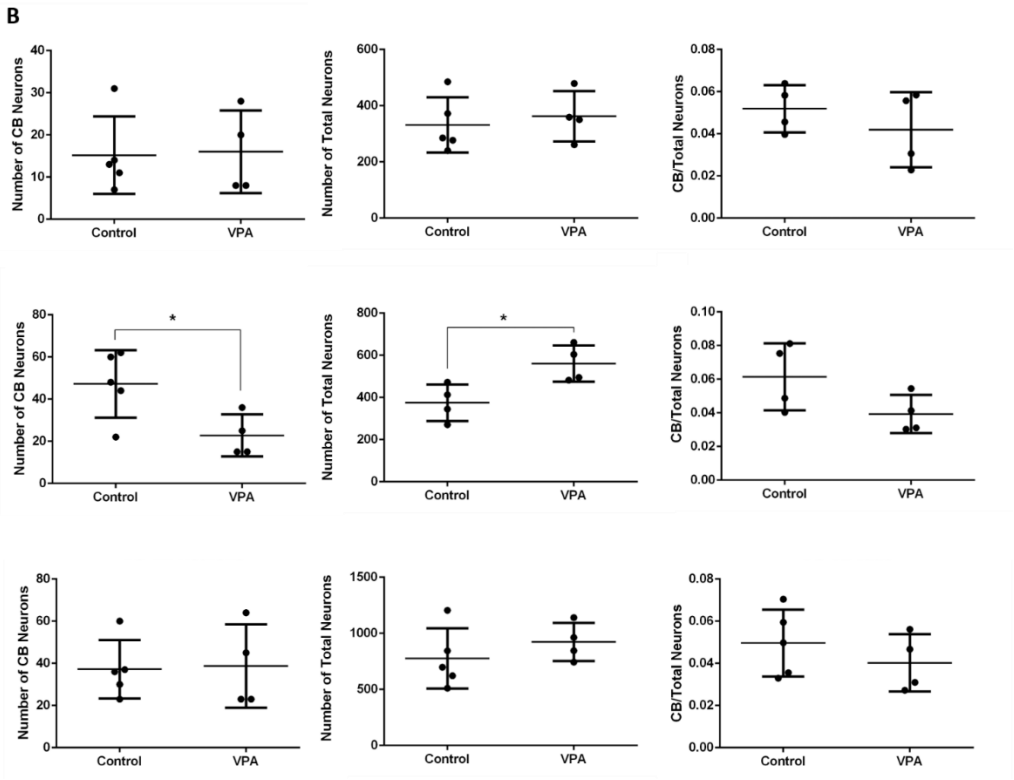
Figure S1 | **VPA group presents abnormal proportion and distribution impairments of PV^+ (A) and CB^+ (B) interneurons across aCC layers.** Values are plotted as mean ± standard deviation. n= 3-5 (in litters). Statistical analysis: Student's *t* Test followed by Welch's correction. *p<0.05. ***p<0.001.

Figure S2 | **VPA group presents abnormal proportion and distribution impairments of PV^+ (A) and CB^+ (B) interneurons across prelimbic layers.** Values are plotted as mean ± standard deviation. n= 3-5 (in litters). Statistical analysis: Student's *t* Test followed by Welch's correction. *p<0.05. **p<0.01.

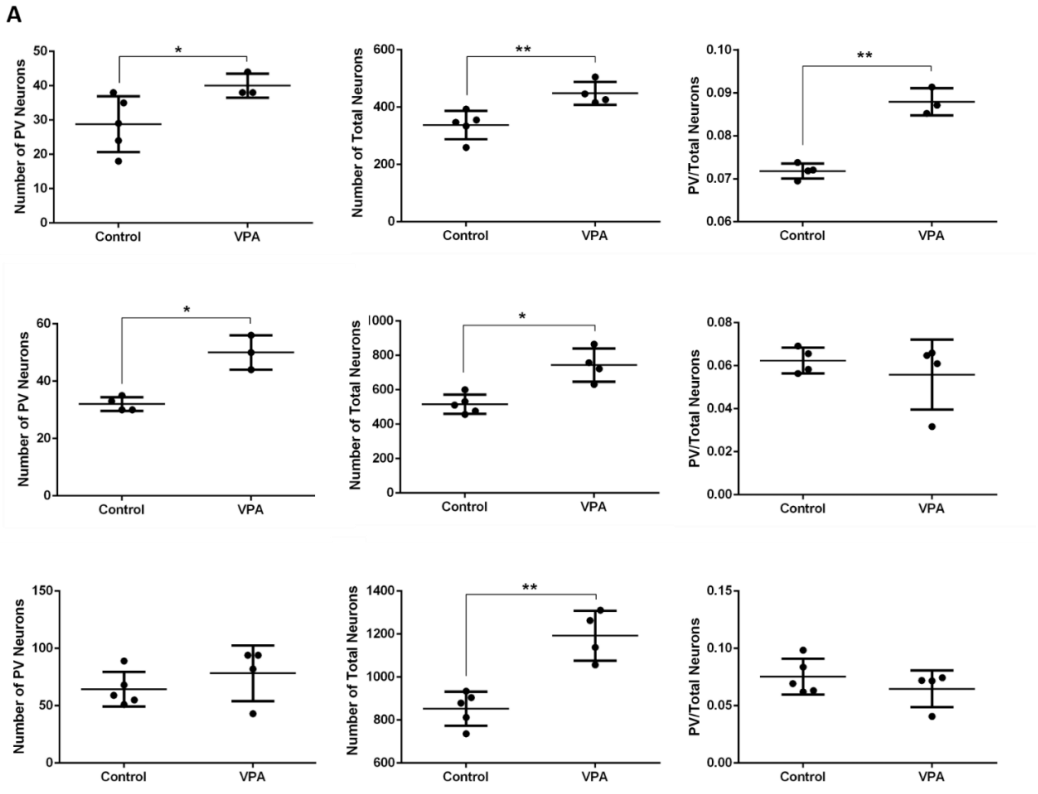
Figure S3 | **VPA group presents abnormal proportion and distribution impairments of PV^+ (A) and CB^+ (B) interneurons across infralimbic layers.** Values are plotted as mean ± standard deviation. n= 3-5 (in litters). Statistical analysis: Student's *t* Test followed by Welch's correction. *p<0.05. **p<0.01.

Figure S1

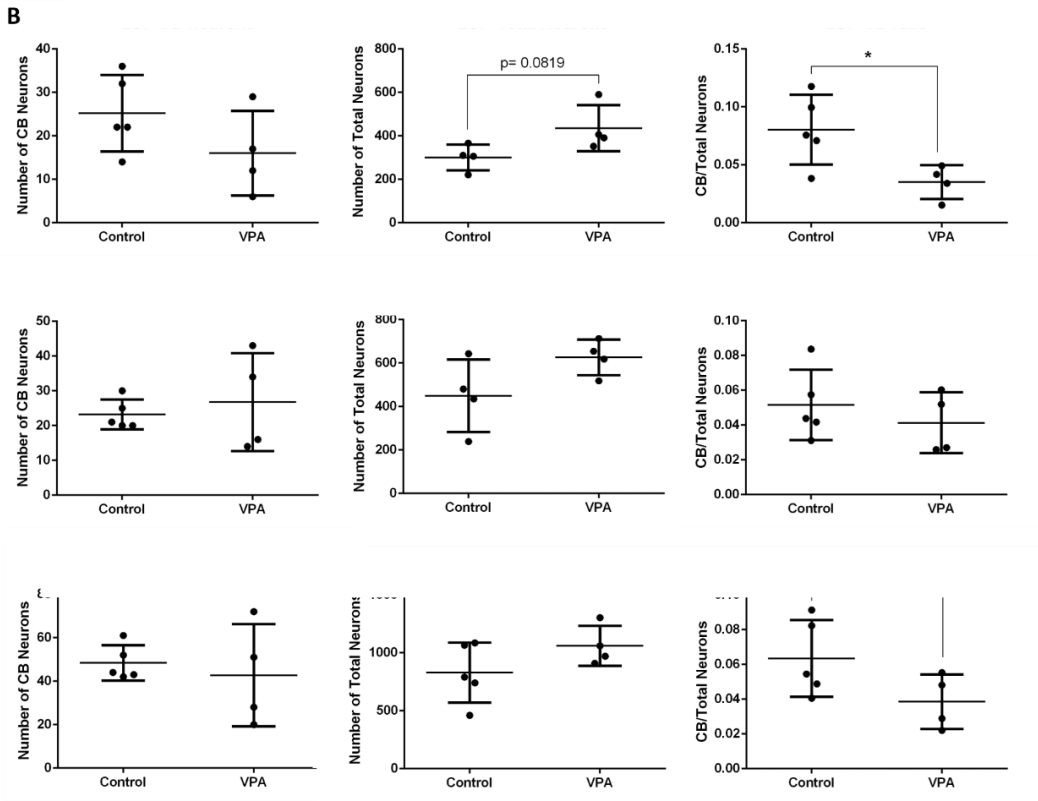

A
Number of PV Neurons
Number of Total Neurons
Ratio of PV/Total Neurons
***
p=0.0521
*
Control
VPA


B
Number of CB Neurons
Number of Total Neurons
CB/Total Neurons
*
Control
VPA

779 Figure S2

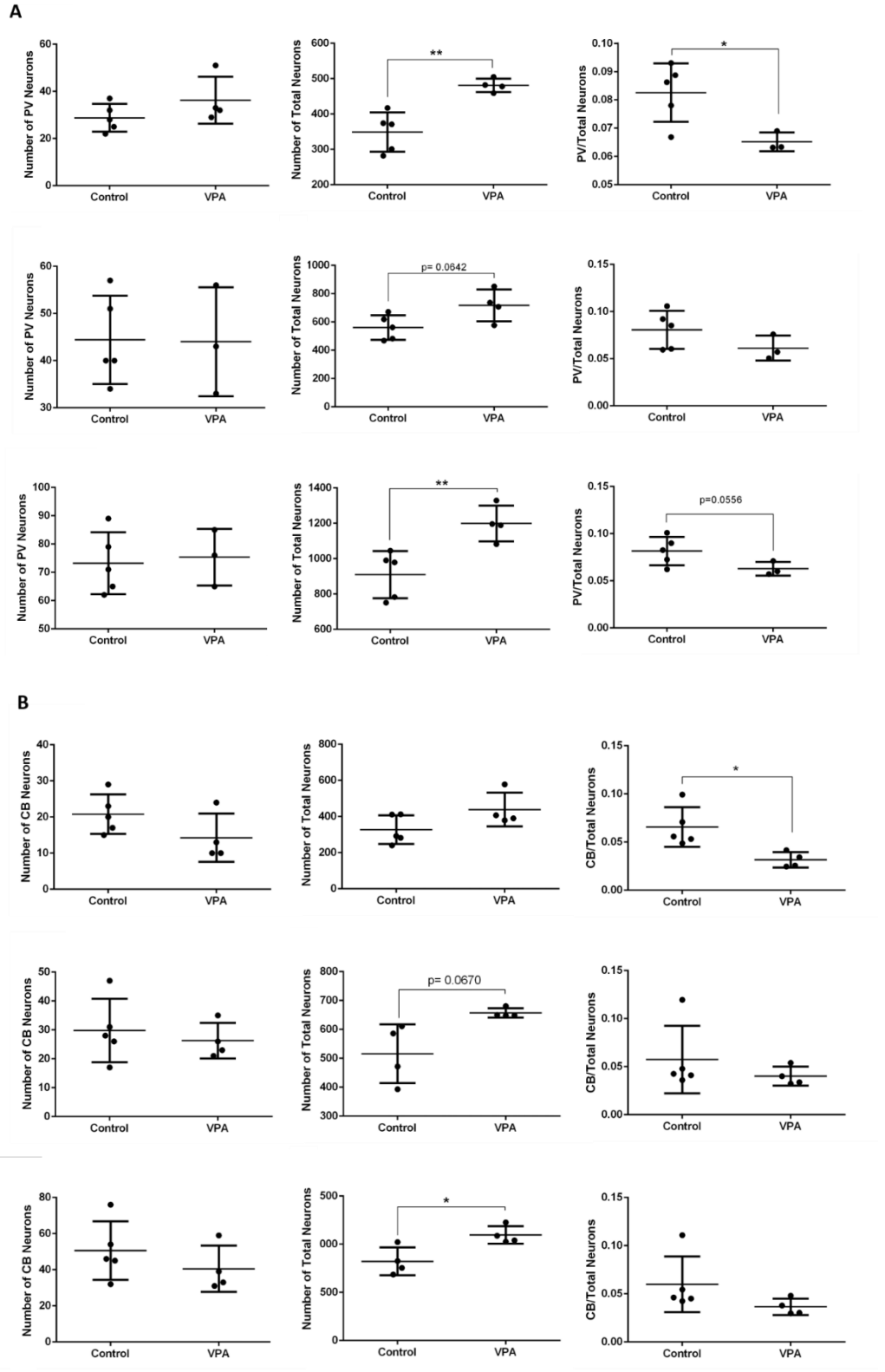


780



781

782 Figure S3



783

784

785

3 CONCLUSÕES E PERSPECTIVAS

Os resultados deste estudo mostraram que a exposição pré-natal ao VPA conduziu a alterações pós-natais importantes no CPFm tanto em número como em distribuição de interneurônios CB^+ e PV^+ e neurônios totais $NeuN^+$. Os dados sugerem que a exposição pré-natal ao VPA possa estar relacionada com alterações no processo de migração dos interneurônios em direção ao córtex cerebral e intracortical, uma vez que os animais do grupo VPA apresentaram alterações quantitativas dos interneurônios CB^+, PV^+ e neurônios totais $NeuN^+$ no CPFm, bem como alterações na proporção desses interneurônios em relação aos neurônios totais $NeuN^+$ (Figura 7). A composição de todos os resultados nos permitiu observar diferentes padrões de alterações nas sub-regiões, além de laminação cortical também alterada.

A complexidade dos resultados encontrados mostra a necessidade de expansão das análises, com um foco maior na ontogenia dos interneurônios no contexto do TEA, na tentativa de elucidar possíveis mecanismos envolvidos com os prejuízos quantitativos e de distribuição de interneurônios evidenciados neste estudo. Os esforços a partir de agora serão centrados na análise das moléculas cruciais para o processo de migração dos interneurônios, com especial atenção para os fatores de transcrição Sox6 e Lhx6 e os receptores $GABA_A$ e $GABA_B$. Assim, será possível observar se a exposição pré-natal ao VPA contribui para possíveis prejuízos nestes componentes tão fundamentais para uma migração adequada. Ainda, os outros subtipos de interneurônios também serão estudados, uma vez que as análises acerca dos PV^+ e CB^+ fazem parte de uma varredura da população GABAérgica inibitória total.

O entendimento acerca dos mecanismos pelos quais ocorre um desequilíbrio E/I no TEA ainda permanece com muitos questionamentos. Alterações neste balanço no CPF durante períodos críticos de desenvolvimento embrionário podem estar relacionadas com o fenótipo cognitivo, social e sensorial encontrado no TEA. Os resultados do presente trabalho contribuem para a tentativa de elucidar possíveis elementos-chave relacionados a alterações neurais observadas no TEA, bem como o entendimento de vias envolvidas no desequilíbrio E/I.

Figura 7 – Representação esquemática dos resultados obtidos neste trabalho

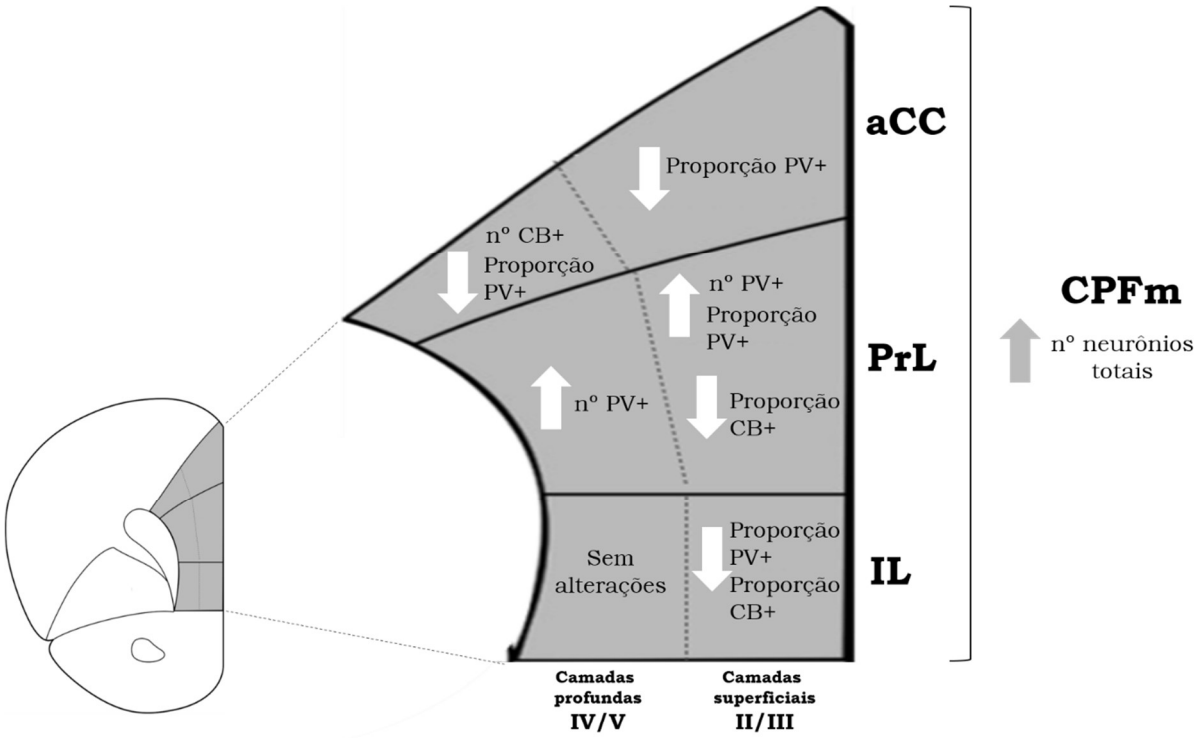


Imagem ilustrativa de um hemisfério encefálico, com destaque para o CPFm (área cinza) e com ampliação se suas sub-regiões (aCC, PrL e IL). Fonte: elaborada pela autora.

REFERÊNCIAS

ABDUL-MONIM, Z.; NEILL, J. C.; REYNOLDS, G. P. Sub-chronic psychotomimetic phencyclidine induces deficits in reversal learning and alterations in parvalbumin-immunoreactive expression in the rat. **Journal of Psychopharmacology**, v. 21, n. 2, p. 198–205, 16 mar. 2007.

ALLEN, G. V. *et al*. Organization of visceral and limbic connections in the insular cortex of the rat. **The Journal of Comparative Neurology**, v. 311, n. 1, p. 1–16, 1 set. 1991.

AMERICAN PSYCHIATRIC ASSOCIATION. **Manual Diagnóstico e Estatístico de Transtornos Mentais - DSM-5**. [s.l: s.n.].

ANDERSON, S. A. *et al*. Distinct cortical migrations from the medial and lateral ganglionic eminences. **Development** , v. 128, n. 3, p. 353–63, fev. 2001.

ASCOLI, G. A. *et al*. Petilla terminology: Nomenclature of features of GABAergic interneurons of the cerebral cortex. **Nature Reviews Neuroscience**, v. 9, n. 7, p. 557–568, 2008.

ASPERGER, H. Die "Autistischen Psychopathen" im Kindesalter. **Archiv für Psychiatrie und Nervenkrankheiten**, v. 117, n. 1, p. 76–136, jun. 1944.
ASPERGER, H. **'Autistic psychopathy' in childhood**. [s.l: s.n.].

ATALLAH, B. V.; SCANZIANI, M. Instantaneous Modulation of Gamma Oscillation Frequency by Balancing Excitation with Inhibition. **Neuron**, v. 62, n. 4, p. 566–577, 28 maio 2009.

ATLADOTTIR, H. O. *et al*. Association of Family History of Autoimmune Diseases and Autism Spectrum Disorders. **PEDIATRICS**, v. 124, n. 2, p. 687–694, 2009.

BALDINO, F.; GELLER, H. M. Effect of sodium valporate on hypothalamic neurons in vivo and in vitro. **Brain Research**, v. 219, n. 1, p. 231–237, 1981.

BAMBINI-JUNIOR, V. *et al*. Resveratrol prevents social deficts in animal model of autism induced by valproic acid. **Neuroscience Letters**, v. 583, p. 176–181, 2014.

BARON-COHEN, S.; WHEELWRIGHT, S. The Empathy Quotient: An Investigation of Adults with Asperger Syndrome or High Functioning Autism, and Normal Sex Differences. **Journal of Autism and Developmental Disorders**, v. 34, n. 2, p. 163–175, abr. 2004.

BAZINET, R. P. *et al*. Valproic acid selectively inhibits conversion of arachidonic acid to arachidonoyl-CoA by brain microsomal long-chain fatty acyl-CoA synthetases: Relevance to bipolar disorder. **Psychopharmacology**, v. 184, n. 1, p. 122–129, 2006.

BEASLEY, C. L. *et al*. Selective deficits in prefrontal cortical GABAergic neurons in schizophrenia defined by the presence of calcium-binding proteins. **Society of Biological Psychiatry**, v. 52, n. 7, p. 708–715, 2002.

BELICHENKO, P. V. *et al*. Excitatory-inhibitory relationship in the fascia dentata in the Ts65Dn mouse model of down syndrome. **Journal of Comparative Neurology**, v. 512, n. 4, p. 453–466, 2009.

BELMONTE, M. K. Autism and Abnormal Development of Brain Connectivity. **Journal of Neuroscience**, v. 24, n. 42, p. 9228–9231, 2004.

BELMONTE, M. K.; BARON-COHEN, S. Normal sibs of children with autism share negative frontal but not positive sensory abnormalities: preliminary evidence from fMRI during processing of visual distractors. **Society for Neuroscience**, v. 30, 2004.

BEVERSDORF, D. Q. Phenotyping, etiological factors, and biomarkers: Toward precision medicine in autism spectrum disorders. **Journal of Developmental and Behavioral Pediatrics**, v. 37, p. 659–673, 2016.

BLEULER, E. Die Prognose der Dementia Praecox (Schizophreniegruppe). **Allgemeine Zeitschrift für Psychiatrie und psychisch-gerichtliche Medizin**, 1908.

BLEULER, E. Das autistische Denken. **Jahrbuch für psychoanalytische und psychopathologische Forschungen**, v. 4, n. 1, p. 1–39, 1912.

BOLTON, P. F. *et al*. Epilepsy in autism: Features and correlates. **British Journal of Psychiatry**, v. 198, n. 4, p. 289–294, 2011.

BOURGERON, T. From the genetic architecture to synaptic plasticity in autism spectrum disorder. **Nature Reviews Neuroscience**, v. 16, n. 9, p. 551–563, 2015.

BROCK, J. *et al*. The temporal binding deficit hypothesis of autism. **Development and Psychopathology**, v. 14, n. 2, p. 209–224, 15 jun. 2002.

BROMLEY, R. L. *et al*. Autism spectrum disorders following in utero exposure to antiepileptic drugs. **Neurology**, v. 71, n. 23, p. 1923–1924, 2008.

BRUNI, J.; WILDER, B. J. Valproic Acid: Review of a New Antiepileptic Drug. **Archives of Neurology**, v. 36, n. 7, p. 393–398, 1979.

BUESCHER, A. V. S. *et al*. Costs of autism spectrum disorders in the United Kingdom and the United States. **JAMA Pediatrics**, v. 168, n. 8, p. 721–728, 2014.

BUIE, T. *et al*. Evaluation, Diagnosis, and Treatment of Gastrointestinal Disorders in Individuals With ASDs: A Consensus Report. **Pediatrics**, v. 125, n. 1, p. 1–18, 2010.

BURTON, B. S. On the propyl derivatives and decomposition products of ethylacetoacetate. **American Chemical Society**, v. 3, p. 385–395, 1882.

BUTT, S. J. B. *et al*. The Temporal and Spatial Origins of Cortical Interneurons Predict Their Physiological Subtype. **Neuron**, v. 48, p. 591–604, 2005.

BUXHOEVEDEN, D. P. *et al*. Reduced minicolumns in the frontal cortex of patients with autism. **Neuropathology and Applied Neurobiology**, v. 32, n. 5, p. 483–491, 1 out. 2006.

BUZSAKI, G. *et al*. Neuronal Oscillations in Cortical Networks. **Science**, v. 304, n. 2004, p. 1926–1930, 2004.

CAGLAYAN, A. O. Genetic causes of syndromic and non-syndromic autism. **Developmental Medicine and Child Neurology**, v. 52, n. 2, p. 130–138, 2010.

CAO, W. *et al*. Gamma Oscillation Dysfunction in mPFC Leads to Social Deficits in Neuroligin 3 R451C Knockin Mice. **Neuron**, v. 97, p. 1–8, 2018.

CARDIN, J. A. *et al*. Driving fast-spiking cells induces gamma rhythm and controls sensory responses. **Nature**, v. 459, n. 7247, p. 663–667, 26 jun. 2009.

CARLÉN, M. What constitutes the prefrontal cortex? **Science** , v. 358, n. 6362, p. 478–482, 27 out. 2017.

CARLSON, N. R. **Physiology of Behavior**. 11. ed. [s.l.] Pearson, 2012.

CARLSSON, M. L. Hypothesis: Is infantile autism a hypoglutamatergic disorder? Relevance of glutamate-serotonin interactions for pharmacotherapy. **Journal of Neural Transmission**, v. 105, n. 4–5, p. 525–535, 1998.

CASANOVA, M. F. *et al*. Minicolumnar pathology in autism. **Neurology**, v. 58, n. 3, p. 428–32, 12 fev. 2002.

CASANOVA, M. F. Neuropathological and Genetic Findings in Autism: The Significance of a Putative Minicolumnopathy. **The Neuroscientist**, v. 12, n. 5, p. 435–441, 29 out. 2006.

CASANOVA, M. F. The neuropathology of autism. **Brain Pathology**, v. 17, n. 4, p. 422–433, 2007.

CASANOVA, M. F. *et al*. A Topographic Study of Minicolumnar Core Width by Lamina Comparison between Autistic Subjects and Controls: Possible Minicolumnar Disruption due to an Anatomical Element In-Common to Multiple Laminae. **Brain Pathology**, v. 20, n. 2, p. 451–458, 1 mar. 2010.

CASANOVA, M. F.; BUXHOEVEDEN, D.; GOMEZ, J. Disruption in the Inhibitory Architecture of the Cell Minicolumn: Implications for Autisim. **The Neuroscientist**, v. 9, n. 6, p. 496–507, 29 dez. 2003.

CASANOVA, M. F.; BUXHOEVEDEN, D. P.; BROWN, C. Clinical and Macroscopic Correlates of Minicolumnar Pathology in Autism. **Journal of Child Neurology**, v. 17, n. 9, p. 692–695, 2 set. 2002.

CELLOT, G.; CHERUBINI, E. GABAergic Signaling as Therapeutic Target for Autism Spectrum Disorders. **Frontiers in Pediatrics**, v. 2, n. 70, p. 1–11, 2014.

CHANCE, S. A.; WALKER, M.; CROW, T. J. Reduced density of calbindin-immunoreactive interneurons in the planum temporale in schizophrenia. **Brain Research**, v. 1046, n. 1–2, p. 32–37, 2005.

CHANNELL, M. M. *et al*. Patterns of autism spectrum symptomatology in individuals with Down syndrome without comorbid autism spectrum disorder. **Journal of Neurodevelopmental Disorders**, v. 7, n. 1, p. 1–9, 2015.

CHAPMAN, A. G. *et al*. Acute effects of sodium valproate and gama-vinyl gaba on regional amino acid metabolism in the rat brain: Incorporation of 2-[14C]glucose into amino acids. **Neurochemical Research**, v. 7, n. 9, p. 1089–1105, set. 1982.

CHEN, M. H. *et al*. Comorbidity of allergic and autoimmune diseases in patients with autism spectrum disorder: A nationwide population-based study. **Research in Autism Spectrum Disorders**, v. 7, n. 2, p. 205–212, 2013.

CHRISTENSEN, D. L. *et al*. Prevalence and Characteristics of Autism Spectrum Disorder Among Children Aged 8 Years - Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2012. **Morbidity and Mortality Weekly Report. Surveillance Summaries (Washington, D.C.: 2002)**, v. 65, n. 13, p. 1–23, 16 nov. 2018.

CHRISTENSEN, J. *et al*. Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. **JAMA - Journal of the American Medical Association**, v. 309, n. 16, p. 1696–1703, 2013.

CHRISTIANSON, A. L.; CHESTER, N.; KROMBERG, J. G. R. Fetal Valproate Syndrome: Clinical and Neuro-developmental Features in Two Sibling Pairs. **Developmental Medicine & Child Neurology**, v. 36, n. 4, p. 361–369, 1994.

CHRONICLE, E. P.; MULLENERS, W. M. **Anticonvulsant drugs for migraine prophylaxis**. [s.l: s.n.].

CLINE, H. Synaptogenesis: A Balancing Act between Excitation and Inhibition. **Current Biology**, v. 15, n. 6, p. R203–R205, 29 mar. 2005.

COLVERT, E. *et al*. Heritability of autism spectrum disorder in a UK population-based twin sample. **JAMA Psychiatry**, v. 72, n. 5, p. 415, 2015.

CONDÉ, F. *et al*. Local circuit neurons immunoreactive for calretinin, calbindin D-28k or parvalbumin in monkey prefronatal cortex: Distribution and morphology. **Journal of Comparative Neurology**, v. 341, n. 1, p. 95–116, 1 mar. 1994.

CONNORS, B. W.; GUTNICK, M. J. Intrinsic firing patterns of diverse neocortical neurons. **Trends in Neurosciences**, v. 13, n. 3, p. 99–104, 1990.

CORBIN, J. G.; BUTT, S. J. B. Developmental mechanisms for the generation of telencephalic interneurons. **Developmental Neurobiology**, v. 71, n. 8, p. 710–732, 1 ago. 2011.

COURCHESNE, E. *et al*. Neuron Number and Size in Prefrontal Cortex of Children With Autism. **JAMA**, v. 306, n. 18, p. 2001–2010, 9 nov. 2011.

COURY, D. L. *et al*. Gastrointestinal Conditions in Children With Autism Spectrum Disorder: Developing a Research Agenda. **Pediatrics**, v. 130, n. 2, p. 160–168, 2012.

CRAWLEY, J. N. Mouse Behavioral Assays Relevant to the Symptoms of Autism. **Brain Pathology**, v. 17, n. 4, p. 448–459, 1 out. 2007.

CROEN, L. A. *et al*. The health status of adults on the autism spectrum. **Autism**, v. 19, n. 7, p. 814–823, 2015.

DANI, V. S. *et al*. Reduced cortical activity due to a shift in the balance between excitation and inhibition in a mouse model of Rett Syndrome. **Proceedings of the National Academy of Sciences**, v. 102, n. 35, p. 12560–12565, 2005.

DAVISS, S. R.; LEWIS, D. A. Local circuit neurons of the prefrontal cortex in schizophrenia: selective increase in the density of calbindin-immunoreactive neurons. **Psychiatry Research**, v. 59, n. 1–2, p. 81–96, 29 nov. 1995.

DAWSON, G. *et al*. Neurocognitive and electrophysiological evidence of altered face processing in parents of children with autism: Implications for a model of abnormal development of social brain circuitry in autism. **Development and Psychopathology**, v. 17, n. 03, p. 679–97, 1 set. 2005.

DEFELIPE, J. *et al*. A microcolumnar structure of monkey cerebral cortex revealed by immunocytochemical studies of double bouquet cell axons. **Neuroscience**, v. 37, n. 3, p. 655–673, 1990.

DEFELIPE, J. Neocortical neuronal diversity: chemical heterogeneity revealed by colocalization studies of classic neurotransmitters, neuropeptides, calcium-binding proteins, and cell surface molecules. **Cerebral Cortex**, v. 3, n. 4, p. 273–289, 1993.

DEFELIPE, J. Types of neurons, synaptic connections and chemical characteristics of cells immunoreactive for calbindin-D28K, parvalbumin and calretinin in the neocortex. **Journal of Chemical Neuroanatomy**, v. 14, n. 1, p. 1–19, 1 dez. 1997.

DEFELIPE, J. Chandelier cells and epilepsy. **Brain**, v. 122, n. 10, p. 1807–1822, 1 out. 1999.

DEFELIPE, J. *et al*. New insights into the classification and nomenclature of cortical GABAergic interneurons. **Nature Reviews Neuroscience**, v. 14, n. 3, p. 202–216, 2013.

DEFELIPE, J.; HENDRY, S. H. C.; JONES, E. G. Synapses of double bouquet cells in monkey cerebral cortex visualized by calbindin immunoreactivity. **Brain Research**, v. 503, n. 1, p. 49–54, 1989.

DEL RÍO, M. R.; DEFELIPE, J. A light and electron microscopic study of calbindin D-28k immunoreactive double bouquet cells in the human temporal cortex. **Brain Research**, v. 690, n. 1, p. 133–140, 1995.

DEL RÍO, M. R.; DEFELIPE, J. Colocalization of parvalbumin and calbindin D-28k in neurons including chandelier cells of the human temporal neocortex. **Journal of Chemical Neuroanatomy**, v. 12, n. 3, p. 165–173, 1997.

DEMEULEMEESTER, H. *et al*. Calbindin D-28K and parvalbumin immunoreactivity is confined to two separate neuronal subpopulations in the cat visual cortex, whereas partial

coexistence is shown in the dorsal lateral geniculate nucleus. **Neuroscience Letters**, v. 99, n. 1–2, p. 6–11, 1989.

DENDRINOS, G.; HEMELT, M.; KELLER, A. Prenatal VPA Exposure and Changes in Sensory Processing by the Superior Colliculus. **Frontiers in Integrative Neuroscience**, v. 5, p. 68, 2011.

DEVLIN, B.; SCHERER, S. W. Genetic architecture in autism spectrum disorder. **Current Opinion in Genetics and Development**, v. 22, n. 3, p. 229–237, 2012.

DICHTER, G. S. Functional magnetic resonance imaging of autism spectrum disorders. **Dialogues in clinical neuroscience**, v. 14, n. 3, p. 319–51, set. 2012.

DUFOUR-RAINFRAY, D. *et al*. Fetal exposure to teratogens: Evidence of genes involved in autism. **Neuroscience and Biobehavioral Reviews**, v. 35, n. 5, p. 1254–1265, 2011.

EICHLER, SABRINA A.; MEIER, J. C. E-I balance and human diseases – from molecules to networking. **Frontiers in Molecular Neuroscience**, v. 1, n. 2, p. 1–5, 2008.

EL-FISHAWY, P.; STATE, M. W. The Genetics of Autism: Key Issues, Recent Findings, and Clinical Implications. **Psychiatric Clinics of North America**, v. 33, n. 1, p. 83–105, 1 mar. 2010.

EUSTON, D. The Role of Medial Prefrontal Cortex in Memory and Decision Making. **Neuron**, v. 76, n. 6, p. 1057–1070, 2012.

EVANS, D. W. *et al*. Adaptive and maladaptive correlates of repetitive behavior and restricted interests in persons with down syndrome and developmentally-matched typical children: A two-year longitudinal sequential design. **PLoS ONE**, v. 9, n. 4, p. 939–951, 2014.

FARBER, N. B. *et al*. Antiepileptic drugs and agents that inhibit voltage-gated sodium channels prevent NMDA antagonist neurotoxicity. **Molecular Psychiatry**, v. 7, n. 7, p. 726–733, 2002.

FAUX, C. *et al*. Differential gene expression in migrating cortical interneurons during mouse forebrain development. **The Journal of Comparative Neurology**, v. 518, n. 8, p. NA-NA, 15 abr. 2009.

FAVOROV, O. V.; KELLY, D. G. Minicolumnar Organization within Somatosensory Cortical Segregates: I. Development of Afferent Connections. **Cerebral Cortex**, v. 4, n. 4, p. 408–427, 1 jul. 1994a.

FAVOROV, O. V.; KELLY, D. G. Minicolumnar Organization within Somatosensory Cortical Segregates: II. Emergent Functional Properties. **Cerebral Cortex**, v. 4, n. 4, p. 428–442, 1 jul. 1994b.

FAVRE, M. R. *et al*. General developmental health in the VPA-rat model of autism. **Frontiers in Behavioral Neuroscience**, v. 7, p. 88, 2013.

FERGUSON, B. R.; GAO, W.-J. PV Interneurons: Critical Regulators of E/I Balance for

Prefrontal Cortex-Dependent Behavior and Psychiatric Disorders. **Frontiers in Neural Circuits**, v. 12, n. May, p. 1–13, 2018.

FONTES-DUTRA, M. *et al*. Abnormal empathy-like pro-social behaviour in the valproic acid model of autism spectrum disorder. **Behavioural Brain Research**, v. 364, p. 11–18, 17 maio 2019.

FOUNTOULAKIS, K. N. *et al*. Treatment guidelines for bipolar disorder: A critical review. **Journal of Affective Disorders**, v. 86, n. 1, p. 1–10, 2005.

FUKUDA, T. *et al*. Delayed Maturation of Neuronal Architecture and Synaptogenesis in Cerebral Cortex of Mecp2-Deficient Mice. **Journal of Neuropathology & Experimental Neurology**, v. 64, n. 6, p. 537–544, 1 jun. 2005.

FUNG, S. J. *et al*. Expression of Interneuron Markers in the Dorsolateral Prefrontal Cortex of the Developing Human and in Schizophrenia. **American Journal of Psychiatry**, v. 167, n. 12, p. 1479–1488, 1 dez. 2010.

GAETZ, W. *et al*. Relating MEG measured motor cortical oscillations to resting γ-Aminobutyric acid (GABA) concentration. **NeuroImage**, v. 55, n. 2, p. 616–621, 2011.

GALARRETA, M.; HESTRIN, S. Frequency-dependent synaptic depression and the balance of excitation and inhibition in the neocortex. **Nature Neuroscience**, v. 1, n. 7, p. 587–594, nov. 1998.

GARDENER, H.; SPIEGELMAN, D.; BUKA, S. L. Prenatal risk factors for autism: Comprehensive meta-analysis. **British Journal of Psychiatry**, v. 195, n. 1, p. 7–14, 2009.

GESCHWIND, D. H. Advances in Autism. **Annual Review of Medicine**, v. 60, p. 367–380, 2009.

GESCHWIND, D. H. Genetics of autism spectrum disorders. **Trends in cognitive sciences**, v. 15, n. 9, p. 409–16, 1 set. 2011.

GIDAYA, N. B. *et al*. In utero Exposure to -2-Adrenergic Receptor Agonist Drugs and Risk for Autism Spectrum Disorders. **PEDIATRICS**, v. 137, n. 2, p. 1–8, 2016.

GODIN, Y. *et al*. Effects of di-n-propylacetate, and anticonvulsive compound, on GABA metabolism. **Journal of Neurochemistry**, v. 16, n. 6, p. 869–873, 1969.

GOETZ, C. G. The history of Parkinson's disease: early clinical descriptions and neurological therapies. **Cold Spring Harbor perspectives in medicine**, p. 1–15, 2011.

GOGOLLA, N. *et al*. Common circuit defect of excitatory-inhibitory balance in mouse models of autism. **Journal of Neurodevelopmental Disorders**, v. 1, n. 2, p. 172–181, 2009.

GOTTFRIED, C. *et al*. Valproic Acid in Autism Spectrum Disorder: From an Environmental Risk Factor to a Reliable Animal Model. **Recent Advances in Autism Spectrum Disorders**, v. 1, p. 64, 2013.

GOTTLICHER, M. Valproic acid: an old drug newly discovered as inhibitor of histone deacetylases. **Annals of Hematology**, v. 83, n. 0, p. 91–92, 2004.

GOTTS, S. J. *et al*. Fractionation of social brain circuits in autism spectrum disorders. **Brain**, v. 135, n. 9, p. 2711–2725, 1 set. 2012.

GRELOTTI, D. J.; GAUTHIER, I.; SCHULTZ, R. T. Social interest and the development of cortical face specialization: What autism teaches us about face processing. **Developmental Psychobiology**, v. 40, n. 3, p. 213–225, 1 abr. 2002.

GROSSMANN, T. The role of medial prefrontal cortex in early social cognition. **Frontiers in human neuroscience**, v. 7, p. 340, 2013.

GUINCHAT, V. *et al*. Pre-, peri- and neonatal risk factors for autism. **Acta Obstetricia et Gynecologica Scandinavica**, v. 91, p. 287–300, 2012.

GULYÁS, A. I. *et al*. Subpopulations of GABAergic neurons containing parvalbumin, calbindin D28k, and cholecystokinin in the rat hippocampus. **Journal of Comparative Neurology**, v. 312, n. 3, p. 371–378, 1991.

GUNNES, N. *et al*. Interpregnancy interval and risk of autistic disorder. **Epidemiology**, v. 24, n. 6, p. 906–912, 2013.

GURNEY, J. G.; MCPHEETERS, M. L.; DAVIS, M. M. Parental report of health conditions and health care use among children with and without autism: National survey of children's health. **Archives of Pediatrics and Adolescent Medicine**, v. 160, n. 8, p. 825–830, 2006.

GUY, J.; STAIGER, J. F. The Functioning of a Cortex without Layers. **Frontiers in Neuroanatomy**, v. 11, p. 54, 2017.

HALLMAYER, J. *et al*. Genetic heritability and shared environmental factors among twin pairs with autism. **Archives of General Psychiatry**, v. 68, n. 11, p. 1095–1102, 2011.

HARITON, C. *et al*. Distribution of sodium valproate and GABA metabolism in CNS of the rat. **Biopharmaceutics & Drug Disposition**, v. 5, n. 4, p. 409–414, 1984.

HASHEMI, E. *et al*. The Number of Parvalbumin-Expressing Interneurons Is Decreased in the Medial Prefrontal Cortex in Autism. **Cerebral Cortex**, p. 1–13, 2016.

HASHIMOTO, T. *et al*. Gene Expression Deficits in a Subclass of GABA Neurons in the Prefrontal Cortex of Subjects with Schizophrenia. **Journal of Neuroscience**, v. 23, n. 15, p. 6315–6326, 16 jul. 2003.

HASHIMOTO, T. *et al*. Conserved Regional Patterns of GABA-Related Transcript Expression in the Neocortex of Subjects With Schizophrenia. **American Journal of Psychiatry**, v. 165, n. 4, p. 479–489, 1 abr. 2008.

HERRMANN, C. S.; MECKLINGER, A. Gamma activity in human EEG is related to highspeed memory comparisons during object selective attention. **Visual Cognition**, v. 8, n. 3–5, p. 593–608, jun. 2001.

HERTZ-PICCIOTTO, I.; DELWICHE, L. The rise in autism and the role of age at diagnosis. **Epidemiology**, v. 20, n. 1, p. 84–90, 2009.

HIPPIUS, H.; NEUNDÖRFER, G. The discovery of Alzheimer's disease. **Dialogues in Clinical Neuroscience**, v. 5, n. 1, p. 101–108, 2003.

HIRSCH, M. M. *et al*. Behavioral alterations in autism model induced by valproic acid and translational analysis of circulating microRNA. **Food and Chemical Toxicology**, v. 115, p. 336–343, 1 maio 2018a.

HIRSCH, M. M. *et al*. Data on social transmission of food preference in a model of autism induced by valproic acid and translational analysis of circulating microRNA. **Data in Brief**, v. 18, p. 1433–1440, 1 jun. 2018b.

HIRVIKOSKI, T. *et al*. Premature mortality in autism spectrum disorder. **British Journal of Psychiatry**, v. 208, n. 3, p. 232–238, 2016.

HOLINGUE, C. *et al*. Gastrointestinal symptoms in autism spectrum disorder: A review of the literature on ascertainment and prevalence. **Autism Research**, v. 11, n. 1, p. 24–36, 2018.

HOOVER, W. B.; VERTES, R. P. Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. **Brain Structure and Function**, v. 212, n. 2, p. 149–179, 10 set. 2007.

HOWARD, M. W. *et al*. Gamma Oscillations Correlate with Working Memory Load in Humans. **Cerebral Cortex**, v. 13, n. 12, p. 1369–1374, 1 dez. 2003.

HU, H.; GAN, J.; JONAS, P. Fast-spiking, parvalbumin+ GABAergic interneurons: From cellular design to microcircuit function. **Science**, v. 345, n. 6196, p. 1255263–1255263, 1 ago. 2014.

HUSSMAN, J. P. Suppressed gabaergic inhibition as a common factor in suspected etiologies of autism [1]. **Journal of Autism and Developmental Disorders**, v. 31, n. 2, p. 247–248, 2001.

IDRING, S. *et al*. Parental age and the risk of autism spectrum disorders: Findings from a Swedish population-based cohort. **International Journal of Epidemiology**, v. 43, n. 1, p. 107–115, 2014.

IRITANI, S. *et al*. Calbindin immunoreactivity in the hippocampal formation and neocortex of schizophrenics. **Progress in Neuro-psychopharmacology & Biological Psychiatry**, v. 23, n. 3, p. 409–21, abr. 1999.

ISAACSON, J. S.; SCANZIANI, M. How inhibition shapes cortical activity. **Neuron**, v. 72, n. 2, p. 231–243, 2011.

ISLER, H. Autism, invented 1908 by Eugen Bleuler for the Plight of the Isolated Schizophrenic. **SciFed Journal of Autism**, v. 2, n. 1, p. 1–5, 2018.

ITO, M. *et al*. The enzymatic basis for the metabolism and inhibitory effects of valproic acid:

dehydrogenation of valproyl-CoA by 2-methyl-branched-chain acyl-CoA dehydrogenase. **Biochimica et Biophysica Acta**, v. 1034, n. 2, p. 213–218, 1990.

JAMAIN, S. *et al*. Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. **Nature Genetics**, v. 34, n. 1, p. 27–29, 31 maio 2003.

JASMIN, L.; GRANATO, A.; OHARA, P. T. Rostral agranular insular cortex and pain areas of the central nervous system: A tract-tracing study in the rat. **The Journal of Comparative Neurology**, v. 468, n. 3, p. 425–440, 12 jan. 2004.

JOHANNESSEN, C. U. Mechanisms of action of valproate: A commentatory. **Neurochemistry International**, v. 37, n. 2–3, p. 103–110, 2000.

JOHANNESSEN, C. U.; JOHANNESSEN, S. I. Valproate: past, present, and future. **CNS Drug Reviews**, v. 9, n. 2, p. 199–216, 2003.

JONES, A. P. *et al*. Feeling, caring, knowing: different types of empathy deficit in boys with psychopathic tendencies and autism spectrum disorder. **Journal of Child Psychology and Psychiatry**, v. 51, n. 11, p. 1188–1197, nov. 2010.

KANAI, H. *et al*. Valproic acid inhibits histone deacetylase activity and suppresses excitotoxicity-induced GAPDH nuclear accumulation and apoptotic death in neurons. **Pharmacogenomics Journal**, v. 4, n. 5, p. 336–344, 2004.

KANG, J.; KIM, E. Suppression of NMDA receptor function in mice prenatally exposed to valproic acid improves social deficits and repetitive behaviors. **Frontiers in Molecular Neuroscience**, v. 8, n. 17, p. 1–9, 2015.

KANNER, L. Autistic Disturbances Of Affective Contact. **Nervous Child**, p. 217–250, 1943.
KAWAGUCHI, Y. *et al*. Fast spiking cells in rat hippocampus (CA1 region) contain the calcium-binding protein parvalbumin. **Brain Research**, 1987.

KAWAGUCHI, Y.; KUBOTA, Y. GABAergic cell subtypes and their synaptic connections in rat frontal cortex. **Cerebral Cortex**, v. 7, n. 6, p. 476–486, 1997.

KEIL, A. *et al*. Effects of emotional arousal in the cerebral hemispheres: a study of oscillatory brain activity and event-related potentials. **Clinical Neurophysiology**, v. 112, n. 11, p. 2057–2068, 1 nov. 2001a.

KEIL, A. *et al*. Human large-scale oscillatory brain activity during an operant shaping procedure. **Cognitive Brain Research**, v. 12, n. 3, p. 397–407, 1 dez. 2001b.

KEIL, A. *et al*. Parental autoimmune diseases associated with autism spectrum disorders in offspring. **Epidemiology**, v. 21, n. 6, p. 805–808, 2010.

KELSOM, C.; LU, W. Development and specification of GABAergic cortical interneurons. **Cell and Bioscience**, v. 3, n. 1, p. 1–19, 2013.

KING, M. D.; BEARMAN, P. S. Socioeconomic status and the increased prevalence of autism in California. **American Sociological Review**, v. 76, n. 2, p. 320–346, 2011.

KLAUSBERGER, T. *et al*. Brain-state- and cell-type-specific firing of hippocampal interneurons in vivo. **Nature**, v. 421, n. 6925, p. 844–848, 2003.

KLIN, A. *et al*. Two-year-olds with autism orient to non-social contingencies rather than biological motion. **Nature**, v. 459, n. 7244, p. 257–261, 14 maio 2009.

KOHANE, I. S. *et al*. The co-morbidity burden of children and young adults with autism spectrum disorders. **PloS one**, v. 7, n. 4, p. 1–7, 2012.

KRAKOWIAK, P. *et al*. Sleep problems in children with autism spectrum disorders, developmental delays, and typical development: A population-based study. **Journal of Sleep Research**, v. 17, n. 2, p. 197–206, 2008.

KRÄMER, O. H. *et al*. The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. **EMBO Journal**, v. 22, n. 13, p. 3411–3420, 2003.

KUKINO, K.; DEGUCHI, T. Anticonvulsant activity and effects of sodium dipropylacetate on cerebral 5-hydroxytryptamine and GABA in reserpinized mice. **CHEMICAL & PHARMACEUTICAL BULLETIN**, v. 26, n. 11, p. 3551–3555, 1978.

KURODA, M. *et al*. Synaptic relationships between axon terminals from the mediodorsal thalamic nucleus and layer III pyramidal cells in the prelimbic cortex of the rat. **Brain Research**, v. 708, n. 1–2, p. 185–190, 5 fev. 1996.

LAUBACH, M. *et al*. What, If Anything, Is Rodent Prefrontal Cortex? **eNeuro**, v. 5, n. 5, 2018.

LAVELLE, T. A. *et al*. Economic Burden of Childhood Autism Spectrum Disorders. **PEDIATRICS**, v. 133, n. 3, p. 520–529, 2014.

LAWRENCE, Y. A. *et al*. Parvalbumin-, calbindin-, and calretinin-immunoreactive hippocampal interneuron density in autism. **Acta Neurologica Scandinavica**, v. 121, n. 2, p. 99–108, 2010.

LEE, B. K. *et al*. Maternal hospitalization with infection during pregnancy and risk of autism spectrum disorders. **Brain, behavior, and immunity**, v. 44, p. 100–105, 2015.

LEE, E.; LEE, J.; KIM, E. Excitation/Inhibition Imbalance in Animal Models of Autism Spectrum Disorders. **Biological Psychiatry**, v. 81, n. 10, p. 838–847, 2017a.

LEE, E.; LEE, J.; KIM, E. Excitation/Inhibition Imbalance in Animal Models of Autism Spectrum Disorders. **Biological Psychiatry**, v. 81, n. 10, p. 838–847, 15 maio 2017b.

LETINIC, K.; ZONCU, R.; RAKIC, P. Origin of GABAergic neurons in the human neocortex. **Nature**, v. 417, n. 6889, p. 645–649, jun. 2002.

LEVITT, P.; EAGLESON, K. L.; POWELL, E. M. Regulation of neocortical interneuron development and the implications for neurodevelopmental disorders. **Trends in Neurosciences**, v. 27, n. 7, p. 400–406, 1 jul. 2004.

LI, X.; ZOU, H.; BROWN, W. T. Genes associated with autism spectrum disorder. **Brain Research Bulletin**, v. 88, n. 6, p. 543–552, set. 2012.

LI, Y.-M. *et al*. Association Between Maternal Obesity and Autism Spectrum Disorder in Offspring: A Meta-analysis. **Journal of Autism and Developmental Disorders**, v. 46, n. 1, p. 95–102, 9 jan. 2016.

LIN, H. C. *et al*. The Amygdala Excitatory/Inhibitory Balance in a Valproate-Induced Rat Autism Model. **PLoS ONE**, v. 8, n. 1, 2013.

LIU, K.; KING, M.; BEARMAN, P. S. Social Influence and the Autism Epidemic. **American Journal of Sociology**, v. 115, n. 5, p. 1387–1434, 2010.

LORENZ, J.; MINOSHIMA, S.; CASEY, K. L. Keeping pain out of mind: the role of the dorsolateral prefrontal cortex in pain modulation. **Brain**, v. 126, n. 5, p. 1079–1091, 1 maio 2003.

LÖSCHER, W. Anticonvulsant and biochemical effects of inhibitors of gaba aminotransferase and valproic acid during subchronic treatment in mice. **Biochemical Pharmacology**, v. 31, n. 5, p. 837–842, 1982.

LÖSCHER, W. Valproic acid: mechanisms of action. **Antiepileptic drugs**, p. 767–779, 2002.
LÖSCHER, W.; HÖSTERMANN, D. Differential effects of vigabatrin, -acetylenic GABA, aminooxyacetic acid, and valproate on levels of various amino acids in rat brain regions and plasma. **Naunyn-Schmiedeberg's Archives of Pharmacology**, v. 3, n. 349, p. 270–278, 1994.

LOTTER, V. Epidemiology of autistic conditions in young children - Prevalence. **Social Psychiatry**, v. 1, n. 3, p. 124–135, 1966.

LUNDQVIST, M. *et al*. Gamma and Beta Bursts Underlie Working Memory. **Neuron**, v. 90, n. 1, p. 1–13, 2016.

LYALL, K. *et al*. The Changing Epidemiology of Autism Spectrum Disorders. **Annual Review of Public Health**, v. 38, n. 1, p. 81–102, 2017.

MABUNGA, D. F. N. *et al*. Exploring the Validity of Valproic Acid Animal Model of Autism. **Experimental Neurobiology**, v. 24, n. 4, p. 285–300, 2015.

MACDONALD, A. W. Dissociating the Role of the Dorsolateral Prefrontal and Anterior Cingulate Cortex in Cognitive Control. **Science**, v. 288, n. 5472, p. 1835–1838, 9 jun. 2000.

MALOW, B. A. *et al*. A Practice Pathway for the Identification, Evaluation, and Management of Insomnia in Children and Adolescents With Autism Spectrum Disorders. **Pediatrics**, v. 130, n. 2, p. 106–124, 2012.

MARÍN, O. Interneuron dysfunction in psychiatric disorders. **Nature Reviews Neuroscience**, v. 13, n. 2, p. 107–120, 18 fev. 2012.

MARKRAM, H. *et al*. Interneurons of the neocortical inhibitory system. **Nature Reviews**

Neuroscience, v. 5, n. 10, p. 793–807, 2004.

MARKRAM, K. *et al*. Abnormal Fear Conditioning and Amygdala Processing in an Animal Model of Autism. **Neuropsychopharmacology**, v. 33, n. 4, p. 901–912, 16 mar. 2008.

MARKRAM, K.; MARKRAM, H. The Intense World Theory – A Unifying Theory of the Neurobiology of Autism. **Frontiers in Human Neuroscience**, v. 4, n. 224, p. 1–29, 2010.

MARSH, E. D. *et al*. FACS-array gene expression analysis during early development of mouse telencephalic interneurons. **Developmental Neurobiology**, v. 68, n. 4, p. 434–445, 1 mar. 2008.

MATSON, J. L.; CERVANTES, P. E. Commonly studied comorbid psychopathologies among persons with autism spectrum disorder. **Research in Developmental Disabilities**, v. 35, n. 5, p. 952–962, 2014.

MATSON, J. L.; RIESKE, R. D.; WILLIAMS, L. W. The relationship between autism spectrum disorders and attention-deficit/hyperactivity disorder: An overview. **Research in Developmental Disabilities**, v. 34, n. 9, p. 2475–2484, 2013.

MCCORMICK, D. A. *et al*. Comparative electrophysiology of pyramidal and sparsely spiny stellate neurons of the neocortex. **Journal of Neurophysiology**, v. 54, n. 4, p. 782–806, 1985.

MCDONALD, A. J.; BETETTE, R. L. Parvalbumin-containing neurons in the rat basolateral amygdala: Morphology and co-localization of Calbindin-D28k. **Neuroscience**, v. 102, n. 2, p. 413–425, 2001.

MCLEAN, M. J.; MACDONALD, R. L. Sodium valproate, but not ethosuximide, produces use- and voltage-dependent limitation of high frequency repetitive firing of action potentials of mouse central neurons in cell culture. **The Journal of pharmacology and experimental therapeutics**, v. 237, n. 3, p. 1001–1011, 1986.

MEADOR, K. J. Effects of in Utero Antiepileptic Drug Exposure. **Epilepsy Currents**, v. 8, n. 6, p. 143–147, 2008.

MEGÍAS, M. *et al*. Total number and distribution of inhibitory and excitatory synapses on hippocampal CA1 pyramidal cells. **Neuroscience**, v. 102, n. 3, p. 527–540, 2001.

MEHTA, M. V.; GANDAL, M. J.; SIEGEL, S. J. mGluR5-antagonist mediated reversal of elevated stereotyped, repetitive behaviors in the VPA model of autism. **PLoS ONE**, v. 6, n. 10, p. 260–277, 2011.

MELLIOS, N. *et al*. Molecular Determinants of Dysregulated GABAergic Gene Expression in the Prefrontal Cortex of Subjects with Schizophrenia. **Biological Psychiatry**, v. 65, n. 12, p. 1006–1014, 15 jun. 2009.

MEUNIER; H. Pharmacodynamic properties of N-dipropylacetic acid. **Therapie**, v. 18, p. 435–438, 1963.

MEYER, U. *et al*. Adult brain and behavioral pathological markers of prenatal immune

challenge during early/middle and late fetal development in mice. **Brain, Behavior, and Immunity**, v. 22, n. 4, p. 469–486, 1 maio 2008.

MEZZACAPPA, A. *et al*. Risk for autism spectrum disorders according to period of prenatal antidepressant exposure: A systematic review and meta-analysis. **JAMA Pediatrics**, v. 171, n. 6, p. 555–563, 2017.

MILLER, E. K.; COHEN, J. D. An Integrative Theory of Prefrontal Cortex Function. **Annual Review of Neuroscience**, v. 24, n. 1, p. 167–202, 28 mar. 2001.

MINSHEW, N. J.; KELLER, T. A. The nature of brain dysfunction in autism: functional brain imaging studies. **Current Opinion in Neurology**, v. 23, n. 2, p. 124–30, abr. 2010.

MIYAZAKI, C. *et al*. Allergies in Children with Autism Spectrum Disorder: a Systematic Review and Meta-analysis. **Review Journal of Autism and Developmental Disorders**, v. 2, n. 4, p. 374–401, 2015.

MOREIRA, S. Epilepsia: concepção histórica, aspectos conceituais, diagnóstico e tratamento. **Mental**, v. 2, n. 3, p. 107–122, 2004.

MOUNTCASTLE, V. The columnar organization of the neocortex. **Brain**, v. 120, n. 4, p. 701–722, 1 abr. 1997.

MÜLLER, M. M.; GRUBER, T.; KEIL, A. Modulation of induced gamma band activity in the human EEG by attention and visual information processing. **International Journal of Psychophysiology**, v. 38, n. 3, p. 283–299, 1 dez. 2000.

MURRAY, E. A.; WISE, S. P.; GRAHAM, K. S. **The evolution of memory systems: ancestors, anatomy, and adaptations**. [s.l: s.n.].

NAKASATO, A. *et al*. Swim stress exaggerates the hyperactive mesocortical dopamine system in a rodent model of autism. **Brain Research**, v. 1193, p. 128–135, 8 fev. 2008.

NELSON, R. J.; TRAINOR, B. C. Neural mechanisms of aggression. **Nature Reviews Neuroscience**, v. 8, n. 7, p. 536–546, 1 jul. 2007.

NELSON, S. B.; VALAKH, V. Excitatory/Inhibitory Balance and Circuit Homeostasis in Autism Spectrum Disorders. **Neuron**, v. 87, n. 4, p. 684–698, 2015.

NERY, S.; FISHELL, G.; CORBIN, J. G. The caudal ganglionic eminence is a source of distinct cortical and subcortical cell populations. **Nature Neuroscience**, v. 5, n. 12, p. 1279–1287, 4 dez. 2002.

OBLAK, A. L. *et al*. Altered posterior cingulate cortical cyctoarchitecture, but normal density of neurons and interneurons in the posterior cingulate cortex and fusiform gyrus in autism. **Autism Research**, v. 4, n. 3, p. 200–211, 1 jun. 2011.

OFNER, M.; COLES, A.; DECOU, M. L. **Autism Spectrum Disorder Among Children and Youth in Canada 2018: a Report of the National Autism Spectrum Disorder Surveillance System**. [s.l: s.n.].

OREKHOVA, E. V. *et al*. Excess of High Frequency Electroencephalogram Oscillations in Boys with Autism. **Biological Psychiatry**, v. 62, n. 9, p. 1022–1029, 1 nov. 2007.

ORNOY, A. Valproic acid in pregnancy: How much are we endangering the embryo and fetus? **Reproductive Toxicology**, v. 28, n. 1, p. 1–10, 2009.

OSTERLING, J.; DAWSON, G. Early recognition of children with autism: A study of first birthday home videotapes. **Journal of Autism and Developmental Disorders**, v. 24, n. 3, p. 247–257, jun. 1994.

PACKER, A. M.; YUSTE, R. Dense, Unspecific Connectivity of Neocortical Parvalbumin-Positive Interneurons: A Canonical Microcircuit for Inhibition? **Journal of Neuroscience**, v. 31, n. 37, p. 13260–13271, 2011.

PAN, C. Y.; TSAI, C. L.; CHU, C. H. Fundamental Movement skills in children diagnosed with autism spectrum disorders and attention deficit hyperactivity disorder. **Journal of Autism and Developmental Disorders**, v. 39, n. 12, p. 1694–1705, 2009.

PAULA, C. S. *et al*. Brief report: Prevalence of pervasive developmental disorder in Brazil: A pilot study. **Journal of Autism and Developmental Disorders**, v. 41, n. 12, p. 1738–1742, 2011.

PEARCE, J. M. S. Kanner's infantile autism and Asperger's syndrome. **Journal of Neurology, Neurosurgery and Psychiatry**, p. 200–205, 2005.

PELPHREY, K. A. *et al*. Research Review: Constraining heterogeneity: the social brain and its development in autism spectrum disorder. **Journal of Child Psychology and Psychiatry**, v. 52, n. 6, p. 631–644, 1 jun. 2011.

PENSCHUCK, S. *et al*. Decrease in parvalbumin-expressing neurons in the hippocampus and increased phencyclidine-induced locomotor activity in the rat methylazoxymethanol (MAM) model of schizophrenia. **European Journal of Neuroscience**, v. 23, n. 1, p. 279–284, 1 jan. 2006.

PETERS, A.; SETHARES, C. Myelinated axons and the pyramidal cell modules in monkey primary visual cortex. **The Journal of Comparative Neurology**, v. 365, n. 2, p. 232–255, 5 fev. 1996.

PLEASURE, S. J. *et al*. Cell Migration from the Ganglionic Eminences Is Required for the Development of Hippocampal GABAergic Interneurons. **Neuron**, v. 28, n. 3, p. 727–740, 1 dez. 2000.

POLLEUX, F.; LAUDER, J. M. Toward a developmental neurobiology of autism. **Mental Retardation and Developmental Disabilities Research Reviews**, v. 10, n. 4, p. 303–17, 2004.

POUILLE, F.; SCANZIANI, M. Enforcement of temporal fidelity in pyramidal cells by somatic feed-forward inhibition. **Science**, v. 293, n. 5532, p. 1159–1163, 2001.

POWELL, E. M. *et al*. Genetic disruption of cortical interneuron development causes region-

and GABA cell type-specific deficits, epilepsy, and behavioral dysfunction. **The Journal of Neuroscience**, v. 23, n. 2, p. 622–631, 2003.

QIN, S. *et al*. Acute Psychological Stress Reduces Working Memory-Related Activity in the Dorsolateral Prefrontal Cortex. **Biological Psychiatry**, v. 66, n. 1, p. 25–32, jul. 2009.

R. MUHLE, S. V. TRENTACOSTE, I. R. The genetics of autism. **Pediatrics**, v. 113, n. 5, p. 472–486, 2004.

RAMAMOORTHI, K.; LIN, Y. The contribution of GABAergic dysfunction to neurodevelopmental disorders. **Trends in Molecular Medicine**, v. 17, n. 8, p. 452–462, 2011.

REYNOLDS, G. P. *et al*. Selective deficits of frontal cortical gabaergic neuronal subtypes defined by calcium binding proteins in psychotic illness. **Schizophrenia Research**, v. 41, n. 1, p. 255, jan. 2000.

REYNOLDS, M. F.; SISK, E. C.; RASGON, N. L. Valproate and neuroendocrine changes in relation to women treated for epilepsy and bipolar disorder: a review. **Current Medicinal Chemistry**, v. 14, n. 26, p. 2799–812, 2007.

RINALDI, T. *et al*. Elevated NMDA receptor levels and enhanced postsynaptic long-term potentiation induced by prenatal exposure to valproic acid. **Proceedings of the National Academy of Sciences**, v. 104, n. 33, p. 13501–13506, 2007.

RINALDI, T.; SILBERBERG, G.; MARKRAM, H. Hyperconnectivity of Local Neocortical Microcircuitry Induced by Prenatal Exposure to Valproic Acid. **Cerebral Cortex**, v. 18, n. 4, p. 763–770, 1 abr. 2008.

RISCH, N. *et al*. Familial recurrence of autism spectrum disorder: Evaluating genetic and environmental contributions. **American Journal of Psychiatry**, v. 171, n. 11, p. 1206–1213, 2014.

RODIER, P. M. *et al*. Embryological origin for autism: Developmental anomalies of the cranial nerve motor nuclei. **Journal of Comparative Neurology**, v. 370, n. 2, p. 247–261, 1996.

RODIER, P. M. *et al*. Linking etiologies in humans and animal models: Studies of autism. **Reproductive Toxicology**, v. 11, n. 2–3, p. 417–422, 1997.

RODRIGUEZ, E. *et al*. Perception's shadow: long-distance synchronization of human brain activity. **Nature**, v. 397, n. 6718, p. 430–433, fev. 1999.

ROGERS, J. H.; RÉSIBOIS, A. Calretinin and calbindin-D28k in rat brain: Patterns of partial co-localization. **Neuroscience**, v. 51, n. 4, p. 843–865, 1992.

ROJAS, D. C. *et al*. Reduced neural synchronization of gamma-band MEG oscillations in first-degree relatives of children with autism. **BMC Psychiatry**, v. 8, n. 1, p. 66, 1 dez. 2008.

ROJAS, D. C.; WILSON, L. B. γ-band abnormalities as markers of autism spectrum

disorders. **Biomarkers in Medicine**, v. 8, n. 3, p. 353–368, 8 mar. 2014.
ROSENBERG, G. The mechanisms of action of valproate in neuropsychiatric disorders: Can we see the forest for the trees? **Cellular and Molecular Life Sciences**, v. 64, n. 16, p. 2090–2103, 2007.

ROULLET, F. I. *et al*. Behavioral and molecular changes in the mouse in response to prenatal exposure to the anti-epileptic drug valproic acid. **Neuroscience**, v. 170, n. 2, p. 514–522, 13 out. 2010.

ROULLET, F. I.; LAI, J. K. Y.; FOSTER, J. A. In utero exposure to valproic acid and autism - A current review of clinical and animal studies. **Neurotoxicology and Teratology**, v. 36, p. 47–56, 2013.

RUBENSTEIN, J.L.R & MERZENICH, M. M. Model of autism: increased ratio of excitation/inhibition in key neural systems. **Genes, Brain and Behavior**, v. 2, n. 5, p. 255–267, 2003.

RUBENSTEIN, J. L. R. Three hypotheses for developmental defects that may underlie some forms of autism spectrum disorder. **Current Opinion in Neurology**, v. 23, p. 118–123, 2010.

RUDY, B. *et al*. Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons. **Developmental neurobiology**, v. 71, n. 1, p. 45–61, 1 jan. 2011.

SANDIN, S. *et al*. Advancing maternal age is associated with increasing risk for autism: A review and meta-analysis. **Journal of the American Academy of Child and Adolescent Psychiatry**, v. 51, n. 5, p. 477–486, 2012.

SANDIN, S. *et al*. The familial risk of autism. **JAMA - Journal of the American Medical Association**, v. 311, n. 17, p. 1770–1777, 2014.

SCHECHTER, P. J.; TRANIER, Y.; GROVE, J. Effect of n-dipropylacetate on amino acid concentrations in mouse brain: correlations with anti-convulsant activity. **Journal of Neurochemistry**, v. 31, n. 5, p. 1325–1327, 1 nov. 1978.

SCHENDEL, D. E. *et al*. Association of psychiatric and neurologic comorbidity with mortality among persons with autism spectrum disorder in a danish population. **JAMA Pediatrics**, v. 170, n. 3, p. 243–250, 2016.

SCHMITT, L. I. *et al*. Thalamic amplification of cortical connectivity sustains attentional control. **Nature**, v. 545, n. 7653, p. 219–223, 3 maio 2017.

SCHNEIDER, T. *et al*. Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. **Psychoneuroendocrinology**, v. 33, n. 6, p. 728–740, 2008.

SCHNEIDER, T.; PRZEWŁOCKI, R. Behavioral alterations in rats prenatally to valproic acid: Animal model of autism. **Neuropsychopharmacology**, v. 30, n. 1, p. 80–89, 2005a.

SCHNEIDER, T.; PRZEWŁOCKI, R. Behavioral alterations in rats prenatally to valproic acid: Animal model of autism. **Neuropsychopharmacology**, v. 30, p. 80–89, 2005b.

SCHNEIDER, T.; TURCZAK, J.; PRZEWŁOCKI, R. Environmental enrichment reverses behavioral alterations in rats prenatally exposed to valproic acid: Issues for a therapeutic approach in autism. **Neuropsychopharmacology**, v. 31, p. 36–46, 2006.

SCHUMACHER, H. J. *et al*. The teratogenic activity of α thalidomide analogue, EM12, in rabbits, rats, and monkeys. **Teratology**, v. 5, n. 2, p. 233–240, 1972.

SEALEY, L. A. *et al*. Environmental factors in the development of autism spectrum disorders. **Environment International**, v. 88, p. 288–298, 2016.

SEAMANS, J. K.; LAPISH, C. C.; DURSTEWITZ, D. Comparing the prefrontal cortex of rats and primates: Insights from electrophysiology. **Neurotoxicity Research**, v. 14, n. 2–3, p. 249–262, jun. 2008.

SEWARDS, T. V; SEWARDS, M. A. The medial pain system: Neural representations of the motivational aspect of pain. **Brain Research Bulletin**, v. 59, n. 3, p. 163–180, 30 nov. 2002.

SHU, Y.; HASENSTAUB, A.; MCCORMICK, D. A. Turning on and off recurrent balanced cortical activity. **Nature**, v. 423, n. 6937, p. 288–293, maio 2003.

SOHAL, V. S. *et al*. Parvalbumin neurons and gamma rhythms enhance cortical circuit performance. **Nature**, v. 459, n. 7247, p. 698–702, 26 jun. 2009.

SOKOLOVA, E. *et al*. A causal and mediation analysis of the comorbidity between attention deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD). **Journal of Autism and Developmental Disorders**, v. 47, n. 6, p. 1595–1604, 2017.

SOMOGYI, P.; COWEY, A. Double Bouquet Cells. **Cerebral Cortex**, v. 1, p. 337–360, 1984.

SPERDIN, H. F.; SCHAER, M. Aberrant Development of Speech Processing in Young Children with Autism: New Insights from Neuroimaging Biomarkers. **Frontiers in Neuroscience**, v. 10, p. 393, 25 ago. 2016.

SSUCHAREWA, G. E. Die schizoiden Psychopathien im Kindesalter - Parte 1. 1926a.

SSUCHAREWA, G. E. Die schizoiden Psychopathien im Kindesalter - Parte 2. 1926b.

STOCKHAUSEN, M. T. *et al*. Effects of the histone deacetylase inhibitor valproic acid on Notch signalling in human neuroblastoma cells. **British Journal of Cancer**, v. 92, n. 4, p. 751–759, 2005.

STONER, R. *et al*. Patches of Disorganization in the Neocortex of Children with Autism. **New England Journal of Medicine**, v. 370, n. 13, p. 1209–1219, 27 mar. 2014.

STRÖMLAND, K. *et al*. Autism in thalidomide embryopathy: a population study. **Developmental Medicine & Child Neurology**, v. 36, n. 4, p. 351–356, 1994.

STRÖMLAND, K.; MILLER, M. T. Thalidomide embryopathy: Revisited 27 years later. **Acta Ophthalmologica**, v. 71, n. 2, p. 238–245, 1993.

STUSS, D. T.; BENSON, D. F. Neuropsychological studies of the frontal lobes. **Psychological Bulletin**, v. 95, n. 1, p. 3–28, 1984.

SÜDHOF, T. C. Neuroligins and neurexins link synaptic function to cognitive disease. **Nature**, v. 455, n. 7215, p. 903–911, 2008.

SZATMARI, P. *et al*. Prospective Longitudinal Studies of Infant Siblings of Children with Autism: Lessons Learned and Future Directions. **Journal of the American Academy of Child and Adolescent Psychiatry**, v. 55, n. 3, p. 179–187, 2016.

TABUCHI, K. *et al*. A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. **Science**, v. 318, n. 5847, p. 71–76, 2007.

TALLON-BAUDRY, C. *et al*. Induced gamma-band activity during the delay of a visual short-term memory task in humans. **The Journal of Neuroscience**, v. 18, n. 11, p. 4244–54, 1 jun. 1998.

TAMÁS, G.; SOMOGYI, P.; BUHL, E. H. Differentially Interconnected Networks of GABAergic Interneurons in the Visual Cortex of the Cat. **The Journal of Neuroscience**, v. 18, n. 11, p. 4255–4270, 1998.

TOMMERDAHL, M. *et al*. Minicolumnar Activation Patterns in Cat and Monkey SI Cortex. **Cerebral Cortex**, v. 3, n. 5, p. 399–411, 1 set. 1993.

TSUJINO, N. *et al*. Abnormality of circadian rhythm accompanied by an increase in frontal cortex serotonin in animal model of autism. **Neuroscience Research**, v. 57, n. 2, p. 289–295, 2007.

TUCHMAN, R.; CUCCARO, M. Epilepsy and autism: Neurodevelopmental perspective. **Current Neurology and Neuroscience Reports**, v. 11, n. 4, p. 428–434, 2011.

TUCHMAN, R.; RAPIN, I. Epilepsy in autism. **Lancet Neurology**, v. 1, n. 6, p. 352–358, 2002.

TURRIGIANO, G. G.; NELSON, S. B. Homeostatic plasticity in the developing nervous system. **Nature Reviews Neuroscience**, v. 5, p. 97–107, 2004.

US FOOD AND DRUG ADMINISTRATION. **FDA Drug Safety Communication: Children born to mothers who took Valproate products while pregnant may have impaired cognitive development | FDA**. Disponível em: <https://www.fda.gov/drugs/drug-safety-and-availability/fda-drug-safety-communication-children-born-mothers-who-took-valproate-products-while-pregnant-may>. Acesso em: 28 maio. 2019.

US FOOD AND DRUG ADMINISTRATION. Information for healthcare professionals: risk of neural tube birth defects following prenatal exposure to valproate. **Food and Drug Administration**, 2009.

UYLINGS, H. B. M.; GROENEWEGEN, H. J.; KOLB, B. Do rats have a prefrontal cortex? **Behavioural Brain Research**, v. 146, n. 1–2, p. 3–17, 30 nov. 2003.

VOLK, D. W. *et al*. Deficits in Transcriptional Regulators of Cortical Parvalbumin Neurons in Schizophrenia. **American Journal of Psychiatry**, v. 169, n. 10, p. 1082–1091, 1 out. 2012.

WANG, P. *et al*. Enriched expression of genes associated with autism spectrum disorders in human inhibitory neurons. **Translational Psychiatry**, v. 8, n. 1, p. 13, 10 dez. 2018.

WELSH, J. P.; ESTES, A. M. Exploring the social brain. **eLife**, v. 7, 1 mar. 2018.

WERLER, M. M. *et al*. Use of Antiepileptic Medications in Pregnancy in Relation to Risks of Birth Defects. **Annals of Epidemiology**, v. 21, n. 11, p. 842–850, 2011.

WERLING, D. M.; GESCHWIND, D. H. Sex differences in autism spectrum disorders. **Current Opinion in Neurology**, v. 26, n. 2, p. 146–153, 2013.

WHITNEY, E. R. *et al*. Cerebellar Purkinje Cells are Reduced in a Subpopulation of Autistic Brains: A Stereological Experiment Using Calbindin-D28k. **The Cerebellum**, v. 7, n. 3, p. 406–416, 28 set. 2008.

WIGGS, L.; STORES, G. Sleep patterns and sleep disorders in children with autistic spectrum disorders: Insights using parent report and actigraphy. **Developmental Medicine and Child Neurology**, v. 46, n. 6, p. 372–380, 2004.

WILLIAMS, G. *et al*. Fetal valproate syndrome and autism: Additional evidence of an association. **Developmental Medicine and Child Neurology**, v. 43, n. 3, p. 202–206, 2001.

WILLIAMS, P. G.; HERSH, J. H. A male with fetal valproate syndrome and autism. **Developmental Medicine & Child Neurology**, v. 39, n. 9, p. 632–634, 1997.

WILSON, T. W. *et al*. Children and Adolescents with Autism Exhibit Reduced MEG Steady-State Gamma Responses. **Biological Psychiatry**, v. 62, n. 3, p. 192–197, 1 ago. 2007.

WÖHR, M. *et al*. Lack of parvalbumin in mice leads to behavioral deficits relevant to all human autism core symptoms and related neural morphofunctional abnormalities. **Translational Psychiatry**, v. 5, n. 3, p. e525–e525, 10 mar. 2015.

WONDERS, C. P.; ANDERSON, S. A. The origin and specification of cortical interneurons. **Nature reviews Neuroscience**, v. 7, n. 9, p. 687–96, set. 2006.

WOO, T.-U. W. *et al*. N-Methyl-D-Aspartate Receptor and Calbindin-Containing Neurons in the Anterior Cingulate Cortex in Schizophrenia and Bipolar Disorder. **Biological Psychiatry**, v. 64, n. 9, p. 803–809, 1 nov. 2008.

WORLD HEALTH ORGANIZATION. **The world health report 2000: Health System - Improving Performance**. Genebra: World Health Organization, 2000.

WORLD HEALTH ORGANIZATION. Epilepsy. 2019.

XUE, M.; ATALLAH, B. V.; SCANZIANI, M. Equalizing excitation–inhibition ratios across visual cortical neurons. **Nature**, v. 511, n. 7511, p. 596–600, 22 jul. 2014.

YENKOYAN, K. *et al*. Advances in understanding the pathophysiology of autism spectrum disorders. **Behavioural Brain Research**, v. 331, p. 92–101, 2017.

YIZHAR, O. *et al*. Neocortical excitation/inhibition balance in information processing and social dysfunction. **Nature**, v. 477, n. 7363, p. 171–178, 27 set. 2011.

ZEISE, M. L.; KASPAROW, S.; ZIEGLGÄNSBERGER, W. Valproate suppresses N-methyl-d-aspartate-evoked, transient depolarizations in the rat neocortex in vitro. **Brain Research**, v. 544, n. 2, p. 345–348, 29 mar. 1991.

ZERBO, O. *et al*. Immune mediated conditions in autism spectrum disorders. **Brain, Behavior, and Immunity**, v. 46, p. 232–236, 2015a.

ZERBO, O. *et al*. Interpregnancy Interval and Risk of Autism Spectrum Disorders. **PEDIATRICS**, v. 136, n. 4, p. 651–657, 2015b.

ZERBO, O. *et al*. Maternal Infection During Pregnancy and Autism Spectrum Disorders. **Journal of Autism and Developmental Disorders**, v. 45, n. 12, p. 4015–4025, 2015c.

ZHOU, Y. *et al*. Atypical behaviour and connectivity in SHANK3-mutant macaques. **Nature**, p. 1, 12 jun. 2019.

ZIKOPOULOS, B.; BARBAS, H. Changes in prefrontal axons may disrupt the network in autism. **The Journal of Neuroscience**, v. 30, n. 44, p. 14595–609, 3 nov. 2010.

ZIKOPOULOS, B.; BARBAS, H. Altered neural connectivity in excitatory and inhibitory cortical circuits in autism. **Frontiers in Human Neuroscience**, v. 7, p. 609, 27 set. 2013.

ZWAIGENBAUM, L. *et al*. Behavioral manifestations of autism in the first year of life. **International Journal of Developmental Neuroscience**, v. 23, n. 2–3, p. 143–152, 2005.

ANEXO A – CARTA DE APROVAÇÃO DO COMITÊ DE ÉTICA NO USO DE ANIMAIS (CEUA-UFRGS)

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: 140367
Data da Versão do Projeto: 13/08/2014

Pesquisadores:
RUDIMAR DOS SANTOS RIESGO
GUSTAVO DELLA FLORA NUNES
KAMILA CASTRO GROKOSKI
MELLANIE FONTES DUTRA DA SILVA
CARMEM GOTTFRIED
DIEGO MOURA BARONIO

Título: Modelo animal de autismo por exposição pré-natal ao ácido valpróico: Análise de sinapses inibitórias e excitatórias

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, 07 de outubro de 2014.

Profª Iraci Lucena da Silva Torres
Coordenadora CEUA/HCPA

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

IMPACT FACTOR 3.877

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

All Frontiers articles from July 2012 onwards are published with open access under the CC-BY Creative Commons attribution license (the current version is CC-BY, version 4.0 http://creativecommons.org/licenses/by/4.0/ (https://creativecommons.org/licenses/by/4.0/)). This means that the author(s) retain copyright, but the content is free to download, distribute and adapt for commercial or non-commercial purposes, given appropriate attribution to the original article.

Upon submission, author(s) grant Frontiers an exclusive license to publish, including to display, store, copy and reuse the content. The CC-BY Creative Commons attribution license enables anyone to use the publication freely, given appropriate attribution to the author(s) and citing Frontiers as the original publisher. The CC-BY Creative Commons attribution license does not apply to third-party materials that display a copyright notice to prohibit copying. Unless the third-party content is also subject to a CC-BY Creative Commons attribution license, or an equally permissive license, the author(s) must comply with any third-party copyright notices.

Preprint Policy

Frontiers' supportive preprint policy encourages full open access at all stages of a research paper, to share and generate the knowledge researchers need to support their work. Authors publishing in Frontiers journals may share their work ahead of submission to a peer-reviewed journal, as well as during the Frontiers review process, on repositories or pre-print servers (such as ArXiv, PeerJ Preprints, OSF and others), provided that the server imposes no restrictions upon the author's full copyright and re-use rights. Also note that any manuscript files shared after submission to Frontiers journals, during the review process, must not contain the Frontiers logo or branding.

Correct attribution of the original source in repositories or pre-print servers must be included on submission, or added at re-submission if the deposition is done during the review process.

If the article is published, authors are then strongly encouraged to link from the preprint server to the Frontiers publication to enable readers to find, access and cite the final peer-reviewed version. Please note that we cannot consider for publication content that has been previously published, or is already under review, within a scientific journal, book or similar entity.

2.2. Registration with Frontiers

Please note that the corresponding and all submitting authors MUST register

(https://www.frontiersin.org/Registration/Register.aspx) with Frontiers before submitting an article. You must be logged in to your personal Frontiers Account to submit an article.

For any co-author who would like his/her name on the article abstract page and PDF to be linked to a Frontiers profile on the Loop network (http://loop.frontiersin.org/about), please ensure to register (https://www.frontiersin.org/Registration/Register.aspx) before the final publication of the paper.

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 attentiontothewordcountlimits.**

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).

Note that sending your manuscript for language editing does not imply or guarantee that it will be accepted for publication by a Frontiers journal.
Editorial decisions on the scientific content of a manuscript are independent of whether it has received language editing or proofing by the partner services, or other services.

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 Affiliations

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.

The Corresponding Author(s) should be marked with an asterisk. Provide the exact contact email address of the corresponding author(s) in a separate section.

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 TableRequirements

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 onrequest:**

   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 thedatasets:**

   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 RequiredRepositories

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	dbSN P 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	OpenNeur o 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 RNAseqData

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. Conflicts 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 O±ce at editorial.o±ce@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 auect the objectivity of the review.

AFFILIATION	6. Are you a±liated with the same institution as the editor? Are you a±liated 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 a±liation 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 auect 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).