UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

CURSO DE GRADUAÇÃO EM BIOMEDICINA

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ANÁLISE DE NEURÔNIOS GABAÉRGICOS PARVALBUMINA-POSITIVOS EM ÁREAS SENSORIAIS NO MODELO ANIMAL DE AUTISMO INDUZIDO POR EXPOSIÇÃO PRÉ-NATAL AO ÁCIDO VALPROICO

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

Área de Habilitação: Bioquímica

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Porto Alegre

2017

" […] E a normalidade era precisamente o mais terrível daquela guerra infinita: não acontecia nada."

Gabriel García Márquez, Cem Anos de Solidão.

"O espírito sem limites é o maior tesouro do homem."

J. K. Rowling, Harry Potter e a Ordem da Fênix,

AGRADECIMENTOS

Aos meus pais Jesus e Véra, agradeço por toda dedicação, apoio e amor incondicional. Cada passo, etapa e conquista da minha vida deve ser dedicado a vocês, que têm paciência pra aguentar um dos filhos mais mau humorados da história (mas que os ama do fundo do coração).

À minha avó Maria (da qual herdei as fortes convicções que muitos insistem em chamar de "teimosia", vai entender), por demonstrar que a humildade e a dedicação podem te levar a qualquer lugar – sem sobrepujar ninguém.

Ao meu avô Hercílio, por mostrar que amizade e honestidade te fazem dignos qualquer que seja a posição onde estejas.

Aos meus avós Zé Terra e Nina (in memorian) por terem me mostrado, de diversas formas, que o amor à família lança suas raízes para muito além.

Às minhas tias Mair, Leda, Izabel, Sônia e Simone e tios Pedro Antônio, Heraldo, Zezinho e Paulinho por apoiarem minha educação e, principalmente, por serem as melhores tias e tios que qualquer sobrinho poderia ter, seja nos momentos de dificuldade, seja nos momentos de alegria.

A todos os meus primos, por serem meus primeiros e eternos amigos e por me proporcionarem os melhores almoços e madrugadas de conversa.

À minha orientadora, Professora Carmem, agradeço por todo apoio, dedicação e entusiasmo dedicados a esse trabalho e a todos os outros do grupo. É apenas graças a pessoas focadas, críticas e engajadas como ela que temos os avanços apresentados atualmente (e, se depender, teremos o infinito e muito mais).

À minha co-orientadora e amiga Mellanie Dutra, por me mostrar que sempre há um solução para os piores problemas – sejam eles na imunofluorescências ou na vida. Te agradeço de um jeito que nem o confocal (nem eu) é capaz de mensurar – mas tu sabes.

Aos meus companheiros de laboratório Mauro, Iohanna, Brum, Gui, Bruna, Giovanna e Marília, por todo o apoio durante os tempos árduos do TCC, especialmente pelos menes que me auxiliaram na grande jornada que é não perder a sanidade. Não desistam de mim apesar de eu não conseguir resolver um mísero sudoku, pelo menos eu levo café! <3

À minha melhor amiga, Vitória Lemos, não há o que agradecer em especial – apenas dizer que sem ti nada disso teria sentido. Mais do que Watson, tu dá significado às coisas (desde 2006?) e me faz crescer junto contigo. É maravilhoso.

Às amigas Mariana e Taís, agradeço por todos os momentos maravilhosos e principalmente nunca terem desistido de me arrastar pras festas das quais certamente teria me arrependido de não ir. Muito amor!

Ao meu grande amigo, Marcos Braz, agradeço pela capacidade de compartilhar os melhores e piores momentos e por me mostrar que vinho na concha, plantagrama e placa do brechó são apenas uma prévia dos acertos que ainda estão por vir.

Aos meu grandes amigos Vitor, Frê e André, por tudo que fizeram por mim desde que eu era piá. No mundo de um filho único vocês são os irmãos com quem eu posso contar na boa, na ruim e, principalmente, no vinho.

Às minhas queridas e desaparecidas amigas Marina e Fernanda, por também fazerem parte da minha vida desde o princípio dos tempos e por serem muito especiais (apesar do sumiço)!

Aos amigos, Dudu Cleney, Lorenzo, Laura, Julia Momo, Betina, Ana, Júlia Constante, Manu, Pavão e Felipe agradeço por todos os momentos de alegria, diversão, trago e carnaval em SS! Que a gente sempre possa compartilhar desses momentos sensacionais.

Aos amados amigos Maria Eduarda, Anna Carolina, Lucio, Sanvitto, Eugênio, Yan e João Vicente por serem a melhor parte do ensino médio e por me proporcionarem momentos de alegria, alimentação vasta, festas e, digamos, um pouco de vergonha

À grande amiza Izabela Espíndula, por me acompanhar durante todos os anos de graduação, me ajudando a manter as forças pra continuar, compartilhando bons momentos e bloqueando todos os efeitos destrutivos dos meus choros.

Aos novíssimos e incríveis amigos Jessica, Paulinho, Gabriel e Lennon, por fazerem a Fernandes ou qualquer outro lugar um ambiente maravilhoso e por estarem comigo em momentos em que eu só precisava esquecer o TCC.

Às minhas eternas professoras Ana Ibraima e Ana Lemos, pelos conhecimentos passados e pela referência de respeito à diversidade que levo sempre comigo. Ah, e pela MELHOR carona pra Praia Nova da história!

À família Lemos, por serem pessoas sensacionais, especialmente ao tio Kico, por todos os resgates e papos reflexivos, à tia Iza pela irreverência, carinho e divertimento de sempre e à tia Lêda, tio Paulo e Tina, por terem a casa com maior densidade de pessoas maravilhosas da praia e pela sopa salvadora (certamente sem ela eu não teria chegado até aqui).

À praia de São Simão, por ser o lugar onde tudo que é maravilhoso acontece.

SUMÁRIO

RESUMO

O Transtorno do Espectro Autista é uma desordem do neurodesenvolvimento caracterizada, segundo o DSM-V, por uma díade comportamental: déficits de comunicação e interação social além de comportamentos repetitivos ou estereotipados. Outro aspecto de extrema importância para o transtorno são as alterações sensoriais presentes em mais de 90% dos indivíduos, tais como hiper-responsividades a estímulos não nocivos (visuais, táteis, auditivos) e hiporresponsividade a estímulos nocivos, que implicam em prejuízo na qualidade de vida. Apesar da etiologia do autismo não ser conhecida, tanto fatores genéticos quanto ambientais já foram associados à desordem, incluindo a utilização de ácido valproico (VPA) durante a gestação – situação que aumenta significativamente a chance do desenvolvimento de autismo nos filhos. Baseado nessas observações, desenvolveu-se um modelo animal de autismo induzido por exposição pré-natal ao VPA, validado pela identificação de diversas características (comportamentais, morfológicas e moleculares) do tipo autista. No presente trabalho, realizou-se uma análise quantitativa e organizacional de interneurônios GABAérgicos positivos para parvalbumina em regiões associadas com processamento sensorial – Área Somatossensorial Primária (ASSP) e Região da Amígdala (RAM). A obtenção dos animais passou por um processo inicial de acasalamentos, onde a verificação da fecundação foi comprovada através da identificação de espermatozoides no líquido vaginal na manhã posterior ao pareamento. As fêmeas do grupo VPA foram induzidas com uma injeção intraperitoneal de VPA (600mg/kg) no E12,5. Filhotes machos com 30 dias foram anestesiados e perfundidos com paraformaldeído 4% para promover a fixação tecidual, após a eutanásia relizou-se a remoção do encéfalo, posteriormente preservado em Tissue-Tek® a -80ºC. O tecido foi cortado em criostato e as fatias contendo as estruturas de interesse foram identificadas através do atlas Paxinos. Realizou-se imunofluorescência com objetivo de obter três marcações: DAPI, NeuN e Parvalbumina (PV). As imagens foram obtidas por microscopia confocal e processadas no software ImageJ com o plug—in Cell Counter. A análise estatística foi realizada no software Graph Pad Prims 5, utilizando o Teste t de Student seguido de correção de Welch, considerando p<0,05 como significativo. Na ASSP o grupo VPA exibiu, além de severa desorganização laminar, redução significativa na quantidade de células não-neuronais na camada IV-V (1,26x), demonstrando um prejuízo em células gliais, essenciais para a manutenção da homeostase local, além disso, a quantidade e a densidade de neurônios PV-positivos também demonstraram redução significativa na camada IV-V (3,06x), enquanto na camada II-III (1,69x) houve um aumento significativo. Complementarmente, a análise de todas as camadas em conjunto não apresentou diferenças significativas para nesses parâmetros, demonstrando uma possível alteração na migração desses interneurônios entre as camadas corticais. Na Região da Amígdala, houve redução significativa do número e densidade de interneurônios PV+ no grupo VPA (2,66x) Considerando os dados apresentados, nós demonstramos pela primeira vez que a exposição pré-natal ao VPA induziu importantes alterações nas camadas corticais da ASSP e na RAM no que se refere a células não neuronais e neurônios inibitórios PV+. Esses prejuízos no componente inibitório provavelmente resultam em expressivas consequências no balanço excitatório/inibitório que poderiam explicar diversas alterações comportamentais identificadas.

Palavras-chave: TEA, sistema sensorial, modelo animal, parvalbumina, imunofluorescência, equilíbrio excitatório-inibitório.

ABSTRACT

Autism Spectrum Disorder is a neurodevelopmental disorder characterized, according to DSM-5, by a behavioral dyad: impairments in communication and social interaction in addition to repetitive or stereotyped behaviors. Another extremely important aspect for ASD are the sensory alterations present in more than 90% of the individuals with ASD like hyper-responsiveness to non-harmful stimuli (visual, tactile and auditory) and hypo-responsiveness to harmful stimuli, which implies a great loss in life quality. Although etiology is not known, both genetic and environmental factors have been associated with the disorder, including the use of valproic acid during pregnancy – situation that significantly increases the chances of autism development in the children. Based on these observations, an animal model of autism by prenatal exposure to valproic was developed and validated by the findings (behavioral, molecular and morphological) of several autistic-like characteristics. In the present study, a quantitative and organizational analysis of parvalbumin-positive GABAergic interneurons was carried out in sensory processing related regions – Primary Somatosensory Area and Amygdala Region. Obtaining the animals underwent an initial process of mating, in which the fecundation check was confirmed by the presence of spermatozoa in vaginal smear in the morning after pairing. The female were separated and the VPA group received a intraperitoneal injection of VPA (600m/kg) in E12.5. The 30 days old male pups were anesthetized and transcardiacally perfused with paraformaldehyde 4% to promote tissue fixation, after euthanasia the brain was removed and preserved in Tissue-Tek® at -80ºC. The tissue was sliced in cryostat at -20ºC and the slices containing the structures of interest were identified through Paxinos atlas. Immunofluorescence was made in order to identify three markers: DAPI, NeuN and Parvalbumin (PV). The images were obtained by confocal microscopy and processed in ImageJ software with Cell Counter plug-in. Statistical analysis realized in Graph Pad Prism 5 software, using Student's T test followed by Welch's Correction and considering $p<0.05$ as significant. In Primary Somatosensory Area, VPA group exhibited, besides severe laminar disorganization, significant reduction in non-neuronal cells in layer IV-V (1,26x), demonstrating impairment in glial cells, essential for maintaining local homeostasis, moreover, the quantity and density of PV-positive neurons also showed significant reduction in layer IV-V (3,06x), while in layer II-III (1,69x) was a significant increase. In addition to this, the analysis of all layers together didn't exhibited significant differences for PV+ quantity and density, demonstrating a possible alteration in the migration of these interneurons between the cortical layers. In Amygdala Region, the number and density of PV+ interneurons were significant reduced (2,66x) in VPA group. Considering the present data we demonstrated for the first time that prenatal exposure to VPA induced important alteration in PSSA cortical layers regarding to non-neuronal and inhibitory PV neurons. These impairments in the inhibitory component, probably have expressive consequences in the excitatory/inhibitory balance which may explain some behavioral alteration.

Key-words: ASD, sensory system, animal model, parvalbumin, immunofluorescence, excitatory/inhibitory balance.

LISTA DE ABREVIATURAS

AMR – Amygdala Region

ASD – Autism Spectrum Disorder

ASSP – Área Somatossensorial Primária

CDC - Centers for Disease Control and Prevention

Desequilíbrio E/I – Desequilíbrio Excitatório/Inibitório

DSM - Manual Diagnóstico e Estatístico de Desordens Mentais, do inglês *Diagnostic and Statistical Manual of Mental Disorder*.

fMRI - Ressonância Magnética Funcional

GABA – Ácido gama-aminobutírico

GAD - Glutamato Descarboxilase

PSSA – Primary Somatosensory Area

PV+ - Positivo para Parvalbumina

RAM – Região da Amígdala

TEA – Transtorno do Espectro Autista

VPA – Ácido Valproico

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1. INTRODUÇÃO

1.1 Transtorno do Espectro Autista

O Transtorno do Espectro Autista (TEA) é uma desordem do neurodesenvolvimento caracterizada atualmente, segundo o DSM-5, por uma díade comportamental composta por:

1) Comportamentos repetitivos ou estereotipados;

2) Déficits de comunicação e interação social.

Historicamente, o termo "autismo" foi utilizado pela primeira vez pelo cientista Paul E. Bleuler em 1911 (ASHOK *et al.*, 2012), sendo posteriormente descrito em maiores detalhes pelo estudo de 11 crianças realizado por Leo Kanner (KANNER *et al.*, 1943) e, na sequência, por Hans Asperger (ASPERGER *et al.*, 1944) – bases históricas do conhecimento atual sobre o transtorno.

Os dados epidemiológicos demonstram um aumento significativo na prevalência do transtorno ao longo dos anos – fato explicado somente em parte pela migração de diagnósticos em decorrência das alterações do DSM-V (FOMBONNE *et al.*, 2009), demonstrando a necessidade crescente de ampliação das pesquisas para entendimento dos fatores relacionados ao desenvolvimento do autismo. Segundo dados de 2010 do Centro de Controle de Prevenção de Doenças dos EUA (CDC) a prevalência atual do Transtorno é de 1:68 crianças com 8 anos de idade, sendo cinco vezes mais prevalente entre indivíduos do sexo masculino. No Brasil, o único estudo epidemiológico piloto realizado ocorreu na cidade de Atibaia (RIBEIRO *et al.*, 2007), sendo a prevalência identificada de aproximadamente 0,3% - porém o dado obtido provavelmente não corresponde à realidade brasileira, já que, por exemplo, o tamanho amostral era muito reduzido para poder indicar dados consistentes sobre a prevalência do transtorno.
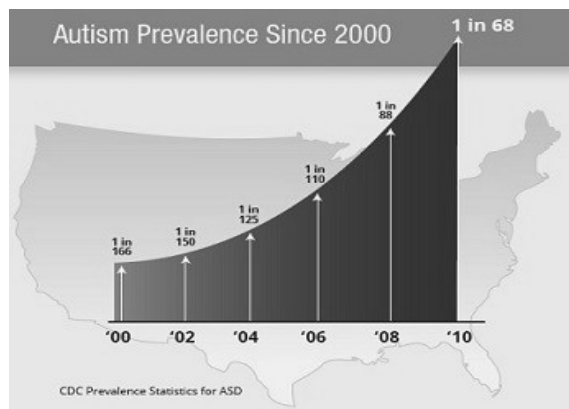
Figura 1: Aumento na prevalência do Transtorno do Espectro Autista nos EUA entre os anos 2000 e 2010.

Apesar dos amplos estudos sobre o tema, a etiologia do TEA ainda não foi propriamente elucidada, porém já se sabe que tanto fatores genéticos quanto ambientais (além de suas interações) são essencias para a determinação do fenótipo identificado em cada indivíduo (GOTTFRIED *et al.*, 2015). Estudos envolvendo gêmeos indicam uma alta herdabilidade do transtorno (alcançando valores superiores a 90% de concordância em gêmeos monozigóticos) – demonstrando um importante aspecto genético (EL-FISHAWY *et al.*, 2010), porém diversos fatores ambientais importantes já foram descritos, tais como a utilização de talidomida, álcool, e ácido valproico durante a gestação (DIETERT *et al.*, 2011) .

A presença de uma ampla diversidade de sintomas, além da heterogeneidade entre os indivíduos e ausência de biomarcadores validados torna o diagnóstico complexo, demandando uma equipe multidisciplinar para correta identificação das características comportamentais associadas ao transtorno. Além da díade supracitada, alguns outros sintomas incluem alterações gastrointestinais, desordens do sono, agressividade, epilepsia e ansiedade (MANNION A *et al.*, 2013).

Um dos aspectos mais importantes associados com o TEA são as alterações no processamento e integração dos estímulos sensoriais (que chegam a afetar mais de 90% dos indivíduos com o transtorno), condições que acabam trazendo grande prejuízo na qualidade de vida (GESCHWIND *et al.*, 2009). Os exemplos mais comuns dessas alterações (já identificadas em humanos e modelos animais) incluem hiper-responsividades a estímulos sonoros, táteis e visuais, hiporresponsividade a estímulos nocivos e preferência restrita por determinado

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padrão de estímulo, além de déficits na integração sensorial (DIETERT *et al.*, 2011). Ainda que muitas teorias tenham sido estabelecidas a fim de explicar esse aspecto do transtorno, diversos âmbitos do processamento sensorial no TEA ainda permanecem, em sua maioria, desconhecidos.

1.2 Ácido Valproico

O ácido valproico é um fármaco amplamente utilizado atualmente no tratamento de epilepsia, enxaqueca, dor neuropática e transtornos de humor, porém sua administração durante o período gestacional está associada ao desenvolvimento de algumas desordens como a Síndrome Fetal do Valproato e o Transtorno do Espectro Autista (CHRISTENSEN *et al.,* 2013) – ambas com consequências deletérias para o neurodesenvolvimento.

Historicamente, o ácido valproico foi sintetizado pela primeira vez em 1882, sendo utilizado como solvente para líquidos lipofílicos (BURTON *et* al., 1882). Posteriormente, em 1962, um grupo de cientistas que realizava testes com derivados da substância kelina verificou que o veículo do composto apresentava, por si, efeito anticonvulsivante (LÖSCHER *et al.*, 1999), consagrando o uso moderno do ácido valproico.
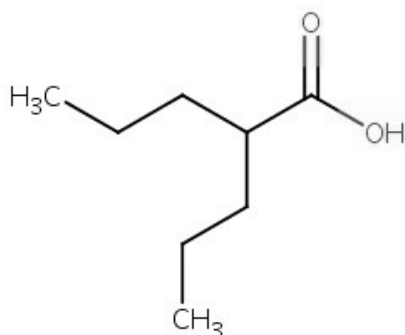
Figura 2: Representação da estrutura molecular do ácido valproico. Fórmula molecular: $C_8H_{16}O_2$ Peso molecular: 144.214 g/mol. A característica lipofílica do VPA permite sua passagem pela barreira hematoencefálica e pela barreira placentária.

As investigações sobre potenciais efeitos adversos do ácido valproico

demonstraram, primeiramente, sua característica teratogênica relacionada ao desenvolvimento da Síndrome Fetal do Valproato. A descrição das características dessa alteração inclui malformações em ossos cranianos, atraso no desenvolvimento psicomotor, estrabismo e outros sintomas (DILIBERTI *et al.*, 1984). Assim, estabeleceu-se a necessidade de ampliar o controle da dose e avaliar o custo/benefício da utilização do fármaco durante o período gestacional.

A relação entre utilização de ácido valproico e Transtorno do Espectro Autista passou a ser bem descrita e consolidada em um estudo realizado na Dinamarca que avaliou todos os nascidos vivos entre 1996 e 2006 (CHRISTENSEN *et al.,* 2013). Nesse trabalho, verificou-se que o uso de ácido valproico durante a gravidez, principalmente no primeiro trimestre gestacional, aumentava significativamente o risco de desenvolvimento de autismo nos filhos. Apesar de diversas hipóteses terem sido propostas, ainda não se sabe exatamente como o ácido valproico acaba induzindo as alterações que resultam nessa desordem neuropsiquiátrica, de forma que a ampliação dos estudos (em modelos animais, por exemplo) é essencial para trazer clareza sobre as vias etiológicas do TEA.

1.3 Modelo Animal

Os modelos animais, de forma geral, são ferramentas importantes no estudo de diversas patologias e desordens. Dentro desse contexto, algumas validades identificadas por Paul Wilner em seus estudos de depressão (WILLNER *et al.*, 1984) podem ser expandidas para modelos de outras desordens neuropsiquiátricas a fim de garantir consistência na translacionalidade entre comportamento humano e animal, sendo elas:

1) **Validade de Face:** replicação, em animais, de características ou sintomas ligados à desordem;
2) **Validade de Construto:** os fatores de risco que levam às alterações em humanos também devem realizar o mesmo em animais;
3) **Validade Preditiva:** a resposta obtida em humanos com a desordem a alguma intervenção (medicação, por exemplo) deve ser semelhante nos animais.

No contexto do TEA, o modelo animal baseado na exposição pré-natal ao ácido valproico teve início em 1996, quando um estudo demonstrou que essa intervenção, em ratos, causava alterações no cerebelo e no tronco encefálico semelhantes às descritas em pacientes com autismo (RODIER *et al.,* 1996). A partir daí, o modelo passou a ser validado e amplamente explorado no estudo do TEA e em 2005 foi realizada a primeira descrição de comportamentos associados ao autismo (défcts sociais, comportamentos estereotipados) em animais expostos ao VPA, consolidando o modelo animal (SCHNEIDER *et al.*, 2005).

Atualmente, existem diversos modelos animais de TEA, incluindo os baseados na exposição a fatores de risco ambiental como o ácido valproico e os desenvolvidos a partir de alterações genéticas tais como modificações no gene codificante da proteína SHANK3 (SPEED *et al.,* 2015) e mutações nos genes codificantes de neuroliguinas e neuroxinas (SHINODA *et al.,* 2013). Apesar de não haver um modelo que replique com total fidelidade as características do transtorno em humanos, os modelos animais são essenciais para seu estudo, já que permitem análises bioquímicas, fisiológicas e comportamentais em grande escala, além serem mais facilmente replicáveis e acessíveis.

### 1.4 Desequilíbrio Excitatório-Inibitório e Alterações Sensoriais

Uma das hipóteses mais abordadas em relação ao desenvolvimento e consolidação das características do Transtorno do Espectro Autista se refere às alterações eletrofisiológicas, principalmente no desequilíbrio entre o delicado balanço excitatório-inibitório (E/I) em algumas estruturas encefálicas. A presença de epilepsia ou episódios de convulsão em aproximadamente 1/3 dos indivíduos com TEA (SPENCE *et al.,* 2009) é uma das evidências mais consolidadas da associação do transtorno com esse tipo de desequilíbrio eletrofisiológico, além disso, patologias como Esclerose Tuberosa, que possuem o autismo como comorbidade frequente (20%–60% dos pacientes (NUMIS *et al*, 2011)) também compartilham da alta taxa de alterações eletrofisiológicas, porém as relações de causa e efeito ainda não foram propriamente identificadas.

Os desequilíbrios elétricos identificados no TEA são de grande importância não só para o diagnóstico de comorbidades como também para entendimento geral

dos comportamentos apresentados pelos indivíduos com o transtorno. A descrição de uma ausência de "coerência central", ou seja, falta da capacidade de integrar itens formando um todo coerente é descrita em pacientes com autismo desde a década de 1980 (FRITH *et al.*, 1989), porém apenas recentemente estudos passaram a buscar compreender mais essa alteração na conectividade sob ponto de vista eletrofisiológico.

A presença de alta conectividade local associada à hipoconectividade em regiões encefálicas distantes (BELMONTE *et al.*, 2004) já comprovada através de estudos com ressonância magnética funcional (fMRI) em pacientes com autismo (CLERY *et al.*, 2013) é umas das evidências mais consolidadas para demonstrar os problemas associados à integração de estímulos, visuais, táteis, auditivos e outros. Nesse contexto, supõe-se que haja uma compensação geral baseada no aumento isolado da percepção de cada estímulo uma vez que a coerência é prejudicada, de forma que o resultado final pode ser percebido como a ausência de um "filtro" sensorial – situação observada em alguns indivíduos com TEA, onde estímulos não nocivos acabam causando grande sofrimento.
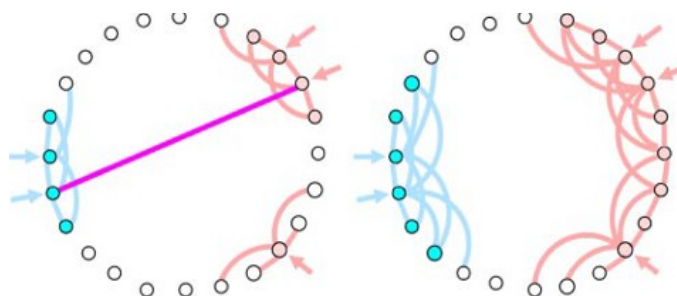
Figura 3: à esquerda, observa-se uma representação da conectividade usual entre regiões do encéfalo, de forma que a integração é rapidamente realizada e facilmente conduzida. À direita, observa-se a representação da conectividade alterada presente no TEA, onde a dificuldade de integração supostamente aumenta a percepção do estímulo primário. (imagem adaptada de BELMONTE *et al.*, 2004).

A neurotransmissão glutamatérgica e GABAérgica é outro fator de grande importância dentro da eletrofisiologia do autismo, sendo o predomínio disfuncional da ação glutamatérgica sobre a GABAérgica uma das hipóteses mais frequentes para explicar o desequilíbrio entre excitação e inibição (RUBENSTEIN *et al.*, 2003). Esse conceito, apesar de ser simplificação geral, já que diversos microcircuitos

possuem suas próprias formas de estabelecer esse equilíbrio para realizarem suas funções corretamente, representa a ideia geral da "ausência de filtro" que acaba deturpando a percepção sensorial.

A ampliação de estudos abordando os neurotransmissores-chave no balanço elétrico do encéfalo só foi possível através da utilização de modelos animais, principalmente pela possibilidade do controle e padronização dos experimentos. Em nível genético, estudos com animais nocaute possibilitaram uma análise isolada do impacto das alterações sinápticas. Dentre os achados mais relevantes, destaca-se o realizado com modelo animal de Síndrome de Dravet (HAN *et al.*, 2012), onde os animais heterozigotos para o gene que codifica a subunidade Scn1a de canais de sódio presentes em neurônios GABAérgicos apresentam comportamentos do tipo autista possivelmente associados com a falha na ação dessas células, já que a retomada farmacológica da ação GABAérgica foi capaz de reverter a mudança comportamental – interessantemente, esse tipo de canal iônico é encontrado em diversos interneurônios corticais, incluindo os positivos para parvalbumina e somatostatina (KEARNEY *et al., 2015)*, indicando o papel crucial dessas células na regulação do equilíbrio E/I. Além disso, em modelos animais de síndrome de Rett (também intimamente associada com o TEA), onde é realizada deleção de gene que codifica a proteína Mecp2 já observou-se, juntamente com alteração comportamental tipo autista, redução na síntese de enzimas da rota de produção de GABA, além de perda substancial do potencial inibitório (NELSON *et al.*, 2015).

Outros aspectos associados à desorganização da arquitetura cerebral identificados em modelos também corroboram a ideia geral de desequilíbrio eletrofisiológico. Já foi demonstrada a perda do padrão organizacional das minicolunas no neocórtex (CASANOVA *et al.*, 2015), inclusive em regiões associadas à percepção sensorial, como o córtex sensorial primário (dados não publicados), indicando que, possivelmente, a alteração no posicionamento dos interneurônios nesses locais possa resultar em queda no refinamento do processamento sensorial.

Os estudos realizados em humanos corroboram muitos dos achados em modelos animais: estudos post mortem demonstram redução nos níveis das enzimas GAD65/GAD67 (essenciais para a conversão de glutamato em GABA) no córtex parietal e cerebelo de indivíduos com o autismo (FATEMI *et al.*, 2002), além

de outras alterações como redução na expressão dos receptores GABAA(FATEMI *et al.*, 2009) and GABAB (OBLAK *et al.*, 2010).

Observando o todo demonstrado pelas evidências citadas é possível verificar uma questão central voltada para os problemas de conectividade e desequilíbrios eletrofisiológicos, indicando uma possível via na busca do entendimento das questões relacionadas aos déficits sensoriais identificados no TEA.

1.5 Neurônios GABAérgicos positivos para parvalbumina

Os interneurônios são células essenciais para promover organização, ritmicidade e filtro na transmissão de estímulos elétricos, possibilitando a correta interpretação das inúmeras aferências sensoriais que ocorrem a todo momento. Dentre os diversos tipos de interneurônios (RAGHANTI *et al.*, 2010) (normalmente identificados pela expressão de alguma molécula proteica específica como somatostatina, calretinina, calbindina e etc.) destacam-se os neurônios positivos para parvalbumina (PV+), que correspondem a 40% dos interneurônios presentes no córtex.
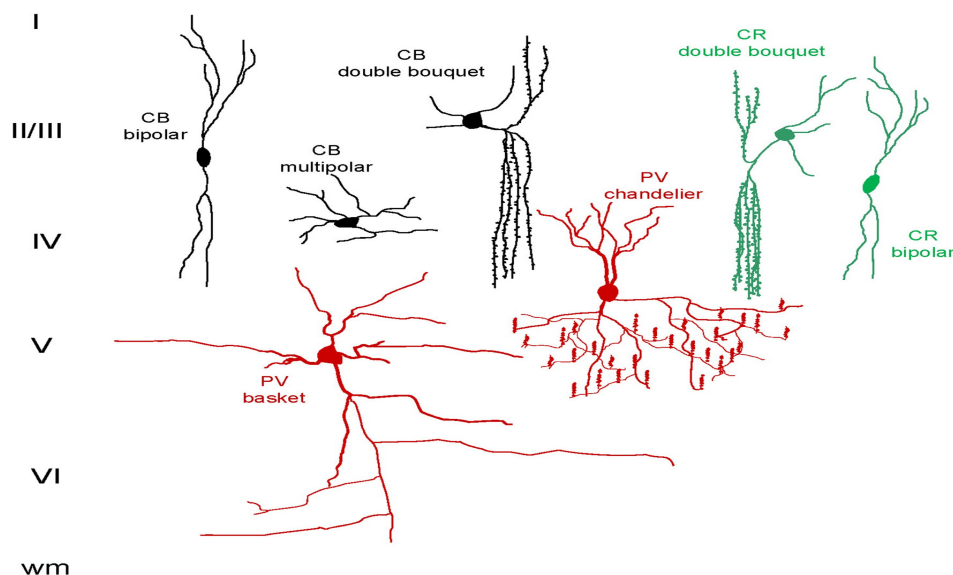
Figura 4: Representação da organização de diversos interneurônios no neocórtex. Em vermelho estão representados os dois tipos de neurônios PV+, diferenciados por sua morfologia: "basket cell" e "chandelier cell". Deve-se notar a presença de grande quantidade de contatos sinápticos na célula chandelier, característica essencial para o desempenho de suas funções (de

RAGHANTI et al, 2010).

Os neurônios PV+ são gerados na porção medial da Eminência Gangliônica (KELSOM *et al.,* 2013), estrutura presente durante a embriogênese e, partir daí, iniciam sua migração para diversas regiões do encéfalo, incluindo o córtex, no dia embrionário 12,5 em roedores (MARÍN *et al.*, 2003). Existem diversos fatores de transcrição, receptores e outras moléculas envolvidas nesse processo, de forma que alterações pontuais podem acabar prejudicando muito o processo.

Apesar de diversos estudos descreverem alterações na migração de neurônios GABAérgicos dentro do TEA, ainda não há relatos de mudanças associadas especificamente à migração dos neurônios PV+. Em outras desordens como a esquizofrenia, entretanto, há mais dados sobre o tema: já se observou a redução de Lhx6 e Sox6 (VOLK *et al*., 2012) fatores de transcrição relacionados com desenvolvimento e migração dos PV+, em análises post mortem, além de reduzida capacidade de síntese por essas células de GAD67, levando à falha na função GABAérgica (VOLK *et al*., 2014).

Outro fator importante associado aos neurônios PV+ é a sua capacidade de estabelecer um ritmo de ondas gama no encéfalo. Essa habilidade é proporcionada pela presença de sinapses elétricas entre os PV+ (SOHAL *et al.,* 2009), promovendo rápida sincronização e pela característica de disparo rápido dessas células, essencial para gerar as condições necessárias para a ocorrência de ritmos gama. Um ponto importante associado a esse padrão de ondas é a sua relação com diversos fatores como memória, atenção e, principalmente, integração de estímulos através da conectividade de diversas regiões do encéfalo (JIANG *et al.,* 2011). Já foi observado que a inativação seletiva dos neurônios PV+ induz uma desorganização severa da circuitaria cortical (GALARRETA *et al.,* 2001), indicando mais uma função importante dessas células e demonstrando a necessidade de ampliação de estudos que possam avaliar esse característica específica em desordens como o autismo.
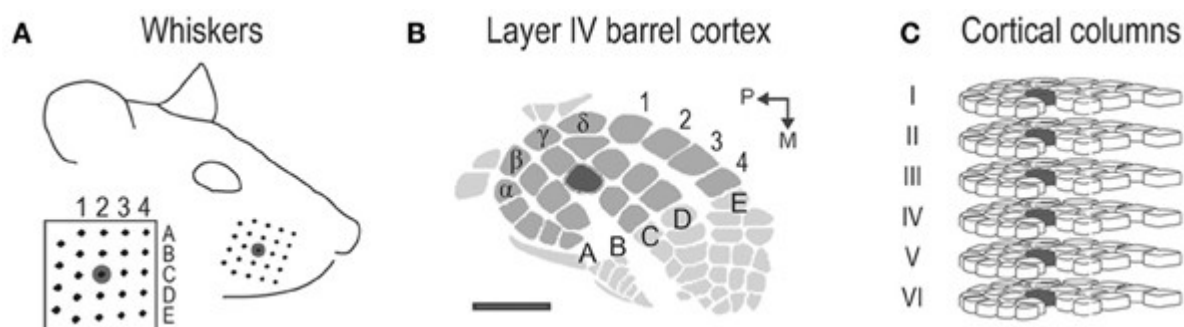
No Transtorno do Espectro Autista, apesar de existirem dados extensos sobre neurotransmissão GABAérgica, há poucas evidências sobre os neurônios PV+, principalmente em suas etapas iniciais como origem e migração. Os principais trabalhos demonstram, por exemplo, redução de PV+ no córtex pré-frontal medial em análises post mortem (HASHEMI *et al.,* 2016) e em hipocampo, córtex cingulado

anterior e córtex pré-límbico de modelos animais. Outras descrições de alteração englobam os PV+ juntos com outros neurônios, demonstrando os problemas de conectividade classicamente associados ao autismo. Assim, é necessário buscar entender mais o papel dessas células no autismo de forma semelhante ao que já vem sendo feito com a esquizofrenia.

1.6 Área Somatossensorial Primária

A percepção de estímulos sensoriais é essencial para diversos aspectos da sobrevivência dos seres vivos, pois possibilita uma ampla interpretação do ambiente externo e interno onde estão inseridos. Ao longo da evolução diversas especializações ocorreram no sentido de aprimorar a função de regiões específicas no encéfalo, gerando, por exemplo, a Área Somatossensorial Primária, região responsável pelo processamento de estímulos sensoriais somáticos (dor, tato, temperatura e propriocepção). Em humanos, essa região é situada no giro pós-central, correspondendo às Áreas de Brodmann 1, 2 e 3, enquanto em roedores não há uma delimitação anatômica bem definida, porém a identificação pode ser realizada de maneira simples através da utilização de atlas padronizados (PAXINOS *et al.,* 1997).

Alguns roedores possuem uma organização peculiar da Área Somatossensorial Primária denominada "corpos em barril" (CHEN-BEE *et al.*, 2012) destinados exclusivamente para o processamento tátil proveniente dos movimentos das vibrissas. Essa especialização é essencial para esses animais, que exploram seu ambiente majoritariamente através do movimento dessas estruturas de forma que cada vibrissa é processada por um grupo específico de neurônios constituintes de um corpo em barril. Esse mapeamento somatotópico pode ser visualizado através de técnicas de coloração (principalmente quando é realizada a marcação da enzima citocromo c oxidase) e fornece grandes informações sobre a qualidade do processamento sensorial desses animais.

Figura 5: Representação do refinado processamento cortical destinado a cada uma das vibrissas do animal nos campos em barril (de CHEN-BEE et al, 2012).

No contexto do Transtorno do Espectro Autista, as alterações sensoriais são componente importante no cotidiano dos indivíduos, porém os estudos avaliando possíveis regiões envolvidas ainda são incipientes. Em animais, uma das formas mais promissoras de observar essas modificações é através dos testes comportamentais, tais como avaliação do limiar nociceptivo, resposta a estímulos táteis não nocivos e discriminação olfatória. Nos modelos animais de autismo já foi observada, por exemplo, redução no limiar nociceptivo (hiporresponsividade ao estímulo doloroso), característica encontrada em humanos com o transtorno, além de falhas em neonatos na diferenciação de odores conhecidos (maravalha do ninho materno) de odores neutros (maravalha limpa) (SCHNEIDER *et al.,* 2005).

Em indivíduos com autismo, há evidências de alteração no córtex somatossensorial, incluindo alterações na organização somatotópica de regiões associadas aos dedos e à face (COSKUN *et al.,* 2009), indicando uma possível desorganização na rede neuronal local. Além disso, diversos outros estudos envolvendo exames de imagem já demonstraram déficits em regiões corticais associadas à audição (GOMOT M *et al.,* 2008), inclusive com descrições de alterações em nível de neurônios PV+ no córtex auditivo primário (ANOMAL *et al.,* 2015) e visão (CLERY H *et al.,* 2016), por exemplo.

Modificações na citoarquitetura envolvendo especificamente a Área Somatossensorial Primária já foram descritas no modelo animal de autismo induzido por VPA e incluem alterações na celularidade, principalmente no conteúdo de neurônios GABAérgicos da camada V e na organização de minicolunas (dados não publicados do grupo). Futuros estudos avaliando aspectos eletrofisiológicos dessa

região associados a testes comportamentais serão capazes de fornecer informações importantes no entendimento do papel da Área Somatossensorial Primária no TEA.

1.7 Região da Amígdala

O sistema límbico é um conjunto de estruturas encefálicas situadas no telencéfalo e diencéfalo, responsáveis pelo processamento de emoções e comportamentos sociais. Uma das regiões mais estudadas nesse sistema é a amígdala, que possui papel fundamental em diversos aspectos como formação de memórias emotivas associadas à olfação e à gustação, desenvolvimento de memórias condicionadas por medo, interação social e integração de estímulos sensoriais – demonstrando o papel chave dessa região no processamento sensorial aplicado às relações sociais (RAJMOHAN V *et al.,* 2007).

Diversas desordens neuropsiquiátricas de grande prevalência apresentam alterações importantes na amígdala tais como distúrbios de ansiedade (ZALLA *et al.,* 2013), esquizofrenia (SHIN *et al.,* 2010), Transtorno do Espectro Autista (MIER *et al.,* 2014) e outras. No TEA especificamente, uma das primeiras evidências interessantes é a alta porcentagem de indivíduos que possuem ansiedade como comorbidade (cerca de 40%), já que as alterações na amígdala estão intimamente relacionadas ao medo e ao comportamento do tipo ansioso (VAN STEENSEL *et al.,* 2011). Além disso, estudos realizados com ressonância magnética funcional indicam respostas atípicas dessa região a um treinamento de condicionamento por medo em humanos (TOP *et al.,* 2016).

Outros aspectos importantes identificados em pacientes com TEA são as alterações a nível estrutural, incluindo aumento no peso relativo da amígdala (ECKER *et al.,* 2015) e hiperativação bilateral antecipadamente ao contato social (DICHTER *et al.,* 2012), indicando que um possível desequilíbrio excitatório-inibitório nesse local possa estar atuando como fator determinante para as alterações comportamentais identificadas.

Em modelos animais, a realização de testes comportamentais associados à ansiedade fornecem dados interessantes que podem ser associados de certa forma com a função da amígdala. Nos modelos de autismo já se observou, por exemplo, através do teste de labirinto em cruz elevado, que há um aumento geral na

ansiedade (identificado pela permanência do animal majoritariamente nos braços fechados do aparato). Avaliações eletrofisiológicas já demonstraram uma sobreposição do componente excitatório em relação ao inibitório principalmente em no núcleo lateral (LIN *et al.,* 2013), sendo essencial a ampliação de estudos para estabelecer as relações de causa e efeito nessas alterações.

A integração sensorial nessa região é essencial para a formação de memórias associadas a estímulos como, por exemplo, o desenvolvimento de comportamento de vínculo entre mãe e prole neonata (MORICEAU *et al.,* 2005) a partir da associação do aroma, texturas e sons presentes no ambiente ou a formação de memória aversiva a partir da ocorrência de algum evento traumático. Todas essas alterações são de suma importância para o estudo do TEA, já que possivelmente as alterações sensoriais presentes podem acabar trazendo modificações nas formas de processar e interpretar as relações sociais.

2. JUSTIFICATIVA

Observando-se os diversos prejuízos na qualidade de vida associados ao Transtorno do Espectro do Autismo, além da crescente prevalência da desordem e relativa ausência de tratamentos especifícos, se faz necessário ampliar os estudos com enfoques em alterações celulares e modificações moleculares com possíveis reflexos comportamentais a fim de expandir a compreensão do TEA, promovendo mudanças positivas, em último nível, no panorama geral dos indivíduos afetados.

3. OBJETIVOS

3.1 OBJETIVOS GERAIS

Avaliar na Área Somatossensorial Primária e na Região da Amígdala, organização celular e distribuição de neurônios GABAérgicos positivos para parvalbumina em ratos Wistar de 30 dias pós natal, no modelo animal de autismo induzido por exposição pré-natal ao ácido valproico.

3.2 OBJETIVOS ESPECÍFICOS

1) Quantificar neurônios totais, células não neuronais e neurônios PV+ na Região da Amígdala e na Área Somatossensorial Primária;

2) Avaliar a distribuição dos neurônios PV+ nas camadas da Área Somatossensorial Primária;

3) Avaliar a organização das camadas corticais e possível comprometimento da organização colunar na Área Somatossensorial Primária.

5. TRABALHO EXPERIMENTAL NA FORMA DE ARTIGO CIENTÍFICO

A ser submetido no periódico Neuroscience

ANALYSIS OF PARVALBUMIN-POSITIVE GABAERIC NEURONES IN SENSORY AREAS IN THE ANIMAL MODEL OF AUTISM INDUCED BY PRE-CHRONIC EXPOSURE TO VALPROIC ACID

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ABSTRACT

Autism Spectrum Disorder is a high prevalent neurodevelopmental disorder characterized by impairments in communication and social interaction in addition to repetitive or stereotyped behaviors. Another important aspect for ASD - present in more than 90% of the individuals - is the sensory alterations, which cause great loss in life quality. In this study, we used the already consolidated and validated animal model of autism induced by prenatal exposure to valproic acid (VPA) in order to evaluate quantity and distribution of parvalbumin-positive GABAergic interneurons in two sensory related regions: Primary Somatosensory Area and Amygdala Region. The analysis was made by immunfluorescence (DAPI, NeuN and Parvalbumin) of brain slices identified with Paxinos Atlas from 30 days old male animals in two groups: control and VPA. The images were obtained by confocal microscopy and processed using Cell Counter plug-in from ImageJ software. In Primary Somatosensory Area, VPA group exhibited alterations in laminar organization and significant reduction in the number of non-neuronal cells in the layer IV-V – indicating impairments in glial cells, moreover, PV+ cells quantity and density were significant reduced in layer IV-V and increased in layer II-III, demonstrating a possible impairment in migrations between layers since the data from the same parameters considering all the layers together didn't showed differences. In amygdala region, both quantity and density of PV+ were reduced in VPA, demonstrating again the loss of an important inhibitory component, probably causing an excitatory/inhibitory imbalance that reflects on behavioral alterations.

Key words: ASD, VPA, Animal Model, Sensory, Parvalbumin, Immunofluorescence, Primary Somatosensory Area, Amygdala Region.

Highlights: ►Animal model of autism induced by prenatal exposure to valproic acid (VPA) ►Quantitative analyzes of non-neuronal cells, total neurons and PV+ neurons in Primary Somatosensory Area (PSSA) and Amygdala Region (AMR) ► PV+ cells distribution altered in cortical layers of PSSA and in AMR in VPA group ► Possible impairment in migration of interneurons ► Connectivity alterations in autism ►Excitatory/Inhibitory Imbalance in sensory related regions.

INTRODUCTION

Autism Spectrum Disorder (ASD) is a high prevalent neurodevelopmental disturb (1:68 children aged 8 years in USA) characterized, according to DSM-V, by a behavioral dyad:

1) Impairments in communication and social interaction;

2) Repetitive or stereotyped behaviors.

Although many studies have been proposing interesting theories, the ASD etiology remains unknown and, in addition to that, the lack of a validated biomarker allied to the difficulty of finding proper treatments turns autism into a big challenge for all the individuals affected by this disorder.

Many factors involved with autism have already been described: genetic background can bedemonstrated by the high agreement of ASD development in monozygotic twins (reaching values of up to 90%) (DIETERT *et al.*, 2011), while in dizygotic twins the heritability reaches the value of 10% (CASANOVA *et al.,*2006). Moreover, some environmental factors are also being related with autism, like the use of alcohol, thalidomideand valproic acid during pregnancy – this last one has shown important increase in the risk of autism in the children whose mothers took valproic acid, especially in the first trimester of gestation (CHRISTENSEN *et al.,* 2013).

Besides de classic features associated with autism, one of the most important alterations are the sensory impairments, identified in more than 90% of individuals with ASD (GESCHWIND *et al.*, 2009). The most commons deficits include hyper-responsiveness to non-harmful stimuli (e. g., visual, tactile and auditory) and hyporresponsiveness to harmful stimuli. Although these alterations bring great impairment in life quality, little is known about how the sensory stimuli are processed and integrated in ASD patients.

Another characteristic identified in ASD is the excitatory/inhibitory imbalance, observed in clinical terms by the high prevalence of epilepsy in individuals with autism (1/3 of the patients) (SPENCE *et al.*, 2009). Along with that, many studies with animal models and humans have been describing several alterations in connectivity and electrical features in neurons of different brain regions. One of the most interesting discoveries about the pattern of connectivity in the ASD brain, made using

fMRI, showed an increase in the local connections and a deficit in "long distances" connections between different regions of the brain (BELMONTE *et al.* 2004), suggesting a possible explanation for the deficits in the integration of stimuli. Therefore, the content and organization of excitatory and inhibitory components along neuronal circuits may be a key factor in the establishment of some behavioral peculiarities found in ASD especially in areas related to sensory functions as Amygdala and Primary Somatosensory Area.

Interneurons have a major role in the circuits of the brain, acting as switches and pattern generators, providing refinement to the countless connections present in the brain. The positive parvalbumin GABAergic neurons are the most common interneurons in the cortex (40% of the total interneuron population) (XU *et al.*, 2010) and they demonstrate important participation in synchronization of rhythms, memory, attention and integration of different sensory areas. The study of these neurons in schizophrenia showed important evidences of alteration in migration and distribution, however in ASD studies are still incipient. In view of all the previous evidence, the goals of this study are quantify and analyze the organization of PV neurons in Amygdala Region (RAM) and Primary Somatosensory Area (PSSA). Non-neuronal cells exhibited significant reduction in PSSA of VPA model and PV+ cells showed complex patterns of density alterations in PSSA (increase density in layer II-III and reduction in layer IV-V in VPA) and in RAM (decrease in VPA model). Moreover, cortical columnar organization seemed to be very impaired and disrupted. The data suggested a major action of VPA in sensory related areas, altering neuronal networks and excitatory/inhibitory balance – alterations that may underlie the great amount of sensory deficits identified in individuals with ASD.

EXPERIMENTAL PROCEDURES

Animals

Wistar rats were obtained from the facilities of HCPA (Hospital de Clínicas de Porto Alegre) and Biochemistry Department – ICBS (Instituto de Ciências Básicas da Saúde) and maintained according to the following conditions: 12:12 light cycle (lights on at 7:00 and lights off at 19:00), controlled temperature (22±1°C) and water and food *ad libitum* – in line with the recommendations of Ethics Committee in Animals

Use (CEUA).

The animals were mated overnight and the confirmation of fertilization was made in the following morning by the visualization of spermatozoa in the vaginal smear (embrionary day 0). Females of VPA group received a single intraperitoneal injection of VPA (600 mg/kg, 250 mg/ml diluted in NaCl 0.9%) in the 12.5th day of (BAMBINI-JUNIOR *et al.*, 2011; SCHNEIDER *et al.*, 2005), during the days E6.5-E18.5 the females also received subcutaneous injections of vehicles (saline 0.9% and DMSO 3.6mg/kg). The justificative for using DMSO (RSV vehicle) is based on our research lab strategy of using Resveratrol (RSV) as a prenatal treatment to evaluate possible pathways related to autism. In this case, for a matter of time and monograph structure, only two groups were used (Control-DMSO and VPA), but the work is part of a bigger project that soon will be completed with the other groups.

1: Control (DMSO)   2: VPA   3: RSV   4: RSV+VPA
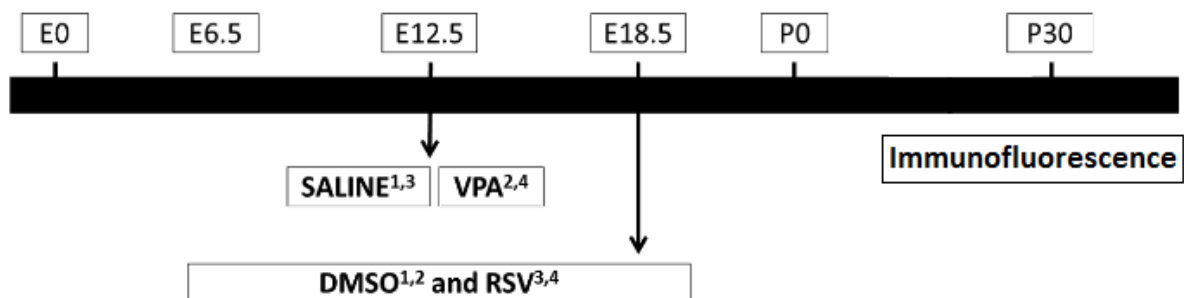
Image 1: timeline of experiment with all groups demonstrated. In this work, however, only the first two groups (Control-DMSO and VPA) were used according to previous justificatives.

Females were housed individually and were allowed to raise their own litters. The offspring rats were separated by sex at P21. Male pups at P30 (n=3,4) were anaesthetized (75 mg/kg ketamine + 10 mg/kg xylazine) and perfused transcardiacally with 0.9%-NaCl solution followed by first 1,5%-paraformaldehyde and after 4%-paraformaldehyde solution before the removal of their brain. In order to perform immunofluorescence analysis, the brains were preserved in Tissue-Tek® and kept in −80°C ultrafreezer. It were used 12 animals from different litters, being 6 control and 6 VPA.

The project was approved by CEUA-HCPA with the protocol number 140367.

Parvalbumin and NeuN immunofluorescence

Before freezing in the ultrafreezer, brains are post-fixed during 4 hours in a 4%-paraformaldehyde solution and were subsequently prepared for freezing in 15% and 30%-sucrose PBS solutions until completely submersion.

Coronal brain slices (25µm) were obtained in −20ºC cryostat (Leica Microsystems GmbH) using a rat brain atlas (PAXINOS *et al.*, 1997) to identify the sections containing primary somatosensory area and amygdala regions. The slices were arranged in microscopy slides covered with poly-l-lysine to improve fixation.

Slices were exposed to the following steps: 1) final tissue fixation with 4%-paraformaldehyde (10 minutes); 2) 3 washes with PBS 0.1M buffer (5 minutes each); 3) permeabilization with PBS-Triton 0.1% (10 minutes); 4) 3 washes with PBS 0.1M buffer (5 minutes each); 5) antigen re-exposure in citrate buffer at 60ºC (1 hour); 6) 2 washes with PBS-Triton 0.1% (5 minutes each); 7) blockage with PBS-Triton 0.1% BSA 5% (1 hour); 8) Incubation with primary antibodies (PV anti-mouse Sigma-Aldrich® (P3088) and NeuN Abcam® anti-rabbit (ab104225) – both diluted to 1:500 in blockade solution) for 48h at 4ºC; 9) 5 washes with PBS 0.1M buffer (3 minutes each); 10) incubation with secondary antibodies (AlexaFluor 488 Abcam® (ab150077) anti-mouse and Alexa Fluor 546 Invitrogen® (A-11030) anti-rabbit - both diluted to 1:2000 in blockade solution) for 2h at room temperature; 11) 5 washes with PBS 0.1M buffer (3 minutes each); 12) incubation with DAPI solution (10 minutes); 13) final 5 washes with PBS 0.1M buffer (3 minutes each) followed by addition of mounting medium and coverslip.

Images were obtained in the confocal microscope (Olympus FluoView 4.0 Viewer) of Electronic Microscopy Center (CME-UFRGS) and cell types and fluorescence were analyzed using the Image J software with the Cell Counter plug-in. The number of parvalbumin positive neurons, total neurons and total cells were counted manually.

Statistical Analysis

Data were analyzed in Graph Pad Prism 5 software using Student's T Test followed by Welch's correction and considering $p < 0.05$ as statistically significant. The results are expressed as mean±SD.

RESULTS

Primary Somatosensory Area (Figures 1, 2, 3, 4 and 5)

There was no difference between groups both in the quantity of total neurons in the layer II-III (Control: 274,3±10,92; VPA: 265,0±30,04 p=0.7817) and IV-V (Control: 320,3±33,20; VPA: 335,5±41,81 p=0.7824). The number of PV neurons showed difference only in the layer IV-V (Control: 36,25±3,172; VPA: 24,58±1,287 p = 0.0270).

Significant differences related to density of PV neurons were found in the layer II-III showing increased density in VPA group (Control: 0,09319±0,006055; VPA: 0,1574±0,01342 p=0.0036) and in the layer IV-V, in which the density was reduced in VPA group (Control: 0,1147±0,008883; VPA: 0,03757±0,001790 p=0.0025). No alteration was found when considering all the layers (Control: 0,1047±0,003848; VPA: 0,1115±0,008866 p=0.5099).

There was no difference between the groups in the quantity of non-neuronal cells in the layer II-III (Control: 752,8±47,57; VPA: 659,3 ± 38,49 p=0.1737), while in the layer IV-V, VPA showed a significant reduction in relation to control group (Control: 907,0±50,60; VPA: 717,6±31,20 p=0.0233).

Cortical layer organization exhibited impairment in VPA group with disruption of the classic columnar structures when comparing to control group.

Amygdala Region (Figures 6 and 7)

Significant difference was found in the total number of neurons demonstrating increase in VPA group (Control: 221,8±41,35; VPA: 402,6±43,80 p=0.0265). However, the number of PV neurons wasn't different between groups (Control: 33,83±3,844; VPA: 24,20±3,813 p=0.1320).

The density of PV neurons was significantly reduced in VPA group (Control: 0,1593±0,02466; VPA: 0,05991±0,007587 p=0.0459).

Non-Neuronal cells exhibited no differences between groups (Control: 658,8±

138,4; VPA: 449,0±48,06 p=0.2648).

DISCUSSION

The most interesting and challenging characteristic of ASD is its heterogeneity of behaviors, molecular patterns and biochemical parameters, so it's necessary to find points of convergence to better understand this disorder. One of the most commons features related to ASD is the sensory impairment, present in more than 90% of the individuals (GESCHWIND *et al.*, 2009), so is very important to expand knowledge about it in animal models validated and consolidated. The previous presented data showed important alterations in animals from VPA model of autism in primary somatosensory area and amygdala, regions with major role in sensory processing and integration, demonstrating modifications in both non-neural and neural cells.

In the IV-V layer of Primary Somatosensory Area, glial cells appeared significant reduced in the VPA group, suggesting a cell loss or an important impairment in the migration. Otherwise, the lack of proper glia function in this area may cause great imbalance in the local homeostasis since these cells are essential to synapse formation, neurotransmitter reuptake and recycle, myelination, formation and permeability regulation of brain blood barrier and many other functions (ZEIDN-CHULI *et al.*, 2014). Many evidences in ASD have been highlighting the central role of microglia and astrocytes in the development of disorder – microglial activation was reported in the brains of autistic children by positron emission tomography (SUZUKI *et al.*, 2013) and in the hippocampus of animal model of autism induced by VPA (LUCCHINA *et* al., 2014). It's very likely that the pro inflammatory environmental induce not only alteration in microglia but also in astrocytes, leading to multiple outcomes like increase in the production of reactive oxygen species, deficits in metabolism neurotransmitters and enhancement of inflammatory mediators. (PETRELLI *et al.*, 2016) In the layer II-III of PsSA and in Amygdala, there was no significant differences in the number of non-neuronal cells, but it's very possible that some subpopulations are reduced and compensated by others, homogenizing the differences.

Neuronal quantity showed significant differences ionly in Amygdala, but many

studies in ASD demonstrated important alterations in subpopulations (TAKANO *et al.*, 2015), creating an imbalance that is not seen when observing all neurons as a whole. Our group verified in still unpublished data that GABAergic neurons are reduced in the PSSA of VPA animal model, besides that some studies also demonstrated loss of Purkinje cells in the cerebellum (WHITNEY *et al.*, 2008), reduction in the number of pyramidal neurons (CAMACHO *et* al., 2013) and signals of neurodegeneration in amygdala (SCHUMANN *et* al., 2006) – all of them obtained by post mortem analysis. So, in order to refine analysis we choose to quantify a specific subset of inhibitory interneurons related with several impaired characteristics in ASD: the parvalbumin positive neurons.

The task of organizing 86 billions of neurons and a similar number of glial cells (AZEVEDO *et al.*, 2009) is not trivial and requires specialized filters and countless checkpoints. One of the most interesting mechanisms to perform these adjustments are interneuron networks, which act from the most basic regulations like nociceptive flexion reflex (BEAR *et al.*, 2007) to higher brain functions as sensory processing. Our results showed an important alteration in parvalbumin positive neurons in both areas analyzed, leading to many insights about the triad excitatory/inhibitory imbalance, interneuron connectivity and sensory related areas. Not only the number of parvalbumin positive neurons demonstrated significant differences, but also the density of them expressed important patterns, besides that, the absolute number is a tricky data to analyze, because no matter how standardized the microscopy images are captured, there will always be some positions differences – so the density eliminates these bias, improving conclusions.

In the Primary Somatossensory Area, density of PV+ neurons are signficant increased in the VPA group in the layer II-III and reduced in the same group in the layer IV-V, however, the density considering all PV+ neurons in all layer didn't expressed differences between groups – a study made with VPA model in mice demonstrated a similar result regarding to general density: in analysis of all somatosensory cortex they have found no differences, but the layer analysis wasn't performed (LAUBER *et al.*, 2016). So, observing the "big picture" we can see that there is no loss of parvalbumin positive neurons in this area, but the distribution of them is completely altered in layer suggesting that VPA promotes an over migration

to superior layer II-III, while layer IV-V end up deficient. Since parvalbumin positive neurons correspond to 40% of interneurons in cortical regions (XU *et al.*, 2010) and have innumerable synaptic contacts an alteration in that level in such an important area certainly leads to major outcomes. Evidence from other areas like superior colliculus (DENDRINOS *et* al., 2011) and medial prefrontal cortex, both essential for functions like visual stimulus processing and inhibitory control, in the VPA model, demonstrated that reduction in density of PV+ neurons has an important outcome observed by impairment in social, stereotyped and sensory behaviors. Finally, only recently were described alterations in PV+ neurons in humans: a post mortem analyses of 11 brains of autistic-children showed an important reduction of these cells in the prefrontal cortex (HASHEMI *et al.*, 2016), confirming the data present in animal models and reassuring the necessity to expand experiments on this area.

Neuronal migration is a subject that combines both curiosity and mystery in view of the great number of genes and factors acting in a organized sequence to position each cell on its correct place. With parvalbumin positive neurons this is not different: we know little about the typical migration and much less about how it occurs in the context of psychiatric disorders. All GABAergic interneurons are generate in an embryonic region called Ganglionic Eminence and then migrate during embryo development towards cortex, amygdala, hippocampus and many other regions (JIANG *et al.*, 2016) – in that context, parvalbumin positive neurons are originated from Nkx2.1 lineage after signalizing of Shh and start migration to cortex at E12.5 (MARÍN *et al.*, 2003) (the same day animals of our model receive VPA injection). Many other factors as lhx6/8, sox6 and dlx1/5 play their role in movement and expression of PV (Kelsom C *et al.*, 2013). The great majority of data describing failures in this migration process are obtained in animal models and human studies of schizophrenia: in a genetic model (Disc1-L100P mutants) was found many impairments in the PV+ migration, causing altered distribution in the neocortex and hippocampus and decreased GAD67/PV co-localization (LEE *et* al., 2013), besides that, human studies have already shown, for example, lower expression of lhx6, an important migration regulator and reduction in PV+ cells in Dorsolateral Prefrontal Cortex (VOLK *et* al., 2012). Moreover, a paper in which PV+ neurons were disabled by knocking down Gad1 gene demonstrated curious ASD and Schizophrenia like

behaviors as sensorimotor gating deficits, increased novelty seeking and reduced fear extinction (BROWN *et al.*, 2015).

In the amygdala region PV+ neurons exhibited lower density in the VPA group when compared with control. In this region, it's already known that PV+ have a complex network in basolateral nuclei (WOODRUFF *et al.*, 2007) that can be altered by fear stimulation in the context of fear learning – a major function related to amygdala (LUCAS *et al.*, 2016). Besides that, a behavioral study demonstrated that environmental enrichment increases PV+ number in amygdala at the same time that reduces anxiety – an important comorbidity of ASD (URAKAWA *et* al., 2013). Moreover, reduction in PV+ can be behind the eletrical imbalance found in the amygdala of VPA model (LIN *et* al., 2013), raising the importance of these cells.

Finally, the disruption in cortical columns organization identified qualitatively by observing the images of Primary Somatosensory Area confirm the columnar neuropathy (CASANOVA *et al.*, 2011) present in individuals with ASD. These alteration allied with the modified densities of PV+ neurons demonstrate a great impairment in cortical organization, probably causing major deficits in sensory processing and integration. Since behavior is the final key to clarify data related with autism, is essential to perform tasks like whisker nuisance task to conclude the incredible narrative of sensory impairments of autism, uniting interneurons, cortical organization and E/I imbalance.

Conclusions

The prenatal exposure to VPA seemed to cause great impact in the two sensory related structures evaluated. In Primary Somatosensory Area, the loss of laminar organization and non-neuronal cells in layer IV-V in addition to the differences in distribution of PV+ between layers denote an important impact of the VPA in this area, altering the refined structure and probably influencing in the migration of PV+ cells. In Amygdala, the reduction in quantity and density of PV+ cells in VPA group confirms the alteration found in PSSA, demonstrating the important loss of the inhibitory component that may contribute to the general excitatory-inhibitory imbalance identified in ASD. Since these areas are essential to process and integrate many types of stimuli and also perform specific actions like consolidation of fear

memories the alteration found in this study may have great impact in behavioral features, so is crucial to expand the studies in order to clarify the "big picture" of the sensory questions in autism.

ACKNOWLEDGEMENTS

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding this article.

5. REFERENCES

Ashok AH, Baugh J, Yeragani VK. Paul Eugen Bleuler and the ori-gin of the term schizophrenia. Indian J Psychiatry (2012) 54:95–6.

Azevedo, F. A. C., Carvalho, L. R. B., Grinberg, L. T., Farfel, J. M., Ferretti, R. E. L., Leite, R. E. P., … Herculano-Houzel, S. (2009). Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *Journal of Comparative Neurology, 513*(5), 532–541.

Bambini-Junior, Victorio, et al. "Animal model of autism induced by prenatal exposure to valproate: behavioral changes and liver parameters." *Brain research* 1408 (2011): 8-16.

Bear, M., Connors, B., & Paradiso, M. (2007). *Neuroscience. Sunderland (MA): Sinauer Associates*. http://doi.org/978-0878937257

Belmonte, M. K., Allen, G., Beckel-Mitchener, A., Boulanger, L. M., Carper, R. A., & Webb, S. J. (2004). Autism and abnormal development of brain connectivity. *The Journal of Neuroscience, 24*(42), 9228–31.

Brown, Jacquelyn A., et al. "Inhibition of parvalbumin-expressing interneurons results in complex behavioral changes." *Molecular psychiatry* 20.12 (2015): 1499-1507.

Camacho J, Combs Z, Schumman C, Amaral D, Martinez-Cerdeno V: post mortem analysis of cell density in autistic temporal cortex. Soc Neurosci Kern et al. Translational Neurodegeneration 2013,2:17

Casanova, M. F., Buxhoeveden, D. P., Switala, A. E., & Roy, E. (2011). Minicolumnar pathology in autism Minicolumnar pathology in autism. *Neurology, 58*, 428–432.

Christensen, J., Grønborg, T. K., Sørensen, M. J., Schendel, D., Parner, E. T., Pedersen, L. H., & Vestergaard, M. (2013). Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. *Jama, 309*(16), 1696–703.

Dendrinos, Georgia, Marie Hemelt, and Asaf Keller. "Prenatal VPA exposure and changes in sensory processing by the superior colliculus." *Frontiers in integrative neuroscience* 5 (2011): 68.

Dietert, R. R., Dietert, J. M., & DeWitt, J. C. (2011). Environmental risk factors for autism. *Emerging Health Threats Journal*.

Geschwind, Daniel H. "Advances in autism." Annual review of medicine 60 (2009): 367.

Hashemi, Ezzat, et al. "The number of parvalbumin-expressing interneurons is decreased in the medial prefrontal cortex in autism." *Cerebral cortex* (2016): bhw021.

Jiang, Xiao, Mathieu Lachance, and Elsa Rossignol. "Involvement of cortical fast-spiking parvalbumin positive basket cells in epilepsy." Progress in brain research 226 (2016): 81-126

Kelsom, C., & Lu, W. (2013). Development and specification of GABAergic cortical interneurons. *Cell & Bioscience*, *3*(1), 19.

Lauber, E., Filice, F., & Schwaller, B. (2016). Prenatal Valproate Exposure Differentially Affects Parvalbumin-Expressing Neurons and Related Circuits in the Cortex and Striatum of Mice. *Frontiers in Molecular Neuroscience*, *9*.

Lee, F. H. F., Zai, C. C., Cordes, S. P., Roder, J. C., & Wong, A. H. C. (2013). Abnormal interneuron development in disrupted-in-schizophrenia-1 L100P mutant mice. *Molecular Brain*, *6*(April), 20.

Lin, Hui-Ching, et al. "The amygdala excitatory/inhibitory balance in a valproate-induced rat autism model." *PLoS One* 8.1 (2013): e55248.

Lucas, E. K., Jegarl, A. M., Morishita, H., & Clem, R. L. (2016). Multimodal and Site-Specific Plasticity of Amygdala Parvalbumin Interneurons after Fear Learning. *Neuron*, *91*(3), 629–643.

Lucchina, L., & Depino, A. M. (2014). Altered Peripheral and Central Inflammatory Responses in a Mouse Model of Autism. *Autism Research*, *7*(2), 273–289. http://doi.org/10.1002/aur.1338

Petrelli, F., Pucci, L., & Bezzi, P. (2016). Astrocytes and Microglia and Their Potential Link with Autism Spectrum Disorders. *Frontiers in Cellular Neuroscience*, *10*.

Soares, Ana Maria Araújo. *Implementações metodológicas para o estudo eletrofisiológico e comportamental em um modelo animal de autismo*. MS thesis. Universidade Federal do Rio Grande do Norte, 2015.

Schumann, C. M., & Amaral, D. G. (2006). Stereological Analysis of Amygdala Neuron Number in Autism. *Journal of Neuroscience*, *26*(29), 7674–7679.

Spence, S. J., & Schneider, M. T. (2009). The role of epilepsy and epileptiform EEGs in autism spectrum disorders. *Pediatric research*, *65*(6), 599-606.

Takano, T. (2015). Interneuron dysfunction in syndromic autism: Recent advances. *Developmental Neuroscience*. http://doi.org/10.1159/000434638

Urakawa, Susumu, et al. "Rearing in enriched environment increases parvalbumin-positive small neurons in the amygdala and decreases anxiety-like behavior of male rats." *BMC neuroscience* 14.1 (2013): 13.

Volk, D. W., Matsubara, T., Li, S., Sengupta, E. J., Georgiev, D., Minabe, Y., … Lewis, D. A. (2012). Deficits in transcriptional regulators of cortical parvalbumin neurons in schizophrenia. *American Journal of Psychiatry*, *169*(10), 1082–1091.

Whitney, E. R, Kemper, T. L., Bauman, M. L., Rosene, D. L., & Blatt, G. J. (2008). Cerebellar Purkinje cells are reduced in a subpopulation of autistic brains: A stereological experiment using calbindin-D28k. *Cerebellum*, *7*(3), 406–416.

Woodruff, Alan R., and Pankaj Sah. "Networks of parvalbumin-positive interneurons in the basolateral amygdala." *Journal of Neuroscience* 27.3 (2007): 553-563.

Xu X, Roby KD, Callaway EM (2010) Immunochemical characterization of inhibitory mouse cortical neurons: three chemically distinct classes of inhibitory cells. J Comp Neurol 518:389 – 404. CrossRef Medline

Zeidn-Chuli, F., Salmina, A. B., Malinovskaya, N. A., Noda, M., Verkhratsky, A., & Moreira, J. C. F. (2014). The glial perspective of autism spectrum disorders. *Neuroscience and Biobehavioral Reviews*.

5. LIST OF FIGURES
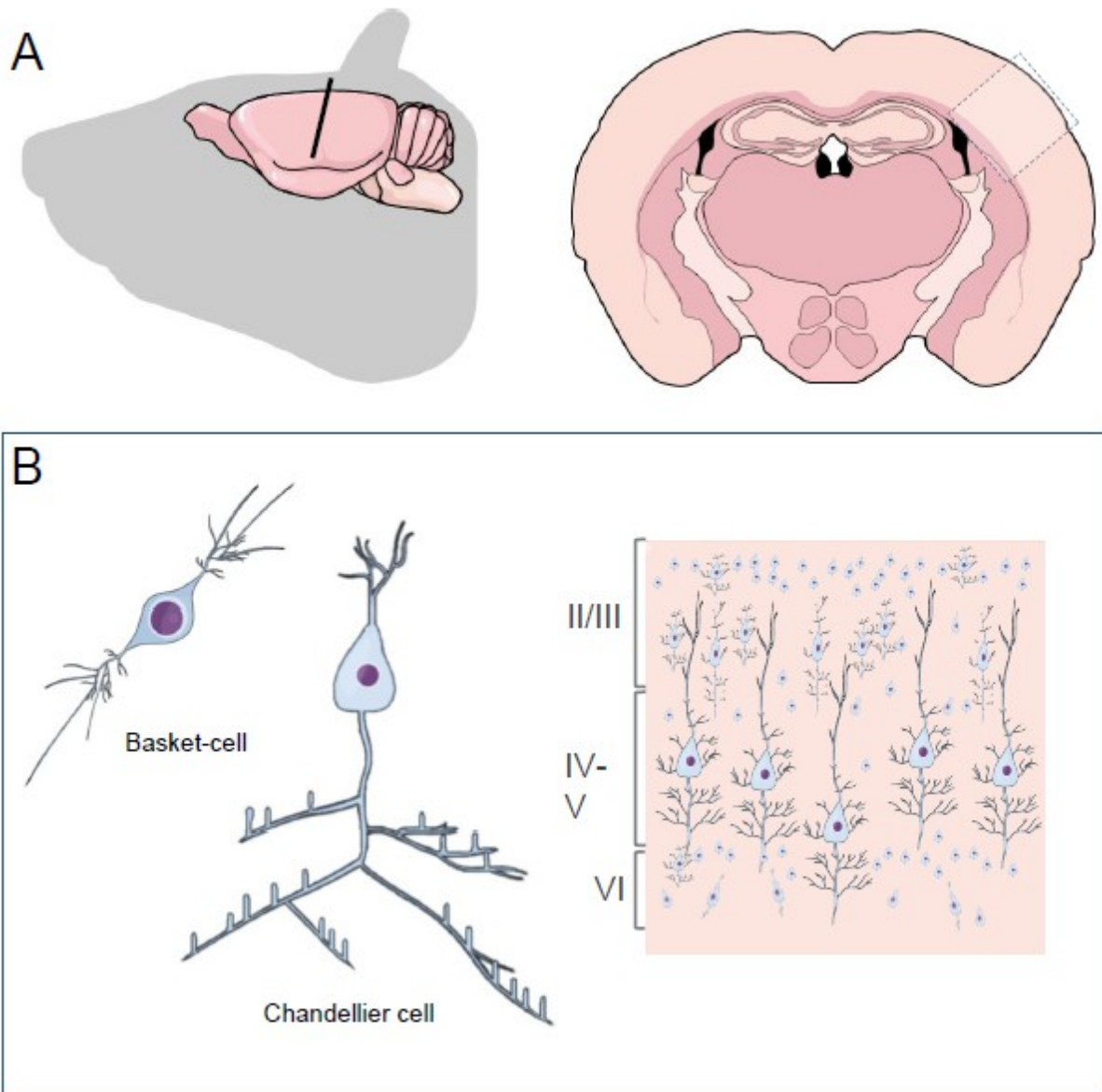
Figure 1: Schematic representation of Primary Somatosensory Area location in rat brain (A) and laminar organization of the cortex, highlighting not only the differences in cytoarchitecture between layer II-III and IV-V but also the morphological features of PV+ neurons (B).
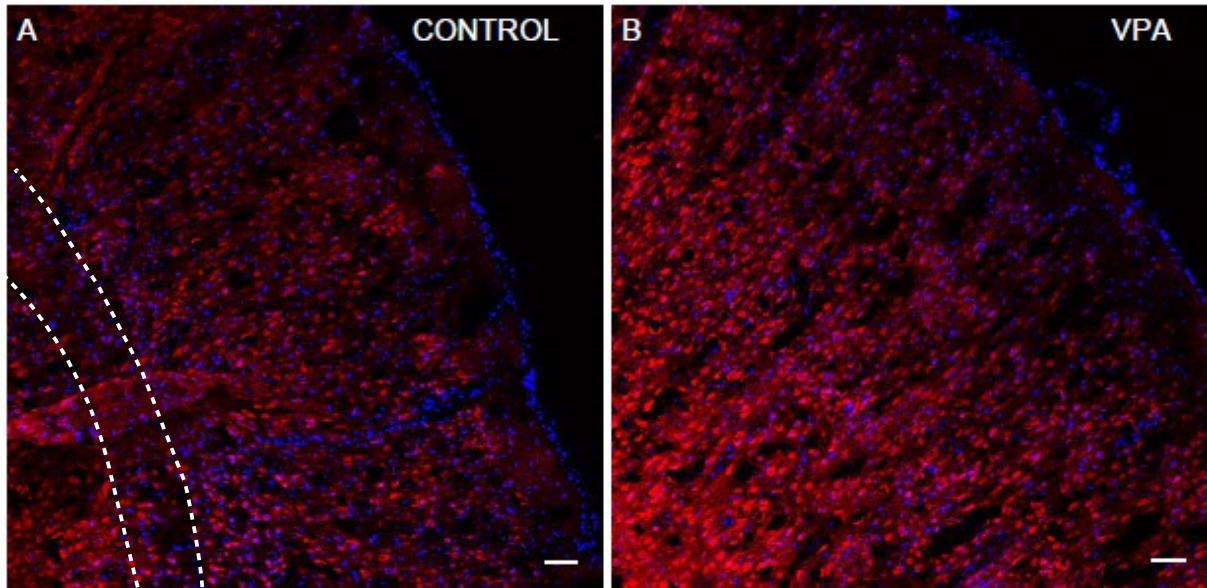
Figure 2: immunofluorescence image of Primary Somatosensory Area (magnification: 10x). Layer disorganization can be perceived in VPA group, in which the gap between layer II-III and IV-V (represented in control by the white dashed line) can no longer be noticed. In addtion to that, columnar arrangement seemed to be impaired in VPA, since the neurons appeared to form disoriented groups, while in the control the vertical disposition is more easily seen.
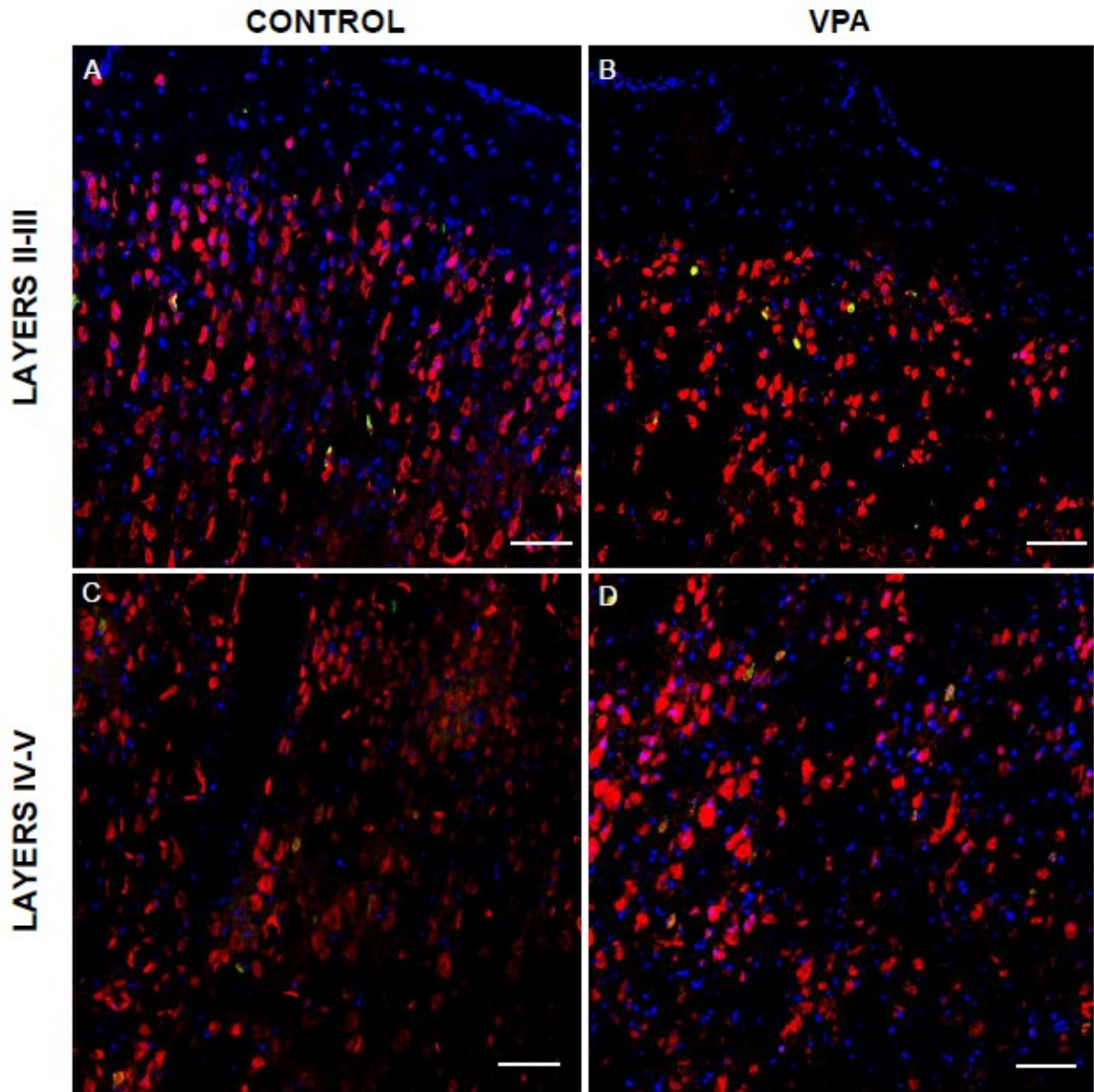
Figure 3: Immunofluorescence images from Primary Somatosensory Area. The increase in PV+ neurons (yellowish marked) number and density can be visualized in VPA group in layer II-III, while in the layer IV-V the same parameters appeared reduced in VPA group. Besides that, the laminar organization in VPA animals can also be visualized.
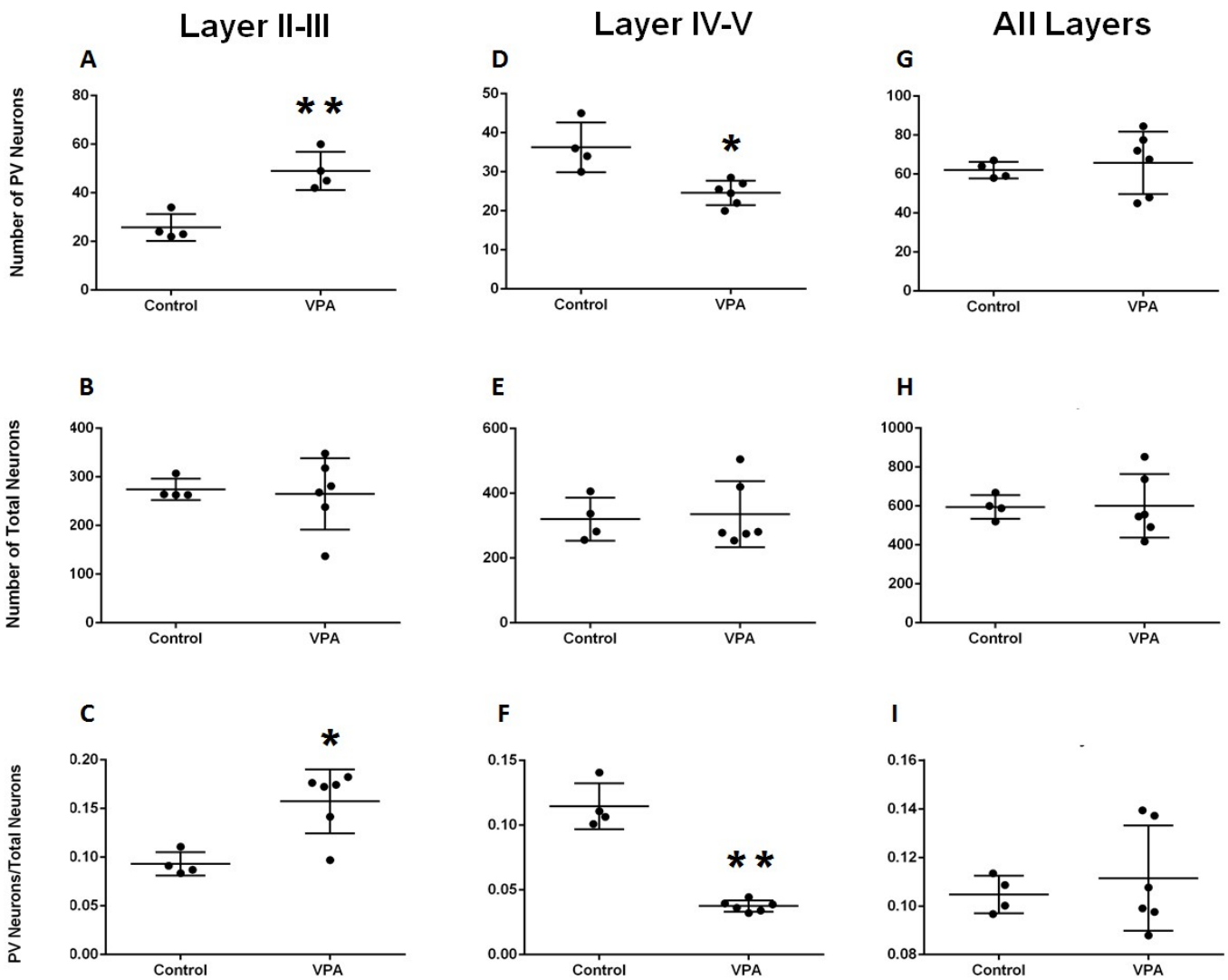
Figure 4: Results from immunofluorescence of Primary Somatosensory Area separated by layer. The number of total neurons showed no significant differences in layer II-III (B), IV-V (E) and in all layers together (H), demonstrating no neuronal loss in this region. Although number (G) and density (I) of PV neurons demonstrated no significant differences when analyzing all layers together, the isolation of II-III and IV-V layers showed: Increase in PV neuron number (A) and density (C) in VPA group in layer II-III, while in the layer IV-V VPA exhibited the opposite result with both reduction in PV number (D) and density (F). These data together suggest that PV cells may be affected by migration impairments promoted by VPA, since neuronal numbers remain unchanged observing the whole area Data expressed as mean±SD . *p<0.05, **p<0.01 (T Student's test, Welch).

Layer II-III

A

Layer IV-V

B
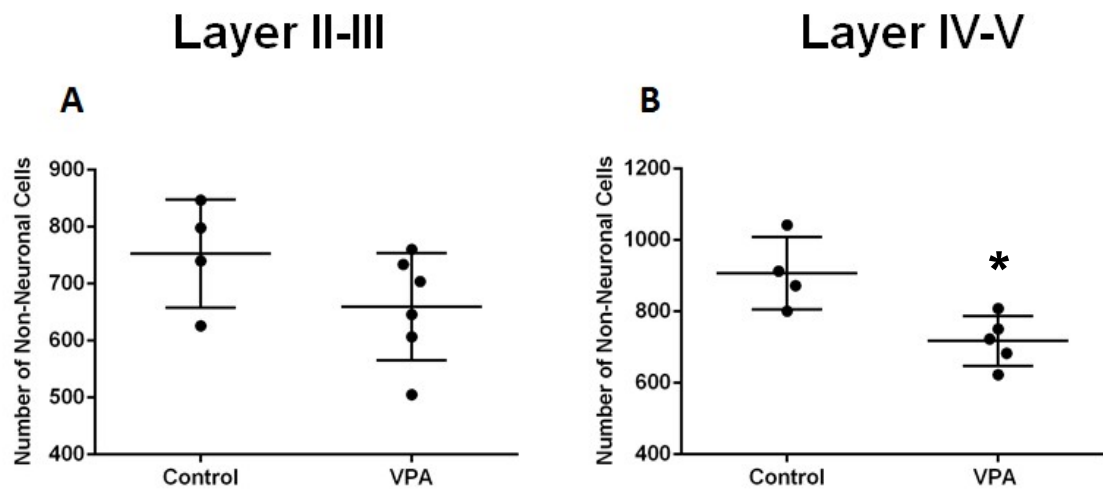
Figure 5: Results from immunofluorescence of non-neuronal cells. Layer II-III showed no alteration (A), while a significant reduction was identified in VPA group in layer IV-V (B). Since glial cells have important roles in countless activities, this impairment may cause several homeostasis disruptions. Data expressed as mean±SD . *$p < 0.05$ (T Student's test, Welch).
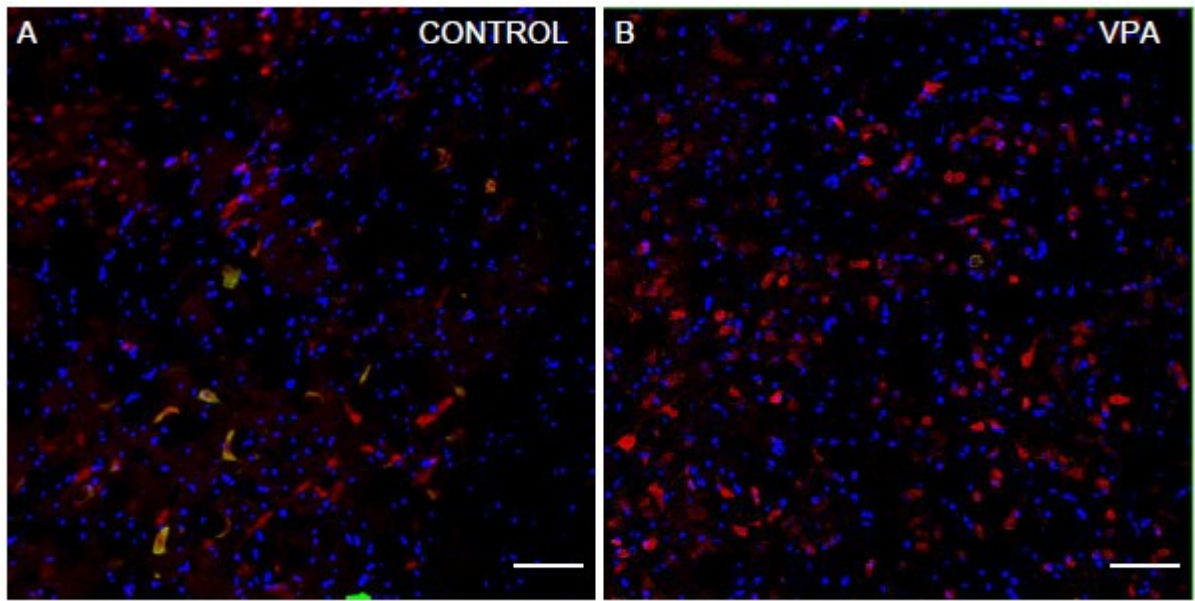
Figure 6: Immunofluorescence image of Amygdala Region (magnification: 20x) demonstrating the reduction of PV+ neurons (yellowish marked) number and density in VPA group compared to control.
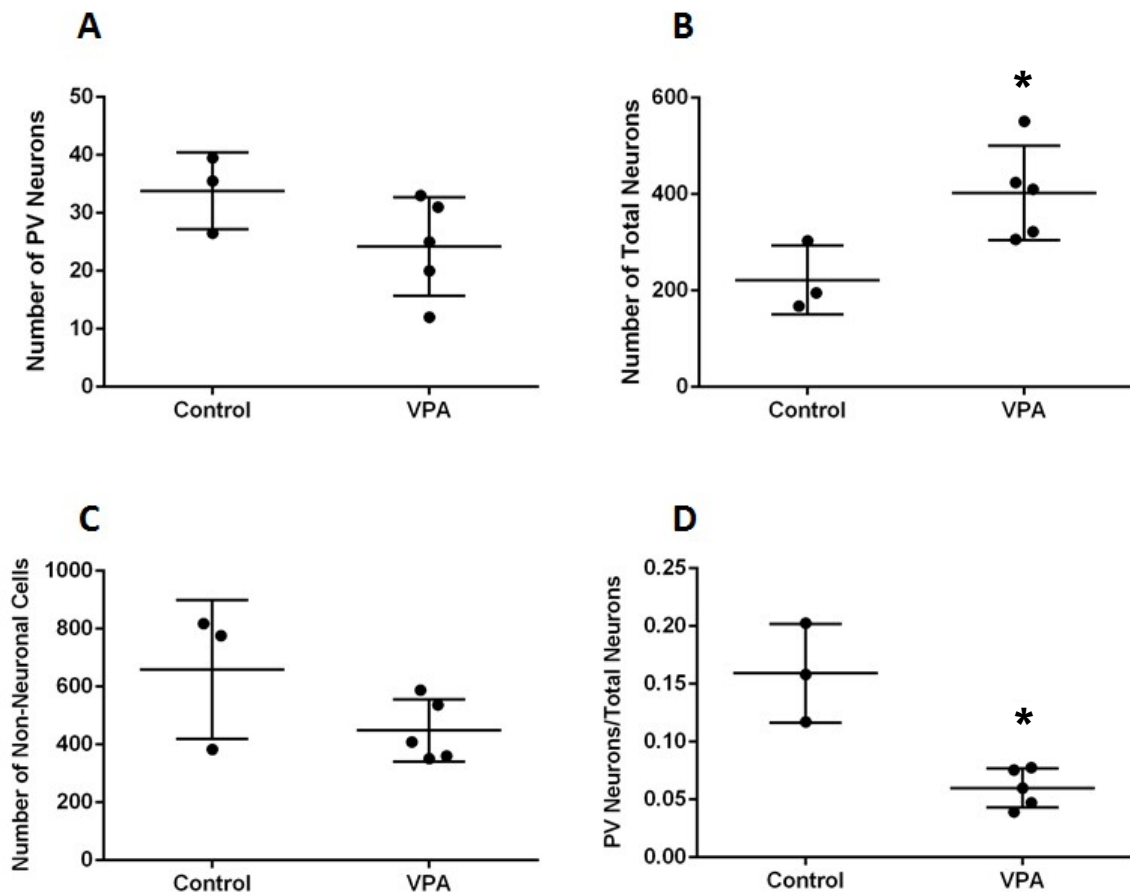
Figure 7: Results from immunofluorescence of Amygdala Region. The number of PV neurons (A) and non-neuronal cells (C) demonstrated no differences, while an important and significant reduction in the total number of neurons (B) and PV neurons density (D) was identified, contributing to clarify the role of VPA in sensory related structures. Data expressed as mean±SD . *$p < 0.05$ (T student's test, Welch).

6. CONCLUSÕES E PERSPECTIVAS

O presente trabalho demonstrou uma importante ação do tratamento pré-natal com ácido valproico em áreas relevantes para o processamento primário e integração de estímulos sensoriais. As alterações a nível de células gliais e de interneurônios inibitórios, mais especificamente os positivos para parvalbumina, na Área Somatossensorial Primária e na Região da Amígdala indica uma possível explicação para as hiper-responsividades e hiporresponsividades a diversos estímulos sensoriais identificadas na maioria dos indivíduos diagnosticados com TEA. Sob o ponto de vista eletrofisiológico, a depleção de neurônios GABAérgicos observada contribui para sustentar a hipótese geral de um desbalanço excitatório/inibitório, amplamente estudada no autismo. Assim, é necessário realizar testes de performance sensorial como o de estimulação de vibrissas a fim de correlacionar os possíveis dados obtidos. Além disso, avaliações de eletrofisiologia dessas regiões bem como detalhamento dos contatos sinápticos a nível de análise morfológica e quantitativa de espinhos além de quantificação proteica permitirão uma consolidação das conclusões desse trabalho no contexto do desequilíbrio excitatório-inibitório associado a alteração em interneurônios em regiões chave para o processamento sensorial.

Tabela 1: Resumo geral dos resultados obtidos nos animais do modelo animal de autismo induzido por exposição pré-natal ao ácido valproico em comparação com o controle.

Região	Células Gliais	Neurônios totais	Densidade de Neurônios PV+
Camadas II-III da Área Somatossensorial Primária	Sem diferença	Sem diferença	Aumento na densidade
Camadas IV-V da Área Somatossensorial Primária	Aumento na quantidade	Sem diferença	Redução na densidade
Amígdala	Sem diferença	Redução na quantidade	Redução na densidade

7. REFERÊNCIAS ADICIONAIS

American Psychiatric Association. (2013). *DSM-V.* ***American Journal of Psychiatry.***

ANOMAL, Renata Figueiredo et al. Impaired processing in the primary auditory cortex of an animal model of autism. **Frontiers in systems neuroscience**, v. 9, 2015.

Ashok AH, Baugh J, Yeragani VK. Paul Eugen Bleuler and the ori-gin of the term schizophrenia.**Indian   J Psychiatry** (2012) 54:95–6. doi:10.4103/0019-5545.94660

Asperger, H. (1944), Die 'Autistischen Psychopathen' im Kindesalter, **Archiv fur Psychiatrie und Nervenkrankheiten**, 117, pp.76-136.

Belmonte, M. K., Allen, G., Beckel-Mitchener, A., Boulanger, L. M., Carper, R. A., &   Webb, S. J. (2004). Autism and abnormal development of brain connectivity. ***The Journal of Neuroscience***, *24*(42),9228–31.

Burton BS (1882) On the propyl derivatives and decomposition products of ethylacetoacetate. ***Am Chem J****3*: 385–39

Casanova, M. F. (2015). The neuropathology of autism. In ***The Molecular Basis of Autism*** (pp. 153–171).

Christensen, J., Grønborg, T. K., Sørensen, M. J., Schendel, D., Parner, E. T., Pedersen, L. H., & Vestergaard, M. (2013). Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. ***Jama***, *309*(16), 1696–703.

Clery, H., Andersson, F., Bonnet-Brilhault, F., Philippe, A., Wicker, B., & Gomot, M. (2013). FMRI investigation of visual change detection in adults with autism. ***NeuroImage: Clinical***, *2*(1), 303–312.

Coskun, M. A., Varghese, L., Reddoch, S., Castillo, E. M., Pearson, D. A., Loveland, K. A., … Sheth, B. R. (2009). How somatic cortical maps differ in autistic and typical brains. ***NeuroReport***, *20*(2), 175–179

Dichter, G. S. (2012). Functional magnetic resonance imaging of autism spectrum disorders. ***Dialogues in Clinical Neuroscience***, *14*(3), 319–351.

Dietert, R. R., Dietert, J. M., & DeWitt, J. C. (2011). Environmental risk factors for autism. ***Emerging Health Threats Journal.***

DiLiberti, J. H., Farndon, P. a, Dennis, N. R., & Curry, C. J. (1984). The fetal valproate syndrome. ***American Journal of Medical Genetics***, *19*(3), 473–481.

Ecker, C., Bookheimer, S. Y., & Murphy, D. G. M. (2015). Neuroimaging in autism spectrum disorder: Brain structure and function across the lifespan. ***The Lancet Neurology***.

El-Fishawy, P., & State, M. W. (2010). The genetics of autism: key issues, recent findings, and clinical implications. *The Psychiatric Clinics of North America*, *33*(1), 83–105.

Fatemi SH, Halt AR, Stary JM, Kanodia R, Schulz SC, Realmuto GR. Glutamic acid decarboxylase 65 and 67 kDa proteins are reduced in autistic parietal and cerebellar cortices. **Biological psychiatry.** 2002; 52(8):805–10.

Fatemi SH, Reutiman TJ, Folsom TD, Thuras PD. GABA(A) receptor downregulation in brains of subjects with autism. **J Autism Dev Disord**. 2009; 39(2):223–30.

Fombonne, E. (2009). Epidemiology of pervasive developmental disorders. *Pediatric Research*.

Frith, U. (1989). *Autism: Explaining the enigma* (Vol. 1989). **Blackwell Scientific Publications: Oxford**.

Galarreta, M., & Hestrin, S. (2001). Electrical synapses between GABA-releasing interneurons. *Nature Reviews. Neuroscience*, *2*(6), 425–433.

Geschwind, Daniel H. "Advances in autism." **Annual review of medicine 60** (2009): 367.

Gomot, M., Belmonte, M. K., Bullmore, E. T., Bernard, F. A., & Baron-Cohen, S. (2008). Brain hyper-reactivity to auditory novel targets in children with high-functioning autism. *Brain*, *131*(9), 2479-2488.

Gottfried, C., Bambini-Junior, V., Francis, F., Riesgo, R., & Savino, W. (2015). The impact of neuroimmune alterations in autism spectrum disorder. *Frontiers in Psychiatry*, *6*(SEP).

Han, S., Tai, C., Westenbroek, R.E., Yu, F.H., Cheah, C.S., Potter, G.B., Rubenstein, J.L., Scheuer, T., de la Iglesia, H.O., and Catterall, W.A. (2012). Autistic-like behaviour in Scn1a+/- mice and rescue by enhanced GABA-mediated neurotransmission. **Nature** 489, 385–390

Hashemi, E., Ariza, J., Rogers, H., Noctor, S. C., & Martínez-Cerdeño, V. (2016). The Number of Parvalbumin-Expressing Interneurons Is Decreased in the Medial Prefrontal Cortex in Autism. *Cerebral Cortex (New York, N.Y. : 1991)*, bhw021-.

Jia, X., & Kohn, A. (2011). Gamma rhythms in the brain. **PLoS Biology**, *9*(4).

Kanner, L. (1943), Autistic Disturbances of Affective Contact, **Nervous Child, 2**, pp.217-250.

Kearney, J. A. (2015). Double Trouble: Impairment of Two Interneuron Types in a Dravet Mouse Model. *Epilepsy Currents*, *15*(1), 47-49

Kelsom, C., & Lu, W. (2013). Development and specification of GABAergic cortical interneurons. *Cell & Bioscience*, *3*(1), 19.

Lin, H. C., Gean, P. W., Wang, C. C., Chan, Y. H., & Chen, P. S. (2013). The amygdala excitatory/inhibitory balance in a valproate-induced rat autism model. *PLoS One*, *8*(1), e55248.

Mannion, A., & Leader, G. (2013). Comorbidity in autism spectrum disorder: A literature review. *Research in Autism Spectrum Disorders*.

Marín, O., & Rubenstein, J. L. R. (2003). CELL MIGRATION IN THE FOREBRAIN. *Annual Review of Neuroscience*, *26*(1), 441–483.

Mier, D., Lis, S., Zygrodnik, K., Sauer, C., Ulferts, J., Gallhofer, B., & Kirsch, P. (2014). Evidence for altered amygdala activation in schizophrenia in an adaptive emotion recognition task. *Psychiatry Research - Neuroimaging*,*221*(3), 195–203.)

Moriceau, S., & Sullivan, R. M. (2005). Neurobiology of infant attachment. *Developmental Psychobiology*.

Nelson, S. B., & Valakh, V. (2015). Excitatory/inhibitory balance and circuit homeostasis in autism spectrum disorders. *Neuron*, *87*(4), 684-698

Numis, A. L., Major, P., Montenegro, M. A., Muzykewicz, D. A., Pulsifer, M. B., & Thiele, E. A. (2011). Identification of risk factors for autism spectrum disorders in tuberous sclerosis complex. *Neurology*, *76*(11), 981–987.

Oblak AL, Gibbs TT, Blatt GJ. Decreased GABA(B) receptors in the cingulate cortex and fusiform gyrus in autism. **J Neurochem**. 2010; 114(5):1414–23.

Paxinos, G., & Watson, C. (1997). The Rat Brain in Stereotaxic Coordinates. *Academic Press, San Diego*, *3rd*.

Rajmohan, V., & Mohandas, E. (2007). The limbic system. *Indian Journal of Psychiatry*, *49*(2), 132–9.

Ribeiro, S. H. B. (2007). Prevalência dos transtornos invasivos do desenvolvimento no município de Atibaia: um estudo piloto.

Raghanti, Mary Ann, et al. "A comparative perspective on minicolumns and inhibitory GABAergic interneurons in the neocortex." *Front. Neuroanat* 4.3 (2010).

Rodier, P. M., Ingram, J. L., Tisdale, B., Nelson, S., & Romano, J. (1996). Embryological origin for autism: Developmental anomalies of the cranial nerve motor nuclei. *Journal of Comparative Neurology*, *370*(2), 247–261.

Rubenstein, J. L. R., & Merzenich, M. M. (2003). Model of autism: increased ratio of excitation/inhibition in key neural systems. *Genes, Brain and Behavior*, *2*(5), 255-267.

Schneider, T., & Przewłocki, R. (2005). Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. *Neuropsychopharmacology*, *30*(1), 80–9.

Shin, L. M., & Liberzon, I. (2010). The neurocircuitry of fear, stress, and anxiety disorders. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *35*(1),169–191.

Shinoda, Yo; SADAKATA, Tetsushi; FURUICHI, Teiichi. Animal models of autism spectrum disorder (ASD): a synaptic-level approach to autistic-like behavior in mice. **Experimental animals**

Sohal, V. S., Zhang, F., Yizhar, O., & Deisseroth, K. (2009). Parvalbumin neurons and gamma rhythms enhance cortical circuit performance. *Nature*, *459*(7247), 698–702.

SOUSA, Juliana Alves Brandão Medeiros de. **Caracterização comportamental e distribuição de neurônios inibitórios em um modelo animal de autismo induzido por ácido valpróico**. 2013. Dissertação de Mestrado. Universidade Federal do Rio Grande do Norte.

Speed, Haley E. et al. Autism-associated insertion mutation (InsG) of Shank3 exon 21 causes impaired synaptic transmission and behavioral deficits. **Journal of Neuroscience**, v. 35, n. 26, p. 9648-9665, 2015.

Spence, S. J., & Schneider, M. T. (2009). The role of epilepsy and epileptiform EEGs in autism spectrum disorders. *Pediatric research*, *65*(6), 599-606.

Top, D. N., Stephenson, K. G., Doxey, C. R., Crowley, M. J., Kirwan, C. B., & South, M. (2016). Atypical Amygdala Response to Fear Conditioning in Autism Spectrum Disorder. *Biological Psychiatry: Cognitive Neuroscience and Neuroimaging*, *1*(4), 308–315.

van Steensel, F. J. A., Bögels, S. M., & Perrin, S. (2011). Anxiety Disorders in Children and Adolescents with Autistic Spectrum Disorders: A Meta-Analysis. *Clinical Child and Family Psychology Review.*

Volk, D. W., & Lewis, D. A. (2014). Early developmental disturbances of cortical inhibitory neurons: contribution to cognitive deficits in schizophrenia. *Schizophrenia bulletin*, *40*(5), 952-957.

Volk, D. W., Matsubara, T., Li, S., Sengupta, E. J., Georgiev, D., Minabe, Y., … Lewis, D. A. (2012). Deficits in transcriptional regulators of cortical parvalbumin neurons in schizophrenia. *American Journal of Psychiatry,* *169*(10), 1082–1091.

Willner, P. (1984). The validity of animal models of depression. *Psychopharmacology.*

Xu X, Roby KD, Callaway EM (2010) Immunochemical characterization of inhibitory mouse cortical neurons: three chemically distinct classes of inhibitory cells. **J Comp Neurol** 518:389 – 404. CrossRef Medline

Zalla, T., & Sperduti, M. (2013). The amygdala and the relevance detection theory of autism: an evolutionary perspective. *Frontiers in Human Neuroscience*, *7*(December), 894.

Disponível em: < https://medlineplus.gov/ency/imagepages/19244.htm> Acesso em: 20 de maio de 2017

Disponível em:<https://www.dartmouth.edu/~rswenson/NeuroSci/chapter_9.html>Acesso em: 20 de maio de 2017

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ANEXO A:

Normas de Avaliação do Periódio Neuroscience

NEUROSCIENCE

TABLE OF CONTENTS

DESCRIPTION

Neuroscience publishes papers describing the results of original research on any aspect of the scientific study of the nervous system. Any paper, however short, will be considered for publication provided that it reports significant, new and carefully confirmed findings with full experimental details. IBRO-DEF-02.jpg

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Neuroscientists from all disciplines.

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GUIDE FOR AUTHORS

INTRODUCTION

Neuroscience publishes the results of original research on any aspect of the scientific study of the nervous system. Papers most suitable for publication are those that report new observations that directly contribute to our understanding of how the nervous system works. Any paper, however short, will be considered for publication provided that it reports significant, new and carefully confirmed findings with full experimental details. The Editor-in-Chief, the Associate Editor, and the Section Editors will initially evaluate all submissions. Articles not estimated to represent strong candidates for publication will be returned to the authors without detailed review within 3-5 days. Otherwise, manuscripts will be sent to reviewers for rapid assessment.

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The Chief and Associate Editors seek advice from Section Editors representing all major areas of research: Developmental NeurosciencePain and Sensory NeuroscienceCellular and Molecular Neuroscience Behavioral and Cognitive Neuroscience Disease-Oriented Neuroscience Systems Neuroscience Theory and Innovative Approaches in Neuroscience

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Yuri Bozzi, Center for Mind/Brain Sciences (CIMeC), University of Trento (Mattarello, Italy). *Molecular neuroscience and neuroprotection*.

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Eleanor Coffey, Turku Centre for Biotechnology (Turku, Finland). Molecular neuroscience.

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Artur Kania, Neural Circuit Development Research Unit, Institut de Recherches Cliniques de Montral (Montral, Canada). *Developmental neuroscience, stem cells and repair*.

Marlies Knipper, Hearing Research Centre Tbingen, THRC (Tbingen, Germany). *Sensory neuroscience*.

Rohini Kuner, Interdisciplinary Center of Neurosciences, University of Heidelberg (Heidelberg, Germany). *Pain mechanisms; sensory neuroscience*.

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Maria ngeles Moro, Neurovascular Research Unit, Department of Pharmacology, School of Pharmacy, Universidad Complutense de Madrid (Madrid, Spain). *Disease-oriented neuroscience, neuroprotection, cerebral ischemia*.

Ranulfo Romo, Instituto de Fisiologa Celular, Universidad Nacional Autnoma de Mxico (Mxico DF, Mxico). *Cognitive neuroscience*.

Susan Sesack, University of Pittsburgh , Department of Neuroscience (Pittsburgh, PA, USA). Pharmacology, drugs of abuse, behavior.

Gnther Sperk, Medical University Innsbruck, Department of Pharmacology (Innsbruck, Austria). Systems neuroscience.

Masahiko Takada, Primate Research Institute, Kyoto University (Aichi, Japan). *Behavioral and cognitive neuroscience, primate research, cortical processing*.

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A Special Issue is not a loose collection of topically related articles but a concerted attempt to provide an overview of the status of an emerging field. Cross references between the articles are strongly encouraged.

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Manuscripts are subjected to the review process according to the same high standards of quality as regular issues of Neuroscience. The Guest Editor(s) identify reviewers and take responsibility for the further editorial handling of the manuscripts, supported by the San Diego office. As for regular papers, the final decision on each article is taken by the Chief Editor.

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BEFORE YOU BEGIN

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ANEXO B:

Carta de Aprovação do Comitê de Ética

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