UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

CURSO DE GRADUAÇÃO EM BIOMEDICINA

MELLANIE FONTES DUTRA DA SILVA

MODELO ANIMAL DE AUTISMO INDUZIDO POR EXPOSIÇÃO PRÉ-NATAL AO ÁCIDO VALPROICO: ANÁLISE QUANTITATIVA DE CÉLULAS NEURONAIS, NÃO-NEURONAIS E IMUNOCONTEÚDO GABAÉRGICO CORTICAL

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

Orientador: Prof. Dr Carmem Juracy Silveira Gottfried

PORTO ALEGRE

Julho/2013

“ A necessidade de muitos se sobrepõe a de poucos”

Sr. Spock, Oficial Cientista da nave U.S.S. Enterprise “A Ira de Khan”

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SUMÁRIO

RESUMO

Os Transtornos do Espectro do Autismo (TEA) são um grupo heterogêneo de transtornos do desenvolvimento que apresentam grau de severidade bastante variável. Dados epidemiológicos indicam que fatores ambientais, como a exposição materna ao ácido valpróico (VPA), aumentam o risco do nascimento de filhos com autismo. O desequilíbrio do balanço excitatório/inibitorio no encéfalo, especialmente em regiões corticais envolvidas com processamento sensorial, vem sendo relacionado com um número considerável de transtornos que afetam o sistema nervoso central, como o TEA. Esse desequilíbrio pode estar fortemente associado com características cognitivo-comportamentais, bem como na morfologia e organização dos neurônios em padrões colunares nas regiões corticais. O presente estudo tem como objetivo quantificar o número de células neuronais GABAérgicas, não-GABAérgicas, células não-neuronais, células totais e o padrão de organização colunar nas camadas corticias II/III e V da área somatossensorial primária - região de campos em barris, no modelo de autismo em ratos Wistar induzido por exposição pré-natal ao ácido valpróico (VPA). Ratas Wistar prenhes receberam uma única injeção, intraperitoneal, de VPA (600 mg/kg) no dia 12,5 de gestação. Os encéfalos da prole de ratos machos de 120 dias foram utilizados para os experimentos de imuno-histoquímica para GABA, NeuN e DAPI na região da área somatossensorial primária. Análises foram realizadas com o software FluoView e ImageJ e os resultados foram considerados significativos com um $P<0.05$ pelo teste *t* de Student. Os resultados deste estudo apontam uma desorganização no padrão morfológico e colunar de neurônios na camada II/III e V da área somatossensorial primária, com diferenças na localização do NeuN ao longo do soma neuronal. Houve redução no número de neurônios GABAérgicos na camada V, porém o número de células não-neuronais reduziu em ambas as camadas estudadas. As vias inibitórias nessa região desempenham papéis fundamentais para a organização colunar e processamento neuronal, tendo relações importantes com as células da glia, as quais regulam e são reguladas por neurotransmissores inibitórios. O desbalanço desse grupo neuronal tem consequências importantes, não só na possível explicação de achados de excitotoxicidade no autismo, mas como na organização das minicolunas e no processamento sensorial, encontrado de forma anormal nesses pacientes, destacando uma via biológica significativa e possivelmente envolvida na sua fisiopatologia.

Lista de Abreviaturas do Trabalho em Português

ADI – Entrevista diagnóstica para autismo

ADOS – Protocolo de observação para diagnóstico de autismo

AIDS – Síndrome da imunodeficiência adquirida

CDC – Centro de Controle e Prevenção de Doenças

CNV – *Copy Number Variations* (Variações no Número de Cópias)

CR – células do tipo Cajal-Retzius

DAPI – 4',6-diamidino-2-fenilindol

DISCO – Entrevista Diagnóstica de Distúrbios Sociais e de Comunicação

DSM-V - TR – Manual Diagnóstico e Estatístico de Doenças Mentais V texto revisado

GABA – Ácido gama-aminobutírico

GABA-A – Receptor GABA-A

GABA-t – GABA Transaminase

GAD – Glutamato Descarboxilase

GAT – Transportador de GABA

GLN – Glutamina

GLNase – Glutaminase

GLU – Glutamato

GS – Glutamina Sintetase

Neu-N – Fator Nuclear Neuronal

PBS – Salina tamponada com fosfato

SNC – Sistema Nervoso Central

STAT– Ferramenta de Triagem para autismo aos 2 anos

TEA – Transtorno do Espectro do Autismo

TGD – Transtorno global do desenvolvimento

VIAAT – Transportador vesicular de aminoácidos inibitórios

VPA – Ácido Valproico

Lista de Abreviaturas do Trabalho em Forma de Artigo Científico

AIF1 – Allograft Inflammatory Factor 1

ASD – Autism Spectrum Disorders

CALB1 – calbindin

CNS – Central Nervous System

CR – Cajal-Retzius Cell

DCAMKL1 – serine-threonine kinase of the CAMK family

DCX – Doublecortin

DSM-IV-TR – Diagnostic and Statistical Manual of Mental Disorders-IV-Text Revision

GABA – gamma-aminobutyric acid

GFAP – glial fibrillary acidic protein

IBA1 – Ionized calcium-binding adapter molecule 1

IQSEC3 – IQ (commonly isoleucine and invariably glutamine) motif and Sec7 domain 3 (guanine nucleotide exchange factor)

NES – Nestin

NeuN – Neuronal Nuclear Factor

NKAIN1 – Na+/K+ transporting ATPase interacting 1

P120 – Postnatal Day 120

PDDNOS – Pervasive Development Disorder Not Otherwise Specified

RBM45 – RNA binding motif protein 45

RNA – Ribonucleic Acid

TSPO – Translocator Protein

VPA – Valproic Acid

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1. INTRODUÇÃO

1.1. O TRANSTORNO DO ESPECTRO DO AUTISMO

Descrito pela primeira vez por Hans Asperger em 1938 e, seguido por um relato de uma população de 11 crianças estudada por Leo Kanner em 1943 (Kanner 1968) o Transtorno do Espectro do Autismo (TEA) é caracterizado como um distúrbio do desenvolvimento marcado por diversos fatores complexos e graus de severidades variáveis. Seu diagnóstico, atualmente, baseia-se em um conjunto de alterações comportamentais, que envolvem déficits na comunicação e interação social, comportamentos repetitivos ou estereotipias e um padrão restrito de interesses (Gadia et al., 2004; Rapin and Tuchman, 2008). O espectro pode ser dividido nas classificações de: 1) autismo clássico, 2) Síndrome de Asperger, e 3) transtornos invasivos do desenvolvimento não especificados (DSM IV). Nos manuais de classificação esses quadros estão localizados dentro do capítulo dos transtornos globais do desenvolvimento (TGD), que inclui além dos TEA, a síndrome de Rett e o transtorno desintegrativo da infância. Pelo DSM-V, recentemente lançado, deixa de existir as subdivisões do espectro, passando a chamar-se apenas Transtornos do Espectro do Autismo. O autismo clássico engloba as características mais proeminentes no transtorno, como déficits cognitivos, comunicativos e sociais, bem como comportamentos estereotipados, sendo essas características a base para o diagnóstico clínico (Gadia *et al.* 2004, Rapin & Tuchman 2008). Apesar da heterogeneidade deste transtorno e da tentativa de criação e um padrão para o diagnóstico, não existem relatos de dois indivíduos com autismo apresentando o mesmo conjunto de sintomas, no entanto todos os indivíduos têm déficits na tríade que engloba o comportamento social, a comunicação verbal e não-verbal e os comportamentos repetitivos ou estereotipados, podendo variar em intensidade entre os indivíduos, porém são presentes ao longo de toda vida (Rapin & Tuchman 2008). Na DSM-V, já se incluem, dentro da caracterização do transtorno, alterações sensoriais importantes, como alta ou baixa responsividade a estímulos sensoriais e interesses atípicos na informação

sensorial. Além desses fatores diagnósticos, existem alguns sintomas associados, como reduzido contato visual, déficit gestual, atrasos de linguagem, inabilidade para interpretar emoções a partir de expressões faciais, hipersensibilidade a estímulos sensoriais, movimentos manuais estereotipados e dificuldade para mudanças em rotinas ou rigidez comportamental (Casanova 2007, Geschwind 2009, Rapin & Tuchman 2008). Características adicionais observadas em alguns casos incluem retardo mental, ansiedade, distúrbios do sono e gastrointestinais, além de maior circunferência craniana e volume cerebral quando jovens (Skoyles 2008, Casanova 2007, Rapin & Tuchman 2008). O diagnóstico desse transtorno somente é possível após dois ou três anos de idade, quando a criança já atinge idade suficiente para a comunicação e o começo das interações sociais complexas. Apesar de existirem sintomas que não podem ser notados nesta idade, como a reduzida coordenação motora, muitos pais percebem problemas no progresso social ou comunicativo das crianças. Os déficits sociais não são propriamente claros na infância, porém gradualmente se tornam mais evidentes com o passar dos anos (Dover & Le Couteur 2007).

A Síndrome de Asperger, descrita pela primeira vez por Hans Asperger em 1944, é uma das grandes controvérsias dentro do TEA. Os prejuízos cognitivos não são observados nessa síndrome, porém prejuízos sociais são bastante semelhantes ao que se observa no autismo clássico, com interesses restritos e um perfil de aprendizado particular, também chamado de deficiência de aprendizagem não-verbal (Volkmar *et al.* 2012).

Os transtornos que não satisfazem os critérios específicos de diagnóstico do DSM-V para autismo clássico, Síndrome de Asperger ou outros transtornos mais caracterizados, são alocados dentro do grupo dos transtornos invasivos do desenvolvimento não especificados. Neste grupo, encontram-se também déficits sociais e prejuízos tanto no comportamento restrito, quanto na comunicação, levando a hipótese desses transtornos serem multifacetas da genética e da complexidade do autismo.

1.1.1. DADOS EPIDEMIOLÓGICOS

Quando descrito pela primeira vez, o autismo era considerado uma condição rara afetando em torno de 4 pessoas em cada 10000 indivíduos. No entanto trata-se de um distúrbio muito mais freqüente, ocorrendo em aproximadamente 1% da população. A ocorrência de autismo nos EUA supera os diagnósticos de AIDS, câncer e diabetes, em crianças, somados (Autism Speaks, 2012).

Segundo dados epidemiológicos do ano de 2012 oriundos do Centro de Prevenção e Controle de Doenças (EUA), a densidade de indivíduos com autismo varia entre 1 a cada 88 crianças identificadas (Tabela 1). Nesse mesmo estudo, dentro do período que abrange os anos 2000 a 2008, a prevalência por 1.000 crianças nos EUA aumentou de 6.7 para 11.3, um aumento considerável quando comparado com dados de 15, ou 20 anos atrás (Fombonne 2003, Kogan *et al.* 2009, Fombonne 2009). Entre o período de 1991 a 1997, a prevalência do autismo aumentou em 556%, passando a afetar mais crianças do que, por exemplo, câncer e síndrome de Down (Muhle *et al.* 2004).

Identified Prevalence of Autism Spectrum Disorders
ADDM Network 2000-2008
Combining Data from All Sites

Surveillance Year	Birth Year	Number of ADDM Sites Reporting	Prevalence per 1,000 Children (Range)	This is about 1 in X children...
2000	1992	6	6.7 (4.5-9.9)	1 in 150
2002	1994	14	6.6 (3.3-10.6)	1 in 150
2004	1996	8	8.0 (4.6-9.8)	1 in 125
2006	1998	11	9.0 (4.2-12.1)	1 in 110
2008	2000	14	11.3 (4.8-21.2)	1 in 88

Tabela 1: Prevalência de TEA nos Estados Unidos da América (EUA) anos de 2000 a 2008. Estudo epidemiológico realizado pelo CDC (EUA).

Esse aumento fez com que as desordens do espectro do autismo se tornassem uma questão importante, não só em saúde, em termos de prevalência, morbidade, mas também em questões de impacto familiar e custo para a sociedade (DiCicco-Bloom *et al.* 2006). Estima-se um gasto médio de 1,6 milhão de dólares por individuo portador de autismo durante a vida (Kogan et al. 2009) e, somando com o custo de intervenções comportamentais intensivas, esse valor pode atingir a faixa de U$ 40.000 a U$ 60.000 por criança por ano, nos Estados Unidos (Autism Speaks, 2012).

Ao contrário do que chegou a ser cogitado (Wing & Potter 2002), vacinações, pelo uso do coadjuvante timerosal contendo mercúrio, não tem relação com o aumento da incidência de autismo (Fombonne 2008). Não há também quaisquer evidências de associação entre autismo, imigração, classe social ou etnicidade (Fombonne 1999). Segundo Fombonne, esse aumento se deve, pelo menos em parte, as mudanças nos critérios de diagnóstico, o que fez com que houvesse uma "migração" de indivíduos, com diagnóstico impreciso para o espectro do autismo (Fombonne 2009). Entretanto essa "migração" não explicaria todos os novos casos. Assim, estudos epidemiológicos e com modelos animais indicam que fatores ambientais podem ser responsáveis pelo aumento na ocorrência de autismo (Fombonne 2003, Schneider & Przewlocki 2005).

1.1.2. SINTOMATOLOGIA E COMORBIDADES

Os sintomas principais do autismo consistem em déficits de interação social e na comunicação, além de uma notável rigidez comportamental e de interesses (Manning-Courtney *et al.* 2013). Apesar de esses sintomas serem sobrepostos a outras desordens e doenças psiquiátricas, somente a presença de todas essas características em um mesmo indivíduo compõem a desordem do autismo. O transtorno em questão possui uma vasta gama de comorbidades que podem estar associadas a ele. A identificação precoce dessas diferentes comorbidades podem otimizar o direcionamento farmacológico com alvos terapêuticos, a qualidade de vida desses indivíduos, além de fornecer possibilidades e hipóteses para o

desenvolvimento científico de mecanismos sobrepostos ou subjacentes a essa desordem, em busca de respostas para a etiologia.

Os critérios diagnósticos no TEA podem variar assustadoramente de intensidade, contabilizando em um verdadeiro espectro: ao passo que se é observado um vasto vocabulário e gramática em alguns pacientes diagnosticados com autismo, é observado em outros somente frases repetitivas, podendo em alguns casos, observar a não apresentação da fala. A rigidez comportamental pode ser justificada por dificuldades por parte desses indivíduos em lidar com mudanças em sua rotina (Goldman *et al.* 2009).

Muitas crianças com autismo apresentam prejuízo intelectual e aproximadamente 75% precisa de apoio social e educacional significativo (Mefford, 2012; Bauman, 2010; Bauman, 2010). Esse prejuízo cognitivo pode ser explicado pelo maior risco promovido pela epilepsia, uma comorbidade bastante frequente no TEA , estando presente em pelo menos 30% dos indivíduos. Com esses dois fatores presentes, o indivíduo possui maior risco de desenvolver atraso no desenvolvimento e prejuízo intelectual do que aquelas com um ou outro distúrbio (Nazeer & Ghaziuddin 2012, Silver & Rapin 2012).

Hiperatividade, agressão, auto-mutilação, distúrbios do sono, além de sinais depressivos, psicóticos e comportamento suicida também podem ocorrer (Nazeer & Ghaziuddin 2012, Duchan & Patel 2012, Kaplan & McCracken 2012, Silver & Rapin 2012). Estudos clínicos apontam relatos de pacientes com hipo ou hiper-responsividade a estímulos sonoros, luminosos e táteis (Grandin 2009, Ben-Sasson *et al.* 2009, Kern *et al.* 2007), podendo apresentar graus variáveis de sensibilidade à dor (Hughes 2009, Klintwall *et al.*). Nos últimos anos, pesquisadores têm dado maior atenção ao sistema nervoso entérico e suas relações com o autismo, onde problemas gastrointestinais são evidenciados (Skoyles 2008, Casanova 2007, Rapin & Tuchman 2008). Distúrbios hormonais e metabólicos também podem estar presentes nestes indivíduos (Bauman 2010). Além disso, doenças como Esclerose tuberosa, X frágil e Síndrome de Angelman são frequentemente associadas ao autismo (Silver & Rapin 2012).

1.1.3. DIAGNÓSTICO

O diagnóstico do autismo é clínico, realizado por meio de uma avaliação detalhada do desenvolvimento do paciente e de uma avaliação sistemática, consistindo num processo que requer tempo e uma equipe multidisciplinar para avaliação de dados comportamentais, história familiar e relatos dos pais (Falkmer *et al.* 2013). Devido ao espectro, a identificação de casos mais moderados ou com presença de comorbidades psiquiátricas podem dificultar os critérios diagnóticos. Embora escalas e entrevistas padronizadas como o Plano de Observação do Diagnóstico de Autismo (ADOS- Autism Diagnostic Observation Schedule), a Ferramenta de Triagem para Autismo aos 2 anos (STAT – Screening Tool for Autism in 2-Years-Olds), a Entrevisa de Diagnóstico do Autismo (ADI – Autism Diagnostic Interview) e a Entrevista Diagnóstica de Distúrbios Sociais e de Comunicação (DISCO – Diagnostic Interview of Social and Communication Disorders) (Falkmer *et al*, 2013) auxiliem no estabelecimento do diagnóstico, não há nenhum exame que detecte o transtorno (Huerta & Lord 2012).

Técnicas de imaginologia como tomografia computadorizada e ressonância magnética mostram alterações eletrofisiológicas, anatômicas e funcionais no encéfalo de pacientes com autismo, sendo comumente utilizadas na pesquisa sobre o distúrbio. Contudo, não há relatos de seu uso como potencial diagnóstico (Tchaconas & Adesman 2013).

1.1.4. ETIOLOGIA

Sendo classificado como uma transtorno multifatorial, o TEA possui componentes genéticos e ambientais em sua etiologia, que permanece pouco compreendida.

O fator genético foi evidenciado por meio de pesquisa com gêmeos monozigóticos, no intuito de verificar como se comporta a herdabilidade do

autismo. Estudos revelaram que o risco de autismo entre gêmeos monozigóticos pode atingir o patamar de 12 vezes mais alto do que na população neurotípica. Em gêmeos dizigóticos, esse risco cai para 4 vezes quando comparado com a população neurotípica (Greenberg *et al.* 2001). Apesar desses dados, o fator genético dentro do TEA não parece ser um componente determinante para desencadear o transtorno em uma população, sendo justificado pelo fato de que a concordância dos genes-alvo em indivíduos afetados é em torno de 1% (Levy *et al.* 2011). Ainda sim esses genes podem estar envolvidos como alterações em seu número variado de cópias (*Copy Number Variation* – CNV) nos seus locais gênicos, contribuindo nas alterações em rotas biológicas, convergentes àquelas envolvidas no TEA.

Cada vez mais se evidencia que o fator ambiental é um dos principais responsáveis pelo surgimento do TEA, uma vez que o alto crescimento da prevalência desse transtorno na população não parece ser explicado pelo fator genético. Estudos demonstraram que 30% dos fetos expostos à talidomida entre 20° e o 24° dia de gestação foram diagnosticados com autismo (Miller & Stromland 1999). Entretanto, a talidomida apresenta diferentes efeitos em primatas e em roedores, sendo que em primatas pode gerar entre outros, crescimento aberrante e deficiente dos membros. Outros teratógenos tidos como fatores de risco são o ácido valpróico (VPA) e o etanol (Ingram *et al.* 2000). O VPA tem seu mecanismo teratogênico baseado em sua atuação como indutor de alterações no fechamento do tubo neural, observadas similarmente em roedores e seres humanos (Bambini-Junior *et al.* , Ingram et al. 2000). Outros fatores de risco para o autismo incluem idade avançada dos pais, baixo peso ao nascer, sangramento materno, diabetes gestacional e exposição do feto a altos níveis de androgênios intrauterinos (Gardener *et al.* 2009, Baron-Cohen 2002).

1.1.5. ESTRUTURAS RELACIONADAS

Visto que a linguagem, funções executivas, interação social e comportamento emocional estão prejudicados no autismo, muitos estudos têm como foco o córtex pré-frontal (CPF), com estudos relatando um aumento de 67% no número de neurônios no CPFem encéfalos de pacientes com autismo (Courchesne *et al.* 2001). As alterações de conectividade estão entre os achados mais consistentes. A hipoconectividade a longa distância entre córtices frontal e temporal, e entre esses com outras estruturas foi documentado por alguns grupos de pesquisa (Just *et al.* 2004, Koshino *et al.* 2005, Villalobos *et al.* 2005). Enquanto isso, uma hiperconectividade foi descrita localmente no CPF (Courchesne & Pierce 2005) acompanhada de aumento no número de colunas e diminuição da espessura delas (Chomiak & Hu 2012).

O cerebelo possui um papel fundamental na locomoção, equilíbrio e diversas funções motoras e cognitivas do SNC, e encontra-se alterado dentro do TEA, tanto funcional e morfologicamente, com a redução de volume e no número de células de Purkinje, quanto geneticamente (Tan *et al.* 2010, Hong *et al.* 2000, Aldinger *et al.* 2012).

Alterações em estruturas límbicas são bastante evidentes e documentadas nessa desordem, como o hipocampo e a amígdala. O hipocampo faz parte do sistema límbico, sendo necessário para mecanismos de aprendizado, memória e diversas funções cognitivas, sendo um componente de estudo frequente dentro de modelos animais de autismo. Estudos relatam um aumento no tamanho dessa estrutura em indivíduos com TEA (Groen *et al.* 2010, Rojas *et al.* 2004). A amígdala é composta por um complexo de núcleos, sendo um local bastante associado com medo e agressividade. Estudos sobre o tamanho dessa região no TEA mostram resultados discordantes, havendo encontrado-se aumento (Groen et al. 2010, Nordahl *et al.* 2012) e diminuição na amígdala (Dalton *et al.* 2007). Outros dados interessantes sobre essa região incluem uma conectividade atípica e redução da habituação da amígdala

correlacionada com escala de responsividade social (Swartz *et al.* 2012, Murphy *et al.* 2013).

Dada a presença de comportamentos repetitivos e estereotipados em pacientes com autismo, alterações nos núcleos da base também já foram encontradas (Langen *et al.* 2007, Stanfield *et al.* 2008, Takarae *et al.* 2007). Durante imitação de expressões faciais e tarefas de flexibilidade cognitiva, pacientes com autismo apresentam hipoativação do estriado (Dapretto *et al.* 2006, Shafritz *et al.* 2008).

Outras estruturas alteradas incluem o giro fusiforme, localizado no lobo temporal ventral, o qual tem papel no reconhecimento de faces. Alterações nessa região parecem fundamentais para vários sinais comportamentais observados em pacientes com autismo, uma vez que sua atividade encontra-se reduzida durante processamento de faces (van Kooten *et al.* 2008, Critchley *et al.* 2000, Pierce *et al.* 2001). As áreas de Broca e Wernicke, envolvidas com a linguagem, tambem mostram ativação anormal no autismo (Verhoeven *et al.* 2009).

1.2. VIAS INIBITÓRIAS E O CÓRTEX SOMATOSSENSORIAL PRIMÁRIO

A principal neurotransmissão inibitória do SNC é realizada por populações de neurônios que sintetizam e liberam o neurotransmissor ácido γ-aminobutírico (GABA), que exerce seus efeitos em um grupo de receptores ionotrópicos (GABA-A) e metabotrópicos (GABA-B).

Quando o GABA se liga aos seus receptores GABA-A, em encéfalos maduros, ocorre a hiperpolarização pós-sináptica pelo aumento do influxo intracelular do íon cloreto. Essa classe de receptores pode ser dividida em: 1) receptores sinápticos que produzem uma inibição caracterizada como rápida, em resposta a altas concentrações (mM) do neurotransmissor liberado e 2) receptores sinápticos que produzem uma liberação caracterizada como lenta, com uma condutância tônica persistente em resposta a baixas concentrações

(nM ou μM) do neurotransmissor na fenda sináptica (Farrant & Nusser 2005). As principais vias de síntese, liberação e recaptação do GABA estão ilustradas na figura 1.

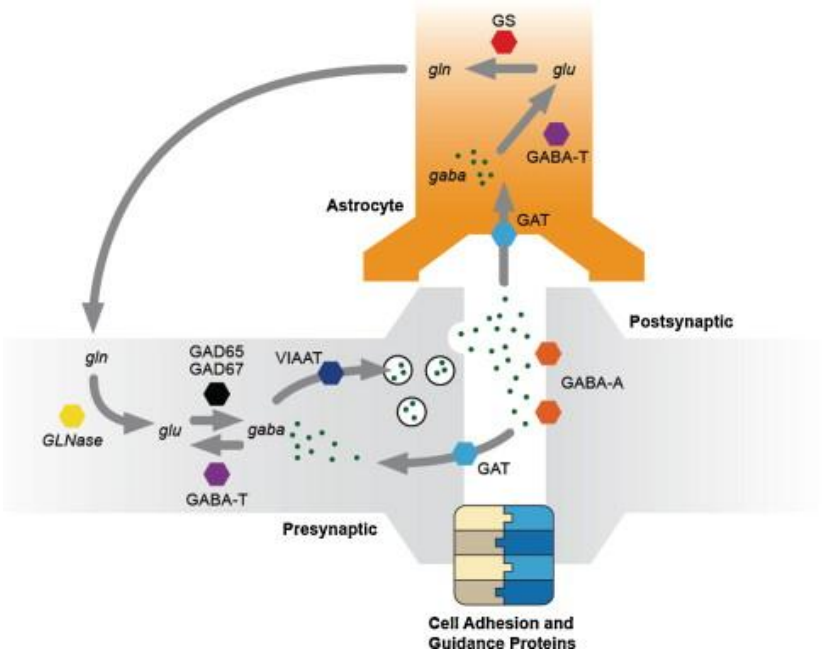

Figura 1.: Vias sinápticas responsáveis pela síntese, liberação e recaptação de GABA. Exemplificando o receptor GABA-A, não sendo mostrados o receptor GABA-B ou os auto-receptores GABA-A e GABA-B (Coghlan *et al.* 2012). O GABA, na fenda, pode ser recaptado, via transportadores específicos, por células gliais ou pelo próprio neurônio pré-sináptico. Nas células gliais, esse neurotransmissor é metabolizado até glutamina e retorna, dessa forma, aos neurônios, que podem reconvertê-lo a GABA e armazenálo em vesículas. No neurônio pré-sináptico, o GABA pode, também, ser re-armazenado em vesículas, bem como ser transformado em glutamato.

Os receptores GABA-B possuem seu mecanismo de hiperpolarização pela ativação de uma proteina G acoplada a canais de íons potássio, produzindo uma corrente inibitória mais lenta do que comparada com a corrente produzida por receptores ionotrópicos GABA-A (Padgett & Slesinger 2010).

A neurotransmissão GABAérgica no SNC é notavelmente refinada, uma vez que esses neurônios exibem grande diversidade morfológica, fisiológica e bioquímica (Somogyi & Klausberger 2005). Essas populações neuronais, podendo se apresentar na forma de interneurônios, possuem a tendência de

formar circuitos inibitórios distintos baseados na conectividade elétrica recíproca (Beierlein *et al.* 2003). Além dessa finalidade, essas células possuem propensão a realizar sinapse em compartimentos subcelulares específicos de neurônios alvos (Muller *et al.* 2006, Muller *et al.* 2007), uma vez que neles ocorre a expressão gênica de diferentes subunidades de receptores GABAérgicos (Fritschy & Brunig 2003, Klausberger *et al.* 2002). Esses fatos corroboram, portanto, circuitos com alto grau de refinamento no SNC maduro.

Devido a vias cruciais desses neurônios inibitórios nas funções cognitivas como o controle da excitabilidade tanto a nível celular quanto a nível de redes neuronais, direcionamento do fluxo de informação e na regulação de processos cognitivos (Cardin *et al.* 2009, Wang *et al.* 2009), estudos com essa via têm apresentado diversas informações relacionadas ao estudo do autismo. Perdas de interneurônios ou disfunções nessa inibição em encéfalos de indivíduos com a Síndrome do X Frágil, autismo, Síndrome de Rett, esquizofrenia e epilepsia são alguns dos exemplos (Chao *et al.* , Cossart *et al.* 2001, Gogolla *et al.* 2009, Selby *et al.* 2007, Marin).

As colunas neocorticais são unidades morfo-funcionais, cuja arquitetura pode ter sofrido pressão evolutiva seletiva em diferentes linhagens de mamíferos, em resposta a encefalização e especialização de habilidades cognitivas (Raghanti et al.). De acordo com o modelo de organização cortical, neurônios, células gliais e suas conexões formam um sistema vertical multi-conectado onde as células de cada minicoluna se unem a uma unidade funcional altamente coordenada (Mountcastle 1997). Nesse contexto, a menor unidade da anatomia cortical é a minicoluna, que é definida por um arranjo radial de neurônios, podendo essas unidades se arranjarem entre si, formando macrocolunas, como encontrados no córtex somatossensorial na forma de "barril" (do inglês *barrel somatosensory cortex*), observado frequentemente na área somatossensorial das vibriças de roedores.

Os interneurônios inibitórios GABAérgicos são um grupo de células que governam a microcircuitaria local cortical, sendo fundamentais para o processamento intra e intercolunar (Casanova *et al.* 2003, DeFelipe *et al.* 1986, Ascoli *et al.* 2008). Seus subtipos morfológicos são altamente conservados

entre os mamíferos (Sherwood *et al.* 2009), mas existe uma significativa variação entre os filos, além de sua diversidade, densidade, distribuição e padrões de desenvolvimento possuírem suas peculiaridades (Hof & Sherwood 2005, Sherwood *et al.* 2007). Em roedores e outras espécies não-primatas, os interneurônios inibitórios ocupam 15% ou menos da população neuronal cortical, enquanto que no córtex de primatas, esse valor pode atingir até 20% da população (Defelipe *et al.* 1999, DeFelipe *et al.* 2002).Ainda, a migração desses interneurônios parece ser diferente entre roedores e primatas, com sítios adicionais de neurogênese no neurepitélio ventricular lateral em primatas (Petanjek *et al.* 2009).

As origens distintas, bem como a distribuição desse grupo de interneurônios espécie-específicas no neocórtex podem estar relacionadas com as diferenças observadas nas habilidades cognitivas. Os interneurônios inibitórios podem ser classificados em subpopulações baseada em sua imunoreatividade para 3 proteínas ligantes de cálcio: CB (calbindina-D28k), CR (calretinina) e PV (parvalbumina). Cerca de 90% de todos os interneurônios GABAérgicos corticais colocalizam com um desses marcadores, com uma pequena sobreposição entre populações separadas (DeFelipe, 1997; Zaitsey *et al*., 2005). A figura abaixo ilustra essas subpopulações, relacionando com a sua orientação ao longo das camadas corticais:

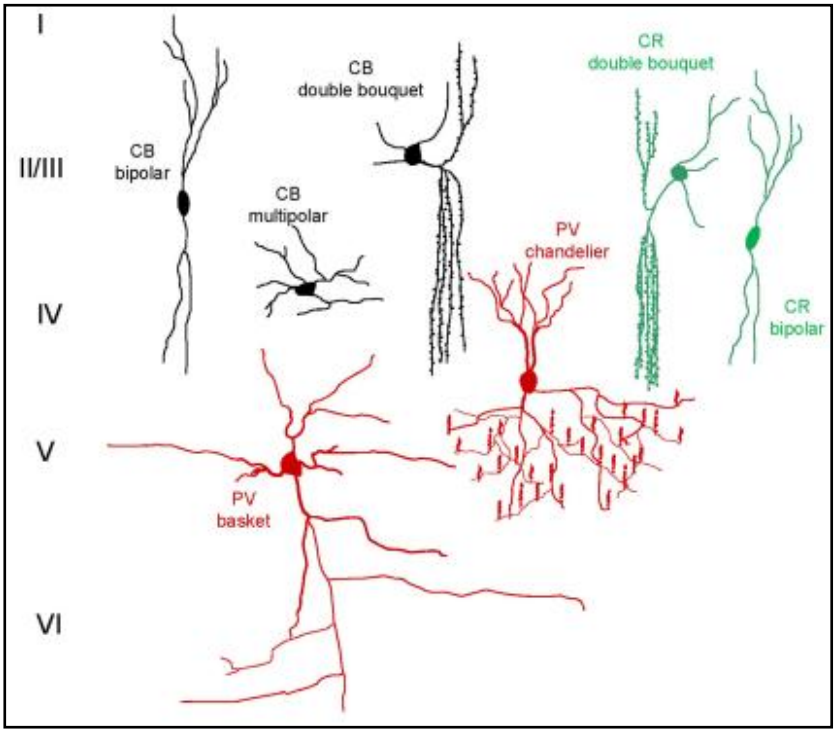

Figura 2: Representação da disposição dos interneurônios GABAérgicos colunares em relação às camadas corticais. A classificação desses interneurônios é realizada a partir da sua imunoreatividade à proteínas ligantes de cálcio (CB - calbidina, CR - calretinina e PV - parvalbumina).

Essas diferentes classes de interneurônios interagem com células piramidais, modulando o processamento do circuito cortical. Um exemplo dessa modulação pode ser observado com os papéis desses interneurônios dentro da coluna: os neurônios CB e CR estão envolvidos praticamente na comunicação intracolunar. Já os imunoreativos a PV, os quais as células multipolares *large basket* e *chandelier* são inclusas, estão envolvidos na sinalização transcolunar (figura 2). As células *large basket* possuem longos axônios, os quais se estendem horizontalmente, tendo como alvo o pericário de células piramidais de diferentes minicolunas (Somogyi *et al.* 1998). As células *chandelier* promovem a inibição lateral por meio de conexões sinápticas com segmentos iniciais de axônios das células piramidais (DeFelipe 1997, Li *et al.* 2002). Esses tipos celulares imunoreativos a PV regulam oscilações rítmicas de populações de células piramidais, e são representados por picos rápidos em seus potenciais de ação breves, com a ausência de picos adaptativos (Zaitsev *et al.* 2005, Sohal *et al.* 2009).

Dentro da supopulação de interneurônios imunoreativos a CR, existe uma grande variabilidade morfológica, diversificando-os em celulas bipolares, *double bouquet* e do tipo Cajal-Retzius (DeFelipe 1997). As células bipolares e do tipo *double bouquet* possuem arborizações axonais que se estendem verticalmente, alcançando dendritos de células piramidais em diferentes camadas do córtex (Figura 2), dentro das colunas vizinhas (DeFelipe 1997, DeFelipe *et al.* 1989)

Os interneurônios GABAérgicos, particularmente aqueles imunoreativos a CB, como a célula *double bouquet*, contribuem significativamente para a morfologia e distribuição das minicolunas no córtex de primatas (Buxhoeveden & Casanova 2002, Casanova *et al.* 2009).

1.3. GABA, CÓRTEX E O AUTISMO

Estudos analisando o balanço entre as vias excitatórias e inibitórias do SNC revelaram a presença de uma excitotoxicidade proveniente do desbalanço entre o glutamato e o GABA, em encéfalos de pacientes com autismo (Essa *et al.* 2012).

Uma vez que o equilíbrio entre a neurotransmissão glutamatérgica e GABAérgica é requerida para a regulação da cognição e de comportamentos emocionais, as primeiras hipóteses de que esse sistema pode estar fortemente ligado com o TEA passou de uma questão para uma confirmação (Ingram et al. 2000). Da mesma forma que alguns dados revelam o aumento da neurotransmissão excitatória no SNC pelo neurotransmissor glutamato, evidências demonstram que essa desregulação glutamatérgica tem relação com um desbalanço GABAérgico, com a consequente redução da neurotransmissão inibitória no SNC de pacientes com autismo (Banerjee *et al.* 2012).

Alguns estudos de expressão gênica revelaram padrões anormais de expressão de genes de receptores e enzimas ligadas à neurotransmissão GABAérgica (Coghlan et al. 2012). Enzimas como GAD65 e GAD67 apresentam seus níveis proteicos reduzidos em 50% nos córtices parietal e cerebelar de pacientes com autismo (Durand *et al.* 2011). Vários alvos genéticos têm sido documentados ao longo dos anos, mas estudos mostram que o cromossomo 15 pode ter uma importante relação com o autismo, devido às suas alterações cromossômicas em determinadas regiões que codificam subunidades do receptor GABA-A (Coghlan et al. 2012).

Muitas anormalidades neuropatológicas parecem afetar a organização e funcionamento das minicolunas e dos interneurônios que as compõem. A diminuição de populações de interneurônios específica (interneurônios imunoreativos a CB) foi observada no córtex pré-frontal de pacientes com esquizofrenia (Sakai *et al.* 2008), bem como alterações no tamanho das

minicolunas (Casanova *et al.* 2008b, Di Rosa *et al.* 2009). Dentro do contexto da doença de Alzheimer, a estrutura das minicolunas é seletivamente desarranjada e a perda da organização colunar é relacionada com o número de emaranhados neurofibrilares (Buldyrev *et al.* 2000). Mudanças morfológicas nas minicolunas parecem ser consistentes com as anormalidades do desenvolvimento em vez de processos patológicos progressivos (Casanova *et al.* 2005, Casanova et al. 2008b). Já é postulado que o controle inibitório GABAérgico das minicolunas corticais está comprometido dentro do autismo (Casanova et al. 2003). Tanto na desordem do autismo, quanto na Síndrome de Asperger, é observado um estreitamento da minicoluna (Casanova et al. 2003, Casanova *et al.* 2002a, Casanova *et al.* 2002b, Casanova *et al.* 2002c). Uma vez que estudos mostram que este espaço é dependente de populações de interneurônios inibitórios, um déficit do controle GABAérgico é esperado. A modulação da atividade das minicolunas pode ser alterada tanto pela conectividade local, quanto pela conectividade a longa distância, resultando em uma super excitação colateral entre as minicolunas, observado no autismo (Casanova & Trippe 2009, Casanova *et al.* 2008a). Essa super-excitação pode estar envolvida na incidência de convulsões em pacientes com o transtorno (Casanova et al. 2003) e, esta relação encontra respaldo em recentes relatos de déficits em ambos interneurônios imunoreativos a PV e a CR com displasias corticais focais associadas com epilepsia (Zamecnik *et al.* 2006, Barinka *et al.*).

Levando em consideração o fato de a epilepsia ser a comorbidade mais frequente dentro do TEA, estudos analisando as fibras GABAérgicas com a prevalência de convulsões em pacientes autistas foram realizados e, não surpreendetemente, mostraram anormalidades (Casanova et al. 2003). Outros estudos utilizando imagenologia mostraram menor ligação de GABA entre os receptores GABA-A na amígdala, verme cerebelar, córtices frontal, parietal e occipital em desordens genéticas com comportamentos autistas presentes (Chugani 2012).

2. OBJETIVOS

2.2. OBJETIVOS GERAIS

Quantificar e analisar a densidade de células neuronais e não neuronais e o imunoconteúdo do principal neurotransmissor inibitório do sistema nervoso central, GABA, na área somatossensorial primária no campo de barris (*Barrel Fields*) do córtex de ratos Wistar prenatalmente expostos ao ácido valproico.

2.3. OBJETIVOS ESPECÍFICOS

Analisar, em amostras de Córtex, na idade pós-natal P120:

1) O conteúdo de GABA intra e extracelular, a partir da co-localização de :
    a. GABA, para a verificação de neurônios GABAérgicos;
    b. Neu-N, para a confirmação do tipo celular neuronal.
2) A relação entre a presença de GABA com o padrão colunar observado em neurônios em camadas corticais do SNC de ratos controle e ratos expostos ao ácido valpróico no modelo animal de autismo

3. TRABALHO EXPERIMENTAL NA FORMA DE ARTIGO CIENTÍFICO

A ser submetido ao periódico Brain Research

ANIMAL MODEL OF AUTISM INDUCED BY PRENATAL EXPOSURE TO VALPROATE: QUANTITATIVE ANALYSIS OF NEURONAL, NONNEURONAL CELLS AND CORTICAL IMMUNOCONTENT OF GABA

Mellanie Fontes Dutra da Silva.[a,b,c], Victorio Bambini Junior[a,b,c], Gabriela Muller de Melo.[b], Guilherme Bauer Negrini.[a,b,c], Carla Moreira Furtado[e], Cecília Hedin-Pereira.[d,e], Carmem Gottfried.[a,b,c*]

[a] Research Group in Neuroglial Plasticity at Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

[b] Department of Biochemistry, Institute of Health's Basic Science at Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

[c] Translational Research Group in Autism Spectrum Disorders (GETEA) at Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

[d] Institute of Biophysics Carlos Chagas Filho at University of Rio de Janeiro 21941-590, Rio de Janeiro, Brazil

[e] Anatomy Program and Morphological Sciences Program, Cellular Neuroanatomy Lab at Biomedical Sciences Institute in Federal University of Rio de Janeiro, Rio de Janeiro 21941-590, Brazil

*Correspondence address: Carmem Gottfried, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio grande do Sul, Rua Ramiro Barcelos 2600, anexo, 90035-003, Porto Alegre, RS, BRAZIL, faz: +55 51 3308 5551. Email address: cgottfried@ufrgs.br

ABSTRACT

Autism spectrum disorders (ASD) are characterized by deficits in social interaction, language and communication impairments and repetitive and stereotyped behaviors, with involvement of several areas of the central nervous system (CNS), including cortical areas, as the primary somatosensory area. Impairments in excitatory/inhibitory rate in CNS, especially in cortical regions related to sensory processing, may be involved in autism disorders, associated with cognitive and behavioral characters, morphology and neuronal organization in columnar patterns in these areas. The present study investigates the cortical layers II/III and V of primary somatosensory area from 120 (P120) days old male rats prenatally exposed to valproic acid (VPA) as an animal model of autism. Herein, we analyzed quantitatively the number of neuronal cells and nonneuronal cells, the columnar cortical organization and the GABA labeling, targeting GABAergic neurons, by immunohistochemistry labeling NeuN and GABA. In the VPA group, results show impairment in columnar organization and in the localization of NeuN in neurons from layer II/III and V as well. Besides, the reduction in nonneuronal cells in both layers and GABAergic neurons in layer V were evidenced in VPA group, representing by a decrease in GABA and NeuN labeling. These data highlight the importance of the balance in excitatory/inhibitory synapses at the cortical level, pointing out important aspects to be considered by a reduction in GABAergic inputs in this area. These results may contribute in both physiopathological and pharmacological approaches in ASD.

Highlights

► Animal model of autism induced by prenatal exposure to valproic acid (VPA). ► Neuronal and nonneuronal cell quantification. ► VPA-impairments in columnar organization at layers II/III and V. ►VPA decreased GABAergic neurons in layer V and nonneuronal cells in both layers ► GABAergic misbalance in primary somatosensory area is possibly involvement in autism.

Keywords

Autism; Valproic acid; Animal model; GABA; Columnar Organization

1. INTRODUCTION

The term Autism Spectrum Disorder (ASD) refers to a group of conditions characterized by deficits in social interaction, language and communication impairments and repetitive and stereotyped behaviors. As stated in the Diagnostic and Statistical Manual of Mental Disorders-IV-Text Revision (DSM-IV-TR), ASD comprises Autistic Disorder (also called ‘‘classic’’ autism), Pervasive Development Disorder Not Otherwise Specified (PDDNOS), and Asperger’s Disorder (Gadia et al., 2004). Although different theories have emerged trying to explain the etiology of this disorder, it remains unknown. Besides, diagnostic criteria still lacks objective information, with limited treatment options for the entire spectrum. Genetic factors such as mutations, deletions and copy number variants are implicated in causation of autism (Abrahams and Geschwind, 2008; Grigorenko, 2009). However, epidemiological studies have evidenced that adverse environmental conditions such as maternal exposure to infections, ethanol, thalidomide and Valproic Acid (VPA) increase the risk of autistic offspring (Arndt et al., 2005; Dufour-Rainfray et al., 2011), explaining the increasing autism prevalence mainly when combined with changes in diagnostic practice. After clinical and animal studies, many encephalic structures have been implicated in autism pathology, including frontal cortex (Courchesne and Pierce, 2005a),, cerebellum (Amaral et al., 2008; Courchesne and Pierce, 2005b), amygdala (Schultz, 2005), cingulate cortex (Oblak et al., 2009; Oblak et al., 2011) and the hippocampus (Courchesne and Pierce, 2005a). Recent studies in postmortem brain samples from individuals with autism have shown that young prefrontal cortex presents impaired developmental pathways while the adult tissue displays arrested growth and degeneration (Chow et al., 2012). There is also much evidence showing that specific neurotransmitter systems may be altered; such as serotonin, GABA (Oblak et al., 2009; Oblak et al., 2010) glutamate (Choudhury et al., 2011) and others (Lam et al., 2006). As a high refined transmission, the GABAergic synapses are crucial not only in the control of brain homeostasis during developing and mature neuronal circuits establishment, but it makes necessary also during the pregnancy. This neuronal population controls the migration and maturation of pyramidal neurons, as well its columnar

organization, essential for the information processing in CNS (DeFelipe et al., 1986; Owens and Kriegstein, 2002; Pizzarelli and Cherubini). Furthermore, a misbalance in the excitatory/inhibitory rate, caused by impairments in GABAergic neurotransmission, can affect cognition abilities and emotional behaviors, as show in autism disorders (Banerjee et al., 2012; Ingram et al., 2000). Cortical neurons are organized as an information processing system of radial structure called columns, and the transmission flow are governed by interneurons and its inhibitory synapses (Ascoli et al., 2008; Casanova et al., 2003; DeFelipe et al., 1986). Altered GABAergic transmission in cortical layers can explain some features observed in autistic patients, as hyper sensibility to sensory stimulus (Rubenstein and Merzenich, 2003) due to the columnar disorganization, causing misprocessing inside the column and in its neighborhood (Ascoli et al., 2008; Casanova et al., 2003; DeFelipe et al., 1986). In the present work we quantified the number of nonneuronal and neuronal cells; the amount of GABAergic cells and evaluated the columnar organization profile in primary somatosensory area from adult male rats prenatally exposed to VPA (Bambini-Junior et al., 2011).

2. RESULTS

2.1. Columnar Organization of neurons and Cellular NeuN profile

Illustrative images show the columnar organization of neurons in layer II/III (Figure 1) and V (Figure 2) of primary somatosensory cortex from P120 rats, respectively. In both layers was evidenced disorganization in the columnar representation in VPA group, compared to the control group. Furthermore, in VPA group, the neurons showed differences in the morphology, losing the pyramidal architecture. The distribution of NeuN, normally observed in the entire nucleus and cytoplasm, (Figures 1-2, panels A-C) changes in VPA group, locating predominantly in the cytoplasm, near to the plasmatic membrane, as showed by the inset of Figures 1-2 (panels B-D).

2.2. Immunostaining of GABA and NeuN

Illustrative images of NeuN and Gaba immunostaining are shown in Figure 3, for layer II/III (A, D) and V (B, D). In layer V, illustrative figures of the NeuN's

fluorescence are shown in Figures 4A-B and GABA's fluorescence are shown in Figures 4D-E. As showed in Figure 3, no statistical significance was found in the immunostaining quantification of NeuN between VPA (18.04 ± 1.434 N=3) and control groups (21.88 ± 4.512 N=3) *p*= 0.463, and of GABA between VPA (18.38 ± 4.037 N=3) and control groups (22.65 ± 5.424 N=3) *p*= 0.5623, at P120 in primary somatosensory area layer II/III. However, as shown in Figure 4, statistical significance was found when comparing the immunostaining of NeuN in layer V. The presence of NeuN+ cells decreased in VPA (16.15 ± 0.9516 N=3) when compared to the control group (27.78 ± 0.3735 N=3) *p*= 0.0003, but no statistical significance was found in the labeling of GABA between VPA (19.75 ± 2.981 N=3) and control group (25.42 ± 2.476 N=3) *p*= 0.2173.

2.3. Number of GABAergic Neurons

Illustrative images of layers II/III and V are shown in Figures 5 (A-F) and 6 (A-F), respectively. No changes were observed in number of GABAergic neurons at layer II/III between control (4.000 ± 2.082 N=3), and VPA (5.000 ± 1.155 N=3) groups, *p*=0.6960, as demonstrated in Figure 5 G. However, a significant decrease (52 %) in the number of GABAergic neurons was observed at layer V in VPA group (6.833 ± 0.6009 N=3) compared to the control (14.33 ± 1.856 N=3) *p*=0.0184 (Figure 6G).

2.4. Number of Neuronal and, Nonneuronal Cells

Illustrative images of layers II/III and V layers II/III and V are shown in Figures 7A-B and 8A-B. There was no difference in the number of neuronal between VPA (202.7 ± 18.84 N=3) and control group (180.7 ± 9.905 N=3) *p*=0.3596, neither in the number of total cells between VPA (314.7 ± 35.10 N=3) and control group (367.3 ± 6.984 N=3), *p*=0.2151. Nevertheless, the number of nonneuronal cells decreased 39.1% in VPA group when compared to the control group (from 184.3 ± 16.05 N=3 to 112.0 ± 16.29 N=3, *p*=0.0341) (Figure 7C). In layer V was observed no difference in the number of neurons between VPA (152.7 ± 2.333 N=3) and control group (136.0 ± 7.810 N=3) *p*=0.1104, neither in total cells between VPA (278.3 ± 3.180 N=3) and control group (295.3 ± 7.688 N=3), *p*=0.1105. However, the number of nonneuronal cells decreased

26.36% in VPA group when compared to the control group (from 165.0 ± 4.223 N=4 to 121.5 ± 5.315 N=4, *p*=0.0007) (Figure 8C).

2.5. The targets for NeuN/Fox-3 transcriptional factor

Figure 9 illustrates the main possible targets for the transcriptional factor NeuN/Fox-3, a RNA-binding protein that regulates alternative splicing events, by text mining search in String 9.05. The description of genes, are summarized in Table 2.

Genes involved in inflammatory processes, shown in red circles: IBA1 (Ionized calcium-binding adapter molecule 1), also known as AIF-1 (allograft inflammatory factor 1), gene position 2-147 and ENSP00000415805, gene position 14-161. These targets play a role in RAC signaling and in phagocytosis and may be involved in macrophage activation and function. They also promote the proliferation of vascular smooth muscle cells and of T- lymphocytes, enhancing lymphocyte migration.

Genes involved in neuronal migration and differentiation, shown in blue circles: GFAP (glial fibrillary acidic protein), a class-III intermediate filament, is a cell-specific marker that, during the development of the central nervous system, distinguishes astrocytes from other glial cells. DCX, (Doublecortin). It seems to be required for initial steps of neuronal dispersion and cortex lamination during cerebral cortex development, may acting by competing with the putative neuronal protein kinase DCAMKL1 in binding to a target protein. It also may in that way participate in a signaling pathway that is crucial for neuronal interaction before and during migration, possibly as part of a calcium ion-dependent signal transduction pathway. NES (Nestin), may play a role in the trafficking and distribution of Initiation Factors proteins and potentially other cellular factors to daughter cells during progenitor cell division, - by similarity and RBM45 (RNA binding motif protein 45), RNA-binding protein with binding specificity for poly(C), which may play an important role in neural development. Another genes-target for NeuN/Fox-3: CALB1 (calbindin 1, 28kDa), buffer of cytosolic calcium. May stimulate a membrane Ca^{2+}-ATPase and a 3',5'-cyclic nucleotide phosphodiesterase. TSPO (translocator protein, 18kDa), responsible for the manifestation of peripheral-type benzodiazepine recognition sites and is most

likely to comprise binding domains for benzodiazepines and isoquinoline carboxamides. It may play a role in the transport of porphyrins and heme. Plays a role in the transport of cholesterol across mitochondrial membranes in steroidogenic cells, by similarity. NKAIN1 (Na+/K+ transporting ATPase interacting 1) and IQSEC3 (IQ motif, commonly isoleucine and invariably glutamine) and Sec7 domain 3, acts as a guanine nucleotide exchange factor (GEF) for ARF1.

3. DISCUSSION

The primary somatosensory area in rodents is an excellent model to study cortical developmental disorders, since its organization is concentrated in a precise somatotopic pattern, reflecting the whiskers localization in the face of these animals, forming a map of its disposition (Woolsey and Van der Loos, 1970). These structures are called *barrels,* formed by neurons that depolarize with similar stimulus, organized in columns disposed in a somatotopic map (Bonhoeffer and Grinvald, 1991; Issa et al., 2000). The misbalance in excitatory and inhibitory synapses in this area is strongly associated with abnormal sensorial processes in neurological disorders, and this could explain important features found in autism, as the hyper sensibility to sensorial stimuli or abnormal sensorial perception to tactile and auditory stimuli (Rubenstein and Merzenich, 2003). Increasing the excitatory/inhibitory rate by knockout in the *Fmr1* gene leads to the epilepsy, hyper sensibility to stimuli and cognitive disorders, features observed in autism (Gibson et al., 2008; Hagerman and Hagerman, 2002).

Here, we demonstrate that VPA group present alteration in columnar organization, losing the cortical layer's delimitations. Recent work showed that the glicoprotein reelin knockout cause disturbance in the normal inside-out pattern, described as an inversion of cortical layering, appeared as a randomly distributed collection of marker-labeled cells (Wagener et al., 2010). Reelin is a secretory serine protease with an embryological role, guiding neurons and radial glial cells to their corrected positions in the developing brain, and a role in adult brain, involved in a signaling pathway which underlies neurotransmission, memory formation and synaptic plasticity (Wagener et al., 2010). This molecule

can be expressed in Cajal-Retzius (CR) Cells and GABAergic cells. These cell types segregate in the cortical marginal zone (MZ) in response to BDNF signaling, leading to an alternating pattern and a columnar cortical organization, affecting the migration of different neuronal populations (Alcantara et al., 2006). These data suggest that both CR cells and GABAergic neurons play a role in directing the radial migration of late-generated cortical neurons, and their distribution in this area is critical for the development of correct cortical organization. In addition, reelin secreted by CR cells in the MZ is not sufficient to direct the migration of late-born neurons to the upper cortical layers, which most likely requires the presence of reelin-secreting interneurons in layers V–VI (Alcantara et al., 2006).

The columnar organization is responsible not only to the morphological display in mammal cortex, but also to play an important role in integrate the information flow of the cortical layers (Casanova et al., 2003). *Post mortem* studies using autistic brains demonstrate minicolumnar impairments in autism (Casanova et al., 2006). The size and morphology of these refined structures can indicate its physiology, with functional implications when altered (Favorov and Kelly, 1994; Gustafsson, 1997; Seldon, 1981), as a language acquisition delay (Casanova et al., 2003).

In the present work we also demonstrate that prenatal exposure to VPA have less GABAergic neurons in layer V, turning the brain more willing to a misbalance in the excitatory/inhibitory rate. These alterations may lead to an inadequate processing of the sensorial inputs which can be detected by behavioral trials to determine whether the decrease in this neuronal population at layer V is responsible for the sensorial misprocessing found in patients with autism. In this layer, studies have demonstrated the contribution of excitatory and inhibitory synapses at somatic level of pyramidal neurons in the global conductance changing. The excitatory compound comprises 20% of this changing and the inhibitory compound comprehends 80% (Le Roux et al., 2006). Therefore, the decrease of GABAergic neurons in layer V indicates a possible answer to the misbalance in excitatory/inhibitory rates, found in this area in autism spectrum disorders. As we have shown in results, the number of GABAergic neurons in layer V is decreased in adult brain. Investigations with

early age's brains are required, in order to enable an explanation to the cortical derangement. Indeed, the involvement of reelin and the GABAergic system is notably stronger in autism.

Glial cells display important roles in the homeostasis of neuronal function and brain plasticity with a number of receptors which can be activate independently of neuronal activity, releasing transmitter, or gliotransmitters (Fiacco and McCarthy, 2006; Rousse and Robitaille, 2006; Volterra and Meldolesi, 2005). *Post mortem* studies verified higher levels of GFAP, the main protein of mature astrocytic cytoskeleton, in frontal, parietal and cerebellar cortex, possibility raising the astroglial activation (Laurence and Fatemi, 2005). Furthermore, in microglia, a myeloid cell resident in CSN, increases in excitatory/inhibitory rate can lead to increases in mobility of its processes, turning the microglial cell more reactive (Banerjee et al., 2012). In this context, we suggest that a decrease in GABAergic neuron may be involved in the microglial reactivation. Besides the decreasing of nonneuronal cells, and knowing that the most part of these cells are represented by glial cells, it did not interfere in their reactivation. Studies must be done to better understand and identify these cell types inside the glial population, that represent this alteration in the primary somatosensory area and how the activity of these cells can modulate possible pathophysiological roles in autism disorders.

The transcriptional factor NeuN is a neuron-specific protein broadly used to target neurons. In fact, the role that NeuN displays in the neuron were discovered recently (Kim et al., 2009). In this context, we also investigated the main described targets for NeuN which includes molecules involved in inflammation and neuronal differentiation and migration. Considering the NeuN act as a transcriptional factor, our results observed in VPA group suggest that with cytoplasmic localization at the cell periphery, their targets may be less expressed. Besides, one of its targets is the the myosin II-B non muscular heavy chain, found only in developing and mature neurons, with a role in regulation of actin, the main compound of neuronal cytoskeleton, related to neuronal migration in CSN (Brown and Bridgman, 2004). These data suggest a possible role for NeuN in the neuronal migration and morphology and more studies must be done to determine its contribution in autism disorders.

The present work demonstrates for the first time to our knowledge alterations in cellular morphology, organization and distribution in the somatosensory cortex in the animal model of autism induced by prenatal exposure to VPA. This is a relevant issue to be investigated in future works, aiming to increase the knowledge of brain impairments induced by autism.

3.1. Conclusions

Our work demonstrates that the neuropathological alterations leading to autism may be due to a primary GABAergic alteration. Since the inhibitory transmission is responsible for neuronal migration, also playing important roles in cortical organization at the columnar level and is involved in many reciprocal interactions with glial cells, our results aim to help filling this gap that exists concerning autistic neuropathology. Our data show that the VPA-induced primary somatosensory area impairments present a decrease in GABAergic neurons at layer V and nonneuronal cells at layer II/III and V. This scenario seems to be responsible for the alterations in the stimuli processing in this area, leading to the main features of autism, as the hyper sensibility to tactile and auditory stimuli. Furthermore, this GABAergic impairment may be involved in the cortical columnar architecture derangement and these changes in the autistic brain may have significant implications in autism physiopathology. Our results also demonstrate a different localization for NeuN in control and VPA groups, and the implications of the many roles of NeuN, as a regulator of the protein expression involved in inflammation and neuronal differentiation/migration. Besides, it may be involved in neuronal morphology alteration, changing from pyramidal in controls, to cylindrical in VPA group, at layer V. In summary, the GABAergic impairment seems to be correlated to all of these findings, and may therefore be a new target to further studies in the etiology and therapeutic strategies in autism.

4. EXPERIMENTAL PROCEDURES

4.1. Subjects

Female Wistar rats were obtained from the local breeding colony (ICBS-Federal University of Rio Grande do Sul), with 12:12 light cycle (lights on at 7:00 and lights off at 19:00), controlled temperature (22±1°C), water and food *ad libitum*. They were handled in accordance to the governmental and Brazilian experimental Biology Societies Federation guidelines. The estrous cycle was monitored and females were mated overnight. The first day of gestation was considered when spermatozoa were found in the vaginal smear. Valproic acid (Acros Organics, New Jersey, USA) was purchased as the sodium salt and dissolved in 0.9% saline for a concentration of 250 mg/ml. Females received a single intraperitoneal injection of VPA (600 mg/kg, 250 mg/ml diluted in NaCl 0.9%) in the 12.5th day of gestation and control females received physiological saline at the same time as previously described (Bambini-Junior et al., 2011; Schneider and Przewlocki, 2005). Females were housed individually and were allowed to raise their own litters. The offspring rats were housed separately by sex at P21. Male pups from at least three different litters at postnatal day 120 were anaesthetized and transcardiacally perfused in order to perform immunofluorescence analysis as described ahead. The brains were removed and were kept in −80°C.

4.2. GABA and NeuN immunofluorescence

Rats were anesthetized (75 mg/kg ketamine + 10 mg/kg xylazine) and transcardiacally perfused with 0.9%-NaCl solution followed by first 1,5%-paraformaldehyde and after 4%-paraformaldehyde solution before the removal of their brain. The brains were further post-fixed during 4 hours in a 4%-paraformaldehyde phosphate buffer saline (PBS) solution (pH 7.4) and were subsequently cryoprotected in 15% and 30%-sucrose PBS solutions until they were completely submerged. After been freezed in -80ºC freezer, coronal slices (25 µm) were obtained using a −20ºC cryostat (Leica Microsystems GmbH). Brain sections containing the primary somatosensory area slices were arranged in microscopy slides (4 slices per lamina) which were washed three times with PBS at room temperature. Rabbit anti-GABA (GE

Healthcare, 1:1000) and mouse anti-NeuN (Sigma, 1:500) were diluted in a 10% bovine serum albumin 0,1% Triton X-100 PBS solution and incubated during 72 h at 4ºC. Secondary antibodies (AlexaFluor 488 anti-rabbit and 568 anti-mouse, Invitrogen, 1:500) at room temperature for 2h. Slides were then incubated with DAPI solution, mounted with n-propilgalate and cover slipped. Images were obtained in a confocal microscope and cell types and fluorescence were analyzed using the Image J software. The number of nonneuronal, neuronal and total cells, as well the amount of GABAergic neurons, were counted in 184 cm^2/sample (n=3-4 rats/group). The images were obtained by Olympus FluoView 4.0 Viewer.

4.4. Statistical analysis

Data are presented as mean±SE and were analyzed statistically by Student`s *t* test and P≤0.05 was considered as statistically significant. All analyses were carried out using the GraphPad Prism 5 software.

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Figure 1. Effect of prenatal exposure to valproic acid on columnar organization pattern, Neuronal morphology and NeuN distribution at layer II/III of the primary somatosensory area – barrel fields. Illustrative fluorescence micrographs of control (A-B) and VPA (C-D) groups. The inset in B and D illustrate differences in NeuN distribution (red, Alexa 546) between groups. Cellular nuclei are labeled with DAPI (blue) Scale Bar = 200 µm

Figure 2. Effect of prenatal exposure to valproic acid on columnar organization pattern, neuronal morphology and NeuN distribution at layer V of the primary somatosensory area – barrel fields. Illustrative fluorescence micrographs of control (A-B) and VPA (C-D) groups. The inset in B and D illustrate differences in NeuN distribution (red, Alexa 546) between groups. Cellular nuclei are labeled with DAPI (blue)Scale Bar = 200 µm.

Figure 3. Prenatal exposure to valproic acid: Analisis of NeuN+ and GABA+ cells at layer II/III of the primary somatosensory area – barrel fields. Illustrative fluorescence micrographs of control (A-B) and VPA (C-D) groups labeled for NeuN (A, D - red, Alexa 546) and GABA (B, E - green, Alexa 488). Fluorescence quantification of NeuN+ and GABA+ cells in control (C) and VPA (F) groups Statistical Analysis by Student's *t* test. Scale Bar = 200 µm.

Figure 4. Prenatal exposure to valproic acid: Analisis of NeuN+ and GABA+ cells at layer V of the primary somatosensory area – barrel fields. Illustrative fluorescence micrographs of control (A-B) and VPA (C-D) groups labeled for NeuN (A, D - red, Alexa 546) and GABA (B, E - green, Alexa 488). Fluorescence quantification of NeuN+ and GABA+ cells in control (C) and VPA (F) groups. Statistical Analysis by Student's *t* test, **p*= 0.0003. Scale Bar = 200 µm.

Figure 5. Effects of pranatal exposure to valproic acid in the number of GABAergic (GABA+) neurons on layer II/III of the primary somatosensory area – barrel fields. A and D, NeuN labeling (red, Alexa 546) in control and VPA groups, respectively. B and E, GABA labeling (green, Alexa

488) in control and VPA groups, respectively. C and F, merge. G, Number of GABAergic (GABA+) neurons. Statistical Analysis by Student's *t* test.

Figure 6. Effects of pranatal exposure to valproic acid in the number of GABAergic (GABA+) neurons on layer V of the primary somatosensory area – barrel fields. A and D, NeuN labeling (red, Alexa 546)) in control and VPA groups, respectively. B and E, GABA labeling (green, Alexa 488) in control and VPA groups, respectively. C and F, merge. G, Number of GABAergic (GABA+) neurons. Statistical Analysis by Student's *t* test, **p*=0.0184

Figure 7. Effect of prenatal exposure to valproic acid on total, neuronal and nonneuronal cells at layer II/III of the primary somatosensory area – barrel fields. A. Merge of ilustrative images NeuN labeling (red, Alexa 546) + DAPI labeling (blue) in control and VPA groups. B, Quantification of cell number. Statistical Analysis by Student's *t* test, **p*=0.0341. Scale Bar = 200 µm.

Figure 8. Effect of prenatal exposure to valproic acid on total, neuronal and nonneuronal cells at layer II/III of the primary somatosensory area – barrel fields. A. Merge of ilustrative images NeuN labeling (red, Alexa 546) + DAPI labeling (blue) in control and VPA groups. B, Quantification of cell number. Statistical Analysis by Student's *t* test, **p*=0.0007. Scale Bar = 200 µm.

Figure 9: The protein-protein interaction network of NeuN/Fox-3 using a search tool String 9.05. The yellow lines represent text mining evidences of possible targets to the protein of interest. Organism of interest: *Homo sapiens*. Red circles represent genes involved in inflammation and blue circles, genes involved in neuronal migration and differentiation. Table 1, Description of targets showed in figure 9.

Table 1

Targets for NeuN/Fox-3

Targets	Description	Actions
IBA1	Allograft inflammatory factor 1 (AIF-1) Ionized calcium-binding adapter molecule 1 (147 aa, gene position 2-147)	Play a role in RAC signaling and in phagocytosis and may be involved in macrophage activation and function. They also promote the proliferation of vascular smooth muscle cells and of T- lymphocytes, enhancing lymphocyte migration.
ENSG00000235588	Allograft inflammatory factor 1 (AIF-1) Ionized calcium-binding adapter molecule 1 (161 aa, gene position 14-161)	Play a role in RAC signaling and in phagocytosis and may be involved in macrophage activation and function. They also promote the proliferation of vascular smooth muscle cells and of T- lymphocytes, enhancing lymphocyte migration.
GFAP	Glial Fibrillary Acidic Protein; GFAP (a class-III intermediate filament)	a cell- specific marker that, during the development of the central nervous system, distinguishes astrocytes from other glial cells
DCX	Doublecortin	Required for initial steps of neuronal dispersion and cortex lamination during cortex development, competes with the putative neuronal protein kinase DCAMKL1 in binding to a target protein neuronal interaction before and during migration, calcium ion-dependent signal transduction pathway
TSPO	translocator protein (18kDa)	Responsible for the manifestation of peripheral-type benzodiazepine recognition sites and is most likely to comprise binding domains for benzodiazepines and isoquinoline carboxamides. It may play a role in the transport of porphyrins and heme. Plays a role in the transport of cholesterol across mitochondrial membranes in steroidogenic cells (By similarity)
CALB1	Calbindin 1, 28kDa;	Buffers cytosolic calcium. May stimulate a membrane Ca(2+)-ATPase and a 3',5'-cyclic nucleotide phosphodiesterase
NES	Nestin	traffick and distribution of Initiation Factors (IF) proteins and potentially other cellular factors to daughter cells during progenitor cell division (By similarity)
RBM45	RNA binding motif protein 45;	RNA-binding protein with binding specificity for poly(C). It may play an important role in neural development)
IQSEC3	IQ motif (commonly isoleucin and invariably glutamine) and Sec7 domain 3;	Acts as a guanine nucleotide exchange factor (GEF) for ARF1)
NKAIN1	Na+/K+ transporting ATPase interacting 1	

Figure 1

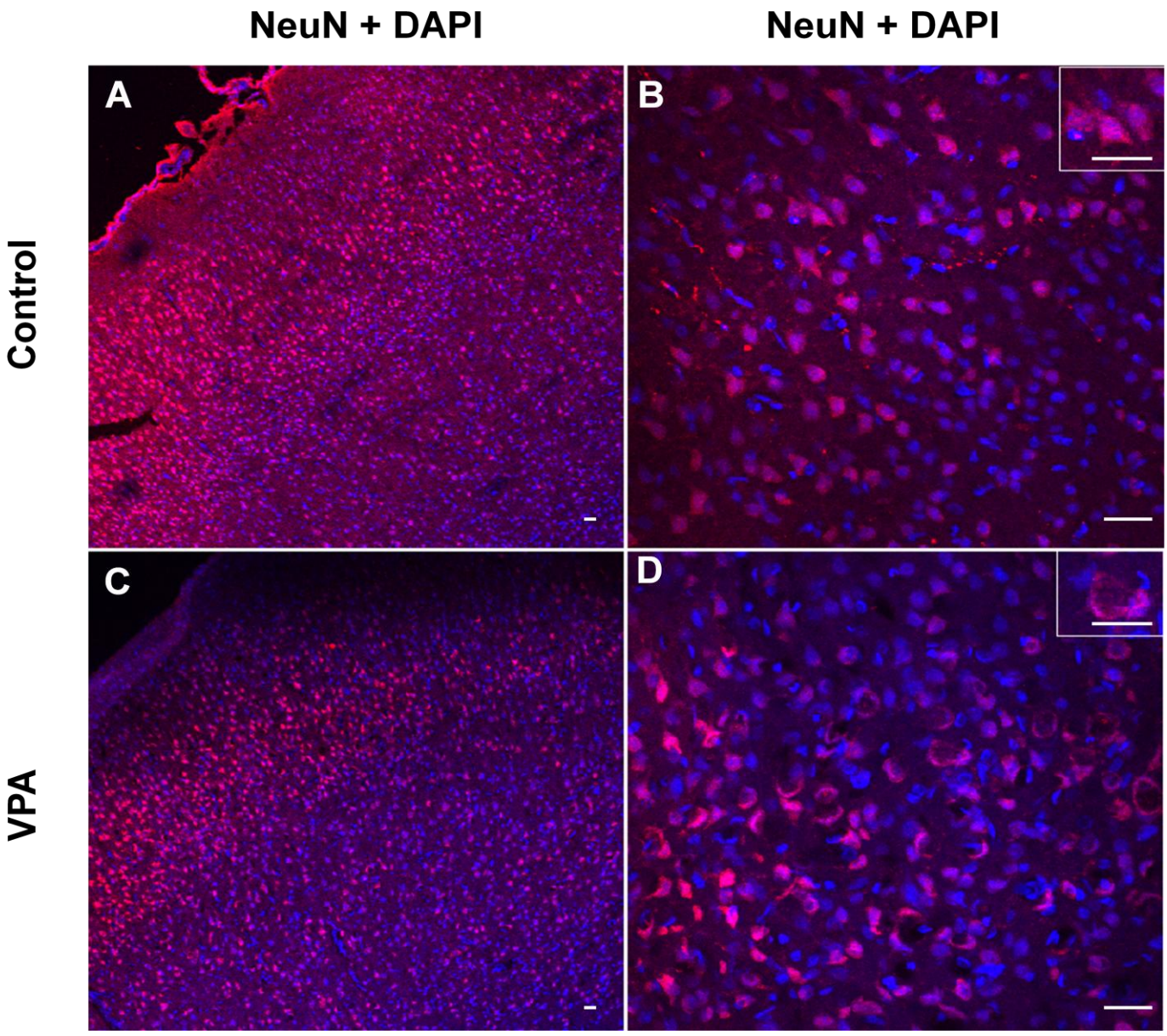


Figure 2

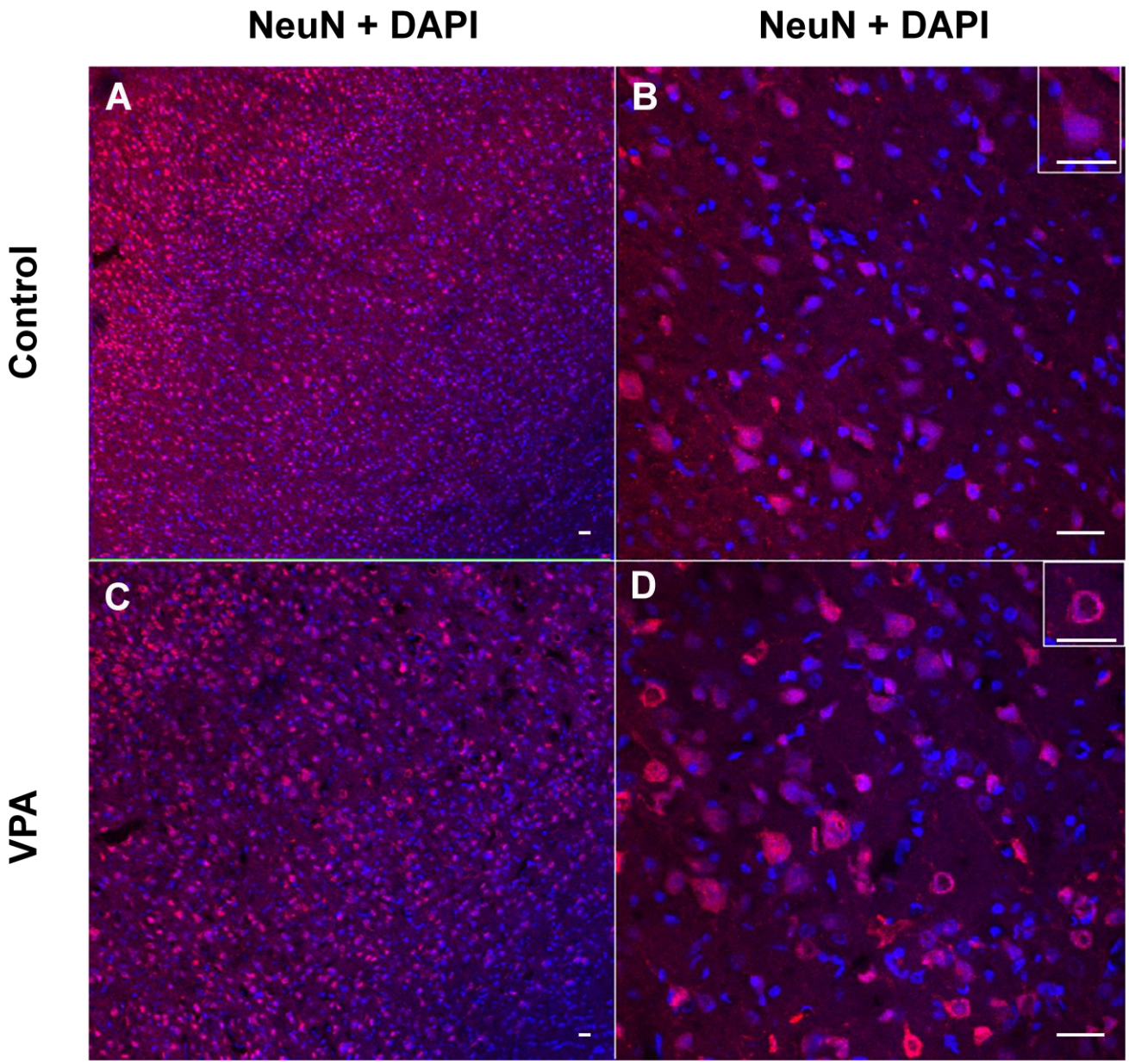


Figure 3

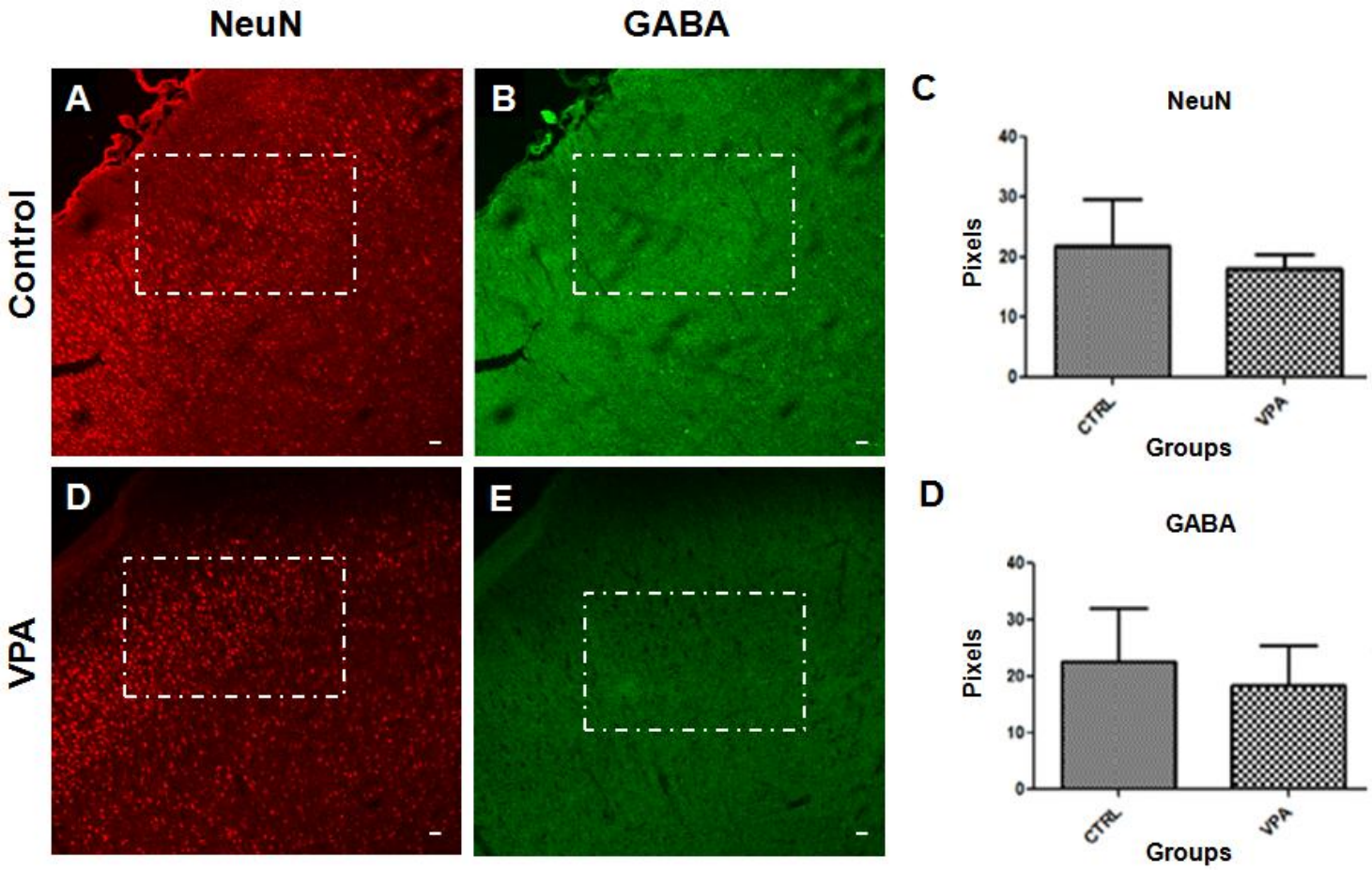
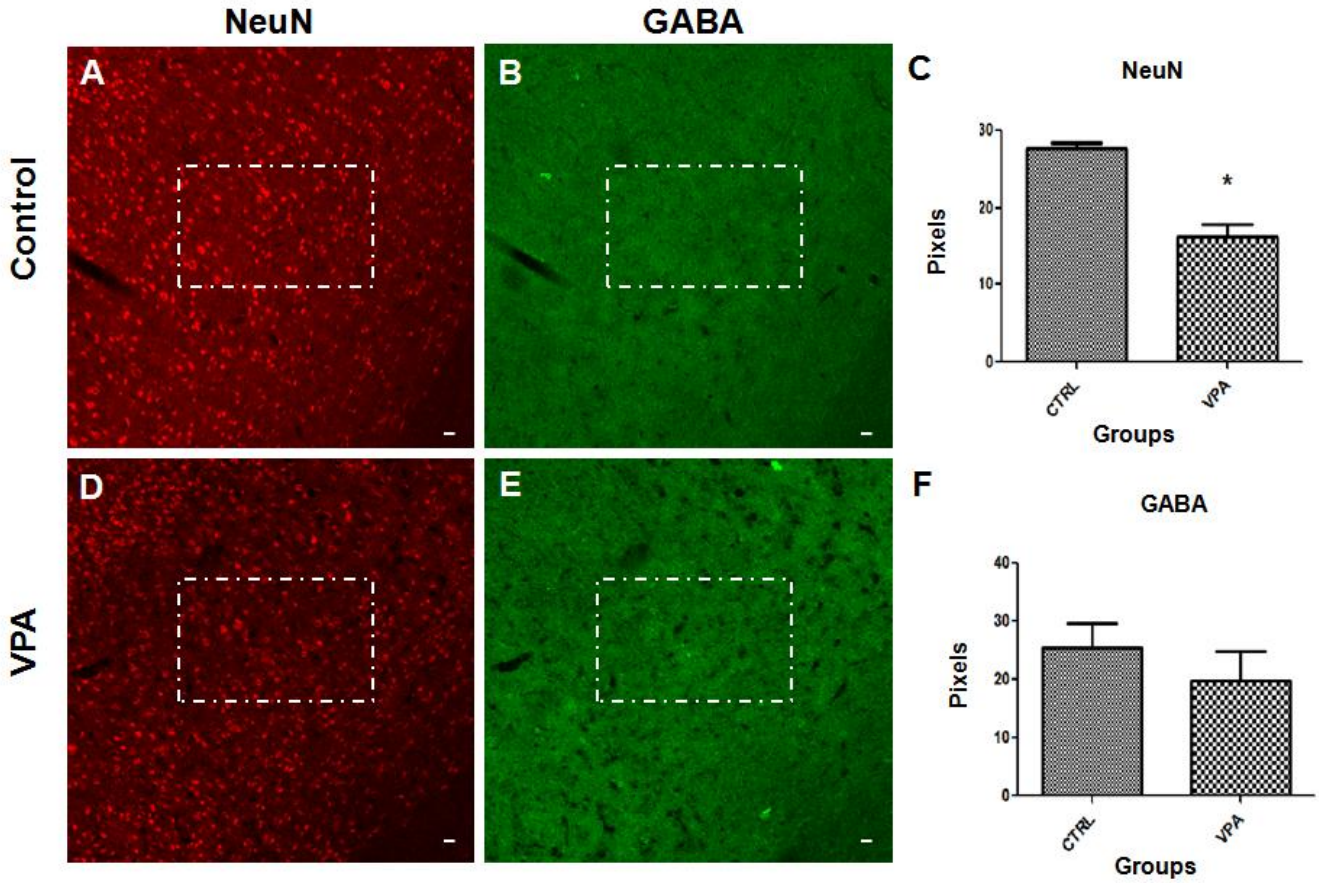

NeuN
GABA
Control
VPA
A
B
C
D
E
NeuN
Pixels
40
30
20
10
0
CTRL
VPA
Groups
D
GABA
Pixels
40
30
20
10
0
CTRL
VPA
Groups

Figure 4


NeuN
GABA
A
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10
0
*
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Groups
D
E
F
VPA
GABA
Pixels
40
30
20
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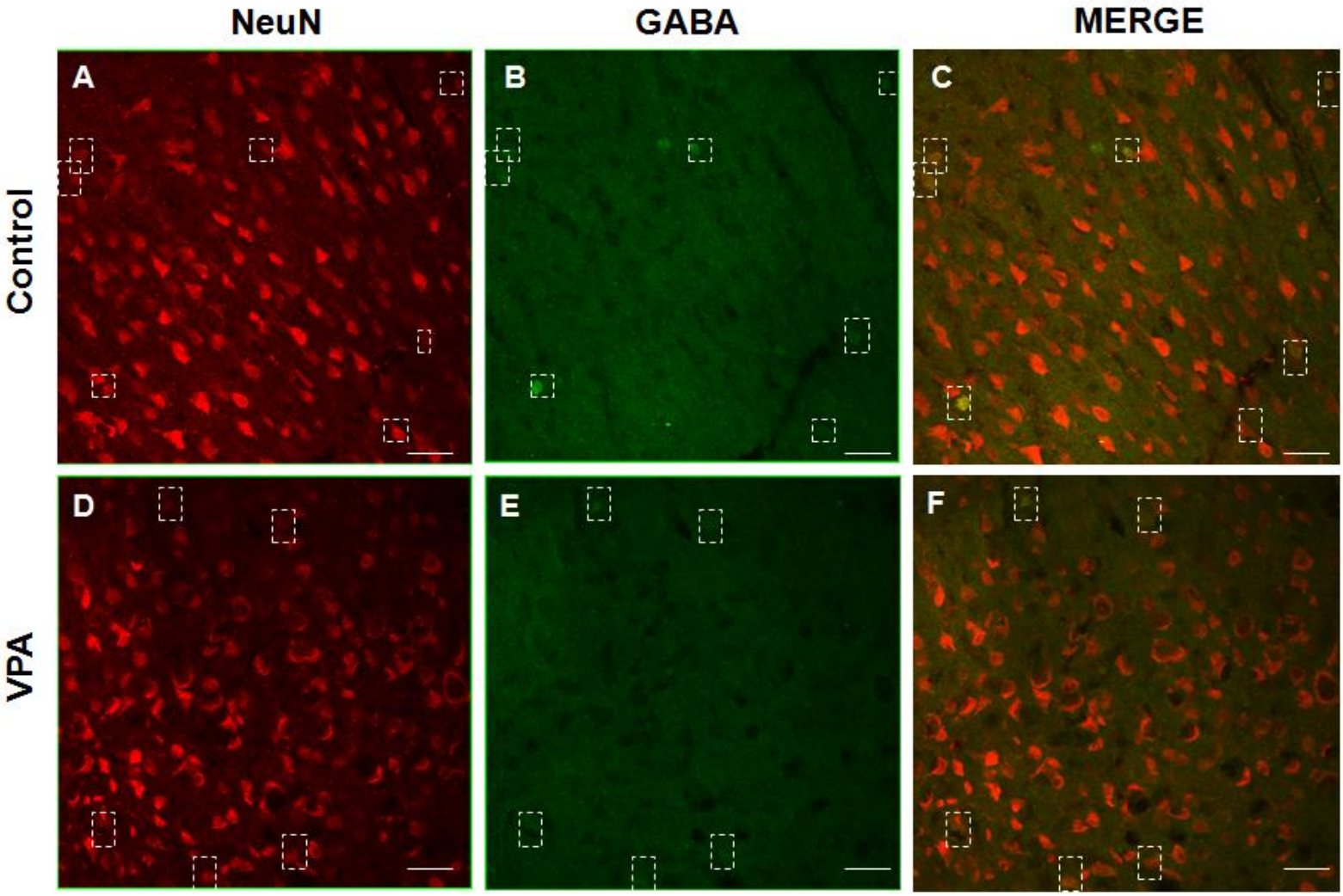
CTRL
VPA
Groups

Figure 5


NeuN
GABA
MERGE
Control
VPA
A
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D
E
F

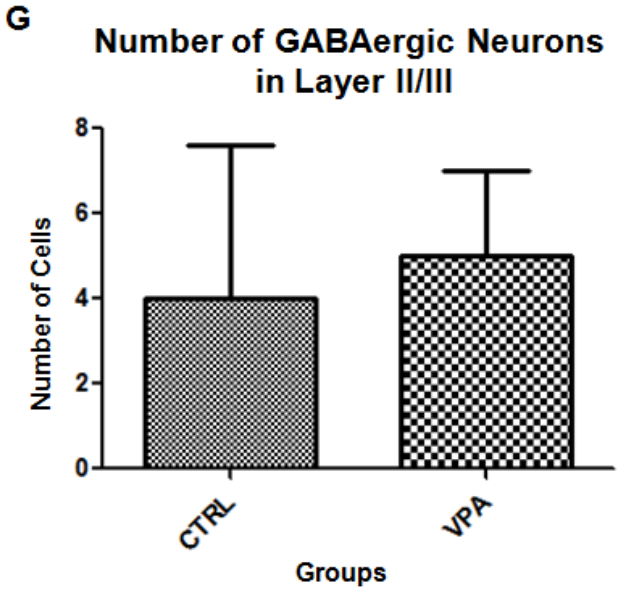
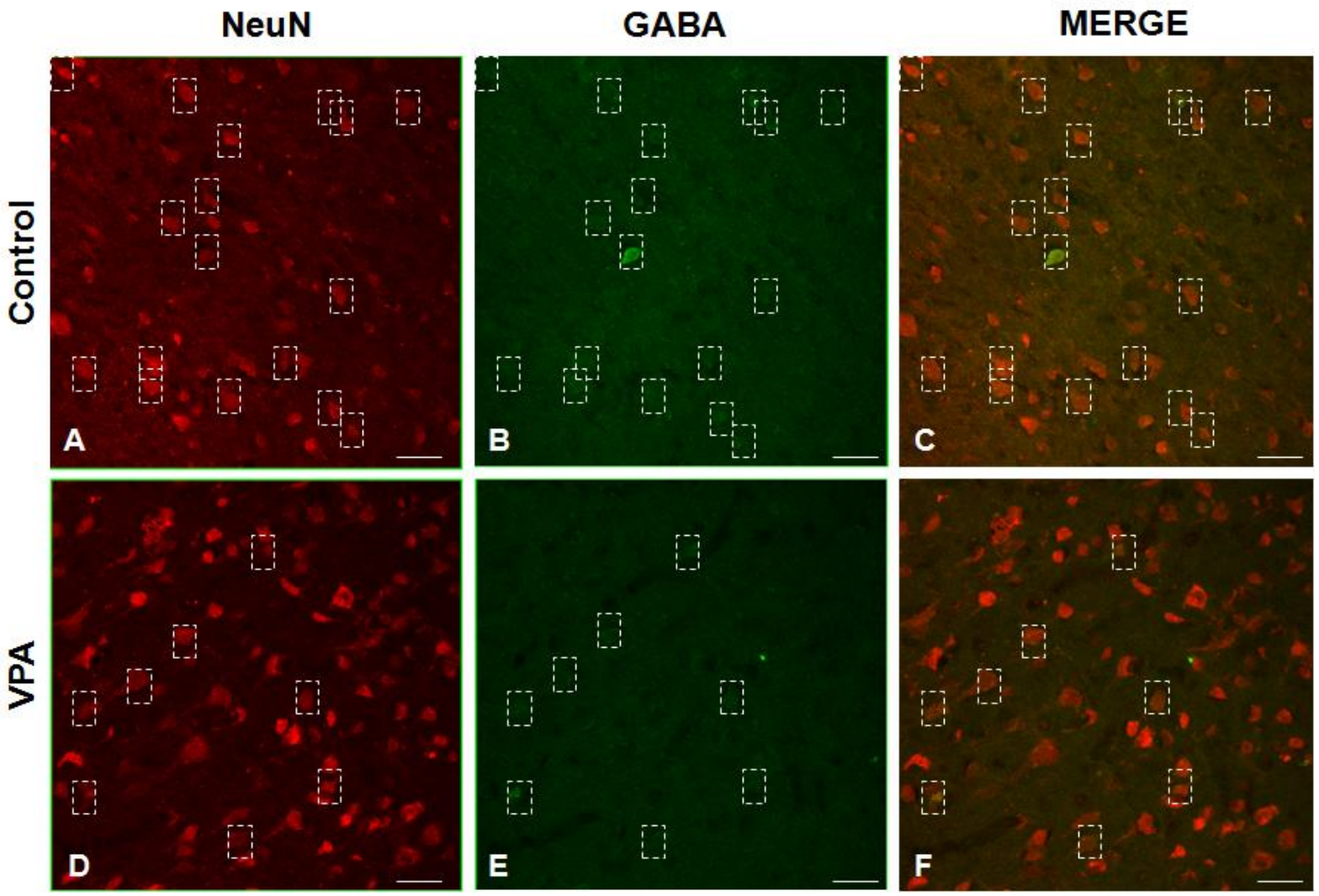

G
Number of GABAergic Neurons in Layer II/III
Number of Cells
0
2
4
6
8
CTRL
VPA
Groups

Figure 6


NeuN
GABA
MERGE
Control
VPA
A
B
C
D
E
F

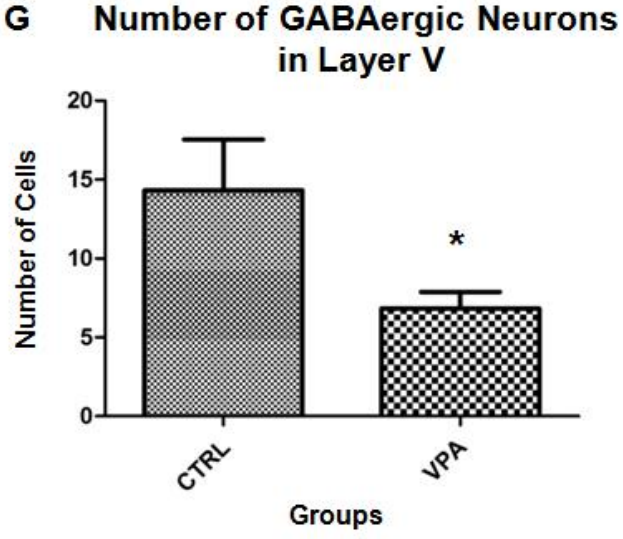

G
Number of GABAergic Neurons in Layer V
Number of Cells
20
15
10
5
0
*
CTRL
VPA
Groups

Figure 7

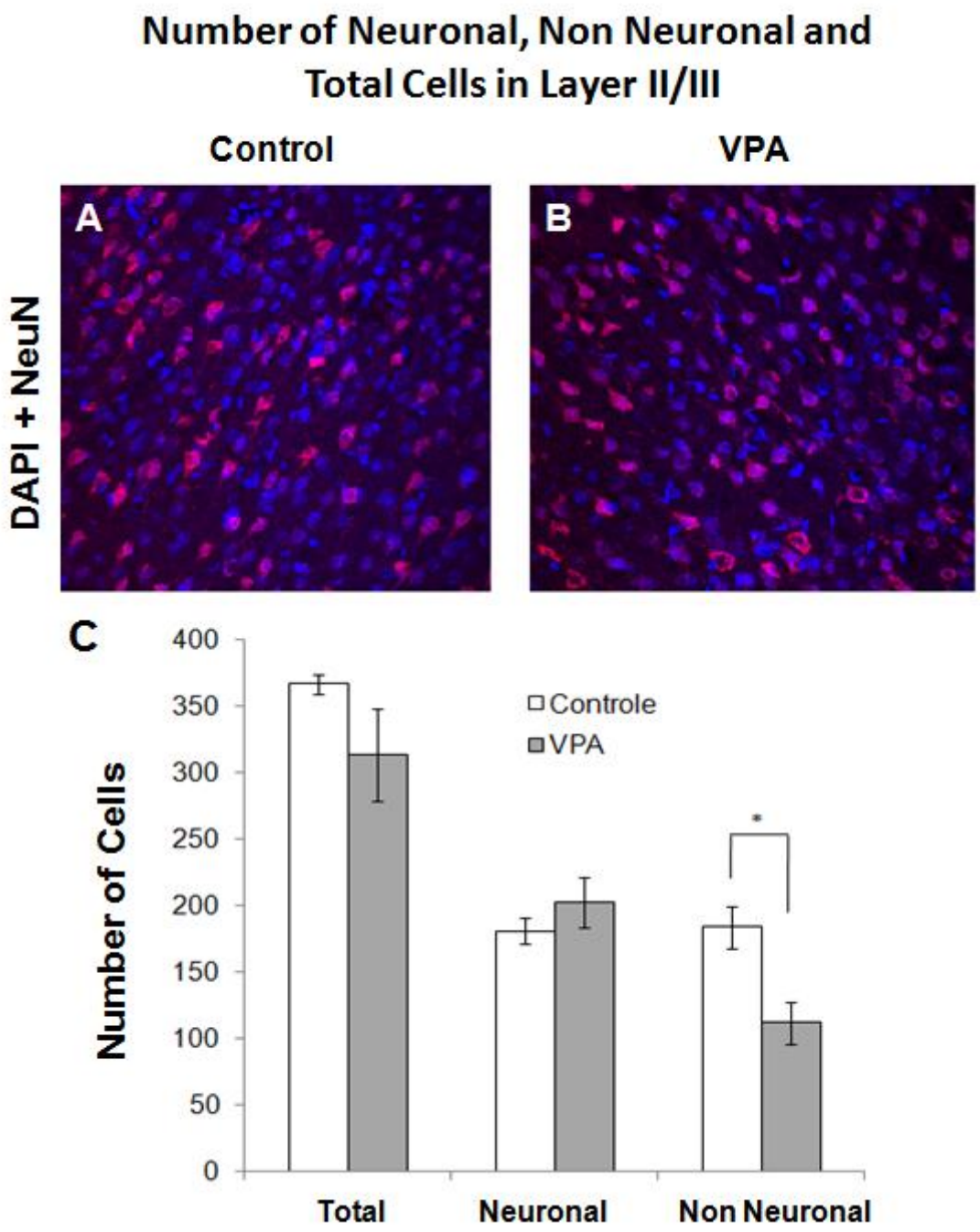

Number of Neuronal, Non Neuronal and Total Cells in Layer II/III
Control
VPA
DAPI + NeuN
A
B
C
Number of Cells
400
350
300
250
200
150
100
50
0
Controle
VPA
*
Total
Neuronal
Non Neuronal

Figure 8

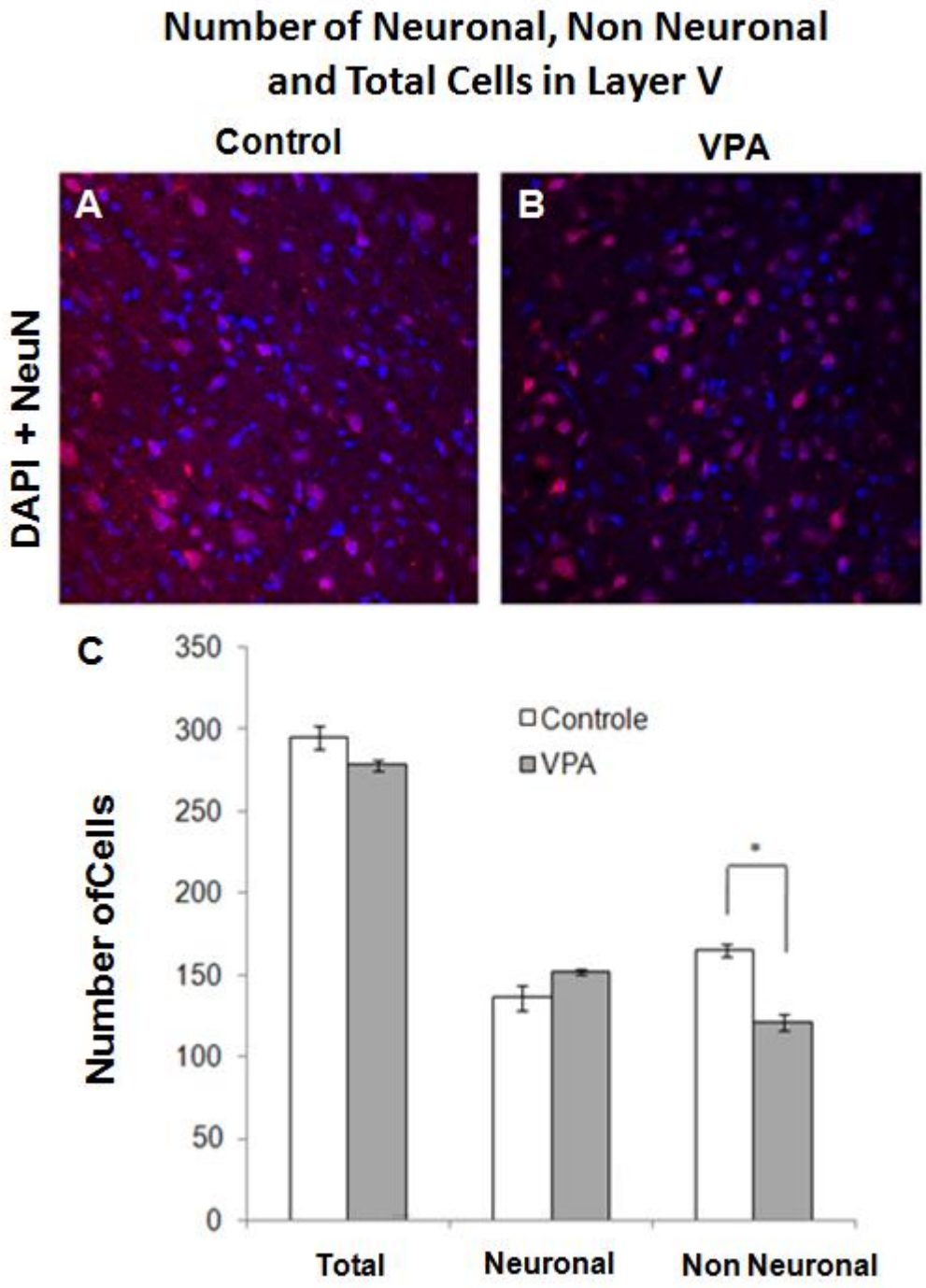
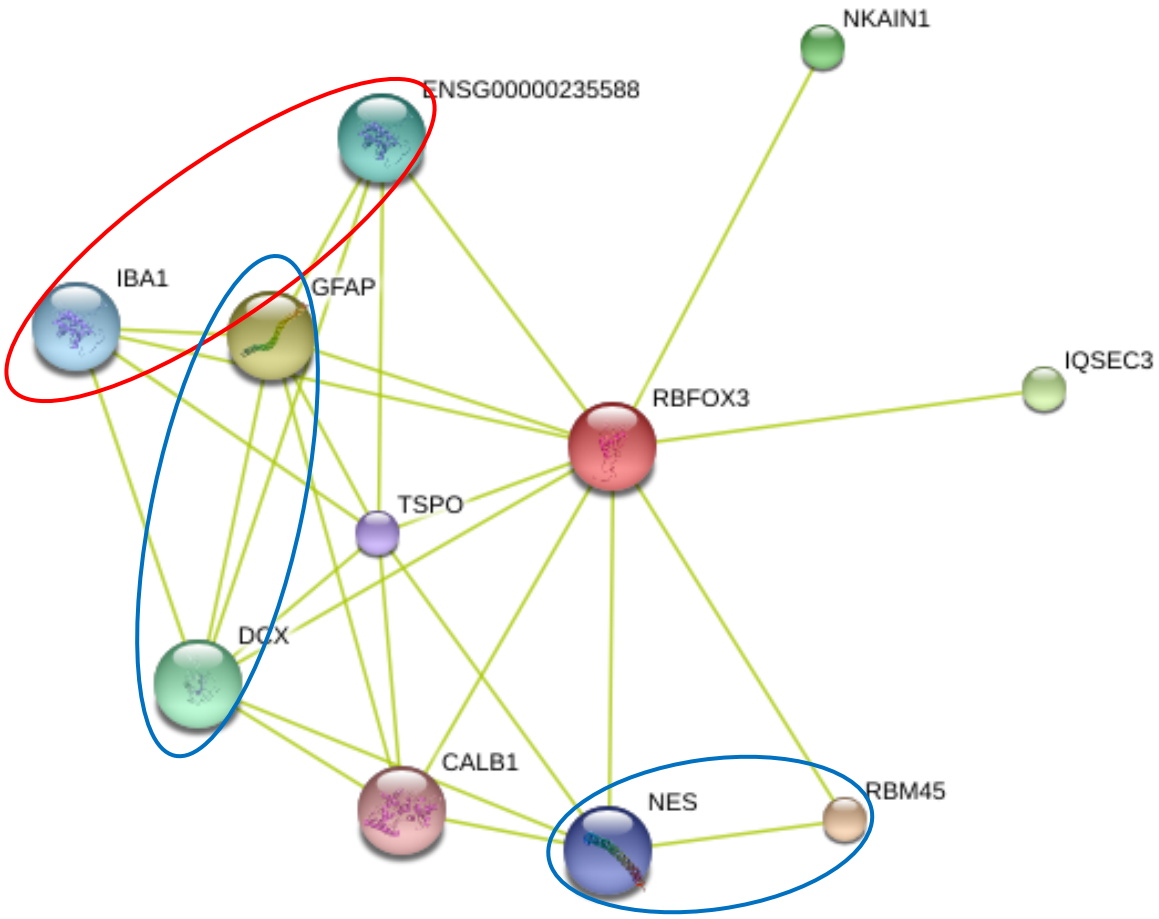

Number of Neuronal, Non Neuronal and Total Cells in Layer V
Control
VPA
A
B
DAPI + NeuN
C
Number ofCells
350
300
250
200
150
100
50
0
Controle
VPA
*
Total
Neuronal
Non Neuronal

Figure 9


NKAIN1
ENSG00000235588
IBA1
GFAP
IQSEC3
RBFOX3
TSPO
DCX
CALB1
NES
RBM45

4. CONSIDERAÇÕES FINAIS

O presente estudo apresenta uma hipótese para o desequilíbrio na excitação/inibição na área somatossensorial primária (região de campos em barril) em ratos expostos pré-natalmente ao ácido valpróico, dentro de um modelo animal reconhecido de autismo. Os resultados são exemplificados na figura abaixo:

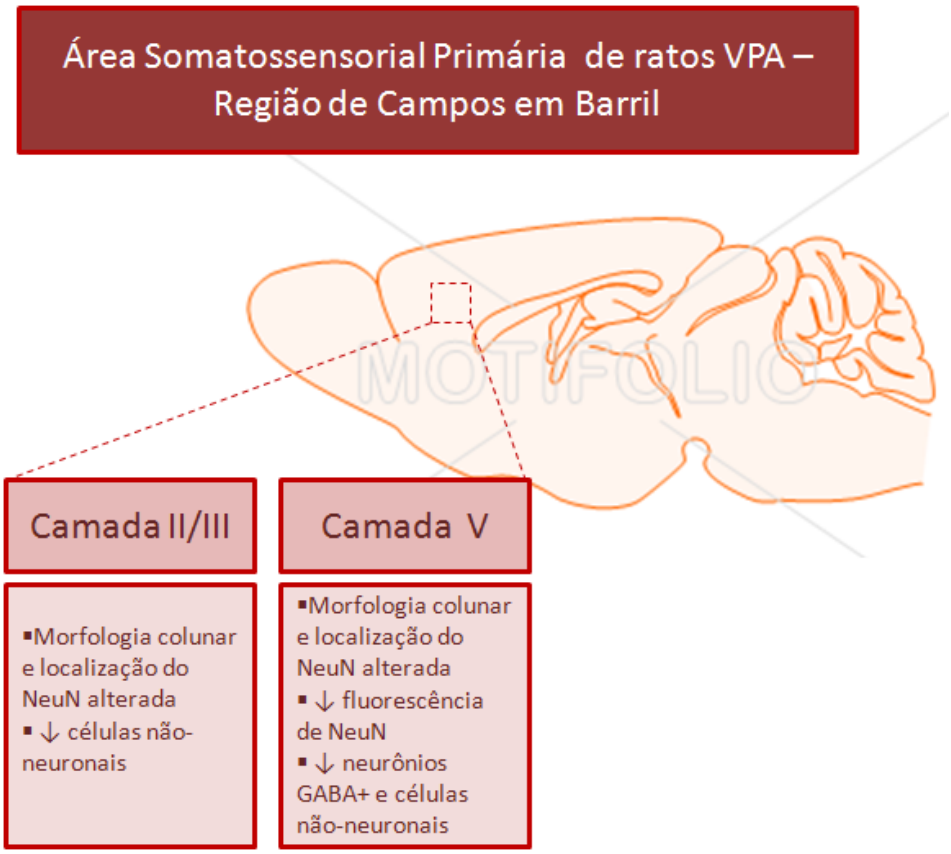


Figura 3: Resumo dos resultados de alterações em ratos expostos pré-natalmente ao ácido valpróico nas camadas II/III e V da área somatossensorial primária.

A disfunção GABAérgica possibilita alicerces para um estudo mais profundo das possíveis causas ou consequências do transtorno do espectro do autismo, podendo explicar porque existe uma hiperconectividade local dentro no córtex pré-frontal desses pacientes. Dados recentes comprovam a diminuição de parâmetros GABAérgicos em pacientes com autismo, como redução da expressão do RNA mensageiro de enzimas GAD65 e GAD67 (Fatemi et al., 2002) nos córtices parietal e cerebelar, também como a diminuição de neurônios GABAérgicos em modelos animais transgênicos

(Sgado et al., 2013), nas regiões do córtex cerebral e hipocampo. Este presente trabalho demonstra pela primeira vez a diminuição de neurônios GABAérgicos na área somatossensorial primária na região dos campos de barril na camada V do cortex, bem como a desorganização colunar em ratos expostos pré-natalmente ao ácido valpróico tanto na camada II/III quanto na camada V. O menor número de neurônios GABAérgios encontrado na camada V pode ser decorrente da desorganização colunar nessa região, possibilitando a relação com componentes-chave do autismo na região somatossensorial primária, uma vez que a hipersensibilidade a estímulos sensoriais está presente no autismo. Embora a quantidade de neurônios GABAérgicos para a camada II/III no grupo VPA não foisignificativamente diferente do grupo controle, houve uma tendência para uma redução, necessitando, portanto, de uma maior amostragem para confirmar ou descartar uma diferença.

Anormalidades estruturais e funcionais de interneurônios GABAérgicos podem representar um substrato/produto anatômico do desequilíbrio excitatório/inibitório no córtex cerebral e em outras regiões encefálicas de um paciente com autismo (Rubenstein and Merzenich, 2003). A maturação dos circuitos GABAérgicos, quando prejudicada, apresenta funções imaturas no córtex cerebral que permanece mais plástico e sensível a alterações de *inputs* sensoriais (Di Cristo, 2007; Hensch, 2005). Um córtex mais excitável (ou menos inibido) possui, por sua natureza, uma diferenciação funcional mais precária (Merzenich, 2001; Merzenich, 1999), levando a anormalidades de percepção, de memória, de cognição e do controle motor. Ainda, alguns pesquisadores acreditam que o córtex de pacientes com autismo possui uma hiperexcitabilidade, levando a instabilidade e susceptibilidade à epilepsia (Rubenstein and Merzenich, 2003).

Evidências apontam que a supressão da inibição GABAérgica é um componente comum no encéfalo de paciente com autismo (Hussman, 2001). Essa redução da inibição poderia ser exacerbada por um controle modulatório anormal de processos de aprendizagem e memória, que permitiriam e regulariam uma diferenciação progressiva normal e a elaboração de processamento da informaçãono encéfalo em desenvolvimento, uma vez que a diferenciação progressiva funcional permite um processamento refinado e, por

tanto, a redução do ruído encefálico (Merzenich, 2001; Merzenich, 1998a; Merzenich, 1999; Merzenich, 1998b). Além disso, esses desbalanços podem ser amplificados por maturação atrasada de sinapses ou mielinização anormal (Merzenich, 2001). Esses dois processos contribuem de maneira crucialpara o desenvolvimento de redes e sistemas neurais notelencéfalo e no aprimoramento da sinalização celular, diminuindo o ruído cortical (Merzenich, 2001). Dessa forma, nossos resultados corroboram com a hipótese de desequilíbrio GABAérgico primário, devido a grande contribuição que a neurotransmissão inibitória proporciona para a migração e diferenciação neuronal, maturação sináptica e controle do tamanho e organização das minicolunas (DeFelipe et al., 1986; Owens and Kriegstein, 2002; Pizzarelli and Cherubini), fatores estes presentes de forma alterada no transtorno do espectro do autismo. Esses dados indicam, portanto, o componente forte que este sistema representa dentro deste contexto, podendo ser um indicativo de possível indutor da desorganização colunar encontrada em córtices de animais expostos pré-natalmente ao ácido valproico.

Anormalidades GABAérgicas são encontradas em doençascomo a esclerose tuberosa e a síndrome do X frágil, as quais apresentam uma alta incidência de autismo associado. Um desbalanço entre excitação e inibição foi encontrada em indivíduos com esclerose tuberosa, uma condição genética multisistêmica que exibe uma variedade de desordens neurológicas, como a epilepsia e desordens do tipo autista (Curatolo et al., 2008). A perda de função GABAérgica, responsável pela hiperexcitabilidade observada em modelos animais da Síndrome do X Frágil é um componente comum causador de retardo mental, com déficits na linguagem, hiperatividade, comportamentos do tipo autista e convulsões (Selby et al., 2007). A possível alteração da taxa entre sinapses excitatórios e inibitórias durante um período critico, como a exposição ao dia pré-natal 12,5 do modelo animal de autismo, pode determinar um desenvolvimento disfuncional de circuitos neurogliais, sendo um possível causador dos sintomas mais característicos do autismo.

É importante ressaltar que a ocitocina possui um papel muito interessante e fundamental dentro do encéfalo fetal durante o parto. Estudos revelam que esta molécula está envolvida no *switch* ou alteração do sentido do

canal de ânios cloreto em receptores GABAérgicos. Durante o desenvolvimento, esses canais, quando ativados, realizam predominantemente despolarização. Essa alteração no sentido de fluxo do receptor, realizada pela ocitocina durante o parto, muda de efluxo de cloretos para influxo, levando ao fenótipo de hiperpolarização, encontrado em neurônios GABAérgicos maduros no encéfalo de indivíduos neurotípicos (Khazipov et al., 2008). Estudos relatam importantes contribuições da ocitocina em interações sociais (Meyer-Lindenberg et al.), podendo ser um fator importante de estudo para alterações GABAérgicas, ainda no desenvolvimento, que podem perdurar até a fase adulta no modelo animal e no autismo.

O crescente avanço no conhecimento de funções desempenhadas pelas células gliais indicam que estas células atuam como elemento-chave no autismo, incluindodisfunções na neurotransmissão ou no próprio ambiente encefálico de sua complexa fisiopatologia.

Estudos *post mortem* indicam níveis maiores de GFAP, a principal proteína de filamentos intermediários de astrócitos maduros,em tecido encefálico decórtexfrontal, parietal e cerebelar de pacientes com autismo, levando a hipótese de que, nessa condição esteja ocorrendoastrogliose reativa e dano ao tecido encefálico (Laurence and Fatemi, 2005). Evidências de ativação glial e neuroinflamação no encéfalo, através do aumento na imunoreatividade de GFAP e níveis elevados de citocinas MCP-1 e TGFB1 foram encontrados no cerebelo, giro do cíngulo e médio-frontal córtex dos mesmos (Vargas et al., 2005). Outros dois marcadores astrocíticos foram pesquisados em amostras detecido encefálico obtidas de necropsias de pacientes com autismo, encontrandoum aumento da expressão da conexina 43 no córtex frontal superior e diminuição da aquaporina 4 no cerebelo(Fatemi et al., 2008).

Dentro do modelo animal de autismo, análises por ELISA, realizadas por nosso grupo, das amostras encefálicas pós-natais de 15 dias de grupos controle e VPA em consonância com a astrogliose reativa encontrada em pacientes, demonstram aumento 58%,(em ng/µg de proteínas) no conteúdo deGFAP (p = 0.002), bem como o conteúdo da proteína S100B, com um

aumento de 88% (em ng/µg de proteínas), ambas marcadoras de ativação astrocítica e astrogliose reativa. Estudos *in vitro* mostram que um aumento de S100B a nível micromolecular pode levar a uma resposta pró-inflamatória e apoptótica sobre neurônios, corroborando a hipótese da astrogliose reativa no autismo, podendoagravar o quadro inflamatório no tecido encefálico.

Sendo caracterizada como uma célula derivada de linhagem mielóide residente no parênquima do sistema nervoso central, a microglia constitui um componente chave e único na composição celular do encéfalo (Ransohoff and Cardona, 2010). A significância funcional da microglia como foco de estudo tem crescido nos últimos 20 anos, procurando-se entender seus inúmeros papéis no desenvolvimento do sistema nervoso central (Streit, 2001) e na resposta imunitária em condições de infecção e injuria (Perry et al., 2010). A microglia, ainda, possui diversos estados em que pode ser encontrada no ambiente encefálico: 1) o estado vigilante, envolvendo um monitoramento dinâmico do status funcional, manutenção e *turn over* das sinapses, com uma grande mobilidade de seus processos, realizando eventos do tipo *screening* do ambiente (Elkabes et al., 1996; Harada et al., 2002), 2) o estado ativo, quando existe algum dano no tecido e essa célula passa a apresentar um fenótipo inflamatório ou clássico, reduzindo a proliferação celular e assumindo forma fagocítica, com retração de processos celulares. Ainda, existe um estado deativação alternativo, onde a microglia apresentau um fenótipo anti-inflamatório, aumentando a expressão de IL-10, TGF-β, BDNF e NGF (Kohman and Rhodes, 2012). Estudos indicam que a microglia pode ser regulada pelo balanço entre a excitação e inibição no sistema nervoso central. Nossos resultados no modelo animal de autismo indicam que uma disfunção GABAérgica, produzindo um balanço positivo entre a taxa excitação/inibição, pode estar envolvidos na ativação microglial, verificada em estudos *post mortem*(Vargas et al., 2005). Uma vez que existem evidências do aumento nos níveis de citocinas pró-inflamatórias no líquor (Chez et al., 2007) e no tecido encefálico (Li et al., 2009), esses dados fortificam a hipótese da participação glial na fisiopatologia do autismo.

Existem poucos estudos relacionando oligodendrócitos e autismo. Estudos demosntram uma relação entre prejuízos maturacionais e funcionais

em encéfalos de murinos expressando a proteína *Fmr1*(*Fragile x Mental Retardation Protein*) de células precursoras de oligodendrócitos no cerebelo, induzindo uma mielinização atrasada (Pacey et al.). Sendo uma região rica em neurônios GABAérgicos (Células de Purkinje), esse achado pode lançar idéias para possíveis prejuízos nesses tipos celulares, podendo estar envolvida com a neurotransmissão GABAérgica, uma vez que correntes inibitórias estão alteradas no autismo (Banerjee et al., 2012; Ingram et al., 2000).

Estudos posteriores serão realizados para avaliar a contribuição dessas células e vias no contexto etiológio e fisiopatológico, possibilitando estratégicas clínicas para aprimoraro direcionamento farmacológico no autismo, bem como na compreensão do envolvimento dessas células no desenvolvimento e maturação neuronal em desordens neurogliais.

5. PERSPECTIVAS

- Avaliar, pela técnica de coloração por DiI, espinhos dendríticos na área somatossensorial primária e outras regiões envolvidas no autismo, utilizando os encéfalos dos animais utilizados para este trabalho;

- Avaliar o sistema GABAérgico em outras regiões encefálicas conhecidas alteradas no Transtorno do Espectro do Autismo;

- Avaliar o sistema GABAérgico no Sistema Nervoso Entérico de ratos do modelo animal de autismo;

- Quantificar receptores GABA-A e GABA-B em encéfalos de ratos do modelo animal de autismo;

- Dosar e quantificar derivados de Adenosina e ATP/Adenosina, além da expressão de receptores purignérgicos, analisando esta via na ativação microglial;

- Analisar possíveis microRNA envolvidos no desenvolvimento neuronal e na modulação de vias excitatórias e inibitórias, bem como na regulação do NeuN;

- Avaliar a contribuição do NeuN no neurodesenvolvimento em idades pré-natais e pós-natais em ratos do modelo animal de autismo;

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