UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS GRADUAÇÃO EM
CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

MODELO ANIMAL DE TRANSTORNO DO ESPECTRO DO AUTISMO

INDUZIDO POR EXPOSIÇÃO PRÉ-NATAL AO ÁCIDO VALPRÓICO:

ESTUDOS COMPORTAMENTAIS, AVALIAÇÕES MOLECULARES E

ESTRATÉGIAS PREVENTIVAS

Victorio Bambini Junior

Porto Alegre, setembro de 2014

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Victorio Bambini Junior

Orientadora: **Carmem Gottfried**

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Dedico aos roedores, que deram a vida

por esse e muitos outros trabalhos

Para criaturas pequenas como nós,

a vastidão só é suportável através do amor.

Carl Sagan

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SUMÁRIO

1. RESUMOS

1.1. Resumo:

O transtorno do espectro do autismo (TEA) representa um grupo de desordens do neurodesenvolvimento, caracterizados por 1) déficits na comunicação e na interação social e 2) comportamentos repetitivos e interesses/atividades restritas. A etiologia do TEA reside em uma interação complexa entre fatores genéticos e fatores de risco ambiental. A exposição pré-natal ao ácido valproico (VPA) tem sido associada com um aumento significativo no diagnóstico de TEA no período pós-natal e, baseado nessas evidências, um modelo animal de autismo foi proposto. Anormalidades neuroquímicas, morfológicas e comportamentais, similares àquelas encontradas em indivíduos com diagnóstico de TEA, têm sido descritas nesse modelo. Ainda, validades preditivas (respostas análogas para tratamentos), de construto (mesma disfunção biológica que origina certa condição em humanos) e de face (características endofenotípicas similares às encontradas no transtorno estudado) estão presentes nesse modelo, garantindo sua eficácia como um método confiável de pesquisa experimental. Considerando os efeitos neuroprotetores, antioxidantes e anti-inflamatórios do resveratrol (RSV), nós investigamos a influência do tratamento pré-natal com RSV nos comportamentos sociais no modelo animal de autismo induzido por exposição pré-natal ao VPA. A administração pré-natal de RSV preveniu as alterações sociais, induzidas pelo VPA, avaliadas nesse estudo. A interação molecular entre o RSV e o VPA é baixa e altamente instável, sugerindo efeitos celulares ao invés de processos químicos independentes. Por fim, os achados da presente tese originaram: (1) um capítulo de livro descrevendo os achados no modelo animal de autismo por exposição pré-natal ao VPA; (2) um artigo onde uma estratégia experimental promissora, utilizando resveratrol (RSV), foi delineada para avaliar alterações no desenvolvimento relacionadas a alterações neurais e comportamentais no TEA; (3) uma patente para o uso dessa estratégia experimental; (4) resultados preliminares sobre os alvos moleculares do RSV e do VPA envolvendo a etiologia do autismo; e (5) um comentário técnico em um artigo de alto impacto ressaltando o conhecimento e domínio adquirido pelo grupo nos estudos dentro do modelo animal de autismo induzido por exposição pré-natal ao VPA.

1.2. Abstract:

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by 1) deficits in social communication and social interaction and 2) restricted repetitive behaviors, interests, and activities (RRBs). Its etiology involves a complex interplay of both genetic and environmental risk factors. Prenatal exposure to valproic acid (VPA) has been associated with significantly increased risks of ASD and, based on this fact, an animal model of ASD was proposed. Neurochemical, morphological, and behavioral abnormalities, similar to those found in individuals with ASD have been described in this model. Construct (the same biological dysfunction that causes the human disorder), face (strong analogies to the endophenotypes of the human disorder), and predictive (analogous response to treatments) validities are also present in this model, ensuring its effectiveness as a trustworthy research tool. Considering the neuroprotective, antioxidant and anti-inflammatory effects of resveratrol (RSV), we investigated the influence of prenatal RSV treatment on social behaviors of in the animal model of ASD induced by prenatal exposure to VPA. Prenatal administration of RSV prevented the VPA-induced social impairments evaluated in this study. The molecular interaction between RSV and VPA is weak and highly unstable, suggesting cellular effects instead of a single chemical process. In summary, the present thesis findings resulted in: (1) a book chapter describing the findings in the VPA animal model of ASD; (2) an article where a promising experimental strategy is design to evaluate developmental alterations implicated in neural and behavioral impairments in ASD; (3) a patent protecting the use of this research strategy; (4) preliminary results about molecular targets of RSV and VPA involved in the etiology of autism; and (5) a technical comment in a high impact article, showing that we are performing our work in the state of the art.

2. LISTA DE ABREVIATURAS

CDC: Centers for Disease Control and preventions

DSM: Diagnostic and Statistical Manual of mental disorders

RSV: Resveratrol

TEA: Transtorno do Espectro do Autismo

TSC: Complexo esclerose tuberosa, do inglês Tuberous Sclerosis Complex

VPA: Ácido valpróico

3. INTRODUÇÃO

3.1 O transtorno do espectro do autismo

O termo autismo, derivado do grego αὐτός (autos), que significa sí próprio, acrescido do sufixo -ismo (que indica orientação ou estado), foi utilizado pela primeira vez em 1911, por Paul Eugen Bleuler (E. Bleuler, 1911; R. Kuhn, 2004), como uma das características necessárias para diagnosticar pessoas com esquizofrenia. Em 1943, em Baltimore nos Estados Unidos da América, Leo Kanner, no paper "Autistic disturbances of affective contact", utilizou o termo para definir um grupo de onze crianças que tinham uma diminuída capacidade de estabelecer contato interpessoal e afetivo (L. Kanner, 1943).

No mesmo, ano Hans Asperger, em Viena na Áustria, submeteu sua tese de doutorado "Die "Autistischen Psychopathen" im Kindesalter" (Psicopatia autista na infância) descrevendo 4 crianças com "psicopatia autista", tendo sido publicada em 1944 (H. Asperger, 1944). Entretanto, em 1938, em uma palestra no Hospital Universitário de Viena, Hans Asperger já havia descrito as características de "autistas psicopatas" baseado em seus estudos de caso (H. Asperger, 1938).

Desde então, diversas tentativas de categorizar adequadamente as pessoas com Transtorno do Espectro do Autismo (TEA; do inglês, autism spectrum disorder; ASD) foram realizadas (A. P. A.- APA, 1994; A. P. A. APA, 2000). Atualmente, o TEA enquadra-se entre os Transtornos do Neurodesenvolvimento. Tipicamente, esses transtornos se manifestam durante a primeira infância e os déficits do desenvolvimento resultam em alterações em habilidades sociais, acadêmicas, pessoais e ocupacionais (A. P. A.- APA, 2013).

O TEA agrupa quatro transtornos anteriormente separados em uma única condição com diferentes níveis de severidade de sintomas (A. P. A.- APA, 2013). Os transtornos agora reunidos no TEA são: os transtornos autistas (autismo), a síndrome de Asperger, o transtorno desintegrativo da infância e os transtornos invasivos do desenvolvimento não-especificados (A. P. A. APA, 2000; A. P. A.- APA, 1994).

Outra mudança significativa descrita no DSM-5 é que a caracterização do espectro anteriormente dividida em três áreas (prejuízo na sociabilidade, na comunicação e com presença de estereotipias e comportamentos repetitivos), passa a ser agrupada em dois domínios comportamentais:

 1) Prejuízo na comunicação e interação social em múltiplos contextos, incluindo déficits em reciprocidade social, comunicação não-verbal utilizada para interação social e em habilidades para iniciar, manter e entender relacionamentos;

2) Comportamentos repetitivos, atividades e interesses restritos.

Ambas as características comportamentais devem estar presentes para o diagnóstico de TEA. Caso comportamentos repetitivos, interesses e atividades restritos não estejam presentes, deve-se considerar o diagnóstico de transtorno de comunicação social (A. P. A.- APA, 2013). Além disso, essas características variam em intensidade, mas são presentes ao longo de toda vida (I. Rapin and R. F. Tuchman, 2008). Uma vez que a apresentação dos sintomas pode mudar com o desenvolvimento ou ser mascarado por mecanismos compensatórios, o histórico do paciente deve ser levado em consideração. Entretanto, a situação atual do paciente deve ser condizente com as características do transtorno (J.

Miller, J. Pandey, and L. Berry, 2014). Somado a atual ausência de marcadores clínicos específicos para o TEA, fica claro que o diagnóstico é quase que exclusivamente clínico (S. D. Mayes et al., 2014).

Apesar de haver um padrão estrito para o diagnóstico, o TEA é uma desordem altamente heterogênea e multifatorial (C. Gottfried and V. Bambini-Junior, 2013). O transtorno pode estar presente em pessoas com manifestações tão distintas que muito raramente dois indivíduos compartilham o mesmo conjunto de sintomas (C. A. Gadia, R. Tuchman, and N. T. Rotta, 2004; I. Rapin and R. F. Tuchman, 2008).

Existem alguns sintomas associados ao TEA, como padrões alterados de contato visual (M. Freeth, T. Foulsham, and A. Kingstone, 2013), déficit gestual (M. Tincani, 2004), atrasos de linguagem, inabilidade para interpretar emoções a partir de expressões faciais (L. Sepeta, N. Tsuchiya, and M. Davies, 2012), anormalidades no processamento de estímulos sensoriais (S. Rogers and S. Ozonoff, 2005), movimentos manuais estereotipados, dificuldade para mudanças em rotinas (M. F. Casanova, 2007; D. H. Geschwind, 2009; I. Rapin and R. F. Tuchman, 2008). Características adicionais, associadas em alguns casos incluem alterações do sistema imunológico (C. Onore, M. Careaga, and P. Ashwood, 2012), retardo mental (J. Matson and M. Shoemaker, 2009), ansiedade (S. White et al., 2009), distúrbios do sono (B. Sivertsen, M. Posserud, and C. Gillberg, 2012) e gastrointestinais (T. Buie et al., 2010), além de maior circunferência craniana e volume cerebral quando jovens (J. R. Skoyles, 2008; M. F. Casanova, 2007; I. Rapin and R. F. Tuchman, 2008).

O TEA está presente ao nascimento, todavia usualmente não é diagnosticado até os dois ou três anos de idade (A. Cox, K. Klein, and T.

Charman, 1999). Apesar de existirem sintomas que não podem ser notados nesta idade, como coordenação motora reduzida, muitos pais percebem problemas no progresso social ou comunicativo das crianças. De fato, os sintomas podem ser completamente manifestados apenas quando as demandas sociais excedem a capacidade limitada da criança. Dessa forma, os déficits sociais não são propriamente claros na infância, porém gradualmente se tornam mais evidentes (C. J. Dover and A. Le Couteur, 2007). Além disso, são comumente relatados casos em que a criança apresentava um desenvolvimento típico e então regride em habilidades sociais e de comunicação (R. Luyster, J. Richler, and S. Risi, 2005).

3.2 Epidemiologia

Em 2014, o CDC (Centers for Disease Control and Preventions) forneceu dados a respeito da incidência de autismo nos Estados Unidos da América, referentes ao levantamento feito no ano de 2010. De acordo com esses dados, uma a cada 68 crianças (aproximadamente 1,5%) é identificada com TEA, sendo cinco vezes mais comum entre meninos (1 em 42) do que em meninas (1 em 189) (*Morbidity and Mortality Weekly Report. Surveillance Summaries (Washington, D.C.: 2002)* 2014). Em levantamento realizado em 2011, a incidência de TEA na Coréia do Sul era de 2,64% em crianças de sete até 12 anos de idade (Y. S. Kim et al., 2011).

No Brasil, dados referentes à incidência de autismo são escassos, e um único estudo piloto, realizado na cidade de Atibaia, analisou 1470 crianças em idade escolar (até 15 anos) e encontrou uma prevalência de aproximadamente

0,3% (C. S. Paula et al., 2011). De fato, países em desenvolvimento apresentam menores taxas de prevalência de TEA (I. D. C. van Balkom et al., 2009). Problemas metodológicos como amostras pequenas, pouca conscientização a respeito do TEA por parte da população e dos profissionais locais, falta de registros adequados e diagnósticos equivocados são hipóteses para explicar essas baixas prevalências (*Morbidity and Mortality Weekly Report. Surveillance Summaries (Washington, D.C. : 2002)* 2014).

O TEA ocorre em todos os grupos étnicos e socioeconômicos (*Morbidity and Mortality Weekly Report. Surveillance Summaries (Washington, D.C. : 2002)* 2014). Apesar da bem estabelecida predominância de TEA no sexo masculino (ocorrência entre quatro ou cinco vezes maior que no sexo feminino), a influência de fatores culturais, étnicos, geográficos e socioeconômicos na incidência de TEA permanece obscura (E. Fombonne, 2009).

Nos últimos anos foi observado um aumento significativo dos diagnósticos de TEA. Por exemplo, entre os anos de 1991 e 1997 a incidência teve um aumento de 556%, passando a afetar mais crianças do que, por exemplo, câncer e síndrome de Down (R. Muhle, S. V Trentacoste, and I. Rapin, 2004). Entre os anos 2000 e 2010 a prevalência nos Estados Unidos da América mais que dobrou, passando de 0,67% (*Morbidity and Mortality Weekly Report. Surveillance Summaries (Washington, D.C. : 2002)* 2007) para 1,47% (*Morbidity and Mortality Weekly Report. Surveillance Summaries (Washington, D.C. : 2002)* 2014).

O timerosal, um conservante utilizado em algumas vacinas, contém mercúrio na sua formulação e foi cogitado como um fator de risco para o autismo (L. Wing and D. Potter, 2002). Porém, estudos recentes do centro de controle e prevenção de doenças (Centers for Disease Control and Prevention – CDC)

indicam que este não seria um fator de risco para o aumento da incidência de autismo (E. Fombonne, 2009). Apesar de conjecturas sobre a possibilidade de os dados publicados pelo CDC estarem equivocados, um novo artigo científico reforça a posição de que o mercúrio não seria um fator de risco (V. M. Yau et al., 2014). Todavia, a organização americana Autism Speaks recomenda mais estudos para que essa hipótese seja realmente descartada.

Segundo Fombonne, o aumento na prevalência do autismo se deve, pelo menos em parte, às mudanças nos critérios de diagnóstico, o que fez com que houvesse uma "migração" de indivíduos com outros transtornos para o TEA, porém esse fato não explicaria completamente o aumento (E. Fombonne, 2009). Além disso, estudos epidemiológicos e com modelos animais indicam, indubitavelmente, que fatores ambientais podem ser responsáveis pelos aumentos na incidência de autismo (E. Fombonne, 2003; T. Schneider and R. Przewłocki, 2005).

Estima-se um custo adicional de pelo menos US$ 17.000 por ano no cuidado de uma criança com TEA quando comparado com uma criança típica (T. A. Lavelle et al., 2014) e o custo total médio de US$ 1.600.000 por individuo com TEA durante a vida (M. D. M. Kogan et al., 2009). Para as famílias, aproximadamente 90% do custo anual de uma criança com TEA vem do menor salário recebido pelos pais (em função, por exemplo, da maior necessidade de auxílio à criança) (C. Horlin et al., 2014). O custo social estimado para os cuidados com crianças com TEA nos Estados Unidos da América foram de mais de US$ 9 bilhões em 2011 (T. A. Lavelle et al., 2014).

É importante ressaltar o impacto emocional que o TEA ocasiona a pessoa com autismo e seus familiares. A falta de marcadores clínicos para o diagnóstico,

aliada a escassez de abordagens terapêuticas adequadas fazem com que organizações não-governamentais e fundações de apoio (mantidas por maciço esforço de voluntários e pais) tenham papel fundamental na divulgação, tratamento e pesquisa do TEA. Instituições americanas como Autism Speaks (www.autismspeaks.org/), Simons Fundation (sfari.org/), Autism Society (www.autism-society.org/) e brasileiras como o Instituto Autismo e Vida (www.autismoevida.org.br) e Autismo e Realidade (autismoerealidade.org/) realizam um formidável trabalho de apoio as pessoas com autismo, seus familiares e as pesquisas sobre TEA.

Devido à defasagem do conhecimento sobre a etiologia do TEA e os motivos de sua crescente prevalência, existe um esforço mundial para o estudo dessa desordem, resultando em aumento considerável da produção científica na área. A figura 1 mostra o número de publicações/ano relacionados ao autismo nos últimos 70 anos.

**Figura 1. Número de artigos, por ano, sobre o tema autismo.**
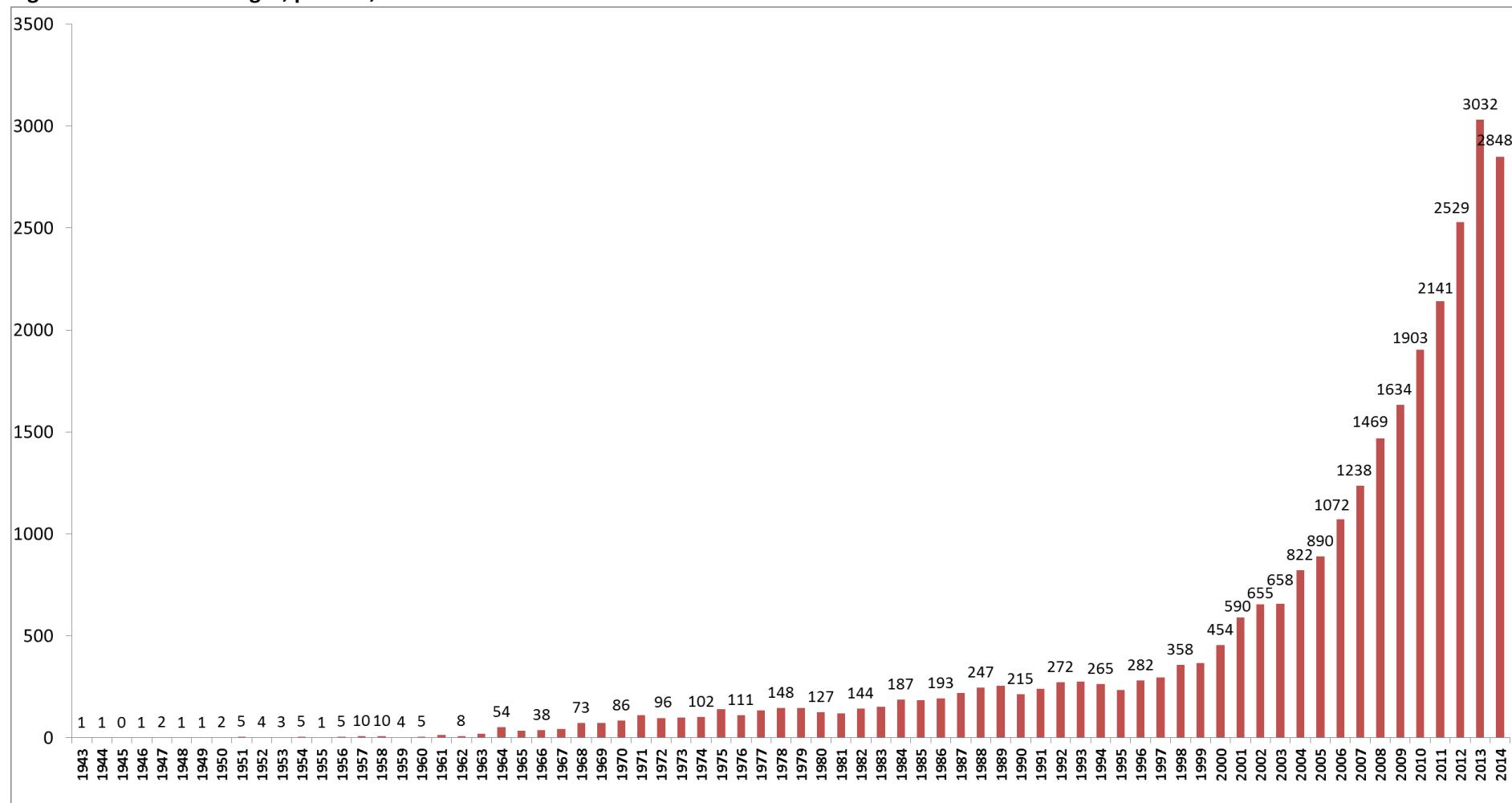
Fig 1: Levantamento do número de artigos, por ano, na plataforma PUBMED utilizando os termos "autism" ou "autistic".

3.3 O desencadeamento do TEA

O autismo apresenta um forte componente genético. Estudo com gêmeos apresenta concordância de 60% no autismo clássico em monozigóticos contra 0% em dizigóticos (R. Muhle, S. V Trentacoste, and I. Rapin, 2004). A alta concordância em monozigóticos é um indicativo de que a herança genética é um fator determinante no desencadeamento do autismo. A reavaliação considerando características mais abrangentes, como déficits em comunicação e alterações sociais, fez essa concordância ir de 60% para 92% em monozigóticos e de 0 para 10% em dizigóticos (M. F. Casanova, 2006). Esses dados sugerem que interações genéticas múltiplas podem ser causadoras do autismo, porém, fatores ambientais podem contribuir para uma maior expressão dos sintomas (R. Muhle, S. V Trentacoste, and I. Rapin, 2004).

Algumas condições genéticas, como Síndrome do X-frágil e Esclerose Tuberosa, tem alta correlação com TEA (W. Brown, E. Jenkins, and E. Friedman, 1982; J. Turk and P. Graham, 1997; M. Belmonte and T. Bourgeron, 2006; A. L. Numis et al., 2011; D. M. Feliciano et al., 2013). A idade avançada dos genitores, independentemente do sexo, parece estar envolvida no desencadeamento do autismo (M. S. Durkin et al., 2008). Porém, fatores como, baixa fertilidade e mudança do estilo de vida em pessoas com mais de 40 anos, são possíveis explicações para esse achado (J. Olsen and J. L. Zhu, 2009).

Outros componentes intimamente envolvidos com o desencadeamento do TEA são as alterações no sistema imunológico durante a gestação (P. Patterson, 2009; P Ashwood, 2006). Evidências epidemiológicas relacionam ativação do sistema imunológico materno durante a gestação e alterações genéticas relacionadas ao sistema imunológico, com o desenvolvimento de TEA pela prole

(H. Atladóttir and P. Thorsen, 2010; E. Korvatska et al., 2002; L. Croen and J. Grether, 2005). Esses achados são corroborados por estudos envolvendo modelos animais (N. Malkova, C. Yu, and E. Hsiao, 2012; M. Bauman, A. Iosif, and S. Smith, 2014) e, juntamente com as alterações imunológicass encontradas em pessoas com TEA (P Ashwood and P. Krakowiak, 2011), ajudam a estabelecer a natureza neuroimune desse transtorno.

Além disso, observações epidemiológicas sugeriram que teratógenos poderiam estar relacionados com o desencadeamento do autismo, destacando-se o ácido valproico (VPA) (F. Roullet, J. Lai, and J. Foster, 2012; V. Smith and N. Brown, 2014) e a talidomida (K. Imai, T. Iida, and M. Yamamoto, 2014). Estudos demonstraram que 30% dos indivíduos expostos à talidomida durante período embrionário (em especial, entre o 20° e o 24° dia de gestação) desenvolveram TEA (M. T. Miller and K. Strömland, 1999). Entretanto, ao contrário do VPA, a talidomida apresenta diferentes efeitos em primatas e em roedores, sendo que em primatas pode gerar entre outros, crescimento aberrante e deficiente dos membros. Já o ácido valproico, induz alterações similares em roedores e seres humanos (C. Gottfried and V. Bambini-Junior, 2013).

Essas observações auxiliaram o estabelecimento do modelo animal para o estudo de autismo, induzidos farmacologicamente pela exposição pré-natal ao ácido valproico, sugerido pela primeira vez em 1997 por Rodier (P. M. Rodier et al., 1997).

3.4 Modelos animais de TEA

Estudos com modelos animais fornecem grandes possibilidades de investigações que não são possíveis em humanos, incluindo análises imunológicas e do desenvolvimento, plasticidade e sinalização neural. Devido à alta prevalência do autismo e seu caráter heterogêneo, um modelo animal torna-se um método de valor inestimável no estudo do TEA. Entretanto, o autismo é uma condição particularmente difícil de ser mimetizada por modelos animais. Por exemplo: a comunicação em roedores não pode ser diretamente avaliada e estes também não possuem regiões cerebrais diretamente comparáveis com aquelas que medeiam a linguagem humana, habilidade essa extremamente relevante ao estudo do autismo. Por outro lado, os roedores em geral apresentam altos índices de interação e comunicação social (J. L. Silverman et al., 2010; J. Crawley, 2004). Essa característica faz com que mudanças no comportamento social de roedores sejam relacionadas a fatores ambientais ou genéticos (J. N. Crawley, 2003). Dentre os modelos animais em roedores destacam-se os induzidos: (1) geneticamente, (2) por lesão, (3) por ativação imunológica da mãe durante a gestação e (4) farmacologicamente (J. Blundell et al., 2009; C. L. Murcia, F. Gulden, and K. Herrup, 2003; P. M. Rodier et al., 1997).

1) Algumas alterações gênicas conhecidas por aumentarem a incidência de TEA, quando reproduzidas em roedores, tendem a mimetizar alguns comportamentos do tipo-autista. Por exemplo, aproximadamente 50% das pessoas com esclerose tuberosa, uma condição relacionada com a perda de função das proteínas TSC1 ou TSC2, enquadram-se no espectro do autismo (A. L. Numis et al., 2011). Roedores com mutações similares às encontradas em humanos com

esclerose tuberosa apresentam alterações do tipo-autista nos padrões de comportamento social (S. M. I. Goorden et al., 2007). Além disso, linhagens de camundongo como BTBR e BALB/c, apresentam validade de face com comportamentos característicos dos TEA (J. L. Silverman et al., 2010).

2) Os modelos induzidos por lesão correspondem a uma parte muito pequena da etiologia do autismo e são mais especificamente utilizados para estudo de fármacos que revertam ou atenuem as condições comportamentais alteradas desses roedores. Os locais de lesões incluem cerebelo, hipocampo e amígdala (E. Courchesne et al., 1995; J. Piven and S. Arndt, 1995). Porém a baixa reprodutibilidade das lesões faz com que cada modelo tenha caráter quase exclusivo, uma vez que incisões minúsculas podem representar alterações significativas no comportamento animal.

3) Os modelos animais de TEA por ativação imunológica materna durante a gestação representam uma abordagem promissora em relação a gatilhos etiológicos do TEA (P. Patterson, 2011; K. Racicot et al., 2014). Em modelos animais, diversas evidências apontam para um papel crucial do sistema imunológico materno no desencadeamento de características do tipo-autista na prole (D. B. Oskvig et al., 2012; N. Malkova, C. Yu, and E. Hsiao, 2012; M. Bauman, A. Iosif, and S. Smith, 2014), ressaltando a importância da interação neuroimune na etiologia do TEA.

4) Quanto à indução química, alguns agentes teratogênicos são tidos como fatores de risco, e dentre eles destacam-se a talidomida e

o ácido valproico (VPA)(J. Ingram and S. Peckham, 2000). O modelo animal baseado na exposição pré-natal ao VPA vem sendo amplamente utilizado para o estudo de autismo (Bambini-Junior et al., 2011; Schneider & Przewłocki, 2005). Esse modelo animal apresenta semelhança real com estudos clínicos, que mostram correlação deste fármaco com o surgimento de TEA (V. Smith and N. Brown, 2014) e foi o modelo eleito para trabalho em nosso laboratório.

3.4.1 O modelo animal de TEA induzido pela exposição pré-natal ao VPA.

Além do gatilho etiológico, inúmeras alterações comportamentais e neuromorfológicas comuns ao roedor induzido por VPA e à pessoa com autismo já foram demonstradas. Por exemplo, o sistema serotoninérgico é comumente afetado em crianças com TEA (G. M. Anderson et al., 1990). Da mesma forma, em ratos a indução por VPA tem um efeito irreversível na migração e maturação dos neurônios 5-HT positivos (K. Miyazaki, N. Narita, and M. Narita, 2005)

No cerebelo, mudanças na forma, no número e no volume das células de Purkinje ocorrem tanto em pessoas com transtornos invasivos do desenvolvimento como no presente modelo animal (P. M. Rodier et al., 1997). O roedor induzido apresenta também, déficits comportamentais correspondentes aos observados em pacientes, fato esse de extrema importância, visto que o diagnóstico de autismo é dado através da análise comportamental. Alterações no ciclo sono-vigília e diminuição nas interações sociais e nas respostas

emocionais são características predominantes no autismo e foram também relatados no modelo animal (C. L. Yochum et al., 2008; N. Tsujino et al., 2007).

Mudanças na concentração de serotonina são observadas igualmente em pacientes autistas e no modelo animal. Tsujino e colaboradores demonstraram através de microdiálise, que no córtex frontal de ratos induzidos, os níveis de 5-HT estão cronicamente elevados (N. Tsujino et al., 2007). Desse modo fica claro que, juntamente com comportamentos aberrantes e com a neuromorfologia do SNC, a serotonina tem sua função alterada pela injeção de VPA. Porém, o mecanismo exato que desencadeia esses fenômenos no SNC não é conhecido nem no modelo animal nem em pessoas com autismo.

O VPA exerce diferentes efeito na arborização dendrítica. O tratamento crônico com VPA é associado com redução no número e na arborização dendrítica em CA1 (C. Sgobio et al., 2010). A exposição pré-natal ao VPA também altera a morfologia e a densidade de espinhos dendríticos. No modelo animal de TEA é relatado: Diminuição na arborização dos dendritos em córtex pré-frontal medial, córtex orbitofrontal, cerebelo e no hipocampo ventral e dorsal (R. Mychasiuk et al., 2012; M. E. Bringas et al., 2013); aumento na arborização dos dendritos no núcleo accumbens, na camada 3 do cortéx pré-frontal e na amigdala basolateral (M. E. Bringas et al., 2013); diminuição na densidade de espinhos em córtex orbitofrontal, cerebelo, córtex pré-frontal medial, hipocampo dorsal e amigdala basolateral (R. Mychasiuk et al., 2012; M. E. Bringas et al., 2013), e; aumento na densidade de espinhos em núcleo accumbens e em hipocampo ventral (M. E. Bringas et al., 2013).

Além desses, um grande número de estudos relata o impacto da exposição pré-natal ao VPA em roedores. Entre os efeitos observados estão:

retardado no crescimento (massa corporal) e do desenvolvimento (abertura dos olhos) dos ratos VPA quando comparado ao controle (T. Schneider and R. Przewłocki, 2005), déficit na discriminação olfativa (T. Schneider and R. Przewłocki, 2005; T. Schneider, J. Turczak, and R. Przewłocki, 2006), menor reação à dor (T. Schneider et al., 2008), atividade exploratória aumentada em campo aberto (T. Schneider et al., 2008; N. Tsujino et al., 2007), aumento de padrões comportamentais estereotipados (T. Schneider et al., 2008) e do tipo ansioso (T. Schneider et al., 2008; K. Markram et al., 2008), rigidez comportamental (Bambini-Junior et al., 2011), aumento da memória de tarefas aversivas (K. Markram et al., 2008) e déficits sociais (Bambini-Junior et al., 2011; Markram et al., 2008; Schneider & Przewłocki, 2005).

Nosso grupo de pesquisa investiga o modelo animal de autismo induzido por VPA desde 2008. Devido à experiência acumulada e os artigos publicados na área (Bambini-Junior et al., 2011; Bristot Silvestrin et al., 2013), fomos convidados a escrever um capítulo no livro "Comprehensive Guide to Autism" sobre a relação da exposição pré-natal ao valproato em roedores e autismo, (V. Patel, V. Preedy, and C. Martin, 2014). Uma vez que os critérios para diagnóstico do TEA são clínicos e resultam de análises comportamentais, atualmente, é impossível estudar esse transtorno em humanos antes da manifestação dos sintomas. Devido as suas peculiaridades, os modelos animais fornecem a oportunidade de análise de alterações do desenvolvimento que podem desencadear as características do TEA (S. Kataoka et al., 2013; M. Favre and T. Barkat, 2013). Dessa forma, surge a possibilidade de estudo e manipulação de vias para compreensão e, até mesmo, prevenção do surgimento das alterações comportamentais típicas do TEA. Uma das principais metas do grupo é a

modulação de gatilhos etiológicos por moléculas com propriedades antiinflamatórias e antioxidantes como tentativa de prevenir ou atenuar o efeito do VPA durante a indução do modelo de autismo. Uma das moléculas neuroprotetoras que o grupo investiga há bastante tempo é o polifenol resveratrol, por suas importantes propriedades moleculares e biológicas.

3.5 Resveratrol

O resveratrol (3,4',5-triidroxi-*trans*-estilbeno; RSV), é um polifenol encontrado naturalmente em frutas e vegetais, isolado pela primeira vez em 1940 das raízes de *Veratrum grandiflorum.* Sua presença em uvas, pinhas e amendoins é relacionada à proteção da planta contra infecções fúngicas e radiações UV (O. Vang et al., 2011; J. Baur and D. Sinclair, 2006). A figura 2 ilustra o estado de protonação, geometria e densidade eletrônica do VPA e do RSV**.**

Nas últimas duas décadas, o papel terapêutico do RSV tem sido evidenciado em diversas enfermidades como câncer, diabetes e doenças cardiovasculares (O. Vang et al., 2011; Y. Shukla and R. Singh, 2011; D. Delmas, B. Jannin, and N. Latruffe, 2005; L. Kang et al., 2010). Entre os diversos alvos moleculares relacionados aos efeitos do RSV, destacam-se as sirtuínas (B. Jian et al., 2014) e a cascatas de sinalização intracelular mTOR (A. L. Widlund, J. A. Baur, and O. Vang, 2013).

Além disso, as propriedades neuroprotetoras e anti-inflamatórias do RSV (O. Vang et al., 2011; J. Chen, I. Alberts, and X. Li, 2014), são relacionadas com conhecidos gatilhos etiológicos do autismo, como alterações de padrões de

migração neuronal e ativação imunológica (B. Gesundheit et al., 2013; M. Bauman, A. Iosif, and S. Smith, 2014; K. Miyazaki, N. Narita, and M. Narita, 2005). Dessa forma, a partir da hipótese de que fatores ambientais possam estar causando modificações no desenvolvimento do sistema neuroimune, torna-se muito importante o estudo de moléculas que apresentem propriedades que possam contrapor estes efeitos.

Figura 2. Estado de protonação, geometria e densidade eletrônica.
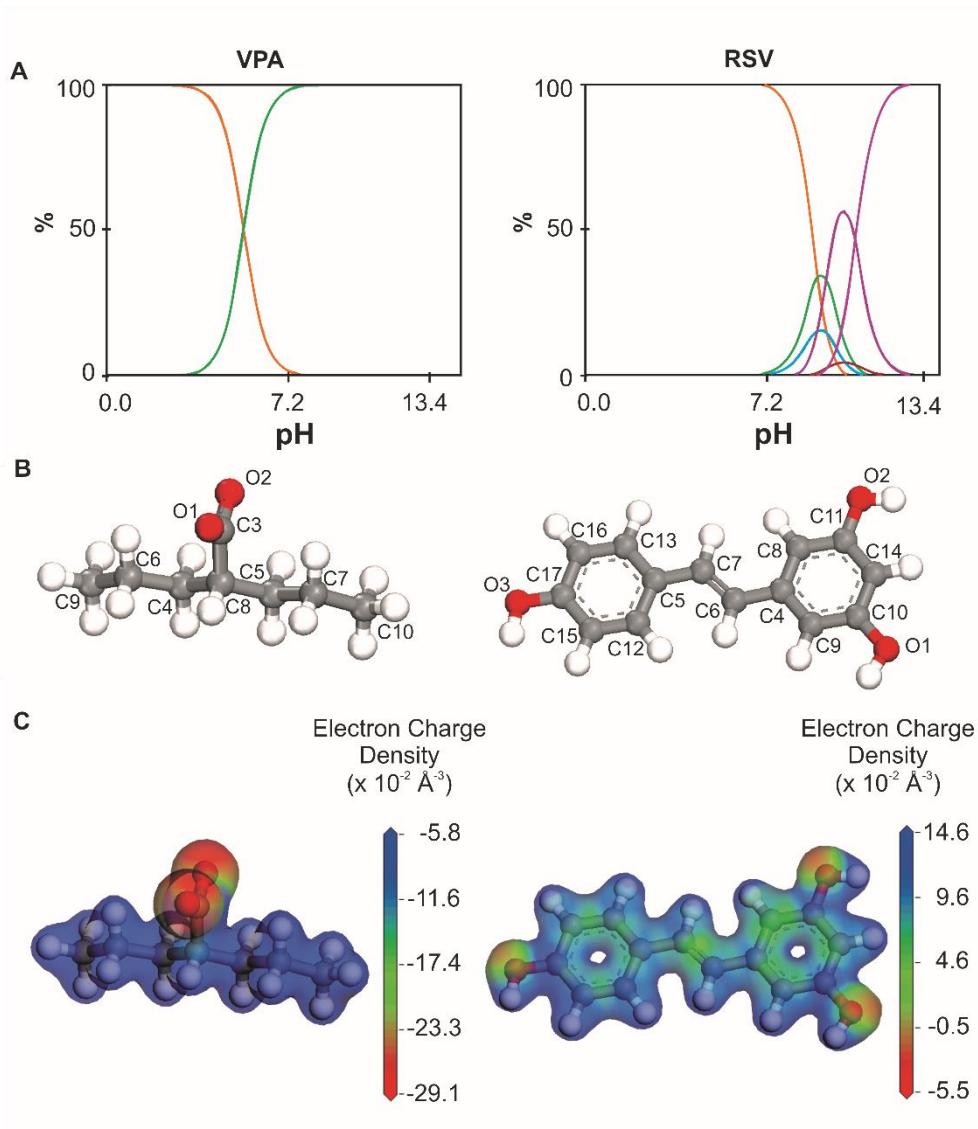
Legenda Fig. 2: Como apresentado na figura 2A, VPA perde o átomo de hidrogênio do grupo carboxil aproximadamente no pH 7,2, ficando com carga molecular -1. No segmento 2B as estruturas otimizadas, no vácuo, de VPA e RSV. A figura 2C ilustra a analise de densidade eletrônica, mostrando cargas negativas no grupamento carboxil do VPA e nos grupos hidroxil do RSV.

4. OBJETIVOS

4.1. Objetivo geral

Analisar parâmetros comportamentais e possíveis alvos etiológicos em modelo animal de TEA induzido por exposição pré-natal ao ácido valpróico (VPA), bem como o possível efeito protetor do polifenol resveratrol (RSV).

4.2. Objetivos específicos:

1- Realizar um amplo levantamento sobre os achados no modelo animal de TEA induzido pela exposição pré-natal ao VPA, para contribuir de forma sistemática com a revisão do conhecimento científico disponível a cerca deste modelo e detectar as áreas com maior potencial para investigação cientifica.

2- Analisar o possível efeito protetor do tratamento pré-natal com RSV no grupo VPA através de avaliação comportamental.

3- Realizar proteção da ideia relacionada ao uso de polifenóis como promotores de prevenção de sintomas do tipo autista no modelo do VPA, com fins de garantir a continuidade dos estudos.

4- A partir dos resultados indicando efeito protetor do polifenol RSV no grupo VPA, decidiu-se:

4.1 Avaliar expressão gênica de membros da família de sirtuinas, importantes alvos deste polifenol, nos grupos controle e VPA, com e sem tratamento pré-natal com RSV

4.2. Avaliar função mitocondrial e estresse oxidativo em hipocampo e cerebelo, inicialmente nos grupos controle e VPA e a partir dos dados obtidos, avaliar a possibilidade de ampliar os estudos para os grupos tratados pré-natal com RSV, bem como em outras regiões encefálicas.

5. CAPÍTULOS

5.1. Capítulo de livro "PRENATAL EXPOSURE TO VALPROATE IN ANIMALS AND AUTISM".

5.1.1. Status

Publicado no livro "Comprehensive Guide to Autism, Springer, 2014, pp 1779-1793"

5.1.2. Publicação

Prenatal Exposure to Valproate in Animals and Autism

Victorio Bambini-Junior, Diego Baronio, Janine MacKenzie,
Geancarlo Zanatta, Rudimar dos Santos Riesgo, and
Carmem Gottfried

Introduction

Scientific research clearly shows that environmental factors play a primary role in the development of *Autism Spectrum Disorders (ASD)* (Fombonne 2009). Prenatal exposure to several factors such as infections, alcohol, thalidomide and valproic acid (VPA) may predispose that child to developing *ASD* features (Dufour-Rainfray 2011). The following overview emphasizes the main findings obtained using an animal model induced by prenatal exposure to VPA, and highlights the next challenges in ASD research.

V. Bambini-Junior (✉)
Departamento de Bioquimica, Universidade Federal do Rio Grande do sul, Instituto de Ciencias
Basicas da Saude, Porto Alegre, RS, Brazil
e-mail: victoriobambini@gmail.com

D. Baronio
Programa de Pós-Graduação em Saúde da Criança e do Adolescente, Universidade Federal do Rio
Grande do Sul, Faculdade de Medicina, Porto Alegre, RS, Brazil
e-mail: diego@baronio.com.br

J. MacKenzie
Department of Phsychology, University of Manitoba, BC, Canada
e-mail: janine.m.mackenzie@gmail.com

G. Zanatta • C. Gottfried
Departamento de Bioquimica, Universidade Federal do Rio Grande do Sul, Instituto de Ciencias
Basicas da Saude, Porto Alegre, RS, Brazil
e-mail: geancarlo.zanatta@gmail.com; cgottfried@ufrgs.br

R.d.S. Riesgo
Unidade de Neuropediatria, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil
e-mail: rriesgo@hcpa.ufrgs.br

Valproic Acid

Valproic acid (Fig. 1) is a simple eight-carbon branched-chain fatty acid, which is dissociated in a physiological environment (pH 7.4) (Fig. 2) and is used medicinally as an anticonvulsant and mood-stabilizing agent for the treatments of epilepsy and bipolar disorder (Bowden and Singh 2005). Valproic acid was first synthesized by B.S. Burton in 1882, who was studying organic solvents (Burton 1882).

In 1963, VPA was found to be an efficient seizures inhibitor (Meunier et al. 1963) and in 1966, it was described as effective for treatment of bipolar disorder (Lambert et al. 1966). Depending on the severity of the patient's condition, the daily dose for VPA ranges between 200 mg and 3,000 mg (Perucca 2002). After intravenous administration, VPA rapidly crosses the blood brain barrier reaching a plateau value after 10–15 min (Lücke et al. 1994).

Research has demonstrated that VPA increasesbrain concentrations of gamma-aminobutyric acid *(GABA)* by decreasing degradation, and increasing synthesis, thereby stimulating its inhibitory activity in some specific brain regions. Furthermore, the effect of VPA on neuronal excitation mediated by the N-methyl-D-aspartate *(NMDA)* subtype of glutamate receptors might be important for its anticonvulsant effects (Johannessen 2000). In addition, VPA has physiological activity as an inhibitor of histone deacetylase *(HDAC)* (Phiel et al. 2001). *Histone proteins* are rich in positive charges that bind to the negative charges of DNA, keeping it wrapped and thus decreasing *gene transcription* (Williamson and Pinto 2012). Acetylation of the lysine residues at the N-terminus of histone proteins removes the positive charges, thereby reducing the affinity between histones and DNA. This allows *RNA polymerase* and transcription factors to access the promoter region. Once HDAC is active, it decreases the transcription of certain genes. As VPA diminishes HDAC activity, it promotes altered gene transcription (Fig. 3), followed by distinct protein expression through the autism spectrum.

Developing the Concept of an Animal Model of Autism

In 1984, fetal *valproate* syndrome (FVS) was proposed after evaluating seven children exposed to VPA *in utero* who presented craniofacial anomalies, such as midface hypoplasia and epicanthal folds (DiLiberti et al. 1984). Exposure to VPA during the first *trimester* of pregnancy was found to be associated with significantly increased risks of several congenital malformations, such as spina bifida, *atrial septal defect*, *cleft palate* and *polydactyly* (Jentink et al. 2010). Demonstration of a link between autism and VPA began with *case reports* of patients with FVS demonstrating autism-like behavior (Table 1). One of the case reports described a 5 year old boy who presented with several anatomical alterations, such as a small midface, mild *micrognathia*, and clinodactyly. His speech development was delayed and his language consisted of a few single words. He exhibited *echolalia*, used *gestures* to communicate, did not interact with other children, and preferred to
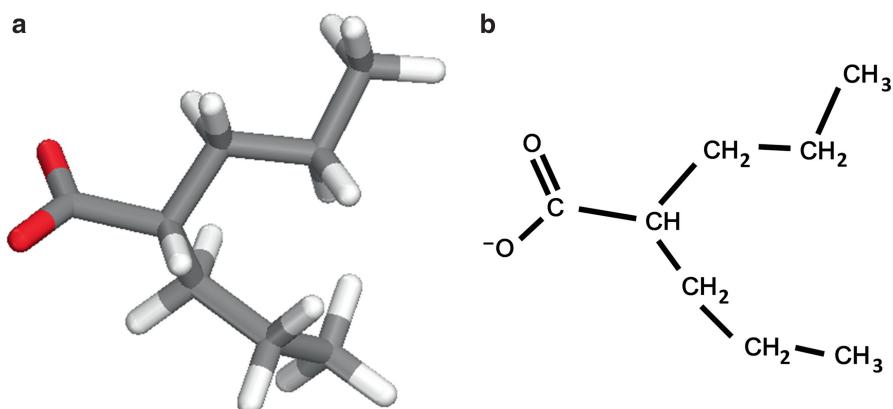
Fig. 1 Valproic acid structure. (**a**) Structural representation of VPA. Oxygen atoms are represented by *red sticks*. Carbon and carbon-carbon bonds are represented by *grey sticks*. Hydrogens are represented by *white sticks*. (**b**) Chemical and structural representation of VPA
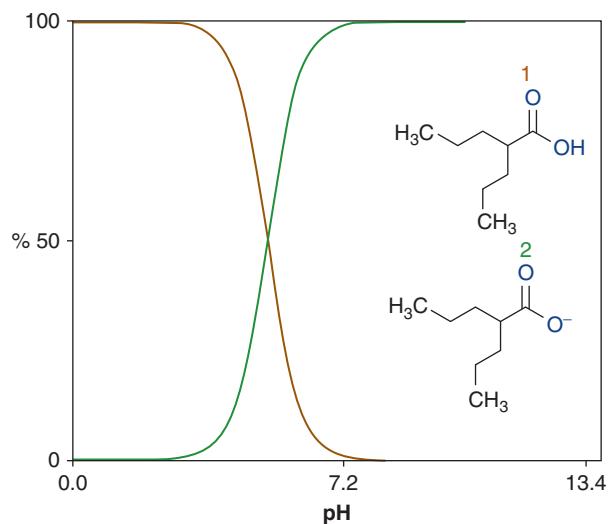
Fig. 2 Valproic acid response to pH variation. Different concentration of VPA, dissociated or not, depending on the pH

play alone. The boy's mother began taking VPA 2–3 years before pregnancy, and kept using it (500 mg – four times per day) until the end of the fifth month of pregnancy (Williams and Hersh 1997). After observing the consequences of prenatal exposure to VPA, a relationship was proposed between exposure and the development of autism (Table 2).

In this context, examination of the triggers of autism has been used to elucidate the role of VPA in the etiology of autism. Therefore, an animal model of autism
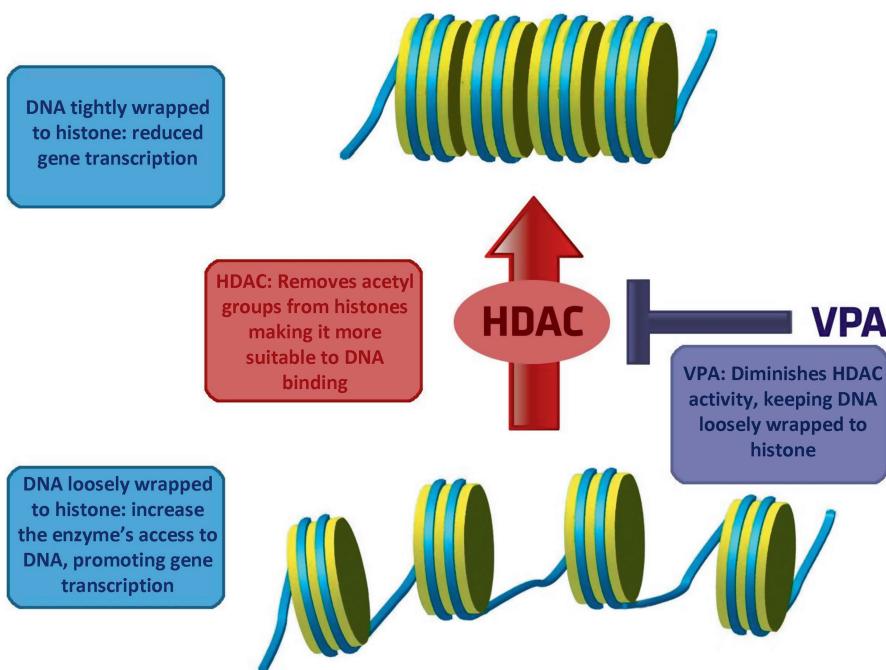
Fig. 3 *Histone deacetylase* **inhibition by VPA**. Valproic acid acting as an HDAC inhibitor, leading to enhanced gene transcription. *HDAC* histone deacetylase

Table 1 First evidence of an association between prenatal exposure to VPA and autism

Valproic acid (VPA)	Valproic acid was serendipitously discovered by Burton in 1882 while he studied organic solvents. Until today it is largely used to prevent seizures and to treat bipolar disorder
Fetal valproate syndrome	After reports of malformations in children prenatally exposed to VPA, a Fetal Valproate Syndrome was proposed in 1984 by DiLiberti and colleagues. Several *autistic* characteristics were found in patients with FVS
Autistic patients prenatally exposed to xenobiotics	In 1994, Strömland observed that a number of autistic patients in Sweden were prenatally exposed to xenobiotics between gestational days 20–24. These days matches with the neural tube closure
Rats prenatally exposed to VPA	In 1996 Rodier et al. observed morphological alterations in rats prenatally exposed to VPA that were similar to those observed in autistic patients. The first work reporting behavioral alterations in VPA model was performed by Schneider and Przewlocki in 2005
Animal model of autism induced by prenatal exposure to VPA	The VPA animal model is becoming a well explored instrument in autism research and has been showing several behavioral, neural and biochemical alterations corresponding to those found on autistic patients

Table 2 Key facts. Prenatal exposure to VPA and autism

Study	Year	Children's outcomes
Winter	1987	Low response to stimulation, infrequent smile, no attempt to communicate
Ardinger et al.	1988	Neurologic abnormalities
Laegreid et al.	1993	Mental retardation, communication disability
Thisted and Ebbesen	1993	*Irritability*, jitteriness, seizures
Christianson et al.	1994	Difficult behavior, developmental delays, speech disabilities
Williams and Hersh	1997	Autism
Moore et al.	2000	Autism, Asperger's Syndrome, poor social interaction, poor *communication skills*
Bescoby-Chambers et al.	2001	Autism, Asperger's Syndrome
Kozma	2001	Mental retardation, speech disabilities
Williams et al.	2001	Autism
Adab et al.	2001	Additional educational needs
Adab et al.	2004	Low *verbal IQ*

induced by prenatal exposure to VPA became an alternative tool of research. Although the model does not exactly replicate a human disorder, there are several behavioral and anatomical similarities, which are useful to improve the understanding of *autism spectrum disorder*. In this context, translational studies of neuropsychiatric conditions employing animal models are reliable and allow a wide range of research possibilities such as the search for an etiology, *molecular targets*, and biomarkers.

It should be emphasized that a trustworthy animal model would be based on: (1) Construct validity, which means to reproduce a circumstance that would lead to a certain state, for example, inducing a genetic disease by manipulating a specific gene; (2) *Face validity*, is to induce similar patterns found in the studied condition, for example, observing the same behavioral alterations found in a particular impairment; and (3) *Predictive validity*, which is how well a test answers a future performance, for example, if an animal model reacts similarly to a human when exposed to certain treatment (Crawley 2008). Considering this, the creation of an animal model to study autism is particularly important and provides a challenge in *ASD* research. These difficulties led, for example, the National Institute of Health to *sponsor* in 1998 the conference Building Animal Models for Autism through Translational Neuroscience Research.

Research suggests that prenatal exposure to VPA is directly related to autism, in this context, exposing rats prenatally to the same chemical agent (construct validity) should give an indication about *what is happening* and *how it happens* in the autism's pathophysiology. Autism is diagnosed exclusively by behavioral alterations in three main areas: sociability, communication and behavioral stereotypes and narrow range of interests. Therefore, a consistent animal model should

demonstrate similar behavioral abnormalities (face validity), which might indicate common neural alterations. Animal models using VPA as an environmental factor generally use prenatal exposure, but postnatal exposure to VPA was also used to induce autism-like behavior (Yochum et al. 2008).

In rats, postnatal day (PND) 14, corresponds to the third *trimester* of human development when cerebellar and hippocampal granule cells migrate and differentiate, therefore it is considered a *critical period* in *neural development* (Rice and Barone 2000). Pre and postnatal exposure to VPA were used in a same experiment, and the animals of this model displayed autistic-like behaviors. One of the major findings of this article is retardation in the maturation of negative *geotaxis* and water maze performance (Wagner et al. 2006). In a Swedish group of patients with thalidomide embryopathy, researchers found several patients with autism who were exposed *in utero* to thalidomide between gestational days (GD) 20 and 24 (Strömland et al. 1994). During this time period in humans the neural tube closes, the corresponding gestational period in rats is day 11.5 (Altman and Bayer 1980), these evidences contributed to the formulation of a reliable animal model using prenatal exposure to VPA.

Examination of the effects of various VPA dosages is extremely important to confidently develop an effective ASD animal model. In a 1988 study, pregnant rats were exposed to VPA doses from 200 mg/kg to 800 mg/kg (5–20× the therapeutic dose), from GD 8–17. The offspring showed dose-dependent effects, with the highest dose causing 100 % maternal lethality. A VPA dose of 600 mg/kg, a very often used dosage to induce autism-like behavior, has a *half life* of 2.3 h and reaches 900 μg/mL in maternal plasma in less than 1 h (Binkerd et al. 1988).

Recently, Kim and colleagues examined the critical period necessary to induce autism-like behavior in rats using prenatal VPA exposure. Pregnant rats received injections of 400 mg/kg VPA at GDs 7, 9.5, 12 and 15. Rats' sociability was measured at 4 weeks of age. The results of the sociability experiment showed that rats exposed to VPA at GD 12 were less sociable than both the control group and animals exposed at GDs 7, 9.5, and 15 (Kim et al. 2011). Although researchers have exposed rodents to VPA as early in development as GD 7 and as late as GD 15, the most utilized exposure time in VPA experiments is day is GD 12.5, a time not evaluated by Kim. Several works used different induction's day, dosage and procedure to injection VPA in pregnant rats. Table 3 summarizes methods employed to induce the VPA animal model.

First Steps in the Implementation of the VPA Model

Prenatal exposure to VPA in rodents is causative of several behavioral and morphological abnormalities similar to those displayed by individuals with autism. For example, abnormalities in the monoamine system, neural hyper-excitability, decreased nociception, deficit in weight gain, anxious-like behavior, deficits in sociability and aberrant *exploratory behavior* are all noted in the animal model (Tsujino et al. 2007; Schneider et al. 2006). These findings indicate that the VPA

Table 3 General methods to VPA induction

Studies	Induction Day	Rodent	Dosage (mg/kg)	Procedure
Bambini-Junior et al. 2011	GD12.5	Rats	600	Injection (i.p.)
Dendrinos et al. 2011	GD12.5	Rats	400–600	Injection (i.p.)
Downing et al. 2010	GD9	Mice	200–800	Injection (i.p.)
Dufour-Rainfray et al. 2010	GD9	Rats	600	Injection (i.p.)
Felix-Ortiz and Febo 2012	GD12.5	Rats	600	Injection (i.p.)
Foley et al. 2012	GD12.5	Rats	600	Injection (i.p.)
Fucic et al. 2010	GD12 to GD14	Mice	100	Injection (i.p.)
Gandal et al. 2010	GD13	Mice	600	Injection (subcutaneous)
Go et al.2011	GD12	Rats	400	Injection
Hara et al. 2012	GD12.5	Mice	500	Injection
Ingram et al. 2000	GD12.5	Rats	600	Injection (i.p.)
Kataoka et al. 2011	GD9, GD12.5 or GD14.5	Mice	500	Injection
Kim et al. 2011	GD7, GD9.5, GD12 or GD15	Rats	400	Injection
Kolozsi et al. 2009	GD11	Mice	800	Oral (mixed with *peanut butter*)
Kuwagata et al. 2009	GD9 or GD11	Rats	800	Oral (gavage)
Lukose et al. 2011	GD12.5	Rats	600	Injection
Mehta et al. 2011	GD13	Mice	600	Injection (subcutaneous)
Miyazaki et al. 2005	GD9	Rats	800	Oral (infant feeding tube)
Narita et al. 2002	GD9	Rats	800	Oral (infant feeding tube)
Rinaldi et al. 2007	GD11.5	Rats	500	Injection (i.p.)
Rodier et al. 1996	GD11.5, GD12 or GD12.5	Rats	350	Injection (i.p.)
Roullet et al. 2010	GD11	Mice	800	Oral (mixed with peanut butter)
Schneider et al. 2006	GD12.5	Rats	600	Injection (i.p.)
Tsujino et al. 2007	GD9	Rats	800	Oral (infant feeding tube)

model of autism provides a close parallel between an experimentally created model to study autism and *ASD*, making the rodent model a well established tool in autism research.

In 1996, Rodier and colleagues performed the first study aiming to induce *autistic* morphological characteristics in rat embryos exposed to VPA during gestation. No test was performed to confirm autism-like behaviors in the animals, however it was stated that with such severe morphological brain alterations, behaviors would be aberrant. Dams received 350 mg/kg of VPA at GDs 11.5, 12, or 12.5. The VPA exposure decreased the number of motor neurons counted in

matched sections in the earliest-forming motor nuclei (V, XII). These effects were more evident at GD 12.5 (Rodier et al. 1996). By determining the time period when exposure to VPA would cause the greatest effect, researchers would be able to target exposure to a specific time period and create a reproducible model.

Exposure to VPA during pregnancy can cause malformations, including neuro-anatomical (Thisted and Ebbesen 1993). Ingram and colleagues described similar changes in the brains of rats exposed to VPA during gestation. Treated rat dams received a single injection of 600 mg/kg VPA at GD 12.5. Pups presented 11.2 % reduction in brain weight when compared to control animals. Examination of the cerebellar vermis revealed that the animals exposed to VPA had a greater reduction of cell number in the posterior lobe than in the anterior lobe. However, a difference in cell density was not observed (Ingram et al. 2000). The *Purkinje cells* form primarily around GD 15, so it is probable that the proliferating cells did not have any contact with VPA itself. The reduction in number of Purkinje cells is one of the most robust findings in both humans with autism and the VPA model, which lends evidence for the efficacy of the model.

Although there were many experiments comparing the anatomical abnormalities of the VPA model to the brains of humans with autism, the first experiment using VPA exposure to verify autism-like behavior was performed in 2005. The elegant paper written by Schneider and Przewłocki produced a series of evidence showing behavior was altered in rats exposed to VPA during gestation. Some of the characteristics exhibited by VPA animals, when compared to controls were: stereotypic-like hyperactivity combined with lower exploratory activity, decreased number of social behaviors and increased latency to social contact (Schneider and Przewłocki 2005).

A Consistent Model

Similar to humans with autism, the VPA animal model exhibits impairments in social behavior, for example, impairments in initiating social interaction. Rodents exposed to VPA demonstrate the same interest exploring an object or an animal, while a control rat normally chooses to spend time socializing (Dufour-Rainfray et al. 2010; Bambini-Junior et al. 2011). The VPA animal model also exhibits additional behavior that is comparable to humans with autism for example, circadian rhythm is often altered in people with autism and this feature is also noted in the animal model (Tsujino et al. 2007). Also, the VPA model shows increased latency to change strategy in the reverse Y-maze task (which could be an indicative of behavioral rigidity) (Bambini-Junior et al. 2011), and repetitive-like behavior (Mehta et al. 2011). Therefore, data is revealing the efficiency of the prenatal exposure to VPA in rodents to generate autism-like behaviors.

A dysregulation in the serotonergic system is considered to be a possible mechanism in the pathophysiology of autism. Serotonin is part of a neurotransmitter family called *monoamines* and among other functions; it is involved in emotional

and mood control. High levels of serotonin have been found in the blood of patients with autism (Anderson et al. 1990). Several alterations in the monoamine system have been found in the VPA model, including increased monoamine levels in brain and blood, and abnormal serotoninergic neurons have been noted (Narita et al. 2002; Tsujino et al. 2007; Miyazaki et al. 2005). Alternatively, a decrease of 46 % in the levels of serotonin in the hippocampus of the animals exposed prenatally to VPA, and unaltered levels in the cerebellum and cortex were also described. The *serotonin transporter* expression was the same in control and VPA exposed animals. Decreased serotonin levels in the hippocampus could reflect a sociability alteration, as it is a *brain structure* that functions in social behaviors (Dufour-Rainfray et al. 2010). The role of the serotonergic system should be more explored in the future.

Brain-derived neurotrophic factor *(BDNF)* is another molecule altered in the blood of patients with autism. BDNF is involved in *neuronal plasticity* and regulation (Nelson et al. 2001). Reduced cortical BDNF *mRNA* expression was detected in mice exposed to VPA and was accompanied by behavioral changes, such as olfactory discrimination deficit (Roullet et al. 2010). A possible role for BDNF in the development of autism-like behavior should be well studied, but is likely that reduced BDNF expression contribute to altered synaptic development.

Alterations in synaptic function have been reported in patients with autism (Laumonnier et al. 2004). This abnormality has also been noted in the VPA model. Significantly lower Neuroligin 3 mRNA expression in VPA mice compared to control animals in the cornu ammonis (CA1) and *dentate gyrus* regions of the hippocampus and somatosensory cortex have been found. *Neuroligins* are postsynaptic cell-adhesion molecules involved in synaptic maturation (Kolozsi et al. 2009). Behavioral alterations found in knockout mice to Neuroligin 3 are similar to those found in VPA rats, such as deficits in response to social *novelty*.

The consequences of prenatal exposure to VPA have been thoroughly studied in the brain by examining behavioral, chemical, and morphological factors. However, information about the effects VPA has on other regions of the body is lacking. In one study, hepatic parameters were evaluated in order to check if metabolic dysfunction could generate some of the behavioral and neurological alterations; however no sign of liver damage was detected (Bambini-Junior et al. 2011). To gain a better understanding of the systemic alterations that might be involved in the pathophysiology of autism, the VPA model should be examined further.

Approaches to Ameliorate Characteristics of Autism

The evaluation of prospective strategies to ameliorate the symptoms of autism is one of the several possibilities enabled by the VPA animal model, making this approach an essential tool in autism research. A variety of therapeutic interventions have been proposed in animal models of autism to *attenuate*, behavioral, neurochemical and anatomical impairments.

One of these interventions is environmental enrichment, which is a well documented influence on *brain plasticity*. Since the 1960s, many experiments have shown that environmental enrichment is a means to enhance cognition. Rats housed in cages equipped with toys, such as ladders and swinging blocks, showed neurochemical changes when compared to controls. These animals had lower cortical activity of the enzyme *cholinesterase*, allowing an increase in the turnover of acetylcholine, a neurotransmitter related with synaptic plasticity (Krech et al. 1960).

In rats prenatally exposed to VPA, environmental enrichment improves behavioral alterations most notably, anxiety (Schneider et al. 2006). Anxiety was measured by verifying the time animals spent in open arms and open arm entries in an elevated plus-maze. Spending time in the open arms is indicative of low anxiety levels, since rodents prefer closed environments. VPA exposed animals subjected to this enrichment spent more time in open arms than the animals that were not placed in the enriched environment. In fact, the VPA animals that did not have access to the enriched environment presented a decrease in time spent in the open arms. It is hypothesized that reduced anxiety would improve exploratory activity and social behavior, as anxiety in animals generally leads to impairments in exploration and social interaction.

The pathophysiology of autism includes several likely mechanisms. It is speculated that oxidative stress, a consequence of toxic environmental insults, injures neural cells in predisposed individuals leading to the characteristic alterations of autism (Frustaci et al. 2012). *Antioxidant capacity* has been shown to be decreased in children with autism. A study examining 80 children with autism showed lower levels of cysteine, glutathione and the ratio of reduced to oxidized glutathione, when compared to 73 controls (James et al. 2006). These are important indicatives of oxidative stress presence in autism's pathophysiology.

Flavonoids, a group of polyphenolic compounds, are known for their antioxidant (Lu and Chen 2008) and anti-inflammatory properties (Meki et al. 2009). The leaves of *green tea* (*Camellia sinensis*) are source of many different antioxidants, including the flavonoid catechin. Mice exposed to VPA (400 mg/kg) on PND 14 and treated with daily doses (300 mg/kg) of green tea extract from PND 13 to PND 40 were compared to animals only treated with VPA and also to control animals. Animals that received the extract displayed improved behavioral characteristics when compared to VPA animals, and spatial *learning and memory* were similar to controls. Animals treated with the extract showed increased exploratory activity on open field and enhanced memory and spatial learning. Upon microscopic examination of the brains of animals exposed to VPA, a damaged Purkinje cell layer was noted, while the animals that received the extract did not present this *histopathological* alteration. Malondialdehyde, a marker of oxidative stress, was also measured. VPA animals were found to have higher plasma levels of malondialdehyde than treated animals and control animals (Banji et al. 2011).

Excitatory activity in the brain is mainly regulated by glutamate. Patients with autism and related disorders display impaired glutamatergic signaling, due a dysfunction of the *metabotropic glutamate receptor* 5 (mGluR5) (Carlson 2012). Exposure to *teratogens*, such as VPA, alters neural circuitry leading to exacerbated excitation and lowered inhibition signals in the brain (Rinaldi et al. 2007). It is

hypothesized that a misbalance in excitatory and inhibitory signaling during certain periods of development could be part of autism pathophysiology (Rubenstein and Merzenich 2003).

Mice exposed to VPA and treated with 2-methyl-6-phenylethyl-pyrididine *(MPEP)* were evaluated for spontaneous grooming time as a measure of *repetitive behavior*. Animals exposed to VPA spent more time grooming than controls. The VPA exposed animals that received treatment with MPEP, an antagonist of mGluR5, showed a significant reduction in time spent grooming compared to the other animals (Gandal et al. 2010), indicating that repetitive behaviors were reduced. To evaluate anxiety-like and repetitive-like behaviors in the animals, marble burying activity was measured. Marble burying activity was measured in order to evaluate anxiety-like and repetitive-like behaviors in the animals. VPA animals treated with MPEP buried significantly less marbles, indicating decreased anxiety and repetitive behaviors (Mehta et al. 2011).

Conclusions and Perspectives

From the initial model established by Rodier et al., which described morphological alterations similar to what is found in autism, to the behavioral alterations studied by Schneider and Przewłocki; and the several evaluations of treatments possibilities, many information has emerged from the animal model of autism induced by prenatal exposure to VPA. There are, though, several challenges in using the VPA animal model. As noted in this chapter, it is a valuable tool, however, like every animal model it is not a perfect replication of a human condition.

It was proposed that *autistic behaviors*, like decreased social interaction, anxious-like behavior and decreased learning, caused by prenatal exposure to VPA, are consequences of the inhibition of classes I and/or II *HDACs* at a certain embryonic stage (Kataoka et al. 2011), as seen in Fig. 3. However, the exactly machinery involved triggering autism-like behavior is not clear yet. The elucidation of *how this is happening* could bring forth very interesting data about the autism etiology and pathophysiology.

From the translational perspective, the VPA animal model can, as an example, link etiological triggers with treatment possibilities for autism. It should be stated that results coming from animal models should be handled carefully, once those observations come from *basic research* and in most cases, are not directly applied to human beings.

Key Terms

Valproic Acid. A drug used to prevent seizures and treat bipolar disorder. At physiological pH it is mostly found as sodium *valproate*.
Prenatal Exposure. When an organism is exposed to any kind of factor while it is still *in utero*.

Teratogen. An agent (chemical, physical, or biological) causative of embryonic developmental impairments.

Animal Models. When animals are used to study a certain condition. They are tools frequently utilized in basic research to gain a better understanding of the pathophysiology of different disorders.

Behavioral Analysis. Behaviors are how an organism responds to and interacts with its environment. There are some human features that are difficult to observe (or even absent) in animals. Therefore, certain tasks are employed to verify animal reactions and then, associate them with human behaviors.

Summary Points

- This chapter focuses on the effects of prenatal exposure to the teratogen, VPA, exposure to which could lead to the development of autism.
- Valproic acid is a simple eight-carbon branched-chain fatty acid.
- Valproic acid is a drug used to prevent seizures and as treatment for bipolar disorder.
- It was observed that women who used VPA during the first *trimester* of pregnancy had an increased chance to have a child with autism.
- An animal model to study autism induced by prenatal exposure to VPA, was proposed in 1996.
- Since 1996, several experiments showed neural and behavioral similar abnormalities in the VPA model of autism and those in human autism.
- The animal model induced by prenatal exposure to VPA demonstrates potential to be a valuable tool to study and better understand the pathophysiology of autism.

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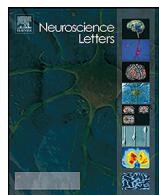
Resveratrol prevents social deficits in animal model of autism induced by valproic acid

Victorio Bambini-Junior [a,b,*], Geancarlo Zanatta [a,b], Gustavo Della Flora Nunes [a,b], Gabriela Mueller de Melo [a,b], Marcus Michels [a,b], Mellanie Fontes-Dutra [a,b], Valder Nogueira Freire [d], Rudimar Riesgo [b,c], Carmem Gottfried [a,b]

[a] Research Group in Neuroglial Plasticity at the Department of Biochemistry, Institute of Health's Basic Science, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Translational Research Group in Autism Spectrum Disorders (GETTEA), Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil
[c] Child Neurology Unit, Clinical Hospital of Porto Alegre (HCPA), Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil
[d] Department of Physics, Federal University of Ceara, Fortaleza, CE, Brazil

HIGHLIGHTS

- We performed a prenatal treatment with resveratrol in a rodent model of autism.
- Resveratrol prevented autistic-like social behaviors.
- Bioinformatics analysis suggests weak and unstable interactions between RSV and VPA.
- These results suggest cellular effects instead of a single chemical process.
- Investigation of VPA and RSV common targets may help to clarify autism etiology.

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ABSTRACT

Autism spectrum disorders (ASD) involve a complex interplay of both genetic and environmental risk factors, such as prenatal exposure to valproic acid (VPA). Considering the neuroprotective, antioxidant and anti-inflammatory effects of resveratrol (RSV), we investigated the influence of prenatal RSV treatment on social behaviors of a rodent model of autism induced by prenatal exposure to VPA. In the three-chambered apparatus test, the VPA group showed a reduced place preference conditioned by conspecific and no preference between exploring a wire-cage or a rat enclosed inside a wire cage, revealing sociability impairments. Prenatal administration of RSV prevented the VPA-induced social impairments evaluated in this study. A bioinformatics analysis was used to discard possible molecular interactions between VPA and RSV during administration. The interaction energy between RSV and VPA is weak and highly unstable, suggesting cellular effects instead of a single chemical process. In summary, the present study highlights a promising experimental strategy to evaluate new molecular targets possibly involved in the etiology of autism and developmental alterations implicated in neural and behavioral impairments in ASD.

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1. Introduction

Autism spectrum disorders (ASD) comprise a set of developmental disabilities characterized by social impairments, communication difficulties, and restricted and stereotyped patterns of behavior [17]. This group of disorders is attracting great public attention because of their high prevalence, elevated social cost and large impact on the family. The US Center for Disease Control and Prevention estimate the prevalence of autism in the United States to be 1 in 68 children, with boys 4.5 fold more affected than girls [1].

In 1943, a landmark paper describing autism was published by Leo Kanner [11]; however, even after 70 years, the etiology of

autism and its molecular basis are not well understood. Genetic studies have revealed a multitude of alterations associated with autism, but the characterized components to date account for only 25% of all cases of autism [15]. On the other hand, environmental factors, like exposure to xenobiotics - e.g. valproic acid (VPA) and thalidomide - can either trigger or contribute to autism development [9].

Considering the association between VPA exposure and ASD in humans [4], an animal model of prenatal VPA administration in rodents was suggested. In the past years, this model has shown to be a reliable research tool, as it presents many morphological and behavioral alterations related to the autism pathophysiology [3,6]. Thus, prevention of complex outcomes, such as behavioral impairments, and investigation of the molecular pathways that underlie these changes in the VPA model can shed light in biological process relevant to autism etiology.

Resveratrol (RSV) is a naturally occurring polyphenolic compound present in grapes, pines, peanuts and red wine [23]. The bulk of an intravenous dose of RSV is mainly converted to sulphate conjugates within approximately 30 min in humans and the serum half-life of total RSV metabolites is approximately 9.2 h [24]. In the last two decades, RSV received special attention from scientific community and has been associated with protective and therapeutic roles in several illnesses [23]. Resveratrol is widely recognized as an anti-oxidant and anti-inflammatory compound besides showing neuroprotective effects [7]. All of these biological activities could be of interest in autism therapeutics [19].

In this context, we investigated preventive effects of RSV in the autistic-like social features of an animal model induced by prenatal exposure to VPA. We performed a three-chamber test to measure social memory and preferences. Additionally, bioinformatics studies were used to evaluate the interaction between VPA and RSV, in order to distinguish whether the effect derivate from a direct molecule-molecule contact or from a broad cellular action.

2. Results

2.1. Behavioral testing

2.1.1. Three-chamber sociability and social novelty test
2.1.1.1. *Sociability test.* Animals of all groups stayed in the central chamber (known chamber) for only short periods of time, frequently less than 100 s. Thus, the animals were engaged in the exploration of the new environment and its content. The Control rats spent significantly more time in the chamber with the conspecific than in the chamber with the object (mean $\pm$ SEM in seconds: 285.14 ± 9.55 and 224.86 ± 12.24, with conspecific and object, respectively; $p < 0.001$). In contrast, VPA-exposed rats showed rather a non-significant tendency to spend more time to the novel object than the conspecific rat. Interestingly, RSV treatment was able to prevent the change induced by VPA (RSV + VPA: 289.5 ± 10.56 and 216.3 ± 21.26; $p < 0.05$) and, in fact the RSV + VPA group showed almost the identical time spent investigating rat 1 and object as the control group. Rats from RSV group behaved similarly to the Control animals, but there was no statistical difference between the times spent in the chamber with the rat and the chamber with the object. These data show that the VPA group animals seem to avoid the rat in favor of the object, an atypical behavioral pattern that was prevented by RSV prenatal treatment (Fig. 1A).

The analysis of exploration time, defined as time sniffing near the enclosed rat or the object and actively interacting with it (Fig. 1B), shows that rodents from both Control and RSV groups preferentially interact with a conspecific than an object (Control: 232.29 ± 10.42 and 117.43 ± 11.67; $p < 0.001$. RSV: 247.43 ± 38.8 and 91.57 ± 19.01; $p < 0.05$. Rat and object, respectively).
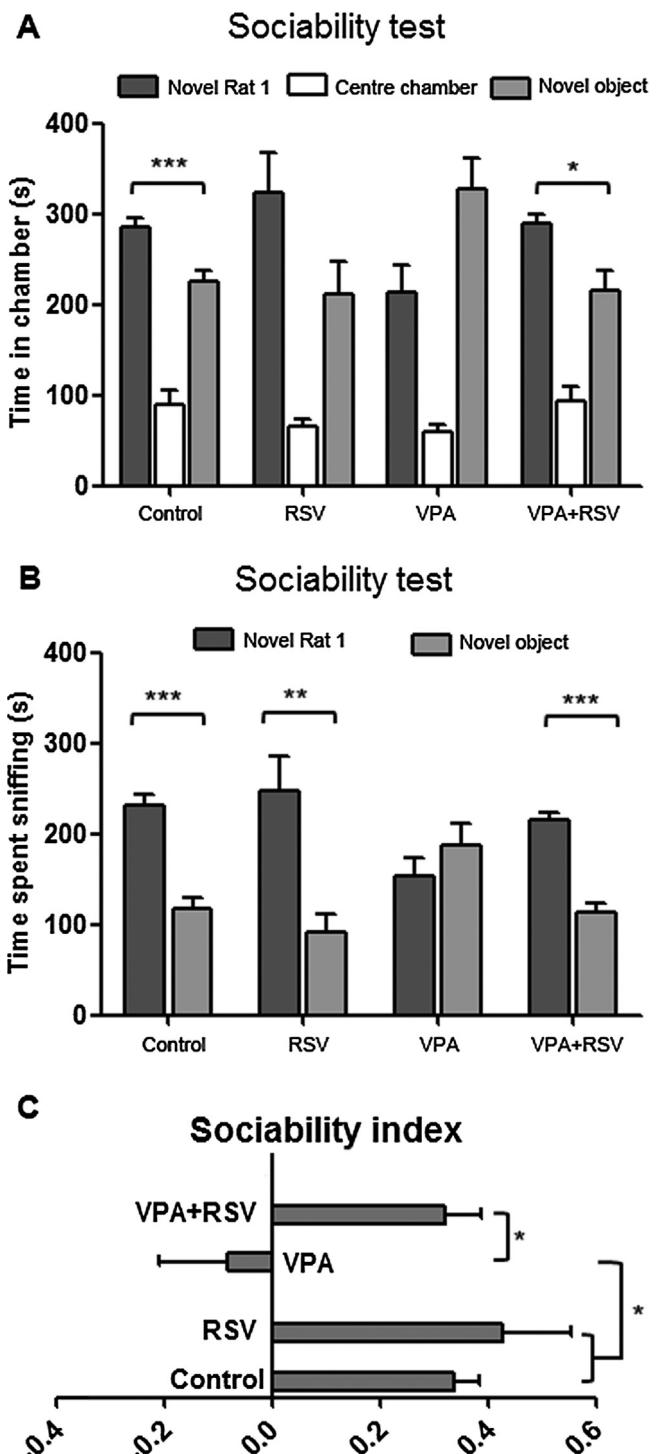
Fig. 1. RSV administration prevents the sociability deficit present in the VPA model of autism in rats. After 5 minutes of acclimatization, male rats were allowed to explore all chambers for 10 min. (A) Time spent in chambers. (B) Time spent exploring novel rat 1 or novel object. (C) Sociability Index. *$p < 0.05$. **$p < 0.01$, ***$p < 0.001$. $n_{Control} = 7$, $n_{RSV} = 7$, $n_{VPA} = 19$, $n_{VPA+RSV} = 10$.

The VPA animals showed no difference between the contact times with the novel rat and the object (147.17 ± 21.54 and 189.44 ± 25.11), which clearly shows decreased sociability. The RSV treatment was again able to counteract the VPA effect by restoring the preference for the novel rat (214.5 ± 9.02 and 112.4 ± 11.43; $p < 0.001$).

Animals from Control and RSV groups showed no mutual difference in the Sociability Index (SI). However, the SI index is significantly reduced in the VPA animals compared to the Control (-0.083 ± 0.127 and 0.337 ± 0.046, respectively; $p < 0.05$). The VPA + RSV group SI (0.319 ± 0.066) was similar to the Control SI (0.337 ± 0.046) and showed a trend to be different from a negative VPA group SI value (-0.083 ± 0.127) (Fig. 1C).

2.1.1.2. Social novelty test. Animals of all groups spent less time in the central chamber than in other chambers (Fig. 2A). The Control animals preferred the chamber with the novel rat 2 to the chamber with the known rat (429.14 ± 34.35 and 127.14 ± 32.32, respectively; $p < 0.001$), indicating an interest in social novelty and/or the formation of social memory. The VPA-exposed rats did not show difference in the time spent in chambers with novel or known rat, while VPA + RSV group showed a behavioral pattern similar to the Control group, although the preference for the novel rat vs the known rat did not reach significance. Both the RSV and the VPA + RSV group rats did not show a statistical significance in preference for novel vs known rat (Fig. 2A). As observed in Fig. 2B, Control (80 ± 19.19 and 213.29 ± 14.73; $p < 0.001$) and RSV (127.14 ± 19.14 and 210.86 ± 28.04; $p < 0.05$) group animals explored the known rat significantly less than the novel rat 2. On the other hand, the VPA-exposed rodents explored both rats to the same extent. RSV once more was able to prevent this alteration (66 ± 18.32 and 174.22 ± 24.52; $p < 0.01$), bringing the behavior back to the Control pattern in VPA + RSV group animals.

The Social Novelty Preference Index (SNI) was at same level in both Control and RSV groups. Nonetheless, there is a reduction in the VPA animals compared to the Control (-0.004 ± 0.073 and 0.485 ± 0.125, respectively; $p < 0.01$). The RSV treatment brought the index back to the same statistical level of the Control in the VPA + RSV animals (0.436 ± 0.17), and was statistically different from the VPA group ($p < 0.05$) (Fig. 2C).

2.1.1.3. Raw data and multiple interactions analysis. The following data are provided as supplementary online material: raw data (Table S1 and Table S2), multiple interactions of all factors for time in chamber and exploration time (Tables S3 and S4, respectively) and descriptive statistics of SI and SNI index (Table S5).

2.2. In silico analyses

2.2.1. Molecular dynamics

Simulations in a cubic box of water through classical molecular dynamics allowed the observation of the VPA and RSV interaction during 5 ns. Simulations were performed in duplicate and no significant differences were observed between them. Molecules moved freely through the box during the simulation and neither aggregates nor long term interactions were observed. As seen in Fig. 3A, in the first simulation only three molecules of VPA moved closer to RSV in distinct times. Nevertheless, these interactions were unstable and molecules quickly moved away from each other, suggesting the inability of complex formation. Fig. 3B–D is representative of distinct VPA molecules interaction with RSV during the simulation.

It is available as supplementary material: Description of VPA and RSV protonation state, optimized structures in vacuum and electron density analysis (Fig. S1); molecular interactions between VPA and RSV molecules in vacuum and in a cubic water box containing Na^+ and Cl^- as counter ions (Fig. S2); and a table showing the calculated interaction energies between distinct VPA molecules and RSV (Table S6).
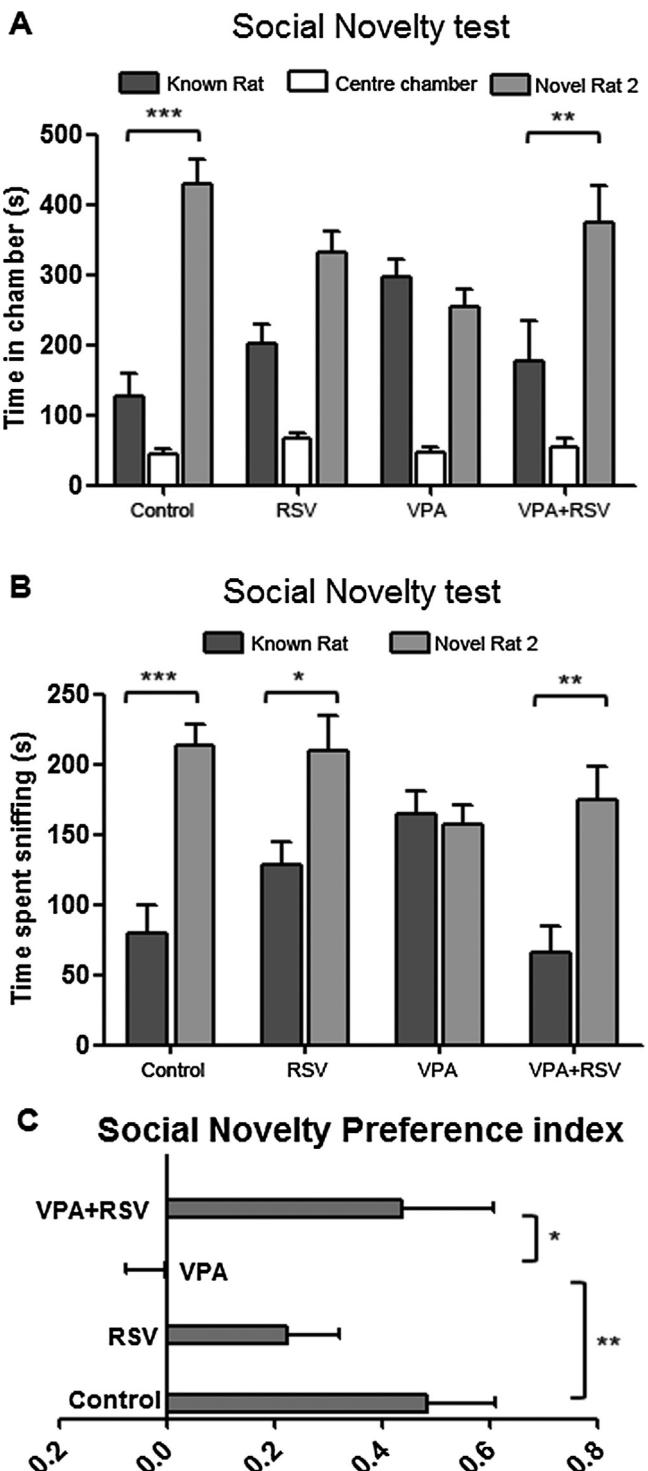
Fig. 2. Prenatal RSV averts VPA detrimental effect on social novelty preference. Immediately after the sociability test, a 10-minute test was performed in the three-chambered apparatus. (A) Time spent in chambers. (B) Time spent exploring the rats. (C) Social Novelty Preference Index. $^*p < 0.05$. $^{**}p < 0.01$, $^{***}p < 0.001$. $n_{Control} = 7$, $n_{RSV} = 7$, $n_{VPA} = 12$, $n_{VPA+RSV} = 9$.

3. Discussion

One of the most crucial areas of ASD research is the role of environmental factors in the development of autism [20]. The present data clearly shows that the development of autistic-like social behaviors induced by prenatal exposure to VPA was highly counteracted by RSV. Several social impairments found in VPA rats, such
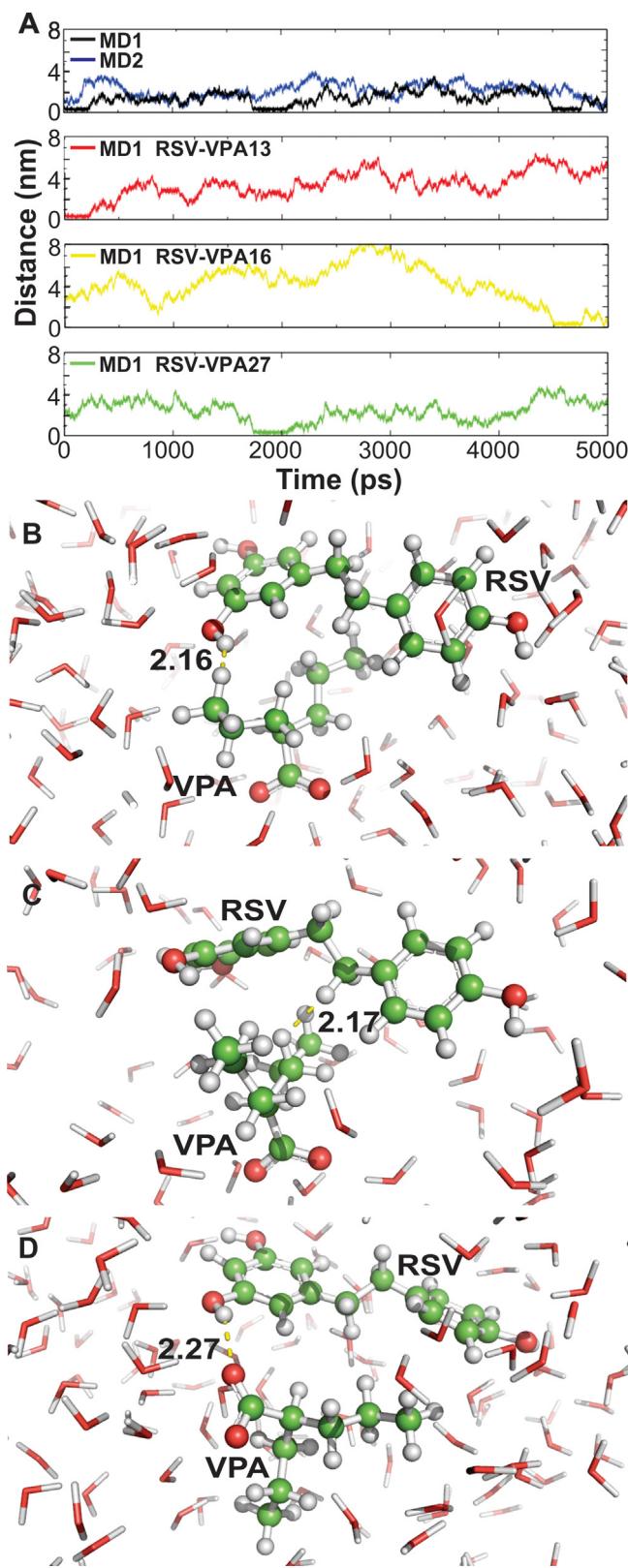
Fig. 3. Representative data of interaction distances as result of molecular dynamics simulations. (A) First box represents the minimum distances between RSV and the closest molecule of VPA (any molecule) during two distinct simulation experiments (blue or black line). Graphs representing the distances of three isolated VPA molecules to RSV are shown in the second, third and fourth boxes. (B) Spatial orientation of VPA13 and RSV during approximation at 100 ps (MD1). (C) Spatial orientation of VPA27 and RSV during approximation at 1916 ps of simulation (MD1). (D) Spatial orientation of VPA16 and RSV during approximation at 4600 ps of simulation (MD1).

as reduced sociability and decreased preference for social novelty, were not present neither in the control group nor in the group that received in utero both VPA and RSV. The examination of pathways and structures that may mediate these social responses, indicate a promising subject for future investigation of the etiological triggers and molecular alterations associated with ASD.

It is worth to note that, in mammalians, social skills require highly orchestrate responses to sensorial inputs, requesting the interplay between several brain areas. Consequently, the prevention of the autistic-like social features in rodents may not involve a specific region of the brain; rather it appears to be the result of a global process. Thus, investigations regarding the physiological effects of both VPA and RSV should have a general approach, embracing different modulatory aspects of the social behavior.

Aiming to verify if residual RSV in the blood stream could interact with VPA, a molecular dynamics study was carried out in a biological fluid. The rate RSV:VPA at the blood stream is supposed to be less than 1:272 (molecule:molecule). During simulations an extreme situation was imposed, using RSV:VPA at a rate of 10:270 (molecule:molecule). As expected, due to the physicochemical features of the molecules, it was observed some degree of interaction between VPA and RSV. Nevertheless, likely due to the effect of the solvent, interactions in a water box demonstrated to be highly unstable. In this way, we can conclude that RSV is having an indirect effect against prenatal exposure to VPA, probably acting at the cellular level.

There is no clear evidence of the molecular mechanisms by which VPA can trigger autism in humans or autistic-like features in the animal model. Although, its histone deacetylase (HDAC) inhibitory activity seems to be important to the effects [12]. On the other hand, RSV is largely believed to perform at least part of its actions by regulating the level and activation of sirtuins (members of the class-III HDAC) in a substrate-specific manner [14]. Thus, further research may investigate the overall epigenetic alterations triggered by RSV and VPA, searching for opposite effects.

Another promising topic to be studied is the well-established immune alterations involved in the pathophysiology of autism [2]. Since similar alterations are also observed in the animal model induced by VPA [21] and RSV exerts anti-inflammatory effects, future studies could analyze its developmental role in the immune system.

In order to advance the knowledge about ASD development, several lines of investigation must be pursued, including research that could merge targets of VPA and RSV to clarify molecules and pathways affected by both. In this respect, we anticipate that further understanding of these molecular targets will be relevant to both therapeutic and etiological aspects of ASD. Similarly, such studies will hopefully help to understand ASD-related epigenetic modulation and developmental alterations implicated in the neural and behavioral impairments.

4. Experimental procedures

4.1. Ethics statement

Experiments were performed according to the *NIH Guide for the Care and Use of Laboratory Animals* and approved by local authorities.

4.2. Animals

Female Wistar rats from the local breeding colony (ICBS-Federal University of Rio Grande do Sul), were kept with a 12:12 light cycle

(lights on at 7:00 and lights off at 19:00), controlled temperature ($22 \pm 1\,^{\circ}$C). Animals were mated overnight and if in the morning spermatozoa were found in vaginal secretion, this day was designated as the first day of pregnancy. Females were kept separated and with free access to their own litters. The offspring was weaned at 21 days old and were housed separately by sex. Rats had free access to food and water. Only male animals were tested. All the experiments were conducted between 14:00 and 18:00. Behavioral tests were performed with 35–50 day old animals. We used at least 3 pregnant female Wistar rats per group, with litter sizes ranging from 3 to 12 animals. The three chambers test was performed for the control group ($n = 7$), RSV group ($n = 7$), VPA group ($n = 12–19$) and VPA + RSV group ($n = 9–10$).

4.3. Treatments

Valproic Acid (Acros Organics, NJ, 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 600 mg/kg VPA or physiological saline on Embryonic day 12.5 (E12.5) [3].

Resveratrol (Fluxome, Stenløse, Denmark) was dissolved in DMSO (Merck, New Jersey, USA) for a concentration of 36 mg/mL. Females received daily subcutaneous injections of 3.6 mg/kg of RSV solution or the correspondent volume of DMSO. Since the biological effects of RSV frequently presents a hormetic dose response curve [5], and high RSV doses are related to both behavioral and physiological pervasive effects in dams and/or offspring [8,18], the 3.6 mg/kg dose (a parallel with 250 mg of RSV to an average 70 kg human, and still a very low dose to rodents) was selected to this work. On E6.5 the pregnant rats were randomly separated in 4 groups: Control (which received only DMSO injections); RSV (which received only RSV injections); VPA (which received DMSO plus VPA injection); and VPA + RSV (which received RSV plus VPA injection). Every day, in the afternoon, pregnant rats were weighed and the treatment was applied according to the groups. The treatment lasted 13 days (E6.5 until E18.5) for each group.

4.4. Behavioral test: three-chamber sociability and social novelty

We performed a 5 min long habituation in the central chamber in the previous day and immediately before the Sociability Test and conducted the experiments as previously described [3]. At the beginning, an object was positioned in one of the lateral chambers, and a set animal + object was placed in the opposite lateral chamber. This animal (called from now on, novel rat 1) was an experimentally naive male Wistar rat with no previous contact with the test animals. The object was an empty cage identical to the one used to enclose the novel rat 1. Time spent in each chamber, as well as the time spent exploring the novel rat 1 or the novel object, was analyzed by two observers during 10 min. Immediately after, the social novelty test began. In this test, the novel rat 1 remained in its wire cage (now it is called the known rat) and a new unfamiliar rat (novel rat 2) was placed in the wire cage in the opposite side (which was previously empty). Time spent in each chamber and time spent sniffing each wire cage was recorded during 10 min. It is important to note that rats that did not explore all three chambers during the sociability test did not perform the social novelty test. In addition, since the social novelty test aims to evaluate the preference for social novelty and the formation of social memories, rodents that did not interact with both the known rat and the novel rat 2 were excluded from the analysis.

We also evaluated a Sociability Index (SI) (according to Kim et al. [13] and modified by us and R. Romcy-Pereira, personal communication), a mathematical transformation designed to allow the direct comparison of social behavior of the groups. It ranges from −1 to 1 and as the score becomes more positive and closer to 1, the more social the animal. The SI was calculated as showed below:

$$SI = \frac{\text{Time exploring}_{\text{novel rat 1}} - \text{Time exploring}_{\text{novel object}}}{\text{Time exploring}_{\text{novel rat 1}} + \text{Time exploring}_{\text{novel object}}}$$

In an analogous manner, we can use a Social Novelty Preference Index (SNI). It also ranges from −1 to 1 and a value closer to 1 indicates an animal more interested in social novelty. The SNI is calculated as showed below:

$$SNI = \frac{\text{Time exploring}_{\text{novel rat 2}} - \text{Time exploring}_{\text{known rat}}}{\text{Time exploring}_{\text{novel rat 2}} + \text{Time exploring}_{\text{known rat}}}$$

4.5. Bioinformatics: molecular dynamics simulations and analysis

Molecular dynamics (MD) simulations were performed with the package GROMACSv.4.5.1 [22], using the SPC water model and the GROMOS 53a6 force field [16]. A cubic box with edges of 12 nm (box volume ≈ 1728 nm^3) containing RSV and VPA molecules in a proportion of 1:27 was built, and filled with 57.084 water molecules. An appropriate number of chloride and sodium counterions were added to neutralize the system, observing the final salt concentration of 0.15 mol l^{-1}. Two independent 5 ns simulations were performed, differing only by the initial system coordinates: in the first (MD_1) the RSV molecule was placed in the center of the simulation box, while in the second (MD_2) it was randomly placed inside the box with the genbox software. The VMD program [10] was used to visualize and manipulate the system, and the g_mindist software was applied to calculate the minimum distance between the molecules during the simulation. Minimum distance plots were generated with XMGrace software (http://plasma-gate.weizmann.ac.il/Grace/).

4.6. Statistical analysis

The values measured for the animals were integrated in a multivariate linear model to predict the impact of the treatment in the behavioral outcome. We used the Generalized Estimation Equations (GEE) in order to enable the comparison between multiple interdependent variables and overcome the necessity of normality and homoscedasticity. Bonferroni post hoc test was used as the final evaluation. Sociability and Social Novelty Preference Indices were compared by Kruskal–Wallis test. Data is reported as mean ± standard error of the mean (SEM). All analyses were performed using the SPSS program, Version 17.0 (SPSS, Chicago, IL).

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2014.09.039.

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5.3. Patente "USO DE PELO MENOS UM POLIFENOL DA FAMÍLIA DOS ESTILBENOS E MÉTODO PARA PREVENÇÃO DO DESENVOLVIMENTO DE AUTISMO EM UM MAMÍFERO DESCENDENTE"

5.3.1. Status

Depositada e aguarda publicação sob o número de protocolo "BR 10 2012 029382 0".

5.3.2. Publicação

Relatório Descritivo de Patente de Invenção

Uso de Pelo Menos um Polifenol da Família dos Estilbenos e Método para Prevenção do Desenvolvimento de Autismo em um Mamífero Descendente

5

Campo da Invenção

A presente invenção se situa no campo de métodos de prevenção de sintomas associados ao autismo em mamíferos, particularmente, composições farmacêuticas e seu uso na preparação.

10

Antecedentes da Invenção

O autismo é um transtorno comportamental de etiologia ainda desconhecida, que se associa a uma alteração no processo de desenvolvimento infantil. Esta desordem é enquadrada no Manual Diagnóstico e Estatístico dos Distúrbios Mentais V revisado (DSMV-TR), como transtorno global do desenvolvimento (TGD), também conhecido como transtorno invasivo do desenvolvimento (TID). O espectro do autismo compreende o autismo clássico, com maior comprometimento intelectual, o autismo não especificado e a síndrome de Asperger.

Algumas características como prejuízo na interação social, severos distúrbios de linguagem, reduzida comunicação e uma preocupação obsessiva pelo que é imutável, podem ser observadas em crianças com TID. O indivíduo adulto com autismo apresenta problemas de interação social, mas em alguns casos, relacionados com o autismo leve, pode se desenvolver profissionalmente se encontrar um entorno favorável.

Há estudos investigando fatores genéticos relacionados, com mais de 100 genes já descritos. O autismo não tem tratamento específico, mas sim, apenas para sintomas associados, como distúrbios do sono e agressividade. O diagnóstico do autismo deve ser o mais precoce possível, no entanto é realizado normalmente após os três anos de idade, quando já se podem observar

características de prejuízo comportamental. Um agravante para o diagnóstico tardio é a falta de marcadores clínicos.

O indivíduo com autismo sofre uma alta dificuldade no tratamento dos sintomas associados, acarretando um alto custo econômico, social e familiar. Portanto a presente invenção envolve profilaxia do autismo, uma vez que se trata de uma intervenção durante períodos iniciais da gestação, e, portanto, visa a prevenção, com caráter inovador e de grande relevância social.

A busca na literatura científica e patentária apontaram alguns documentos relevantes para a presente invenção, os quais são descritos a seguir.

O documento US20110201565A1 revela um método para tratar desordens auto-imunes selecionadas dos grupos do autismo, da esquizofrenia e da diabetes por meio da administração via oral em dose terapêutica efetiva de um ou mais dos seguintes polifenóis: luteolina, diosmina, diosmetina, seus análogos e derivados. O mecanismo de ação desse tratamento ocorre pela inibição da fosforilação da STAT3 induzida pela IL-6. Aumentos acentuados na produção de IL-6 acontecem em condições inflamatórias no cérebro e a IL-6 sinaliza a tradução e ativação da STAT3, além de ativar a JAK A via JAK2/STAT3 induzida por IL-6 atua na proliferação celular, fenômeno que ocorre em nível excessivo de uma proporção significante de crianças com autismo. Foi constatado que camundongos tratados com diosmina demonstraram redução do fenótipo autista. A análise do homogenato de cérebro por Western Blot da prole adulta, cuja mãe foi tratada com diosmina e S31-201 (inibidor de STAT3), além de IL-6, demonstrou uma redução da fosforilação da STAT3. Esse documento se distancia da presente invenção pelo uso de polifenóis de grupos diferentes, enquanto o documento US20110201565A1 se refere a flavonóides, a presente invenção trata de polifenóis da família dos estilbenos.

O documento US20100298402A1 revela a propriedade de estilbenos inibirem a STAT3. Descreve um composto (estrutura abaixo) e seus derivados, análogos, formas tautoméricas, estereoisômeros, polimorfos, solvatos, intermediários, sais, composições farmacêuticas, metabólitos e pró-fármacos, além de seu processo de síntese e uma composição farmacêutica.
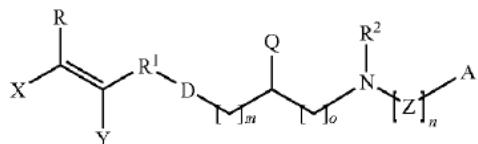
Esse composto pode ser utilizado para profilaxia e tratamento de doenças autoimunes, desordens neurológicas e doenças inflamatórias. Atua inibindo a fosforilação da STAT3, mediado pela IL-6. Além disso, o documento descreve no estado da técnica a propriedade do resveratrol inibir a sinalização da STAT3. O documento US20100298402A1 se distancia da presente invenção, pois esse não apresenta nenhuma evidencia das moléculas da classe dos flavonóides poderem ser utilizadas no tratamento e/ou na prevenção de autismo, além de não demonstrar a possibilidade do tratamento durante o período de gestação.

Do que se depreende da literatura pesquisada, não foram encontrados documentos antecipando ou sugerindo os ensinamentos da presente invenção, de forma que a solução aqui proposta possui novidade e atividade inventiva frente ao estado da técnica.

Sumário da Invenção

Dessa forma, a presente invenção vem propor uma medida profilática para o desenvolvimento do autismo, através do uso de pelo menos um polifenol da família dos estilbenos. Envolve a proposta de preparação de um medicamento para a prevenção do autismo em um mamífero descendente, prevenção esta que consiste em administrar no mamífero genitor, durante a gestação do mamífero descendente, uma quantidade terapeuticamente eficaz de um polifenol da família dos estilbenos.

Em um aspecto da presente invenção o dito polifenol da família dos estilbenos é o resveratrol.

Em um aspecto da presente invenção a dita administração no mamífero genitor é por via subcutânea, intramuscular ou por gavagem.

Em um aspecto da presente invenção a dita quantidade terapeuticamente eficaz de um polifenol da família dos estilbenos é entre 10 µg e 10 g por administração.

Em um aspecto da presente invenção o dito polifenol administrado está na concentração de 0,1 mg/kg de peso corpóreo a 100 mg/Kg de peso corpóreo.

Adicionalmente, a presente invenção apresenta um método para prevenção do desenvolvimento de autismo em um mamífero descendente, o dito método compreende administrar no mamífero genitor, durante a gestação do dito mamífero descendente, uma quantidade terapeuticamente eficaz de um polifenol da família dos estilbenos.

Estes e outros objetos da invenção serão imediatamente valorizados pelos versados na arte e pelas empresas com interesse no segmento, e serão descritos em detalhes suficientes para sua reprodução na descrição a seguir.

Descrição das Figuras

A figura 1 apresenta um gráfico comparando o perfil exploratório social dos ratos descendentes, os quais as mães foram tratadas com ácido valpróico (VPA), sabidamente um indutor de autismo em humanos, e ácido valpróico e resveratrol, em comparação ao grupo controle (sem tratamento).

A figura 2 apresenta quatro gráficos de simulações computacionais da interação entre as moléculas de VPA e de resveratrol. A simulação denota o caráter efêmero das ligações entre VPA e resveratrol, deixando claro que o mecanismo de ação do resveratrol se da através de alterações celulares e não do simples bloqueio da ação do VPA por, por exemplo, quelamento.

Descrição Detalhada da Invenção

A presente invenção revela o uso de pelo menos um polifenol da família dos estilbenos, na preparação de um medicamento para a prevenção do autismo em um mamífero descendente, prevenção esta que consiste em administrar no mamífero genitor, durante a gestação do mamífero descendente, uma quantidade terapeuticamente eficaz de um polifenol da família dos estilbenos.

A prevenção do autismo sugerida na presente invenção é através de um tratamento durante o período gestacional, período esse de risco para o desenvolvimento do autismo no feto. Inicialmente, esse método de tratamento descrito é seria indicado em casos de risco, como, por exemplo, quando o casal já possui um filho autista ou a grávida encontra-se sob tratamento com anticonvulsivantes.

O presente método de tratamento é realizado na genitora durante o período de gestação e previne o desenvolvimento de características comportamentais prejudiciais do autismo no descendente.

Polifenol da família dos estilbenos:

Polifenóis são estruturas químicas caracterizadas por possuírem múltiplos fenóis, anéis aromáticos que possuem uma ou mais hidroxilas ligadas. Os polifenóis podem ser classificados em duas categorias, os flavonoides e os não flavonoides dentre os quais está família dos estilbenos. O resveratrol sendo o estilbeno preferencial da presente invenção. Entretanto, técnicos no assunto poderão reconhecer que outras substâncias com estrutura similar ao do resveratrol poderão ser utilizadas com uma razoável expectativa de similaridade em seu efeito na prevenção do autismo.

Mamífero descendente:

Mamífero descendente é o paciente com autismo ou indivíduo que pode desenvolver o autismo. Esse não recebe o tratamento diretamente, mas sim a fêmea gestante dele.

Mamífero genitor:

Mamífero genitor é a fêmea antecessora do mamífero descendente. O tratamento com o polifenol da família dos estilbenos é realizado como forma de prevenção do autismo e envolve a antecedente direta do possível paciente com autismo, durante sua gestação, ou seja, durante o período de formação e desenvolvimento do mamífero descendente. Dessa forma, o ser vivo que deve

receber o tratamento sugerido na presente invenção é a genitora, durante o período de gestação.

Quantidade terapeuticamente eficaz:

5   Dose do fármaco administrado em determinado intervalo de tempo capaz de apresentar eficácia e depende do peso do indivíduo que receberá o tratamento.

Os exemplos aqui mostrados têm o intuito somente de exemplificar uma das inúmeras maneiras de se realizar a invenção, contudo, sem limitar o escopo
10   da mesma.

Exemplo 1.

Teste utilizando fêmeas de ratos Wistar prenhas foi realizado para comprovar a eficácia do tratamento.

15   As ratas foram divididas em quatro grupos:

- Controle: Expostas ao veículo da solução de polifenol, dimetilsulfóxido

- PLF: Expostas a solução de polifenol

- VPA: Expostas ao ácido valpróico, agente indutor de características do tipo autistas e ao veículo da solução de polifenol, dimetilsulfóxido.

20   - PLF+VPA: Expostas a solução de polifenol e ácido valpróico.

A posologia de polifenol utilizada corresponde a até 10 mg/Kg de peso da rata. Esse foi utilizado na forma de solução injetável, diluído em dimetilsulfóxido, e administrado via subcutânea, por 5 a 21 dias. O ácido valpróico foi administrado entre os dias 9 e 14 de gestação.

25   No trigésimo dia pós-natal, os ratos da prole foram submetidos a diferentes testes comportamentais para avaliar características relacionadas ao autismo. O grupo VPA apresentou alterações sociais esperadas, correlacionadas a comportamentos relacionados ao autismo. Já o grupo PLF+VPA não apresentou tais alterações.

30

Exemplo 2.

Foi realizado teste com fêmeas de ratos no período de gestação. O polifenol utilizado corresponde ao resveratrol. As ratas foram divididas em quatro grupos.

- Controle: Expostas ao veículo da solução de polifenol, dimetilsulfóxido

5   - PLF: Expostas a solução de polifenol

- VPA: Expostas ao ácido valpróico, agente indutor de características autistas e ao veículo da solução de polifenol, dimetilsulfóxido.

- PLF+VPA: Expostas a solução de polifenol e ácido valpróico.

O tratamento pré-natal com o resveratrol foi realizado nas ratas prenhe 10 administrando doses de 0,5 a 5 mg/Kg de peso corpóreo durante os últimos 14 dias da gestação.

O tratamento com ácido valpróico, realizado para induzir características comportais, neuroquímicas e morfológicas correspondentes ao autismo, corresponde a exposição das ratas prenhe a 600 mg/Kg de peso corpóreo de 15 ácido valpróico aos 12,5 dias de gestação.

A prole do grupo PLF+VPA não apresentou características comportamentais comparáveis ao autismo. Isso foi constatado pelo teste denominado labirinto de três câmaras, no qual o rato tem a tarefa de escolher entre explorar um objeto desconhecido ou um rato desconhecido. Os ratos do 20 grupo VPA não demonstraram preferência, já os ratos do grupo PLF+VPA demonstraram preferências semelhantes ao grupo controle, explorando por mais tempo o rato desconhecido do que o objeto desconhecido. Esse resultado pode ser observado na figura 1. As barras cinza claro representam o tempo (em segundos) que cada grupo explorou o rato desconhecido. As barras cinza escuro 25 representam o tempo (em segundos) que cada grupo explorou o objeto desconhecido. O grupo controle recebeu, durante a gestação, tratamento com o veículo do PLF. O asterisco representa diferença estatística dentre Objeto *vs.* Rato, *p*< 0,05. Dessa forma, constatou-se a completa prevenção da característica comportamental observada relacionada ao autismo, por 30 tratamento durante o período gestacional com PLF.

Além disso, foi demonstrado através da utilização de ferramentas de bioquímica quântica, que o efeito deve-se a benefícios fisiológicos e não evitando que a molécula indutora seja ativa. Simulações computacionais demonstraram baixa interação entre as moléculas que previnem com as moléculas que induzem os comportamentos do tipo autista em ratos (Figura 2).

O primeiro gráfico da figura 2 (MD 1 + MD 2) mostra a menor distância (em nanômetros) entre uma molécula de resveratrol (agente protetor) e uma molécula de ácido valpróico (agente indutor), em dois experimentos independentes, ao longo do tempo (em milissegundos). Os gráficos seguintes (MD1 RSV-VPA13, MD1 RSV-VPA16 e MD1 RSV-VPA27) representam as moléculas de RSV que mais se aproximaram (interagiram) com moléculas de VPA. Os experimentos demonstram o caráter transitório da interação entre as moléculas, não podendo, portanto, interagirem suficiente para anularem seus efeitos biológicos. Em todos os experimentos, foi utilizada uma concentração fictícia de 1 molécula de RSV para 27 de VPA, sendo essa uma concentração pelo menos 10x maior que a máxima encontrada em nossos experimentos.

Os versados na arte valorizarão os conhecimentos aqui apresentados e poderão reproduzir a invenção nas modalidades apresentadas e em outros variantes, abrangidos no escopo das reivindicações anexas.

Reivindicações

1. Uso de pelo menos um polifenol da família dos estilbenos, **caracterizado** por ser na preparação de um medicamento para a prevenção do autismo em um mamífero descendente, prevenção esta que consiste em:

a) administrar no mamífero genitor, durante a gestação do mamífero descendente, uma quantidade terapeuticamente eficaz de um polifenol da família dos estilbenos.

2. Uso, de acordo com a reivindicação 1, **caracterizado** pelo polifenol da família dos estilbenos ser o resveratrol.

3. Uso, de acordo com a reivindicação 1, **caracterizado** pela administração no mamífero progenitor ser por via subcutânea, intramuscular ou oral.

4. Uso, de acordo com as reivindicações 1 e 3, **caracterizado** pela quantidade terapeuticamente eficaz de um polifenol estar entre 10 µg e 10 g por administração.

5. Uso, de acordo com as reivindicações 1, 3 e 4, **caracterizado** pelo polifenol administrado estar na concentração de 0,1 mg/kg de peso corpóreo a 100 mg/Kg de peso corpóreo.

6. Método para prevenção do desenvolvimento de autismo em um mamífero descendente, o dito método **caracterizado** por:

a) administrar no mamífero genitor, durante a gestação do dito mamífero descendente, uma quantidade terapeuticamente eficaz de um polifenol da família dos estilbenos.

Resumo

USO DE PELO MENOS UM POLIFENOL DA FAMÍLIA DOS ESTILBENOS E MÉTODO PARA PREVENÇÃO DO DESENVOLVIMENTO DE AUTISMO EM UM MAMÍFERO DESCENDENTE

5

A presente invenção descreve o uso de pelo menos um polifenol da família dos estilbenos, na preparação de um medicamento para a prevenção do autismo em um mamífero descendente, prevenção esta que consiste em administrar no mamífero genitor, durante a gestação do mamífero descendente, uma
10 quantidade terapeuticamente eficaz de um polifenol da família dos estilbenos.

Figuras
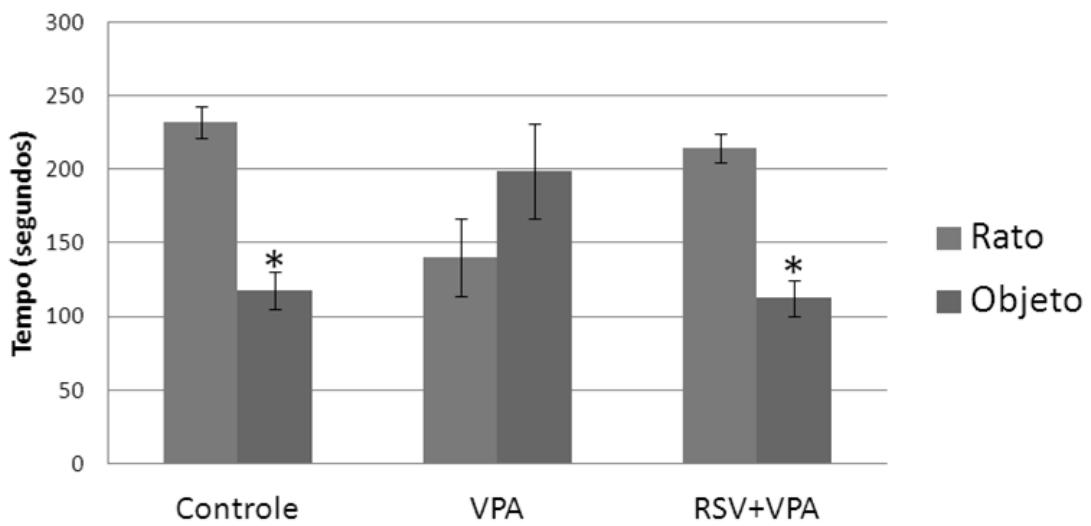
Figura 1
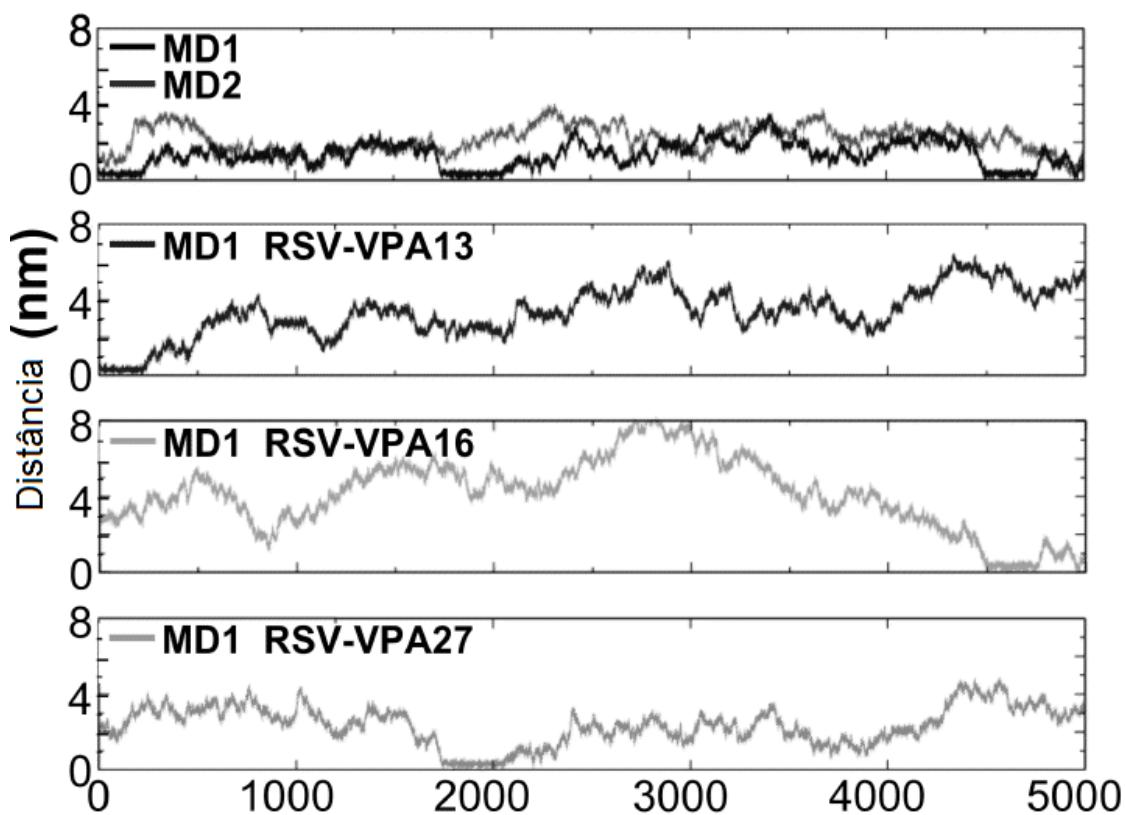
Figura 2

5.4. Resultados adicionais

5.4.1. Status

Em processo de coleta de resultados complementares para posterior publicação.

5.4.2. Resultados adicionais em fase preliminar – Análise da expressão de Sirtuínas

Sirtuins are a family of NAD^+-dependent histone and non-histone deacetylases (S. Y. Roth, J. M. Denu, and C. D. Allis, 2001). This requirement confers sirtuins the ability to detect changes in metabolism and energy homeostasis and to coordinate cellular responses to maintain genome integrity (A. Vaquero, 2009; S. Imai et al., 2000). Thus, sirtuins are crucial in the crosstalk between environment and genome (X.-J. Yang and E. Seto, 2008). In mammals, there are 7 subtypes of sirtuins with different subcellular localizations (R. A. Frye, 2000). Sirt1, Sirt2 and Sirt 3 are a class I type of sirtuins with deacetylation enzymatic activity. Their functions resides on the control of chromatin structure, cell cycle, glucose metabolism, insulin secretion, fatty acid oxidation, oxidative stress and metabolic regulation (D. B. Lombard et al., 2007; A. Vaquero et al., 2004). Sirt 4 are a class II type of sirtuins with ADP-ribosylation enzymatic activity, and studies have shown its involvement in insulin secretion (S. M. Jeong et al., 2014). Sirt5 are a class III type of sirtuin important in the urea cycle regulation with many reported enzymatic activities: desuccinylation, demalonylation and deacetylation (J. Du et al., 2011). Sirt6 and Sirt7 are class IV type of sirtuin but only Sirt6 has its enzymatic activity reported on deacetylation and ADP-ribosylation (E. Michishita et al., 2008). Sirt6 has important roles in glucose

homeostatis, telomeric function and DNA repair, and Sirt7 possibly act on RNA pol I transcription (E. Ford et al., 2006).

Recent studies have demonstrated the role of resveratrol (RSV) in modulation of sirtuin activity (R. Luthi-Carter et al., 2010; B. Jian et al., 2014; H. Schirmer et al., 2012; K. Howitz, K. Bitterman, and H. Cohen, 2003).

The main objective of this work was to analyze the effect of prenatal treatment with RSV on the relative gene expression of 7 subtypes of sirtuins presented in mammals (*Sirt1*, *Sirt2, Sirt3, Sirt4, Sirt5, Sirt6* and *Sirt7*) in animals prenatally exposed to valproic acid.

5.4.2.1. Análise da expressão gênica de sirtuinas em hipocampo e sangue total

Figure 1. Relative gene expression of *Sirt1, Sirt2, Sirt3, Sirt4, Sirt5, Sirt6* and *Sirt7* in hippocampus samples.
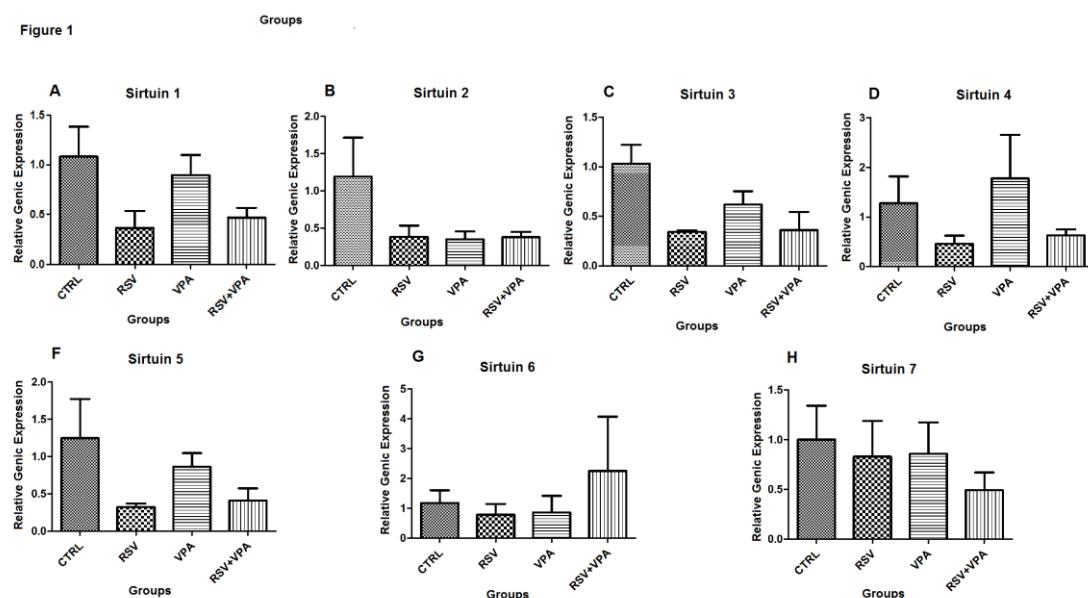
Figure 1. Relative gene expression of five sirtuins (*Sirt1, Sirt2, Sirt3, Sirt4, Sirt5, Sirt6* and *Sirt7*) related to the housekeeping gene GAPDH in

total blood samples. The gene expression was quantified by real time PCR in the control, (CTRL), resveratrol (RSV), valproic acid (VPA) and valproic acid plus RSV (RSV+VPA) groups. The values represent the gene expression of Sirtuins by the gene expression of GAPDH, a housekeeping gene. A-G, there was no significant differences between the experimental groups in relative genic expression of *Sirt4*, S*irt5*, *Sirt6* and *Sirt7*.

Figure 2. Relative gene expression of *Sirt2, Sirt3, Sirt4, Sirt5 and Sirt6* in total blood samples.

Figure 2. Relative gene expression of five sirtuins (*Sirt2, Sirt3, Sirt4,*

Figure 2
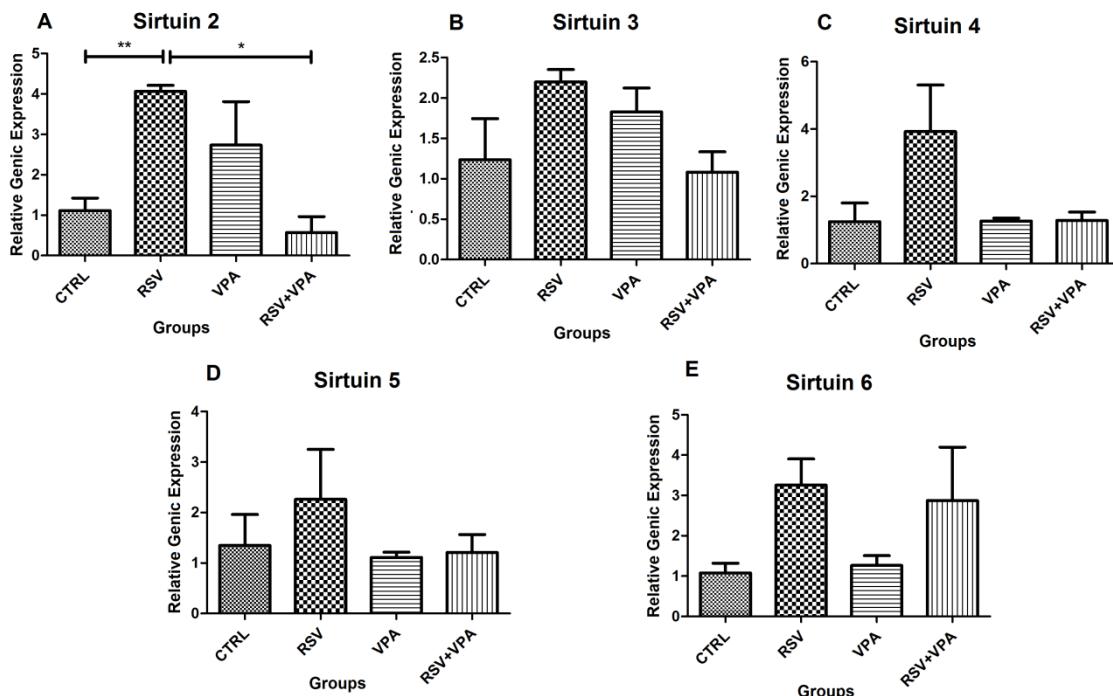
***Sirt5* and *Sirt6*) related to the housekeeping gene GAPDH in total blood samples.** The gene expression was quantified by real time PCR in the control, (CTRL), resveratrol (RSV), valproic acid (VPA) and valproic acid plus RSV (RSV+VPA) groups. The values represent the genic expression of Sirtuins by the gene expression of GAPDH, a housekeeping gene. A, the relative gene expression of *Sirt2* is significantly different between the experimental groups CTRL x RSV and RSV x RSV+VPA (p<0.05). B-E, there was no significant differences among the experimental groups in the relative genic expression of *Sirt3*, *Sirt4*, *Sirt5* and *Sirt6*.

Materials and Methods

Animals

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.

Treatments

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 as previously described (Bambini-Junior et al., 2011; Schneider and Przewlocki, 2005). Resveratrol (Fluxome, Stenløse, Denmark) was dissolved in DMSO (Merck, New Jersey, USA) for a concentration of 36 mg/mL. Females received daily subcutaneous injections of 3.6 mg/kg of RSV solution or the correspondent volume of DMSO. On E6.5 the pregnant rats were randomly separated in 4 groups: Control (which received only DMSO injections); RSV (which received only RSV injections); VPA (which received DMSO plus VPA injection); and VPA+RSV (which received RSV plus VPA injection). Every day, in the afternoon, pregnant rats were weighed and the treatment was applied according to the groups. The treatment lasted 13 days (E6.5 until E18.5) for each group. Females

were housed individually and were allowed to raise their own litters. The offspring rats were housed separately by sex.

Samples

Male pups from at least three different litters at postnatal day 120 were anaesthetized and peripheral blood samples were obtained by cardiac puncture. Also, the brain was removed and the hippocampus and cerebellum were isolated. The blood samples were rapidly homogenized with two-fold volume of TRIzol® (Invitrogen) for total RNA extraction. The tissue samples were weighed and TRIzol® were added according to the 1:10 proportion (10g of tissue homogenized in 100 uL of TRIzol®). After tissues and blood homogenizations, the samples were kept in −80°C until the assays were performed.

RNA Extraction

Samples were left in a clean bench to defrost, in a room temperature. After 4 minutes, chloroform was added in each eppendorf sample to perform phase separation, in a 1:5 proportion (100 uL of TRIzol®, 20 uL of chloroform). After centrifugation, the aqueous phase was replaced to other eppendorf and RNA was precipitated with isopropanol, in a 1:2 proportion (100 uL of TRIzol®, 50 uL of isopropanol). The precipitated RNA was washed with ethanol (1:1 proportion) and resuspended in a RNAse-free water. The RNA samples were stored at −80°C until the cDNA synthesis.

cDNA Synthesis and Quantitative PCR (RT-qPCR)

In this study, we evaluate the genic expression of 7 sirtuins and 2 endogenous controls in hippocampus, cerebellum and blood samples. We synthesized cDNA from total RNA samples by using a well-established protocol

with M-MLV reverse transcriptase (Promega). The mRNA were synthesized according to the following procedure: 5 uL of total RNA (1:50), 1 uL of primer Oligo T (Invitrogen) and 4 uL of RNAse-free water. After exposed to 70ºC for 5 minutes in a thermocicler, we added in each sample: 6 uL of 5x First Strand Buffer (M-MLV buffer, Promega), 2 uL of dNTP (5 mM), 1 uL of M-MLV reverse transcriptase, and 14 uL of RNAse-free water, in a final volume of 30 uL. The synthesis of cDNA was complete after 60 minutes in 40ºC.

The RT-qPCR mix was formed by 12 uL of cDNA (1:15), 1 uL of 5 mM specific forward and reverse primers (Supplemental Table 1), 0.5 uL of 5 mM dNTPs, 2.5 uL of 10x PCR buffer (Invitrogen), 1.5 uL of 50 mM MgCl2, 4 uL of 1X SYBR-Green® (Molecular Probes) and 005 of Platinum Taq DNA polymerase (Invitrogen) to a final volume of 24 uL. SYBR-Green® was used to detect amplification, estimate Ct values and to determine specificity after melting curve analysis. The PCR cycling conditions were standardized to: 95ºC for 5 minutes followed by 40 cycles at 95ºC for 10 seconds, 58ºC for 10 seconds and 72ºC for 10 seconds. After the main amplification, sample fluorescence was measured at temperatures from 60ºC to 95ºC, with an increasing ramp of 0.3ºC, in order to obtain the denaturing curve of the amplified products and assure their homogeneity after peak detection and Tm estimation, using data obtained from Applied Biosystems StepOnePlus® System.

Data Analysis

All the Ct results and efficiencies calculations from RT-qPCR were imported to Microsoft Excel and the average values of Ct values (in triplicate). The PCR efficiency was calculated from the slope of the amplification curve by exponential amplifications analysis using the LinRegPCR algorithm (C. Ramakers et al.,

2003). The data were organized in Microsoft Excel with the Ct mean values and the relative expression was obtained using the -ΔΔCt method, wehere crossing threshold (Ct) values of the groups are subtracted from the average Ct values of control samples. The relative expression of mRNA was calculated considering the PCR efficiency and the -ΔΔCt values for each mRNA, accordingly methodology previously described (M. W. Pfaffl, 2001). To account for possible differences in expression levels of mRNA between groups, statistical analysis was performed by ANOVA 1 way followed by Bonferroni post test, and p-values less than 0.05 were considered significant.

5.4.2.2. Análise da função mitocondrial e do estresse oxidativo em hipocampo e cerebelo.

There is growing evidence that patients with autism present a variety of mitochondrial dysfunctions (D. Rossignol and R. Frye, 2011). In addition, VPA is thought to cause oxidative stress and mitochondrial damage in humans (V Tong et al., 2005). These facts prompted us to investigate the mitochondrial parameters and the oxidative profile on the brain in the animal model of autism induced by prenatal exposure to valproic acid.

We performed in vitro assays in hippocampus and cerebellum to analyze the activities of components of the oxidative phosphorylation: complex II (succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase), complex II-III (succinate/cytochrome c oxidoreductase), Succinate Dehydrogenase (SDH), and complex IV (cytochrome c oxidase). In addition, we tested the enzymatic activity of the Creatine Kinase (CK), which is responsible for generating

phosphocreatine, a reservoir metabolite for buffering and regeneration of ATP *in situ*. Additionally, we evaluated the enzymatic antioxidant system through the analysis of Catalase (responsible for converting hydrogen peroxide to water and oxygen), Superoxide Dismutase (SOD, which generates hydrogen peroxide from the superoxide free radical) and Glutathione Peroxidase (GPx, which converts lipid peroxides to alcohols and hydrogen peroxide to water). In order to investigate the presence of oxidative damage in macromolecules, we also measured the levels of lipid peroxidation through the tiobarbituric acid reactive substances (TBARS) assay.

These analyses allowed us to depict the general landscape of the energy production in hippocampus (Figure 3) and cerebellum (Figure 4), as well as the oxidative profile in these brain regions (Figure 5 and 6, respectively).
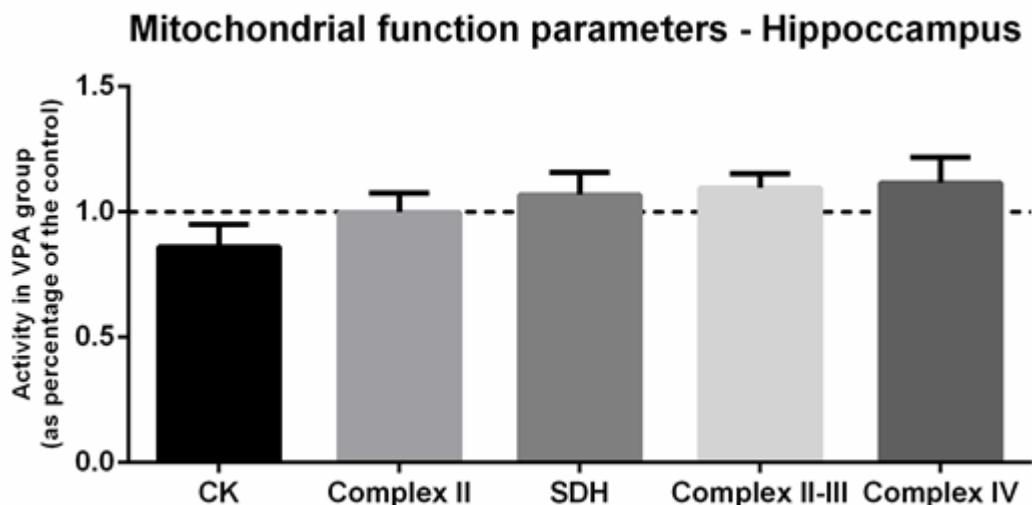
Figure 3. Mitochondrial activity parameters in hippocampus of animals prenatally treated with VPA. The values are expressed as percentage of the control. The dashed line represents 100%, the control value. There is no statistically significant difference between the enzymatic activities in VPA animals in comparison to controls. CK – Creatine Kinase. SDH – Succinate dehydrogenase. $n_{control} = 4$; $n_{VPA} = 5$.

Mitochondrial function parameters - Cerebellum
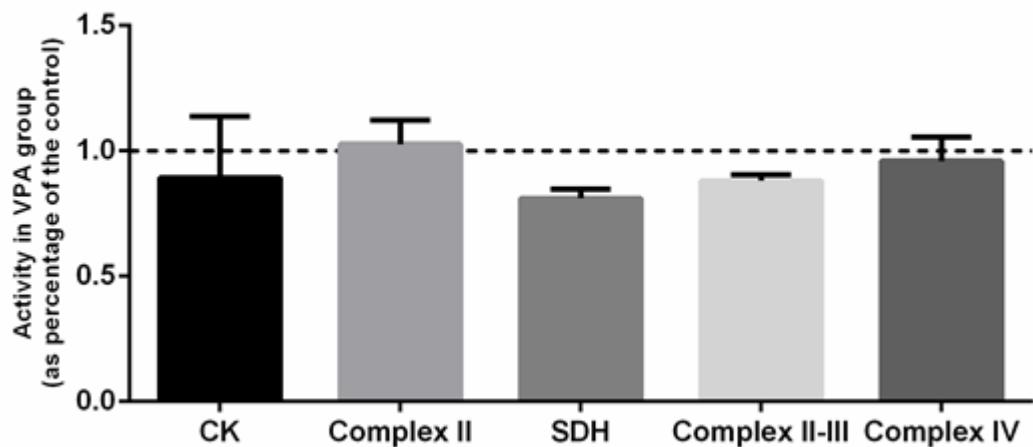
Figure 4. Mitochondrial activity parameters in cerebellum of animals prenatally treated with VPA. The values are expressed as percentage of the control. The dashed line represents 100%, the control value. There is no statistically significant difference between the enzymatic activities in VPA animals in comparison to controls. CK – Creatine Kinase. SDH – Succinate dehydrogenase. For SDH, Complex II e Complex II-III: $n_{control} = 4$; $n_{VPA} = 5$. For CK: $n_{control} = 8$; $n_{VPA} = 8$. For Complex IV: $n_{control} = 9$; $n_{VPA} = 10$.
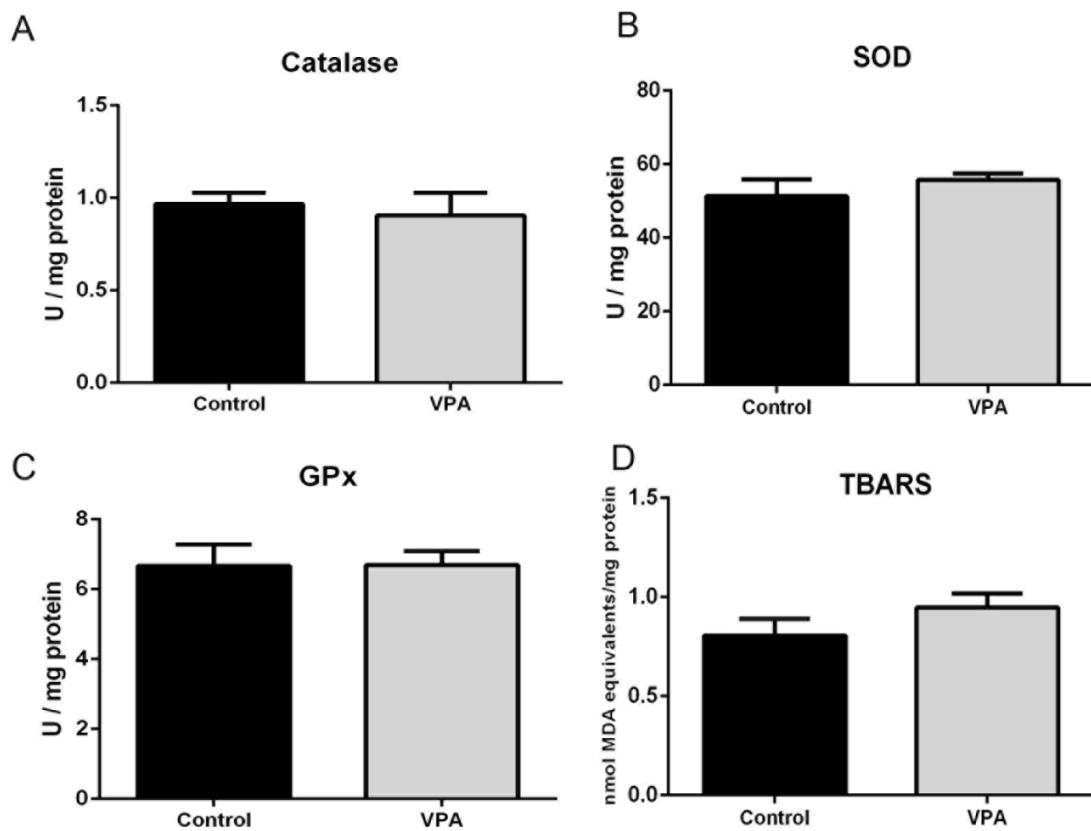
Figure 5. Analysis of the oxidative profile of the hippocampus in the animal model of autism induced by VPA. The activities of the antioxidant enzymes a) Catalase CAT, b) Superoxide Dismutase (SOD) e c) Glutathione Peroxidase (GPx) were evaluated, as well as the levels of d) lipid peroxidation (measured by the technique of the tiobarbituric acid reactive substances– TBARS). There is no statistically significant difference between groups. For Catalase e TBARS: $n_{control} = 4$; $n_{VPA} = 4$. For SOD: $n_{control} = 3$; $n_{VPA} = 4$. For GPx: $n_{control} = 5$; $n_{VPA} = 5$.
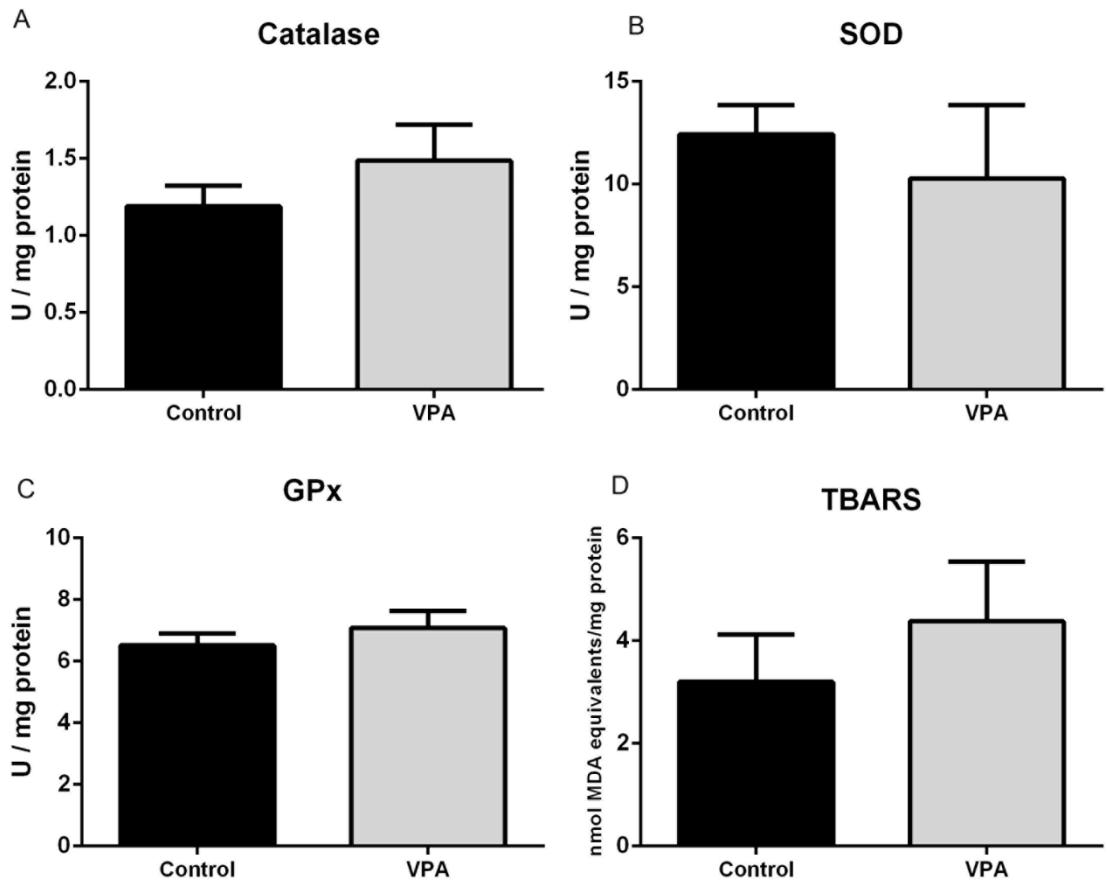
Figure 6. Analysis of the oxidative profile of the cerebellum in the animal model of autism induced by VPA. The activities of the antioxidant enzymes a) Catalase CAT, b) Superoxide Dismutase (SOD) e c) Glutathione Peroxidase (GPx) were evaluated, as well as the levels of d) lipid peroxidation (measured by the technique of the tiobarbituric acid reactive substances–TBARS). There is no statistically significant difference between groups. $n_{control} = 5$; $n_{VPA} = 5$.

Material and Methods

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Sample preparation

Male pups from at least three different litters were euthanized at postnatal day 120. The brain was removed and the hippocampus and cerebellum were isolated. A slice 2mm thick of the cerebellum containing the central region of the vermis and of the two cerebellar hemispheres was removed and stored at -80 °C for activity analysis of mitochondrial complexes. The remaining samples were homogenized in Phosphate Buffer Saline (PBS) and stored at -80 °C until the enzymatic analysis. For the determination of the activities of the respiratory chain complexes I–III, II, II–III and IV, cerebral cortex was homogenized (1:20, w/v) in SETH buffer (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 IU·mL−1heparin), pH 7.4.

Respiratory chain complex I–IV activities

The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate/cytochrome c oxidoreductase (complexes II–III) were determined according to Fischer (J. C. Fischer et al., 1985). The activity of cytochrome c oxidase (complex IV) was assayed according to Rustin (P. Rustin et al., 1994). The methods used to measure these activities were slightly modified, as described in detail in a previous report (C. G. da Silva et al., 2002). The activities of the respiratory chain complexes were calculated as $nmol \cdot min^{-1} \cdot mg$ protein^{-1} and the results were expressed as a percentage of control.

Creatine kinase (CK) activity

The activities of tCK, mCK and cCK were measured according to Hughes (B. P. HUGHES, 1962) with slight modifications (C. G. da Silva et al., 2004). In brief, the reaction mixture consisted of 50 mM Tris buffer, pH 7.5, containing 7.0 mM phosphocreatine, 7.5 mM $MgSO_4$, and cortical preparations in a final volume of 0.1 mL. Sulfite or thiosulfate was added to the medium and submitted to a pre-incubation at 37 °C for 30 min. The reaction was then started by the addition of 4.0 mM ADP and stopped after 10 min by the addition of 0.02 mL of 50 mM p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (B. P. HUGHES, 1962). The color was developed by the addition of 0.1 mL of 20%α-naphthol and 0.1 mL of 20% diacetyl in a final volume of 1.0 mL and read after 20 min at $\lambda = 540$ nm. Results were calculated as μmol of creatine$\cdot min^{-1} \cdot mg$ protein^{-1}and expressed as a percentage of control.

Tiobarbituric acid reactive species (TBARS) assay

As an index of lipid peroxidation, TBARS formation was measured using a hot acid reaction. The homogenates of hippocampus or cerebellum were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and 0.5 mL of 0.67% thiobarbituric acid, and heated in boiling water for 25 min (H. H. Draper and M. Hadley, 1990) . The levels of TBARS were spectrophotometrically determined at 532 nm. Results are expressed as nmol MDA equivalents/mg protein.

Superoxide dismutase (SOD) activity assay

SOD activity was quantified by the inhibition of superoxide dependent adrenaline auto-oxidation to adrenochrome using spectrophotometric measurements at 480 nm, as previously described (H. P. Misra and I. Fridovich, 1972). The results are expressed as absorbance/time (s). The area under the curve of the graph was used for statistical analysis and compared against the control values. Five units of CuZn-SOD (E.C.:1.15.1.1) were used to determine assay specificity.

Catalase (CAT) activity assay

CAT (E.C.:1.11.1.6) activity was assayed as previously described (H. Aebi, 1984) by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 50µgprotein. One unit (U) of the enzyme is defined as 1µmol of H_2O_2 consumed per minute and the specific activity is reported as U/mg protein.

Glutathione Peroxidase (GPx) activity assay

The GPx activity was evaluated as previously described by Flohé, L. and Gunzler (L. Flohé and W. A. Günzler, 1984). Briefly, the rate of NAD(P)H oxidation was measured in a spectrophotometer at 340 nm in the presence of reduced glutathione, tert-butyl hydroperoxide and glutathione reductase.

Statistical analysis

Data are reported as mean ± standard error from mean (SEM) and were analyzed by Student's t-test. Values of $p < 0.05$ were considered significant. All analyses were performed using the SPSS program, Version 17.0 (SPSS, Chicago, IL).

5.5. Comentário técnico - "COMMENT ON "OXYTOCIN-MEDIATED GABA INHIBITION DURING DELIVERY ATTENUATES AUTISM PATHOGENESIS IN RODENT OFFSPRING."

5.5.1. Status

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5.5.2 Publicação

TECHNICAL COMMENT

DEVELOPMENTAL NEUROLOGY

Comment on "Oxytocin-mediated GABA inhibition during delivery attenuates autism pathogenesis in rodent offspring"

Victorio Bambini-Junior,[1,2]* Gustavo Della Flora Nunes,[1,2] Tomasz Schneider,[3] Carmem Gottfried[1,2]

Tyzio *et al.* (Reports, 7 February 2014, p. 675) reported that bumetanide restored the impaired oxytocin-mediated γ-aminobutyric acid (GABA) excitatory-inhibitory shift during delivery in animal models of autism, ameliorating some autistic-like characteristics in the offspring. However, standard practices in the study of these models, such as the use of sex-dimorphic or males-only analyses and implementation of tests measuring social behavior, are lacking to definitely associate their findings to autism.

Autism spectrum disorders (ASD) are a group of neurodevelopmental disabilities characterized by sociability impairments, communication deficits, and stereotyped behavioral patterns (*1*). The etiology of autism is still not known, and current treatment options provide only a mild relief to some aspects of this condition. Nevertheless, there is mounting evidence that an excitation/inhibition imbalance plays a crucial role in the pathology of ASD (*2*). Tyzio *et al.* (*3*) recently reported that bumetanide maternal pretreatment was able to restore physiological levels of intracellular chloride in CA3 hippocampal neurons in two different animal models of autism: rats prenatally exposed to valproate (VPA) and Fmr1 knock-out mice (FRX). As a consequence, excitatory actions of γ-aminobutyric acid–mediated (GABAergic) signaling were reduced and electroencephalographic patterns were normalized. In addition, bumetanide treatment of pregnant females reversed aberrant maternal-separation–induced ultrasonic vocalization in the offspring. The authors also stressed the importance of oxytocin in the developmental GABA switch from excitatory to inhibitory, because prenatal treatment with the oxytocin receptor antagonist SSR126768A in naïve animals triggered alterations similar to those observed in VPA rats and FRX mice. The use of both genetic and drug-induced autism models strengthen their

discovery, which is also supported by their previous finding of bumetanide efficacy in a clinical trial in children with autism (*4*). However, we strongly feel that some technical issues of their work remained unaddressed and that the final conclusions of this work would be greatly improved by resolving those issues.

One of the most relevant features of autism is the difficulty to establish and maintain reciprocal social interactions with peers. These disabilities in social skills are mimicked in both VPA rats (*5*) and FRX mice (*6*) and can be detected by behavioral testing. Tyzio *et al.* did not evaluate social parameters in the rodents used in their work. We understand that, in principle, impaired ultrasonic vocalizations (USV) can lead to sociability deficits, but this has not been proved yet, and changes in USV in pups may be modulated by several factors not related to social domain— for example, temperature, sensitivity to pain, or general distress (*7*). Because social behavior requires the integration of a wide variety of neural circuits related to diverse aspects of sensory and cognitive functions (*8*), they have to be studied in much more elaborate ways—for example, using reciprocal social interaction or social preference tests, possibly in combination with USV recording. We believe that analyses of social behavior and USV emitted during social tasks are necessary to improve the quality and confidence of their data.

It is also important to verify whether the effects of bumetanide and SSR126768A persist over time. To clarify whether those are temporary or long-lasting modifications, the authors should extend the evaluation of the behavioral and electrophysiological phenotypes into different developmental stages, including adulthood and, possibly,

the end of the first postnatal week as a rough equivalent of birth-stage development in humans (*9*).

Another factor that needs to be taken into consideration to fully understand the relevance of Tyzio *et al.*'s results for autism is the use of both male and female rodents in all experiments. The higher prevalence of ASD in males is very well established, and some animal models of autism also show this male bias. The VPA animal model of autism has demonstrated solid evidence of sex-specific behavioral and morphological outcomes. The VPA rats were extensively analyzed, and most of the autistic-like features, including sociability deficits, were not detected in females (*10, 11*). In fact, VPA female rats are sometimes used in comparison to males to determine gender-specific alterations, which are likely to be more relevant to autism pathophysiology (*11, 12*). In addition, there is no complete characterization of autistic-like features in female FRX mice, because the vast majority of researchers use only males in their experiments (*13–15*). Thus, the authors should consider sex, including in embryos, as a factor in their data analysis, and therefore increase the number of animals per sex per group. Without taking this into account, the results are affected by the possibility of misbalanced numbers of males and females per group.

The concepts analyzed by Tyzio *et al.* have an enormous potential to help the development of future studies and can represent a turning point in the research of the etiology of ASD. However, giving the topic's prominence and impact, we believe that our suggestions are important to clarify key aspects discussed by them. It is still premature to think of bumetanide as a prenatal intervention for ASD, but it can be regarded as a meaningful research tool of the molecular underpinnings of this condition.

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[1]Federal University of Rio Grande do Sul, Research Group in Neuroglial Plasticity at the Department of Biochemistry, Institute of Health's Basic Science, Porto Alegre, Rio Grande do Sul, Brazil. [2]Federal University of Rio Grande do Sul, Translational Research Group in Autism Spectrum Disorders (GETTEA), Porto Alegre, RS, Brazil. [3]School of Medicine, Pharmacy and Health, TS17 6BH, Durham University, Durham, UK.
*Corresponding author. E-mail: victoriobambini@gmail.com

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6. DISCUSSÃO

Diversos mecanismos já foram propostos para tentar explicar a patofisiologia do autismo, tais como: gênicos (R. Muhle, S. V Trentacoste, and I. Rapin, 2004), imunológicos (C. Onore, M. Careaga, and P. Ashwood, 2012) e periféricos (Paul Ashwood et al., 2003; S. Fatemi, J. Stary, and E. Egan, 2002; J. White, 2003). Entretanto, parece não existir uma explicação única e os estudos atuais falham ao tentar estabelecer o fator determinante para o desencadeamento do TEA (B. Abrahams and D. Geschwind, 2008; M. Talkowski, 2014; E. Fombonne, 2008; R. Tuchman, S. Moshé, and I. Rapin, 2009; V. M. Yau et al., 2014).

Por exemplo, é inegável a existência de um importante componente genético relacionado ao transtorno (R. Muhle, S. V Trentacoste, and I. Rapin, 2004). Além das evidências de estudos com gêmeos (R. Rosenberg, 2009; J. Hallmayer and S. Cleveland, 2011; A. Ronald, F. Happé, and P. Bolton, 2006), pais que tiveram uma criança com TEA tem uma chance de 2% a 18% de ter uma segunda criança afetada (S. Ozonoff, G. Young, and A. Carter, 2011; S. Sumi et al., 2006). Porém, mesmo os mais de 200 genes e alterações genéticas já relacionadas com TEA são capazes de explicar apenas 25% dos diagnósticos (G. Huguet, E. Ey, and T. Bourgeron, 2013).

Tendo em vista a crescente prevalência de TEA e a falta de informações para justificar esse aumento, um dos campos de pesquisa mais importantes na área do autismo é a elucidação do papel de fatores ambientais em seu desencadeamento (A. Grabrucker, 2012; B. Gadad and L. Hewitson, 2013). Nesse contexto, desde sua proposição inicial por Rodier et al em 1997 (P. M. Rodier et al., 1997), o modelo animal de autismo induzido pela exposição pré-

natal ao VPA vem se estabelecendo como um método de estudos amplamente utilizada (M. Mehta, M. Gandal, and S. Siegel, 2011; M. Favre and T. Barkat, 2013; C. L. Yochum et al., 2008). Nosso grupo de pesquisa investiga o modelo animal de autismo induzido por VPA desde 2008 e tem papel importante nesse contexto, fato evidenciado pelo número de citações que nossos 2 primeiros artigos utilizando o modelo animal de autismo (Bambini-Junior et al., 2011; Bristot Silvestrin et al., 2013) apresentam. Aproximadamente 40 citações pela plataforma "scholar.google.com" e 26 citações pela plataforma "Scopus".

Devido à experiência acumulada e os artigos publicados na área (Bambini-Junior et al., 2011; Bristot Silvestrin et al., 2013), fomos convidados a escrever um capítulo no livro "Comprehensive Guide to Autism" (V. Patel, V. Preedy, and C. Martin, 2014) sobre a relação da exposição pré-natal ao valproato em roedores e autismo. Como um dos objetivos iniciais desta tese era realizar esta ampla revisão bibliográfia em busca de áreas mais promissoras de investigação, este convite possibilitou aproveitarmos este material de pesquisa para ser publicado em forma de capítulo de livro. O capítulo "Prenatal Exposure to Valproate in Animals and Autism", na íntegra como Capítulo 1 da presente tese, discorre sobre o surgimento, a importância e os achados, até o presente momento, no modelo animal de autismo induzido pela exposição pré-natal ao VPA. Dessa forma, representa um importante levantamento histórico e bibliográfico do desenvolvimento e utilização do referido modelo animal (V Bambini-Junior and D. Baronio, 2014).

Destaca-se também que modelos animais confiáveis devem apresentar três características principais: Validade de construto, validade de face e validade

preditiva (J. N. Crawley, 2008). Validade é definida como a extensão na qual uma avaliação mede precisamente o que deveria medir.

Validade de construto significa que o modelo animal reproduz uma circunstância que, em humanos, leva a determinada condição. Validade de face é quando o modelo animal reproduz padrões encontrados na condição estudada. Validade preditiva é a similaridade de reações e respostas fisiológicas entre o modelo e o ser humano, quando expostos a condições correspondentes.

O critério de validade de construto é plenamente satisfeito pelo modelo animal de TEA por exposição pré-natal ao VPA, já que ele baseia-se na utilização de um conhecido fator de risco para o desencadeamento de TEA como o agente indutor das características do tipo-autista em roedores (R. L. Bromley et al., 2013). Estudos analisando o uso de VPA durante a gestação em humanos não conseguem limitar uma faixa estreita relacionando TEA com dose e período crítico de utilização do fármaco. Dessa forma, a concordância nos achados em estudos com roedores, que utilizam inúmeras doses de VPA, períodos gestacionais para indução variados e diversas vias de administração do fármaco, reforçam a extraordinária validade de construto do modelo da exposição pré-natal ao VPA. A tabela 1, abaixo, apresenta resumidamente os métodos utilizados para indução do modelo animal.

Tabela 1. Estudos com modelo animal de TEA induzido pela exposição pré-natal ao VPA plotados conforme o roedor utilizado, o dia e a dose da indução e o procedimento adotado para exposição.

Estudos	Dia de indução	Roedor	Dose	Procedimento
Bambini-Junior et al, 2011	E12,5	Rato	600	Injeção (i.p.)
Cusmano e Mong, 2014	E12,5	Rato	400	Injeção (i.p.)
Dendrinos et al , 2011	E12,5	Rato	400 a 600	Injeção (i.p.)
Downing et al , 2010	E9	Camundongo	200 a 800	Injeção (i.p.)
Dufour-Rainfray et al, 2010	E9	Rato	600	Injeção (i.p.)
Fatemi et al, 2008	E0 a E21	Rato	300	Injeção (i.p.)
Felix-Ortiz and Febo, 2012	E12,5	Rato	600	Injeção (i.p.)
Foley et al, 2012	E12,5	Rato	600	Injeção (i.p.)
Fucic et al, 2010	E12 a E14	Camundongo	100	Injeção (i.p.)
Gandal et al, 2010	E13	Camundongo	600	Injeção (sc)
Go et al, 2011	E12	Rato	400	Injeção
Hara et al, 2012	E12.5	Camundongo	500	Injeção
Ingram et al, 2000	E12.5	Rato	600	Injeção (i.p.)
Kataoka et al, 2011	E9, E12,5 or E14,5	Camundongo	500	Injeção
Kim et al, 2011	E7, E9,5, E12 or E15	Rato	400	Injeção
Kim et al, 2013	E12	Rato	400	Injeção
Kolozsi et al, 2009	E11	Camundongo	800	Oral (em manteiga de amendoim)
Kuwagata et al, 2009	E9 or E11	Rato	800	Oral (gavagem)
Lin et al, 2013	E12,5	Rato	500	Injeção (i.p.)
Lukose et al, 2011	E12,5	Rato	600	Injeção
Manent et al, 2007	E14 a E20	Rato	100	Injeção (i.p.)
Mehta et al, 2011	E13	Camundongo	600	Injeção (sc)
Miyazaki et al, 2005	E9	Rato	800	Oral (tubo para alimentação)
Narita et al, 2002, 2010	E9	Rato	800	Oral (tubo para alimentação)
Rinaldi et al, 2007	E12,5	Rato	500	Injeção (i.p.)
Rodier et al, 1997	E11,5, E12 or E12,5	Rato	350	N/A
Roullet et al, 2010	E11	Camundongo	800	Oral (em manteiga de amendoim)
Schneider et al, 2006	E12,5	Rato	600	Injeção (i.p.)
Tsujino et al, 2007	E9	Rato	800	Oral (tubo para alimentação)
Tyzio et al, 2014	E12,5	Rato	600	Injeção (i.p.)

Em relação à validade de face, testes com os animais VPA demonstraram similaridades em diferentes aspectos diretamente relacionados ao TEA (Victorio Bambini-Junior et al., 2011; M. R. M. Favre et al., 2013; R. Bristot Silvestrin et al., 2013). Dentre os critérios comportamentais utilizados para diagnosticar esse transtorno, já foram descritas extensas análises observando alterações correspondentes tanto aos déficits sociais quanto a presença de estereotipia e comportamentos repetitivos nos roedores VPA (Victorio Bambini-Junior et al., 2011; F. Roullet, J. Lai, and J. Foster, 2012; F. Roullet et al., 2010). Por si só, essas observações justificariam a utilização de um modelo animal (D. B. Jr and D. Hatton, 2001). Entretanto, algumas das variadas nuances do TEA encontram relação direta com o modelo VPA (N. Tsujino et al., 2007; D. M. Cusmano and J. A. Mong, 2014; T. Schneider and R. Przewłocki, 2005). Por exemplo, a bem documentada predominância de autismo em homens encontra paralelo direto no modelo animal, onde as fêmeas nascidas das mesmas ninhadas do modelo animal de TEA não apresentam os padrões comportamentais característicos do transtorno (K. Kim et al., 2013; T. Schneider et al., 2008). De fato, esse dimorfismo sexual é tão saliente que as fêmeas remanescentes da indução, são utilizadas, em alguns trabalhos, como animais controle (C. de Theije and P. Koelink, 2014).

Em um modelo com validade preditiva, uma intervenção bem-sucedida no animal seria também eficiente na situação correspondente em um ser humano. Dessa forma, o modelo animal adequado poderia fornecer informações seguras para a translação interespécie de um tratamento (J. N. Crawley, 2008). É argumentável, porém, que validade de face e validade preditiva tratam-se do

mesmo fenômeno, sendo que a única diferença é se a descoberta inicial ocorreu no modelo animal ou em pacientes.

Dessa forma, diante da enorme quantidade de evidências atestando as similaridades entre o modelo animal induzido pela exposição pré-natal ao VPA e o TEA (T. Schneider and R. Przewłocki, 2005; T. Schneider et al., 2008; Victorio Bambini-Junior et al., 2011; K. Kim et al., 2013), surgiu naturalmente uma tendência a análises de efetividade de tratamentos experimentais em características comportamentais dos animais VPA. Diversos tratamentos administrados pós-natalmente reverteram características comportamentais descritas no modelo VPA. Exemplos: (1) Diferentes inibidores de acetilase de histonas foram eficazes em restaurar déficits em comportamento social (A. G. Foley, A. W. Cassidy, and C. M. Regan, 2014; A. G. Foley et al., 2012); (2) O tratamento com *Bacopa monniera*, uma planta amplamente utilizada em terapias ayurveda (sistema medicinal indiano, desenvolvido a partir do conhecimento empírico-tradicional), foi capaz restaurar padrões alterados de sociabilidade e marcadores de estresse oxidativo (T. Sandhya, J. Sowjanya, and B. Veeresh, 2012); (3) O tratamento subcrônico com donepezil (um inibidor de acetilcolinesterase) foi capaz de recuperar alterações em comportamentos sociais, hiperatividade e estereotipias (J.-W. Kim et al., 2014); (4) O tratamento com d-Cicloserina (um antibiótico utilizado em infecções de *Mycobacterium tuberculosis*, com atividade agonista em receptores NMDA) melhora as alterações sociais (K. A. Wellmann, E. I. Varlinskaya, and S. M. Mooney, 2014); (5) O tratamento com piperina (um alcalóide que dá sensação picante da pimenta preta e com descrito efeito neuroportetor) reverte alterações comportamentais e de marcadores de estresse oxidativo (B. Pragnya, J. S. L. Kameshwari, and B.

Veeresh, 2014); (6) Dados de Ahn *et al.,* corroborados por estudos do nosso grupo (dados não publicados, artigo em preparação), demonstram que dieta cetogênica é capaz de modificar parâmetros sociais e metabólicos (Y. Ahn et al., 2014) e; (7) Baronio *et al.,* demonstrou que o tratamento com o inbidor seletivo do receptor de histamina H3, ciproxifan, é capaz de reverter alterações sociais e estereotipias (artigo submetido).

Ainda, alguns artigos avaliaram a eficácia de tratamentos pré-natais sobre o desenvolvimento de características do tipo-autista nos animais VPA (P. Kim et al., 2013; R. Tyzio et al., 2014). Kim e colaboradores evidenciaram que o extrato de ginseng vermelho coreano exerce um efeito dose dependente, ao evitar tanto o desencadeamento de alterações sociais quanto o fenótipo de "rabo torto" característico dos animais VPA (P. Kim et al., 2013). Apesar da relevante constatação, o tratamento foi executado com um extrato de ginseng, contendo dezenas de possíveis compostos ativos, complicando assim investigações das rotas que medeiam esse efeito protetor. Tyzio e colaboradores (R. Tyzio et al., 2014), elegantemente demonstram que mudanças no padrão de shifting de GABA, mediado por ocitocina, durante o parto estão relacionados com comportamentos do tipo-autista e alterações eletroencefalográficas em dois modelos animais de autismo, sendo um deles, o modelo VPA. Apesar da proeminência e dos importantes achados, alguns aspectos técnicos desse artigo não são completamente apropriados.

O Capítulo 2 da presente tese apresenta um artigo onde foi investigada a influência do tratamento pré-natal com RSV em comportamentos sociais no modelo animal de autismo, induzido pela exposição pré-natal ao VPA. Atualmente, estamos estendendo a quantidade de comportamentos analisados

para compreendermos quais padrões comportamentais são prevenidos pelo tratamento pré-natal com RSV. Cabe destacar que o cunho principal dessa proposta é a possibilidade de análise de vias afetadas por ambos VPA e RSV, facilitando desse modo o entendimento dos gatilhos etiológicos e de outros fatores que modulam os aspectos neurocomportamentais do modelo animal. Como pode ser visto nas tabelas 2, 3 e 4, VPA e RSV possuem diversos alvos intracelulares pelos quais podem exercer os mais variados efeitos. Sendo assim, a avaliação de alvos e processos celulares que demonstram resposta oposta para VPA e RSV é uma perspectiva particularmente promissora. Este trabalho resultou no depósito da patente descrita no capítulo 3, sob número BR1020120293820, visando garantir nossa continuidade do estudo. Essa patente relaciona o uso de RSV na gestação com a prevenção do surgimento das características do tipo-autista na prole.

Tabela 2. Levantamento dos alvos diretos confirmados do VPA e RSV.

Alvos diretos

Alvos	VPA	Resveratrol
Fosfodiesterases (PE1, PE3 and PE4)	↑[1]	↓[2]
Sirt1	-	↑[3]
Sirt2	↑[4]	↑[5]
Sirt3	↑[6]	↓[3]
Sirt4	↑[4]	↓[7] ↑[8]
Sirt5	-	↑[3]
Sirt6	-	
Sirt7	↑[4]	↑[9]
GABA transaminase (ABAT)	↓[10]	-
Succinato semialdeído desidrogenase (ALDH5A1)	↓[11]	-
GAD (glutamic acid decarboxylase)	↓[12]; = or ↑[12]	-
a-cetoglutarato desidrogenase (OGDH)	↓[13]	-
HDAC Clássicas	↓[14]	↓[15]

Alvos diretos descritos do VPA e RSV. Os números em sobrescrito representam a referência e as flechas indicam aumento ou diminuição da atividade ou expressão da molécula descrita. *Devido a incompatibilidade de formatação de citação, as referências das tabelas encontram-se em uma seção a parte (Referências Tabelas)

Tabela 3. Levantamento dos alvos indiretos e das vias intracelulares afetadas por VPA e RSV.

Alvos indiretos e vias intracelulares

Alvos	VPA	Resveratrol
PI3K/Akt/mTOR	↑[16,17] ↓[19]	↑[18] ↓[20]
Adenilato ciclase / PKA/ AMPK	↑[20]	↑[2,21]
NF-κB	↑[22,23] ↓[26,27]	↓[24,25]
ERK – MAPK	↑[28,29]	↓[18,30] ↑[31,32]
p53	↑[33,34]	↑[35–37]
Survivina	↓[38,39]	↓[40–42]
Cox	↓[43,44]	↓[45,46]
Aromatase	↓[47,48]	↓[49,50]
Ativador do plasminogênio tecidual (tPA)	↑[51,52]	↓[53,54]
Jak / STAT	↑[55]	↓[56,57]
Glicogênio sintase cinase 3 β (GSK3-β)	↓[58,59]	↓[60,61]
Wnt/β-catenin	↑[62,63]	↓[64–66]
Receptor de estrogênio	↑[67] ↓[4]	↑[68,69] ↑ or ↓ [70]
Oxido nítrico sintase	↓[71] or =[72]	↑[73,74]
Notch	↑[75]	↑[76]
Sonic hedgehog	↓[77] or =[78]	↓[79,80] ↑[81]

Alvos indiretos e vias intracelulares afetadas por VPA e RSV. Os números em sobrescrito representam a referência e as flechas indicam aumento ou diminuição da atividade ou expressão da molécula descrita. *Devido a incompatibilidade de formatação de citação, as referências das tabelas encontram-se em uma seção a parte (Referências Tabelas)

Tabela 4. Processos celulares influenciados por VPA e RSV.

Processos celulares

Target	VPA	Resveratrol
Proliferação	↓[82–84]	↓[85,86]
Diferenciação	↑[84,87]	↑[88–90]
Neurogênese	↑[91,92]	↓[93,94] ↑[95,96]
Gliogênese	↓[87,97]	↑[98]
Desenvolvimento de neuritos	↑[51,99]	↑[90,100]
Apoptose	↑[101–104]	↑[105–107] ↓[108–110]
Autofagia	↑[111,112] ↓[114]	↓[113] ↑[115,116]
Inflamação	↑[117] ↓[122–124]	↓[118–121]
Angiogenese	↓[125,126]	↓[127–129] ↑[129,130]
Estresse oxidative	↑[111,131,132]	↓[118,133,134] ↑[135,136]
Função mitochondrial	↓[137–140]	↑[141–143]
Mielinização	↑[92,144]	↑[145,146]
Migração neural/glial	↓[147,148]	↑[149]
Sinapse inibitória	↓[150,151] ↑[151,152]	-
Sinapse excitatória	↑[152,153] ↓[151,155]	↓[154] ↑[156]
Ativação microglial	↓[157,158]	↓[159,160]
Astrogliose	↑[161] ↓[157,163]	↓[162]
Hipóxia	↓[164,165]	↓[166–168]

Processos celulares influenciados VPA e RSV. Os números em sobrescrito representam a referência e as flechas indicam aumento ou diminuição da atividade ou expressão da molécula descrita. *Devido a incompatibilidade de formatação de citação, as referências das tabelas encontram-se em uma seção a parte (Referências Tabelas).

Nesse contexto, estamos avaliando o papel das sirtuínas, um dos alvos mais caracterizado do RSV. As sirtuínas são um grupo de proteínas pertencentes à família das deacetilases de histonas NAD$^+$-dependentes (HDACs), sendo altamente conservadas ao longo da evolução (J.-E. Choi and R. Mostoslavsky, 2014). Nos mamíferos, as sirtuínas possuem 7 classes (Sirt 1-7) com localizações celulares específicas. As Sirt1 e Sirt2 se destacam pelo seu papel na diferenciação e migração de certos tipos neuronais. Mecanismos para esse processo foram descritos para Sirt1, a qual promove o crescimento de neuritos e o aumento da sobrevivência celular, via redução da sinalização da mTOR (W. Guo et al., 2011), rota intimamente relacionada ao TEA (A. L. Numis et al., 2011; P. Baker, J. Piven, and Y. Sato, 1998; S. Smalley and P. Tanguay, 1992).

O VPA exerce uma destacada atividade inibidora de HDACs da cromatina (M. Göttlicher et al., 2001; C. J. Phiel et al., 2001) e pelo menos parte de seus efeitos relacionados à indução do modelo animal de TEA devem-se a essa característica. Isso porque dados apontam que a exposição pré-natal a fármacos com ação anti-epiléptica, mas que não possuem ação inibidora de HDAC, não aumentam o risco de desencadear TEA (Caroline Nadebaum et al., 2011; C Nadebaum et al., 2011). Em relação ao RSV, apesar de seu mecanismo de ação ser pouco compreendido, são descritos efeitos sobre as sirtuinas (M. Lagouge et al., 2006; S. Mukherjee et al., 2009; H. Schirmer et al., 2012; K. Suzuki and T. Koike, 2007; O. Vakhrusheva et al., 2008; W. Yu et al., 2009).

Nossos resultados preliminares avaliando a expressão gênica das Sirts em sangue e hipocampo dos animais expostos pré-natalmente ao VPA e ao RSV, são inconclusivos. Assim, objetivamos avaliar um maior número de animais por grupo e também executar análises em estruturas encefálicas adicionais como

cerebelo e estriado. Convergentemente, visamos medir a atividade das Sirts em todos os tecidos citados.

Quando comparados os efeitos antagônicos de VPA e RSV, outro tema promissor remanescente foi a avaliação de parâmetros mitocondriais e de estresse oxidativo nos animais submetidos pré-natalmente a essas moléculas (M. F. B. Silva et al., 2008; Vincent Tong et al., 2005; B. Fromenty and D. Pessayre, 1997; I. Jafarian et al., 2013; J. P. Draye and J. Vamecq, 1987; M. Lagouge et al., 2006; V. Desquiret-Dumas et al., 2013; S. Davinelli et al., 2013; A. Ferretta et al., 2014). Entretanto, nossos achados iniciais indicaram que nem mesmo o modelo animal VPA apresentava alterações significativas nessas vias. Dessa forma, consideramos que o desenvolvimento de características do tipo-autista no modelo animal de TEA induzido por VPA, não esteja diretamente relacionada à atividade mitocondrial em roedores adultos e, por isso, não analisamos os grupos submetidos ao RSV.

Desde os achados comportamentais iniciais, por Schneider e Przewłocki, muitas informações relevantes foram determinadas a partir dos estudos com animais VPA. Porém, apesar de todas as validações pertinentes ao TEA observadas nos roedores expostos pré-natalmente ao VPA, em março de 2013 um interessante levantamento estatístico expôs um problema intrínseco relacionado às análises utilizando esses animais (S. E. Lazic and L. Essioux, 2013). Lazic e Essioux evidenciaram que nesse tipo de experimento, onde o tratamento é administrado na mãe, o "n" amostral deve ser considerado a partir do número de ninhadas, e não do total de animais testados. O efeito de ninhada é muito forte e ignorá-lo contribui para o baixo número de estudos *in vivo* convertidos em terapias efetivas. Dessa forma, estamos reconduzindo (e

incluindo maior número de análises) os experimentos comportamentais com os animais expostos durante o período embrionário ao RSV e ao VPA.

Além de tudo, em parceria com o professor Tomasz Schneider, escrevemos um comentário técnico destacando as limitações do trabalho de *Tyzio et al.*, aceito para publicação na revista Science (Anexo 5). Tomasz Schneider foi o autor do primeiro artigo que descreveu alterações comportamentais do tipo-autista no modelo animal de autismo induzido por VPA (Schneider, 2005) e atualmente é um importante colaborador do grupo. Dentre os problemas encontrados no artigo "Oxytocin-mediated GABA inhibition during delivery attenuates autism pathogenesis in rodent offspring" (R. Tyzio et al., 2014) destacamos: (1) a ausência de avaliações de comportamentos sociais, (2) a falta de avaliações sequenciais para observar o estabelecimento dos efeitos e (3) o fato de ambos machos e fêmeas terem sido avaliados, uma vez que uma parte significativa dos achados em machos do modelo VPA não são encontrados nas fêmeas da mesma ninhada. A publicação desse comentário técnico reforça que estamos de fato integrados a nossa área de pesquisa e que o manejo do modelo animal de TEA induzido por VPA está consolidado em nosso grupo de tal forma, que nos permitiu realizar essa crítica mesmo em uma revista de altíssimo impacto.

Desde os primeiros trabalhos do nosso grupo com os animais pré-natalmente expostos ao VPA, muitas informações a respeito desse modelo foram observadas. Inegavelmente, um modelo como este é um método importante para os estudos do TEA. Porém, diversos desafios relacionados à utilização desses animais ainda perduram. Por exemplo, o mecanismo exato através do qual as características do tipo-autista são desencadeadas permanece

indefinido e a elucidação de como isso ocorre pode trazer dados extremamente úteis sobre a etiologia e patofisiologia do TEA.

De uma perspectiva translacional, a análise de gatilhos etiológicos pode ser relacionada com diversas possibilidades de tratamento para o TEA. Entretanto, todos os dados advindos de modelos animais devem ser tratados com extrema precaução, uma vez que em grande parte dos casos, seus achados não são diretamente aplicados a seres humanos.

7. CONCLUSÕES:

Capítulo 1) Conforme avaliação da literatura feita para a publicação do capítulo de livro, obtivemos dados relevantes sobre potenciais áreas a serem analisadas. Concluímos que seria de suma importância obter estratégias que pudessem prevenir as alterações comportamentais do modelo animal.

Capítulo 2) A partir das análises comportamentais, concluímos que o RSV previne o desencadeamento das características do tipo-autista do modelo animal induzido pela exposição pré-natal ao VPA. Além disso, constatamos que esses efeitos provavelmente se devem a efeitos celulares.

Capítulo 3) Concluímos também que seria de grande importância proteger de interesses puramente financeiros, os achados de trabalhos anteriores. Dessa forma, depositamos um pedido de patente resguardando a utilização ampla e geral dos dados, bem como a continuidade dos estudos.

Capítulo 4) Dados complementares

A abordagem preliminar dos dados relacionados com sirtuínas demonstra que estamos diante de mecanismos complexos que precisam ser melhor investigados, mas que provavelmente os efeitos comportamentais observados no modelo do VPA não tenham influência desta família de proteínas, ao menos do ponto de vista etiológico. Mas uma conclusão definitiva somente poderá ser possível quando pudermos ampliar as análises para outras idades e regiões encefálicas.

Capítulo 5) Comentário técnico

A oportunidade de ter trabalhado neste comentário técnico foi muito valiosa para reforçar a necessidade de manter constante atenção a literatura. Certamente este comentário estará reforçando nossos pensamentos futuros em busca de uma ciência cada vez melhor, com mais qualidade e relevância.

8. PERSPECTIVAS

8.1. Em animais pré-natalmente expostos ao VPA e ao RSV:

Finalizar a obtenção dos dados moleculares preliminares relacionados à expressão gênica, proteica e atividade de sirtuínas.

Analisar o balanço excitatório/inibitório (via expressão proteica de receptores de neurotransmissores) em regiões encefálicas como amigdala, hipocampo e córtex pré-frontal medial.

Avaliar os números absolutos e relativos de linfócitos CD3+CD4+, CD3+CD8+ e CD19+ em sangue, timo, baço e linfonodos.

Avaliar a expressão de receptores de glutamato, serotonina e GABA em células provenientes de sangue, timo, baço e linfonodos.

Avaliar as respostas TH1, TH2 e TH17 (através da aplicação de kit CBA), no SNC, sangue, timo, baço e linfonodos.

8.2. Em avaliações ex vivo de linfócitos

Avaliar em linfócitos expostos diretamente ao VPA *ex vivo*, morte e sinalização celular (rota de sinalização de mTOR) através de técnicas de citometria de fluxo e immunoblotting.

8.3. Em embriões expostos ao VPA e ao RSV:

Avaliar no SNC de embriões machos (sexados por PCR) expostos ao VPA, morte e sinalização celular (rota de sinalização de mTOR) através de técnicas de citometria de fluxo e immunoblotting.

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