UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIÓLOGICAS: BIOQUÍMICA

MAURO MOZAEL HIRSCH

MODELO ANIMAL DE AUTISMO INDUZIDO POR EXPOSIÇÃO PRÉ-NATAL AO ÁCIDO VALPROICO: ESTUDOS COMPORTAMENTAIS, MOLECULARES E ESTRATÉGIAS TERAPÊUTICAS

TESE DE DOUTORADO

Porto Alegre

2018

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIÓLOGICAS: BIOQUÍMICA

MODELO ANIMAL DE AUTISMO INDUZIDO POR EXPOSIÇÃO PRÉ-NATAL AO ÁCIDO VALPROICO: ESTUDOS COMPORTAMENTAIS, MOLECULARES E ESTRATÉGIAS TERAPÊUTICAS

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul como requisito parcial à obtenção do grau de Doutor em Bioquímica.

MAURO MOZAEL HIRSCH

ORIENTADORA: Prof.ª. Dr.ª. Carmem Gottfried

Porto Alegre, 2018

CIP - Catalogação na Publicação

Hirsch, Mauro Mozael
Modelo animal de autismo induzido por exposição pré-natal ao ácido valproico: estudos comportamentais, moleculares e estratégias terapêuticas / Mauro Mozael Hirsch. -- 2018.
154 f.
Orientadora: Carmem Gottfried.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2018.

1. transtorno do espectro autista. 2. comportamento animal. 3. microRNA. 4. resveratrol. 5. suramina. I. Gottfried, Carmem, orient. II. Título.

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

Agradecimentos

Agradeço à minha orientadora Carmem pela confiança e pela oportunidade, pelos conhecimentos que me passou e toda dedicação e amizade.

Agradeço aos meus colegas de laboratório, por todo o auxílio, conhecimentos, momentos de descontração, cultura inútil e por todas as comemorações, às vezes pelos motivos mais improváveis. À Mellanie por primeiramente me trazer para o grupo, e a todos os colegas e amigos pós-graduandos Gustavo, Guilherme, Brum, Bruna, Júlio e Matheus.

Um agradecimento especial para a minha colega PG, amiga e noiva, amor da minha vida, por todo companheirismo durante esse período de Doutorado, dentro e fora do ambiente acadêmico. Obrigado por todo apoio, parceria, amizade, amor e compreensão nos momentos que mais precisei.

Agradeço também aos alunos de graduação, atuais e também os que passaram pelo grupo: Walquíria, Gabriela, Giovanna, Rafael, Tayssa, Pedro, Marília e outros.

Agradeço à minha mãe e irmãos, por me darem todo suporte durante todo esse período na UFRGS. Obrigada pelo amor incondicional, pela paciência e pelo incentivo em tempo integral.

Agradeço ao professor Guilhian e seus alunos, colegas do laboratório 27, por todos os momentos de contribuição e descontração.

Agradeço à professora Florencia e seus alunos no laboratório 310, principalmente o Lucas, por todo apoio, auxílio na compreensão de dúvidas conceituais e de protocolos, por toda ajuda e conhecimento compartilhado.

Agradeço a todos os professores do PPG Bioquímica, e mesmo de outros PPG, por todo conhecimento que adquiri ao longo da pós-graduação, principalmente no curso das disciplinas.

Agradeço aos nossos colaboradores por todo auxílio, seja financeiro ou intelectual, ao longo do desenvolvimento dos trabalhos do grupo: Antonio Rasia-Filho, Rogerio Margis, Victorio Bambini-Junior, Rudimar Riesgo, Henning Ulrich, Tomasz Schneider.

Ao PPG Bioquímica, à secretaria do PPG pela excelente organização e agilidade, facilitando em muito nossa vida de pós-graduando, em especial à Cléia e Giordano.

Agradeço aos professores pesquisadores Renata Abib, Guilherme Baldo e Carlos Alberto Saraiva Gonçalves pelo aceite em ser banca desta tese e pelas excelentes contribuições ao longo dos trabalhos.

Às agências de fomento: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), Instituto Nacional de Ciência e Tecnologia em Neuroimunomodulação (INCT-NIM) e Fundo de Incentivo à Pesquisa e Eventos (FIPE-HCPA).

“A evolução consciente começa assim que tomamos a responsabilidade de remover nossas próprias barreiras.”

Dan Millman, 1946.

SUMÁRIO

PARTE I

RESUMO

O Transtorno do Espectro Autista (TEA), segundo o DSM-5, se enquadra nos Transtornos do Desenvolvimento e é caracterizado por uma díade comportamental: 1) prejuízos na comunicação e interação social e 2) comportamentos repetitivos ou estereotipados. Apesar da etiologia do TEA ser desconhecida, tanto fatores genéticos quanto ambientais já foram associados à desordem, incluindo a utilização de ácido valproico (VPA) durante a gestação. Observando essa relação importante, desenvolveu-se um modelo animal de autismo induzido pela exposição pré-natal ao VPA, o qual já foi amplamente validado em aspectos comportamentais e moleculares. No primeiro capítulo desta tese, utilizamos o modelo animal de autismo obtido através de uma única injeção intraperitoneal de VPA (600mg/kg) no dia E12,5 nas ratas Wistar prenhes e testamos um tratamento pré-natal com resveratrol (RSV), através de injeções subcutâneas (3,6 mg/Kg) administradas nas ratas prenhes nos dias E6,5-E18,5. O tratamento pré-natal com RSV demonstrou ser capaz de prevenir alterações na sociabilidade recíproca, porém não teve impacto nos prejuízos na memória olfativa e comportamentos repetitivos induzidos pelo VPA. No que se refere aos dados de microRNA (miRNA), RSV foi capaz de prevenir a alteração na expressão de miR134-5p, o qual também se observou alterado em pacientes com TEA, juntamente com o miR138-5p, ambos com alvos associados à modulação do citoesqueleto na estrutura de espinhos dendríticos. Em conjunto, esses resultados demonstram que o RSV altera vias importantes no modelo, possivelmente através das suas características antioxidantes e anti-inflamatórias, as quais contrapõem os aspectos inflamatórios do VPA. Este trabalho permitiu o aprimoramento e melhor conhecimento da técnica de RT-qPCR para análise de miRNA, sendo tema do segundo capítulo da presente tese, reunindo diferentes estratégias que auxiliaram a superar potenciais problemas e interferentes no uso dessa técnica. Finalmente, no terceiro capítulo, utilizamos o modelo VPA para avaliar o efeito do tratamento pós-natal com suramina, através de uma única injeção subcutânea (20 mg/kg) administrada nos filhotes machos na idade P30. Este tratamento foi capaz de reverter alterações de sociabilidade, novidade social e comportamento do tipo ansioso, enquanto os prejuízos na sociabilidade recíproca, comportamento exploratório, estereotipia e processamento sensorial não foram revertidos por esse tratamento. Nos dados moleculares, a suramina não foi capaz de reverter o aumento de expressão nos receptores purinérgicos P2X4 (hipocampo e córtex pré-frontal medial) e P2Y2 (hipocampo), porém reverteu o aumento de IL-6 promovido pelo VPA. Assim, possivelmente a modulação comportamental associada à suramina parece não estar associada a interações específicas nos receptores purinérgicos, mas sim com uma modificação neuroimune através da interleucina pró-inflamatória IL-6, demonstrando a importância do sistema imunológico na fisiopatologia do TEA. De forma geral, a tese contribuiu para elucidar mecanismos envolvidos no desenvolvimento das características do tipo autista, demonstrando o papel relevante das alterações neuroimunes e da modulação de alvos por miRNA, as quais, em conjunto, podem contribuir para o desenvolvimento de métodos diagnósticos e adequação de estratégias farmacológicas voltados ao TEA.

Palavras-chave: Comportamento animal, microRNA, neuroimune, PCR, resveratrol, sistema purinérgico, suramina, transtorno do espectro autista.

ABSTRACT

According to DSM-5, Autism Spectrum Disorder (ASD) is a developmental disorder characterized by a behavioral dyad: 1) deficits in communication and social interaction, and 2) repetitive and stereotyped behaviors. Although the etiology of ASD is still unknown, both genetic and environmental factors have been associated with the disorder, including the use of valproic acid (VPA) during gestation. Observing this important relationship, an animal model of autism induced by prenatal exposure to VPA was developed, which has already been widely validated in behavioral and molecular aspects. In the first chapter of this thesis, we used the animal model obtained through a single intraperitoneal injection of VPA (600 mg/kg) at E12.5 in pregnant Wistar rats and tested a prenatal treatment with resveratrol (RSV) by subcutaneous injections (3.6 mg/kg) administered in the pregnant rats at E6.5 to E18.5. The prenatal treatment with RSV was able to prevent changes in the reciprocal sociability, but had no impact on the deficits in olfactory memory and repetitive behavior induced by VPA. Regarding the microRNA (miRNA) data, RSV treatment was able to prevent the alteration in the expression of miR134-5p, which also was altered in ASD patients along with miR138-5p, both with targets associated with cytoskeletal modulation in the structure of dendritic spines. Taken together, these results demonstrate that RSV alters important pathways in the model, possibly through its antioxidant and anti-inflammatory properties, which counteract the inflammatory aspects of VPA. This work allowed the improvement and better knowledge of the RT-qPCR technique for miRNA analysis, which was the theme of the second chapter of this thesis, combining different strategies to overcome potential problems and interferences in this methodology. Finally, in the third chapter we used the same animal model to evaluate the effect of postnatal treatment with suramin after a single subcutaneous injection (20 mg/kg) administered to male pups at P30. This treatment was able to revert VPA-induced deficits in sociability, social novelty, and anxiety-like behavior, whilst present no effect on impairments in reciprocal sociability, exploratory behavior, repetitive behavior and sensory processing. In the molecular data, suramin was not able to reverse the increase of expression in the purinergic receptors P2X4 (hippocampus and medial prefrontal cortex) and P2Y2 (hippocampus), but reversed the VPA-induced increase of proinflammatory interleukin IL-6. Thus, behavioral modulation associated with suramin appears to be related not with specific interactions in purinergic receptors, but with a neuroimmune modification through the IL-6, indicating the importance of the immune system in the ASD pathophysiology. In general, the thesis contributed to elucidate the mechanisms involved in the development of autistic-like features, demonstrating the relevant role of neuroimmune alterations and the modulation of targets by miRNA, which, together, may contribute to the development of diagnostic methods and improvement of pharmacological strategies related to ASD.

Key words: animal behavior, ASD, microRNA, neuroimmune, PCR, purinergic system, resveratrol, suramin.

.

LISTA DE ABREVIATURAS

ANOVA – Análise de Variância.

ASD – Transtorno do Espectro Autista, do inglês *Autism Spectrum Disorder*.

ATP – Adenosina Trifosfato.

CEP – Comissão de Ética em Pesquisa.

CEUA – Comissão de Ética no Uso de Animais.

CONCEA – Conselho Nacional de Controle de Experimentação Animal.

DMSO – Dimetilsulfóxido.

DSM-5 – 5ª Edição do Manual Estatístico e Diagnóstico dos Transtornos Mentais, do inglês *Diagnostic and Statistical Manual of Mental Disorders*.

GEE – Equação de estimativa generalizada, do inglês *Generalized estimating equation*.

HCPA – Hospital de Clínicas de Porto Alegre.

IL – interleucina.

miRNA – microRNA.

PBS – Solução salina tamponada com fosfato, do inglês *phosphate-buffered saline*.

RT-qPCR – Transcrição Reversa seguida da Reação em Cadeia da Polimerase, do inglês Polymerase Chain Reaction.

RSV – Resveratrol.

SNC – Sistema nervoso central.

SUR – Suramina.

TEA – Transtorno do espectro autista.

UTR – Região não traduzida, do inglês *Untranslated Region*.

VPA – Ácido valproico, do inglês *valproic acid.*

1. INTRODUÇÃO

1.1. Transtorno de Espectro Autista

O autismo foi primeiramente descrito por Leo Kanner em 1943 como um distúrbio complexo, definido através de parâmetros comportamentais (KANNER, 1968). Desde então, diversas tentativas de categorizar adequadamente as pessoas com Transtorno do Espectro Autista (TEA) foram realizadas (AMERICAN PSYCHIATRIC ASSOCIATION (APA), 1994; APA, 2000). Os Transtornos do Neurodesenvolvimento, nos quais o TEA se encaixa, se manifestam durante a primeira infância e os prejuízos no desenvolvimento resultam em alterações em habilidades sociais, acadêmicas, pessoais e ocupacionais (AMERICAN PSYCHIATRY ASSOCIATION (APA), 2013).

Segundo a quinta edição do Manual Estatístico e Diagnóstico dos Transtornos Mentais (DSM-5), o TEA atualmente agrupa, em uma única condição, quatro transtornos anteriormente separados - o autismo clássico, a síndrome de Asperger, o transtorno desintegrativo da infância e os transtornos globais do desenvolvimento não especificados (AMERICAN PSYCHIATRY ASSOCIATION (APA), 2013). Outra mudança significativa descrita no DSM-5 em relação a edições anteriores é que a caracterização do espectro, anteriormente dividida em três áreas (prejuízo na sociabilidade, na comunicação e 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 na reciprocidade social, na comunicação não-verbal utilizada para interação social e em habilidades para iniciar, manter e entender relacionamentos;

2) Comportamentos repetitivos, atividades e interesses restritos e estereotipados.

Mesmo que esses sintomas estejam presentes isoladamente em diversas outras doenças psiquiátricas, apenas a presença concomitante destes – agrupados nos dois domínios comportamentais acima citados - em um mesmo indivíduo é que caracteriza o TEA. Apesar de haver um padrão restrito para o diagnóstico, que deve incluir essas duas características principais, o TEA se apresenta como uma desordem heterogênea e multifatorial (GOTTFRIED et al., 2013): enquanto alguns pacientes possuem um vasto vocabulário e gramática, outros utilizam somente frases padronizadas e repetitivas e outros sequer falam. Além disso, comumente se observa na clínica que muitos dos movimentos estereotipados apresentados são em resposta a diversos tipos de estressores, entre eles, as dificuldades de adaptação a mudanças na rotina.

O transtorno pode estar presente em indivíduos com manifestações distintas e abrangentes, de forma que dois indivíduos não compartilham o mesmo conjunto de características (GADIA; TUCHMAN; ROTTA, 2004; RAPIN; TUCHMAN, 2008). Contudo, todos os indivíduos apresentam necessariamente prejuízos nos dois domínios comportamentais já citados, os quais podem variar em intensidade, mas são presentes ao longo de toda vida (TUCHMAN; MOSHE; RAPIN, 2009).

Apesar da crescente investigação em torno do TEA, existem diversas variáveis que podem dificultar a interpretação dos dados obtidos das pesquisas. Primeiramente, há uma dificuldade na obtenção de tecidos encefálicos de pacientes autistas vivos e, em tecidos *post-mortem*, não se sabe

ao certo se as alterações se referem à causa ou à consequência do TEA. Além disso, o quadro clínico de crianças e adolescentes, muitas vezes agravado, dificulta sua participação em estudos, fazendo com que muitos dados se originem de sujeitos autistas adultos. Adicionalmente, a heterogeneidade do TEA e a presença de outras comorbidades e síndromes em pacientes autistas dificultam as investigações e levam a incertezas a respeito dos dados obtidos, se são decorrentes do próprio TEA, ou relacionados a outros distúrbios.

Embora diversas alterações moleculares e fisiológicas possam concorrer para o desencadeamento do TEA, o seu diagnóstico somente será possível após dois ou três anos de idade. Apesar de existirem sintomas que não podem ser percebidos nesta idade, muitos pais percebem problemas no progresso social ou comunicativo das crianças. Os prejuízos sociais não são propriamente claros na infância, porém gradualmente se tornam mais evidentes já que o indivíduo acaba, progressivamente, sendo exposto a situações mais diversas de interação social como o ingresso na vida escolar (DOVER; LE COUTEUR, 2007).

1.2. Comorbidades associadas ao TEA

Além da díade comportamental citada acima, que determina o seu diagnóstico, diversas outras características e comorbidades associadas são frequentemente observadas em indivíduos com TEA, mesmo não sendo necessárias no diagnóstico. Por exemplo, muitas crianças com TEA apresentam prejuízo intelectual, sendo que boa parte precisa de apoio social e educacional (BAUMAN, 2010; MEFFORD; BATSHAW; HOFFMAN, 2012). Além disso, pacientes com TEA podem apresentar hiperatividade, agressividade e automutilação, o que é mais comum entre aqueles que

possuem prejuízo cognitivo. Sintomas depressivos, psicóticos e comportamento suicida também podem ocorrer, e a frequência destes pode ser maior ou menor conforme as diferentes fases da vida (NAZEER; GHAZIUDDIN, 2012). Ainda, impulsividade, déficit de atenção/hiperatividade, transtornos de ansiedade, problemas de conduta, depressão, distúrbios do sono e epilepsia também podem estar presentes (DUCHAN; PATEL, 2012; KAPLAN; MCCRACKEN, 2012; SILVER; RAPIN, 2012).

Somado a essas comorbidades comportamentais, diversas alterações moleculares e fisiológicas podem ser observadas nesses indivíduos, incluindo problemas gastrointestinais, distúrbios hormonais e metabólicos (BAUMAN, 2010), alterações sensoriais, tais como hipo ou hiper-responsividade a estímulos sonoros, visuais e táteis (BEN-SASSON et al., 2009; GRANDIN, 2009; KERN et al., 2007), além de uma menor sensibilidade à dor (KLINTWALL et al., 2011). A epilepsia é uma das comorbidades mais frequentes no TEA, atingindo aproximadamente um terço dos indivíduos, com picos de ocorrência em diferentes fases da vida (DUCHAN; PATEL, 2012; KAPLAN; MCCRACKEN, 2012; NAZEER; GHAZIUDDIN, 2012; SILVER; RAPIN, 2012). Além disso, doenças como Esclerose Tuberosa, Síndrome do X Frágil e Síndrome de Angelman são frequentemente associadas ao autismo (SILVER; RAPIN, 2012).

A identificação de comorbidades associadas ao TEA é importante para melhorar a qualidade de vida desses pacientes, seja mediante tratamento farmacológico seja através de apoio social e educacional. Além disso, há um interesse no progresso da pesquisa em relação aos mecanismos moleculares e genéticos associados a esse distúrbio.

1.3. O desencadeamento do TEA

O TEA tem se apresentado como um desafio para a comunidade científica e para a sociedade, uma vez que a etiologia deste transtorno é desconhecida e até o momento nenhum tratamento ou marcador clínico para diagnóstico foi identificado. Acredita-se que o TEA seja decorrente da associação entre fatores ambientais e genéticos. Pesquisas indicam que alterações genéticas contribuem para o desenvolvimento do TEA, sendo que esses componentes podem estar relacionados com sua etiologia e com o surgimento de sintomas e comorbidades associados (ABRAHAMS; GESCHWIND, 2008; AMEIS; SZATMARI, 2012; FREITAG et al., 2010; GREENBERG et al., 2001; GRIGORENKO, 2009; RONALD; HOEKSTRA, 2011). Estudo com gêmeos apresenta concordância de 60% no autismo clássico em monozigóticos contra 0% em dizigóticos (MUHLE; TRENTACOSTE; RAPIN, 2004). A alta concordância em monozigóticos é um indicativo de que a herança genética é um fator determinante no desencadeamento do autismo. Algumas condições genéticas, como Síndrome do X-frágil e Esclerose Tuberosa, tem alta correlação com TEA (BELMONTE et al., 1995; BROWN et al., 1982; FELICIANO et al., 2013; NUMIS et al., 2011; TURK; GRAHAM, 1997). A idade avançada dos genitores, independentemente do sexo, parece estar envolvida no desencadeamento do TEA (DURKIN et al., 2008). Porém, fatores como baixa fertilidade e mudança do estilo de vida em pessoas com mais de 40 anos também são possíveis explicações para esse achado (OLSEN; ZHU, 2009).

Outros componentes intimamente envolvidos com o desencadeamento do TEA são as alterações no sistema imunológico durante a gestação (PATTERSON, 2009). Observações epidemiológicas sugerem que a exposição

a teratógenos durante a gestação poderia estar relacionada ao desencadeamento do TEA, destacando-se o ácido valproico (VPA) (CHRISTENSEN et al., 2013; CHRISTIANSON; CHESLER; KROMBERG, 1994; MOORE et al., 2000; SMITH; BROWN, 2014; WILLIAMS et al., 2001; WILLIAMS; HERSH, 1997). Essas observações auxiliaram o estabelecimento de um modelo animal para o estudo de autismo induzido farmacologicamente pela exposição pré-natal ao VPA (RODIER et al., [s.d.]; SCHNEIDER; KOCH, 2005). Posterior ao desenvolvimento do modelo, ainda foi demonstrado que o VPA aumenta significativamente a incidência de autismo nos filhos de mães que faziam uso deste medicamento durante a gestação, especialmente no primeiro trimestre (CHRISTENSEN et al., 2013; HARDEN, 2013).

1.4. Modelo animal de autismo induzido pela exposição ao VPA

Entre modelos animais que induzem comportamentos do tipo autista, a exposição pré-natal ao VPA em roedores é um dos mais caracterizados. Os filhotes machos de fêmeas expostas ao VPA apresentam várias características semelhantes às encontradas em pacientes com autismo, assemelhando-se tanto aos sintomas centrais quanto a algumas comorbidades observadas no TEA (BAMBINI-JUNIOR et al., 2011; ROULLET et al., 2010; SCHNEIDER; KOCH, 2005; SCHNEIDER et al., 2008a). Os animais do modelo apresentam prejuízos comportamentais correspondentes aos observados em pacientes (BAMBINI-JUNIOR et al., 2011), fato esse de extrema importância, visto que o diagnóstico do TEA é dado através da avaliação comportamental. Entre os comportamentos alterados, destacam-se atividade exploratória aumentada em campo aberto (SCHNEIDER et al., 2008a; TSUJINO et al., 2007), aumento de padrões comportamentais estereotipados (SCHNEIDER et al., 2008a) e do tipo

ansioso (MARKRAM et al., 2008; SCHNEIDER et al., 2008a), rigidez comportamental (BAMBINI-JUNIOR et al., 2011), aumento da memória de tarefas aversivas (MARKRAM et al., 2008), menor sensibilidade a estímulos nocivos (SCHNEIDER et al., 2008a; WANG et al., 2016) e prejuízos sociais (BAMBINI-JUNIOR et al., 2011; SCHNEIDER; KOCH, 2005; YOCHUM et al., 2008). 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 (TSUJINO et al., 2007).

Os roedores expostos ao VPA expressam também alterações moleculares semelhantes às observadas no TEA (GOTTFRIED et al., 2013), incluindo o funcionamento alterado dos sistemas opioidérgicos (SCHNEIDER et al., 2007), serotoninérgicos (TSUJINO et al., 2007), dopaminérgicos (TSUJINO et al., 2007), gabaérgicos (FUKUCHI et al., 2009) e glutamatérgicos (BRISTOT SILVESTRIN et al., 2013).

O modelo animal já demonstrou apresentar validade de face (fortes semelhanças fenotípicas e fisiopatologia relacionada) e validade de construto (um mesmo fator de risco; neste caso, exposição ao VPA durante o período embrionário). Em relação à validade preditiva, muitos autores divergem nessa questão em relação ao modelo VPA, uma vez que não se conhecem quais rotas biológicas estão envolvidas nessa resposta (ROULLET; LAI; FOSTER, 2013); porém, há fortes indícios que o modelo VPA também possua validade preditiva, ou seja, uma mesma resposta a tratamentos que visam controlar sintomas e comorbidades do TEA (MABUNGA et al., 2015). Portanto, esse modelo pode ser usado para elucidar alvos com a finalidade de aperfeiçoar

intervenções farmacológicas para prevenção, reversão ou atenuação de sintomas semelhantes ao TEA (TYZIO et al., 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 às suas peculiaridades, os modelos animais fornecem a oportunidade de análise de alterações do desenvolvimento que podem desencadear as características do TEA (FAVRE et al., 2013; KATAOKA et al., 2013). Dessa forma, surge a possibilidade de estudo e manipulação de vias biológicas para compreensão e, até mesmo, prevenção do surgimento das alterações comportamentais típicas do TEA. Uma das principais metas do nosso grupo de pesquisa é a modulação de gatilhos etiológicos por moléculas que agem em alguns sistemas de sinalização como tentativa de prevenir ou atenuar o efeito do VPA após a indução do modelo de autismo.

1.5. MicroRNA

Os microRNA (miRNA) são pequenos RNA não-codificantes com 19-25 nucleotídeos que atuam como reguladores da tradução de RNA mensageiros em suas correspondentes proteínas. Assim, eles se destacam como elementos essenciais no controle de diversos processos celulares durante o desenvolvimento e na fase adulta (AMBROS, 2004). Desde sua primeira descrição em 1993 (LEE; FEINBAUM; AMBROS, 1993), muitos estudos têm mostrado que essas moléculas são capazes de controlar seus RNA mensageiros alvos através da interação principalmente com suas regiões 3'UTR (BARTEL, 2009; FILIPOWICZ; BHATTACHARYYA; SONENBERG, 2008).

Quanto à biogênese, os miRNA são gerados a partir de regiões específicas de diversos genes, os quais são transcritos em um miRNA primário longo (pri-miRNA) pela enzima RNA polimerase III (BORCHERT; LANIER; DAVIDSON, 2006). Depois de transcrito, o pri-miRNA é clivado no núcleo por um complexo formado pelas proteínas Dgcr8 e Drosha a uma estrutura mais curta em forma de grampo, com 50-120 nucleotídeos, a qual constitui o miRNA precursor (pré-miRNA)(YANG et al., 2006). O pré-miRNA é então transportado para o citoplasma pela proteína exportina 5 (YI et al., 2005). Uma vez no citoplasma, o pré-miRNA sofre a ação da enzima Dicer, uma RNAase III, produzindo então uma fita dupla (miRNA: miRNA*) de aproximadamente 22 pares de bases. Após a separação desse duplex, uma das fitas (miRNA*) será degradada e outra (miRNA) será acoplada a um complexo silenciador induzido por RNA (do inglês, RISC) (OKAMURA et al., 2008). O complexo RISC, por sua vez, liga-se em sequências localizadas predominantemente na região 3'UTR do RNA mensageiro, podendo bloquear a sua tradução e/ou conduzi-lo à degradação (DOENCH; SHARP, 2004; KEDDE et al., 2007; LIU, 2008). Além de regular o processo de tradução, sabe-se que os miRNA podem influenciar a transcrição gênica, uma vez que interferem nos níveis de fatores de transcrição (KOSIK, 2006).

Para que haja um reconhecimento específico do RNA mensageiro alvo pelo seu miRNA, não é necessária a complementaridade total de bases entre essas duas sequências. Em vez disso, uma pequena sequência de 6–8 nucleotídeos na extremidade 5' do miRNA, conhecida como sequência ''*seed*", proporciona grande parte da regulação gênica via miRNA (GRIMSON et al., 2007). De fato, complementaridades parciais entre sequências de miRNA e as

regiões terminais 3’ UTR dos RNA mensageiros alvos são suficientes para impedir sua translação ou proporcionar sua desestabilização. Pelo fato da exigência desse pequeno número de bases para essa regulação, um grande número de transcritos pode ser alvos de um único miRNA. Por outro lado, um único RNA mensageiro pode ser negativamente regulado por diversos miRNA (FRIEDMAN et al., 2008).

Os miRNA estão possivelmente envolvidos em todos os processos biológicos, por modificar e a modular a tradução de milhares de RNA mensageiros (KAMAL; MUSHTAQ; GREIG, 2015). Assim, podem participar na regulação de vários processos celulares fisiológicos e patológicos, incluindo o desenvolvimento do embrião, proliferação, migração e diferenciação celular, apoptose, sobrevivência, tumorigênese e plasticidade sináptica (BRODERICK; ZAMORE, 2011). Diversas interações entre miRNA e seus alvos já foram validadas experimentalmente em diferentes espécies e diversas interações entre rotas de sinalização já foram descritas (HSU et al., 2011). Assim, há uma crescente evidência que os miRNA possuem funções centrais nessas rotas através da regulação da tradução de seus componentes proteicos. Em contrapartida, foi demonstrado que algumas dessas rotas de sinalização podem, por si só, modular a expressão de diversos miRNA (DAVIS-DUSENBERY; HATA, 2010), resultando em uma dependência funcional bidirecional entre miRNA e rotas de sinalização.

Os miRNA são particularmente abundantes no sistema nervoso central (SNC), sendo que algumas populações se encontram enriquecidas em regiões encefálicas específicas (CAO et al., 2006; SAUGSTAD, 2010). Eles estão envolvidos no desenvolvimento do sistema nervoso, na manutenção do

fenótipo neuronal, bem como na diferenciação celular. Exemplos de miRNA com essas funções são miR-430, miR-9 e miR-124 (BARBATO et al., 2008). Além disso, esses pequenos reguladores também são expressos em dendritos, como o miR-134, que regula negativamente o tamanho de espinhos dendríticos e sua ação é bloqueada pelo fator neurotrófico derivado do cérebro (BDNF) (SCHRATT et al., 2006) e o miR-138, que tem papel na maturação dessas estruturas (SIEGEL et al., 2009). Alguns miRNA, como o miR-132 e o miR-124, influenciam a síntese local de proteínas, estando implicados, assim, na plasticidade sináptica (BARBATO et al., 2008; SAUGSTAD, 2010; WAYMAN et al., 2008).

Níveis alterados de alguns miRNA têm sido implicados em várias desordens do SNC, como doença de Alzheimer, doença de Parkinson, doença de Huntington, atrofia lateral amiotrófica, esquizofrenia e TEA (DE SMAELE; FERRETTI; GULINO, 2010; SAUGSTAD, 2010). No entanto, ainda há poucos estudos demonstrando o papel dos miRNA no contexto de TEA (KAMAL; MUSHTAQ; GREIG, 2015). Em um estudo, 470 miRNA humanos foram avaliados em seis pacientes com TEA e seis indivíduos neurotípicos e verificou-se que nove dos 470 miRNA apresentavam níveis aumentados ou diminuídos nos pacientes autistas, sugerindo o envolvimento dos miRNA na fisiopatologia do TEA (TALEBIZADEH; BUTLER; THEODORO, 2008). Outros estudos também sugerem alterações na regulação de vários miRNA associados ao TEA, dentre eles miR-132, miR-146a, miR-23a, miR-23b (TALEBIZADEH; BUTLER; THEODORO, 2008), miR-106b e miR-23a (ABU-ELNEEL et al., 2008).

A identificação sistemática e a caracterização de miRNA pode auxiliar na elucidação de mecanismos envolvidos no desencadeamento do TEA e também representar uma possível estratégia promissora como potenciais biomarcadores que podem ser obtidos de forma não invasiva para a detecção e diagnóstico precoce do TEA, uma vez que podem ser isolados a partir de amostras biológicas facilmente obtidas de pacientes, como sangue e saliva, as quais, *a priori*, refletem o perfil de expressão de miRNA no SNC.

1.6. Resveratrol

O resveratrol (3,4,5'-trihidroxiestilbeno - RSV) é um composto polifenólico de ocorrência natural, produzido por diversas plantas para protegê-las contra a radiação ultravioleta, ataques de fungos e outros danos (PHILIPPE JEANDET et al., 2002; SCHOUTEN et al., 2002). O RSV foi primeiramente isolado de raízes da planta heléboro-branco (*Veratrum album*) na década de 40. Desde então, soube-se que esta molécula também está presente em uvas, pinhos, amendoins e no vinho tinto, possuindo diversos efeitos biológicos (FRÉMONT, 2000; VANG et al., 2011).

Nas últimas duas décadas, os polifenóis, especialmente o RSV, receberam atenção especial da comunidade científica por meio de estudos mostrando seus papéis protetores e terapêuticos em diversas patologias, incluindo câncer (GUPTA et al., 2011; JANG et al., 1997; NUTAKUL et al., 2011) e diabetes (HUANG et al., 2010; YAO et al., 2015). Adicionalmente, diversos estudos já demonstraram efeitos anti-inflamatórios (LEE et al., 2015; SÁNCHEZ-FIDALGO et al., 2010) e antioxidantes (MOHAMMADSHAHI; HAIDARI; SOUFI, 2014) dessa molécula, os quais poderiam explicar o aumento da expectativa de vida proporcionado pelo tratamento com RSV em

algumas espécies (BAUR et al., 2006; HOWITZ et al., 2003; WOOD et al., 2004). Ainda, estudos ressaltam seus efeitos neuroprotetores (QUINCOZES-SANTOS; GOTTFRIED, 2011; TANG, 2010).

Em estudo recente de nosso grupo de pesquisa, um tratamento pré-natal com RSV foi capaz de prevenir os prejuízos de sociabilidade na prole no modelo animal de autismo induzido pela exposição pré-natal ao VPA (BAMBINI-JUNIOR et al., 2014). Adicionado a isso, um novo estudo do nosso grupo mostrou que essa molécula também é capaz de prevenir prejuízos sensoriais neste mesmo modelo animal (FONTES-DUTRA et al., 2018). Esses resultados possibilitam o uso dessa molécula como ferramenta crucial para a compreensão da fisiopatologia do TEA, bem como no auxílio nos estudos de rotas biológicas e estruturas envolvidas em sua etiologia, tanto em embriões quanto em idades pós-natal.

Apesar desses diversos estudos em modelos animais e culturas celulares, dados de testes clínicos em humanos ainda estão em desenvolvimento (PANGENI et al., 2014; TOMÉ-CARNEIRO et al., 2013), de forma que não se recomenda a suplementação de RSV em quantidades superiores àquelas obtidas através do consumo de alimentos que contenham essa molécula.

1.7. Sinalização purinérgica e TEA

Há uma crescente evidência do papel das disfunções mitocondriais no TEA (FILIPEK et al., 2003; TSAO; MENDELL, 2007), sendo que um estudo mostrou que os níveis de DNA mitocondrial estavam aumentados em crianças com TEA (PICARD et al., 2014). Além disso, um comprometimento do metabolismo energético mitocondrial tem sido proposto para desempenhar um

papel no desencadeamento do TEA (LOMBARD, 1998; PASTURAL et al., 2009; PATOWARY et al., 2017). É importante notar que o ATP extracelular de origem mitocondrial é um sinal de alarme universal liberado pelas células sob estresse. Esse ATP liberado pode, em células adjacentes, desencadear processos inflamatórios (CHAN; GOLD; VON AHSEN, 2011; CORRIDEN; INSEL, 2010; FAAS; SÁEZ; DE VOS, 2017) e induzir uma intensa reação autoimune (THEOHARIDES; ASADI; PATEL, 2013; ZHANG et al., 2012). Aproximadamente 50% do conteúdo mitocondrial que é liberado contém ATP, cuja ação pode ser bloqueada por inibidores de receptores purinérgicos (DUBYAK, 1991; KRISHTAL; MARCHENKO; PIDOPLICHKO, 1983).

Os receptores purinérgicos ionotrópicos P2X são uma classe de canais hetero e homotriméricos permeáveis a cátions que se ligam ao ATP extracelular (FREDHOLM et al., 1994; RALEVIC; BURNSTOCK, 1998). Entre os sete receptores da família dos P2X (P2X1 a P2X7), o receptor P2X4 é de especial interesse como regulador do sistema nervoso e é encontrado em grandes quantidades no encéfalo (BORTOLATO et al., 2013; RUBIO; SOTO, 2001). Além disso, parece estar envolvido na regulação do sistema neuroendócrino (ZEMKOVA et al., 2010), na plasticidade hipocampal (BAXTER et al., 2011), na modulação da atividade dos receptores GABA-A (JO et al., 2011) e nos receptores N-metil-D-aspartato (NMDA) (BAXTER et al., 2011). Outras subunidades P2X, como os subtipos P2X2 e P2X7, estão envolvidas na proliferação neural e na especificação do fenótipo de diferenciação (GLASER et al., 2014; GLASER; RESENDE; ULRICH, 2013), bem como em respostas sensoriais (LEWIS et al., 1995) e inflamatórias (BARICORDI et al., 1999), as

quais foram demonstradas alteradas em pacientes com TEA (TOMCHEK; DUNN, 2007) e no modelo VPA (SCHNEIDER et al., 2008b).

Os receptores P2Y, uma classe de receptores purinérgicos acoplados à proteína G, são determinantes fundamentais em muitas respostas fisiológicas, incluindo neuromodulação (GUZMAN; GEREVICH, 2016; PUCHAŁOWICZ et al., 2015; VON KÜGELGEN; HOFFMANN, 2016), vasodilatação (RALEVIC; DUNN, 2015), inflamação (GUZMAN; GEREVICH, 2016) e migração celular (HARDEN et al., 2010). Em mamíferos com o SNC já desenvolvido, o receptor P2Y2 torna-se expresso em astrócitos corticais e neurônios, sob condições de lesão cerebral, e sua ativação tem um papel neuroprotetor, regulando a proliferação e migração de astrócitos e promovendo a estabilização de neuritos (BURNSTOCK, 2007; PETERSON et al., 2010; WEISMAN et al., 2012).

Publicações recentes demonstraram que a suramina, um antagonista inespecífico de receptores purinérgicos, foi capaz de reverter alterações comportamentais e metabólicas em modelo de TEA induzido por ativação imune materna (NAVIAUX et al., 2014, 2013), bem como em um modelo genético de Síndrome do X Frágil (NAVIAUX et al., 2015). É importante notar que a análise metabolômica identificou vias bioquímicas associadas a melhorias de sintomas que se sobrepõem tanto a pessoas com TEA quanto a modelos animais, as quais foram previamente identificadas como mediadoras funcionalmente relacionadas da resposta ao perigo celular evolutivamente conservada (NAVIAUX et al., 2015). No entanto, os autores não conseguiram explicar como um efeito anti-purinérgico poderia prevenir as mudanças comportamentais no modelo de rato poli (I:C), os quais mostraram ser dependentes dos níveis elevados de interleucina 6 (IL-6) (SMITH et al., 2007),

nem como os efeitos terapêuticos foram alcançados no modelo genético. No entanto, a suramina é inespecífica e pode apresentar outros efeitos, tais como afetar os receptores acoplados a proteínas G (BOLITHO et al., 2007), inibir a secreção de mastócitos (JAFFAR; PEARCE, 1990), proliferação de linfócitos T (NOVALES-LI, 1996) e atividade bactericida de leucócitos polimorfonucleares (ROILIDES et al., 1993).

1.8. Interações neuroimunitárias e TEA

Diversas alterações envolvendo o SNC foram observadas em modelos de autismo em roedores pela exposição pré-natal ao VPA (BAUM; STEVENSON; WALLACE, 2015; JUMAH et al., 2016; YANG et al., 2016). No entanto, uma hipótese existente na pesquisa sobre o autismo que pode explicar seus mecanismos é o envolvimento de componentes do sistema imunitário no desencadeamento deste transtorno (CHOI et al., 2016; GESUNDHEIT et al., 2013; KUGELBERG, 2016). Histórico familiar de doenças autoimunes (SWEETEN et al., 2003) e níveis alterados de citocinas inflamatórias (RATAJCZAK, 2011) são algumas das evidências que sugerem a relação entre sistema imunitário e TEA.

Estudos prévios demonstraram que existem alterações no sistema imunológico durante a gestação que parecem estar intimamente envolvidas com o desencadeamento do TEA (PATTERSON, 2009). 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 (ATLADÓTTIR et al., 2010; CROEN et al., 2005; KORVATSKA et al., 2002). Esses achados são corroborados por estudos envolvendo modelos animais (BAUMAN et al., 2014; MALKOVA et al., 2012) e,

juntamente com as alterações imunológicas encontradas em pessoas com TEA (ASHWOOD et al., 2011), ajudam a estabelecer a natureza imunológica desse transtorno.

Além de tecidos periféricos, alterações imunológicas em tecidos encefálicos já foram descritas em indivíduos com TEA (GOINES; ASHWOOD, 2013). Análises de tecidos encefálicos *post mortem* revelaram indícios de neuroinflamação devido ao aumento nos níveis de citocinas pró-inflamatórias, principalmente IL-1β, IL-6, TNF-α e IFN-y, ativação neuroglial (ROSE; ASHWOOD, 2014) e alterações gênicas diretamente envolvidas com o sistema imunológico (LINTAS et al., 2011).

Adicionalmente, indivíduos diagnosticados com TEA frequentemente apresentam anormalidades linfocitárias. Proporções e quantidades reduzidas de linfócitos T $CD4^+$ foram observadas em estudo com 25 pacientes (YONK et al., 1990). Apesar de não haver relatos de infiltração linfocitária no SNC de pacientes com TEA, existe a possibilidade de isso ocorrer durante a gestação ou durante a primeira infância (GESUNDHEIT et al., 2013). Por outro lado, já foi relatada infiltração linfocitária no sistema gastrointestinal de pacientes com TEA, com um número aumentado de células $CD4^+$ e $CD8^+$ e produção elevada de citocinas inflamatórias por essas células (ASHWOOD et al., 2003; ASHWOOD; WAKEFIELD, 2006).

Além dessas evidências encontradas diretamente em indivíduos com TEA, já foi demonstrado que existe uma relação entre ativação imune materna (alterações pró-inflamatórias maternas durante a gestação) com o nascimento de filhos com TEA, variando a desordem conforme o período de exposição (MEYER et al., 2008). Adicionalmente, já foi demonstrada uma correlação

positiva entre infecção materna viral ou bacteriana nos dois primeiros trimestres de gestação com o desenvolvimento de TEA nos seus filhos (ATLADÓTTIR et al., 2010).

Nesse contexto, variações nos níveis de citocinas maternas destacam-se como prováveis candidatos para mediar a comunicação entre sistema imunitário materno e fetal. Aumentos nos níveis de IL-6 durante a gestação é um dos fatos correlacionados com TEA na prole (SMITH et al., 2007). Estudos demonstram que ativação imune materna altera o desenvolvimento do encéfalo do feto através de receptores de IL-6 (SMITH et al., 2007). Somado a isso, já foi demonstrado que o VPA causa alterações imunitárias (ICHIYAMA et al., 2000; ROSSI et al., 2012) e que as citocinas maternas são capazes de chegar ao feto durante o período crítico da gestação frequentemente utilizado para indução do modelo animal de autismo (DAHLGREN et al., 2006). Dessa forma, o estudo do balanço entre citocinas pró- e anti-inflamatórias no modelo animal de autismo induzido por exposição ao VPA pode gerar respostas que sustentem a hipótese neuroimunológica.

2. OBJETIVOS

2.1. Objetivo Geral

Avaliar parâmetros comportamentais e moleculares no modelo animal de autismo induzido por exposição pré-natal ao ácido valproico e o possível efeito preventivo de resveratrol e reversivo de suramina sobre os parâmetros avaliados.

2.2. Objetivos Específicos

Parâmetros comportamentais:

- Avaliar possíveis alterações comportamentais no modelo VPA e o efeito do tratamento pré-natal com RSV ou tratamento pós-natal com suramina, por meio dos seguintes parâmetros comportamentais: sociabilidade, comportamentos repetitivos, comportamento do tipo ansioso e parâmetros sensoriais;

Quantificação de miRNA:

- Analisar a expressão relativa de um grupo de miRNA no modelo VPA, combinado ou não ao tratamento pré-natal com RSV;

Expressão gênica:

- Quantificar os níveis de RNA mensageiros de receptores purinérgicos no modelo animal de autismo, combinado ou não com o tratamento pós-natal com suramina;
- Quantificar os níveis de RNA mensageiros de citocinas pró-inflamatórias no modelo animal de autismo, combinado ou não ao tratamento pós-natal com suramina.

Análise descritiva sobre a análise de miRNA:

- Revisar algumas técnicas disponíveis para quantificação de miRNA e descrever detalhes importantes na realização da técnica de RT-qPCR para avaliação dessas moléculas.

PARTE II

Capítulo IA:

Artigo Publicado:

“Behavioral alterations in autism model induced by valproic acid and translational analysis of circulating microRNA”

Food and Chemical Toxicology 115 (2018) 336–343.

Food and Chemical Toxicology 115 (2018) 336–343

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Behavioral alterations in autism model induced by valproic acid and translational analysis of circulating microRNA

Mauro Mozael Hirsch[a,b,c,*], Iohanna Deckmann[a,b,c], Mellanie Fontes-Dutra[a,b,c], Guilherme Bauer-Negrini[a,b,c], Gustavo Della-Flora Nunes[a,b,c], Walquiria Nunes[a,b,c], Bruna Rabelo[a,b], Rudimar Riesgo[a,c,d], Rogerio Margis[c,e], Victorio Bambini-Junior[a,c,f], Carmem Gottfried[a,b,c,**]

[a] Translational Group in Autism Spectrum Disorder - GETTEA, Clinical Hospital of Porto Alegre, RS, Brazil
[b] Department of Biochemistry, Federal University of Rio Grande Do Sul - UFRGS, Porto Alegre, RS, Brazil
[c] Brazilian National Institute of Science and Technology on Neuroimmunomodulation (INCT-NIM), Rio de Janeiro, Brazil
[d] Child Neurology Unit, Clinical Hospital of Porto Alegre, Federal University of Rio Grande Do Sul, Porto Alegre, Brazil
[e] Center of Biotechnology and PPGBCM, Laboratory of Genomes and Plant Populations, Federal University of Rio Grande Do Sul - UFRGS, Porto Alegre, RS, Brazil
[f] School of Pharmacy and Biomedical Sciences, University of Central Lancashire, Preston, UK

ARTICLE INFO

Keywords:
ASD
microRNA
Resveratrol
Translational research
Social behavior
Valproate

ABSTRACT

Autism spectrum disorder (ASD) is characterized by difficulties in social interaction, communication and language, and restricted repertoire of activities and interests. The etiology of ASD remains unknown and no clinical markers for diagnosis were identified. Environmental factors, including prenatal exposure to valproic acid (VPA), may contribute to increased risk of developing ASD. MicroRNA (miRNA) are small noncoding RNA that regulate gene expression and are frequently linked to biological processes affected in neurodevelopmental disorders. In this work, we analyzed the effects of resveratrol (an antioxidant and anti-inflammatory molecule) on behavioral alterations of the VPA model of autism, as well as the levels of circulating miRNA. We also evaluated the same set of miRNA in autistic patients. Rats of the VPA model of autism showed reduced total reciprocal social interaction, prevented by prenatal treatment with resveratrol (RSV). The levels of miR134–5p and miR138–5p increased in autistic patients. Interestingly, miR134–5p is also upregulated in animals of the VPA model, which is prevented by RSV. In conclusion, our findings revealed important preventive actions of RSV in the VPA model, ranging from behavior to molecular alterations. Further evaluation of preventive mechanisms of RSV can shed light in important biomarkers and etiological triggers of ASD.

1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder of unknown etiology characterized by a dyad of behavioral alterations: a) social communication and social interaction impairments and b) restricted, repetitive and stereotyped patterns of behavior, interests and activities (American Psychiatric Association, 2013). In addition to these features, several other associated symptoms and comorbidities are more prevalent in individuals with ASD, including sensorial, gastrointestinal, and immune alterations, sleep disturbances and other neurological disorders such as epilepsy and TDAH (Geschwind, 2009; Grandin, 2009; Klintwall et al., 2011).

The development of complex neural circuits relies in the spatial and temporal coordination of genetic and epigenetic processes during embryogenesis. As a consequence, the neurodevelopment is especially susceptible to the influence of environmental factors (Ameis and Catani, 2015; Perera and Herbstman, 2011). The brain of autistic individuals present several structural and functional abnormalities (Hutsler and Casanova, 2016), which could be caused by environmental risk factors, including immune abnormalities during pregnancy, indicating a strong influence of the immune system in the neurodevelopment. (Gottfried et al., 2015).

Valproic acid (VPA) is a drug widely-used as anticonvulsant and mood-stabilizer. Epidemiological observations demonstrated a strong link between the prenatal exposure to VPA, and the onset of ASD in the offspring (Christianson et al., 1994; Moore et al., 2000; Rodier et al.,

* Corresponding author. Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos 2600 – 21111, CEP: 90035-003 Porto Alegre, RS. Brazil.
** Corresponding author. Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos 2600 – 21111, CEP: 90035-003 Porto Alegre, RS. Brazil.
E-mail addresses: mauro.hirsch@ufrgs.br (M.M. Hirsch), cgottfried@ufrgs.br (C. Gottfried).

https://doi.org/10.1016/j.fct.2018.02.061
Received 9 January 2018; Received in revised form 24 February 2018; Accepted 27 February 2018
Available online 03 March 2018
0278-6915/

1997; Williams et al., 2001; Williams and Hersh, 1997). Based on these observations, a prenatal injection of VPA has been used to induce autistic-like features in animal models in rodents (Bambini-Junior et al., 2011; Schneider and Przewłocki, 2005). The VPA animal model of autism presents face validity (strong phenomenological similarities and related pathophysiology), construct validity (the same etiology; in this case, exposure to VPA) and predictive validity (same response to treatments aiming to prevent or revert symptoms) (Mabunga et al., 2015). Therefore, this model is currently used to elucidate new pathways involved in ASD pathophysiology that can be pharmacologically targeted (Tyzio et al., 2014).

Given the importance of social behavior impairments for the diagnosis of ASD, analysis of social behavior is critical in models of autism in rodents. We previously demonstrated that rats of the VPA model present impaired social behavior in a three-chambered social approach task (Bambini-Junior et al., 2011). In addition, we showed that resveratrol (RSV), an antioxidant and anti-inflammatory molecule, prevents social impairments in the VPA model in this same test (Bambini-Junior et al., 2014). Another key component for ASD diagnosis is the presence of repetitive and stereotyped behaviors, which, in animal models, are commonly manifested by excessive and incomplete self-grooming (McFarlane et al., 2008; Schneider and Koch, 2005; Sungur et al., 2014). Animal models of autism also frequently present irregularities in sensorial processing (Dendrinos et al., 2011; Geschwind, 2009), which may interfere with the animals' ability to perceive and interact with their surroundings (Nienborg and Cumming, 2010). The social transmission of food preference (STFP) is an ethologically relevant test of hippocampus-dependent non-spatial olfactory memory (Galef-Jr and Wigmore, 1983) that is commonly used to evaluate social communication in rodents (Bessières et al., 2017; Ryan et al., 2008). Therefore, in this work we evaluated reciprocal social interaction, repetitive self-grooming and the STFP task in rats of the VPA model, as well as the influence of prenatal RSV treatment.

In addition to behavioral assessments, the investigation of common molecular targets of VPA and RSV could help to clarify pathways involved in the pathophysiology of ASD. MicroRNA (miRNA) are a set of small non-coding RNA that control protein expression by binding mainly to the 3′UTR of the messenger RNA (mRNA) and thereby mediating either RNA degradation or translation inhibition (Chandra et al., 2017). The miRNA molecules are able to modulate several cellular functions and events, including cellular proliferation and differentiation, synapse formation/maturation and general metabolism integration (Wahid et al., 2010) and were recently implicated in the pathogenesis of ASD (Wu et al., 2016). The identification of circulating miRNA may also be potentially relevant for the development of clinical diagnostic or prognostic tools (Hu et al., 2017). We hypothesized that the RSV's preventive effect of the VPA-induced impairments might involve modulation of miRNA levels. In addition, we anticipate that some of these results could be conserved inter-species and would also be relevant for patients with ASD.

Thus, the aims of the present study were to investigate possible preventive effects of RSV against VPA-induced impairments on behavior and circulating miRNA alterations in an animal model, as well as to evaluate miRNA alterations in blood from autistic patients.

2. Material and methods

2.1. Animal model of autism induced by VPA and resveratrol treatment

The animal model of autism was induced as previously described (Bambini-Junior et al., 2011; Schneider and Przewłocki, 2005). Briefly, female Wistar rats (UFRGS-Biochemistry Department CREAL), with controlled fertility cycles, were mated overnight. The first day of gestation was determined by the presence of spermatozoa in the vaginal smear (embryonic day 0.5). Pregnant females received a single intraperitoneal injection of 600 mg/kg VPA (sodium valproate, Sigma-Aldrich, USA) or saline solution on embryonic day 12.5 (E12.5), and daily subcutaneous injections of RSV (trans-resveratrol Fluxome, Stenløse, Denmark, 3.6 mg/kg) or DMSO from E6.5 to E18.5 (Bambini-Junior et al., 2014). Only males of the offspring were used in this current study. Blood samples from these animals were obtained by cardiac puncture 30 days after birth. The behavioral analyses were performed in a second cohort of animals. This project was approved by the local animal ethics committee (CEUA-UFRGS 140367/140431) and all animals were handled in accordance with the current guidelines (National Council for the Control of Animal Experimentation - CONCEA).

2.2. Behavioral tests

To investigate possible preventive effect of RSV treatment on VPA animals, we evaluated three important behavioral patterns that correspond to behavioral domains frequently altered in ASD: sociability, olfactory memory and communication, and repetitive behavior.

2.2.1. Reciprocal social behavior

The test was adapted from Schneider and Przewlocki (Schneider and Przewłocki, 2005). Rats were tested at 46 days of age in a 50 × 50 × 50 cm arena. After a 5-min habituation period in the test chamber, the interactions between the animal test and a novel rat (younger male) were recorded for 15 min. The following pro-social interactions were evaluated: nose-to-nose sniffing, anogenital inspection, flank-exploration and following behavior. The number and duration of events were scored using the Anymaze software. We also calculated the total number and time of reciprocal social interaction.

2.2.2. Social transmission of food preference (STFP)

The experiment was adapted from Wrenn et al. protocol (Wrenn et al., 2004). Rats at 47 days of age were habituated for 72 h to eat pelleted food made from powdered chow. In the following day, food was removed 3 h before the test. Next, one animal from each cage (demonstrator) was housed alone in a separate housing box and could eat a randomly assigned flavored food for 1 h: either cinnamon (1% w/w) or cocoa (2% w/w). Then the demonstrator rat was housed with their littermates (the observer rats) and allowed free interaction for 30 min. After this interaction period, the demonstrator animal was removed from the housing box and the observer animals were provided with two choices of powdered food in identical pellets, one with flavor of the cued food presented by demonstrator rat and the other with the alternative food. Rats could eat for one and a half hours, and the amount of cued and non-cued food eaten from each litter was weighed and recorded. The ratio of food preference for each litter was calculated, considering the amount of cued and non-cued food and the total food consumption.

2.2.3. Repetitive self-grooming behavior

Rats at 64 days of age were scored for spontaneous grooming behavior as described (Onaolapo et al., 2017). The test was performed during 10 min in a 50 × 50 × 50 cm arena. Each rat was scored individually in two parts (0–5 min and 5–10 min) and the total time (0–10 min), considering the number of body cleaning with paws and face-washing actions. No habituation was performed.

2.3. Blood samples from autistic and control subjects

Peripheral blood samples from autistic male individuals and from the control group (5-15 years-old range) were obtained at the Clinical Hospital of Porto Alegre (HCPA). Inclusion criteria were age between 5 and 15 years and clinical diagnosis of ASD according to DSM-5 confirmed using the Autism Diagnostic Observation Schedule. Autistic individuals who presented secondary autism or autism as an associated feature of an identified genetic condition (Fragile X Syndrome, Rett

Syndrome, Angelman Syndrome, Prader-Willi Syndrome, Smith-Lemli-Opitz Syndrome and Tuberous Sclerosis) were excluded from the study. This project was approved by the local ethics committee (CEUA-UFRGS 33863).

2.4. RNA extraction and RT-qPCR procedure

After homogenization of blood samples with Trizol® reagent (Invitrogen, USA), chloroform was added to perform phase separation, and RNA was precipitated from the upper aqueous layer using isopropanol. The precipitated RNA was washed with ethanol to remove impurities, resuspended in RNase-free water and stored at −80 °C (Chomczynski, 1993).

Mature miRNA expression was evaluated by reverse transcriptase followed by quantitative polymerase chain reaction (RT-qPCR) (Chen et al., 2005). Complementary DNA (cDNA) was synthesized from mature miRNA using reverse transcriptase reaction containing 2 µg of total RNA, 1 µL of 10 mM dNTP mix (Invitrogen, USA), 3 µL of stem loop RT primer mix, 4 µL M-MLV reverse transcriptase 5X reaction buffer (Invitrogen, USA), 2 µL of 0.1 M DTT (Invitrogen, USA), 1 µL of RNase inhibitor (Invitrogen, USA), 1.0 µL of M-MLV reverse transcriptase (Invitrogen, USA), and sterile distilled water to a final volume of 20 µL. The synthesis of the cDNA was completed after a sequence of three incubations at 65 °C for 5 min, 37 °C for 50 min and 70 °C for 15 min.

The quantitative PCR mix was comprised by 12 µL of cDNA (1:33), 1.0 µL of specific miRNA forward and universal reverse (10 µM) primers, 0.5 µL of 10 µM dNTP mix, 2.4 µL of 10X PCR buffer (Invitrogen, USA), 0.8 µL of 50 mM $MgCl_2$ (Invitrogen, USA), 2.4 µL of 1X SYBR™ Green (Molecular Probes, USA), 0.1 µL of Platinum Taq DNA Polymerase (Invitrogen, USA) and sterile distilled water to a final volume of 24 µL. The fluorescence of SYBR™ Green was used to detect amplification, estimate Ct values, and to determine specificity after melting curve analysis. PCR cycling conditions were standardized to 95 °C for 5 min followed by 40 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. After the main amplification, sample fluorescence was measured from 60 °C to 95 °C, with an increasing ramp of 0.3 °C each, to obtain the denaturing curve of the amplified products and Tm estimation, to assure their homogeneity after peak detection. Data was obtained from an Applied Biosystems StepOne System (USA). The set of 16 miRNA selected for this study includes miRNA involved in immunological and/or synaptic plasticity modulation, processes altered in ASD and includes miRNA commonly altered in many neurodevelopmental disorders.

2.5. Calculation of miRNA relative expression

The RT-qPCR results were imported into Microsoft Excel and the geNorm program was used to assess the variance in expression levels of the miRNA analyzed (Peltier and Latham, 2008; Vandesompele et al., 2002). This program scanned the present miRNA two at a time. Then, the expression stabilities of the set of miRNA were evaluated and ranked accordingly to their stability. A pairwise variation analysis was performed by geNorm to determine the number of miRNA required for accurate normalization and to identify the most suitable miRNA to be used as normalizers.

The average value of crossing threshold (Ct) values (in triplicate) was converted to quantities for geNorm analysis by the difference between Ct value from two groups taken in each comparison. PCR efficiency was calculated from the slope of the amplification curve by exponential amplification analysis using the LinRegPCR algorithm (Ramakers et al., 2003). The relative expression was obtained using the -ΔΔCt method where Ct values of a group are subtracted from the average Ct values of the control group. The relative expression of miRNA was calculated considering the PCR efficiency and the −ΔΔCt values for each miRNA (Pfaffl, 2001) and was normalized to the normalizers identified by the geNorm software.

2.6. Statistical analysis

IBM SPSS Statistics 20.0 (IBM SPSS, Armonk, NY, USA) was used to perform the statistical analysis. Kolgomorov-Smirnov and Shapiro-Wilk tests of normality were applied to determine data distribution. For reciprocal sociability test and self-grooming behavior, we used Generalized Estimating Equations (GEE) to weight both the interventions (VPA exposure and/or RSV treatment) and the litter effect in the behavioral outcome. After a Wald Chi-Square test, we performed pairwise comparisons for the parameters that presented interaction effect between interventions (VPA-by-RSV interaction). If only main effects were observed, the individual effect of VPA or RSV was evaluated. Bonferroni's post hoc test was used as the final evaluation. Data is reported as mean ± standard error of the mean (SEM). The Poisson distribution was used for discrete variables (number of interactions), while gamma distribution was used for time variables. A two-way variance analysis and Bonferroni post hoc test were used to analyze the cued food/total food eaten percentage during the STFP test after normality tests indicated a normal distribution of this variable. The associated data are expressed as mean ± SEM and $P < 0.05$ was considered to indicate a statistically significant difference. On the other hand, the absolute consumption of each food flavor presented non-normal distribution, were compared by non-parametric Kruskal–Wallis test and the results were expressed as median ± interquartile interval.

The miRNA relative expressions of the four animal groups were compared using non-parametric Kruskal–Wallis test followed by Dunn's test for multiple comparisons. The results were expressed as median ± interquartile interval. For the analysis of the human samples, non-parametric Mann-Whitney *U* test was performed and the results were expressed as median ± interquartile interval. All statistical analyses were supervised by the Biostatistics Unit at the Clinical Hospital of Porto Alegre.

3. Results

3.1. Behavioral tests

3.1.1. Reciprocal sociability test

Adult male rats prenatally exposed to VPA were tested in a sociability paradigm considering four pro-social behaviors: nose-to-nose sniffing, anogenital inspection, flank exploration and following (Fig. 1). Wald chi-square test showed a significant interaction effect between VPA exposure and prenatal RSV treatment in all parameters evaluated. Bonferroni's test for multiple comparisons further revealed that animals from the VPA group presented significantly decreased number and time of nose-to-nose sniffing compared to the control group, and RSV was not able to completely prevent these alterations (Fig. 1A). Anogenital inspection presented no significant difference between experimental groups (Fig. 1B). There was no difference between number and time of flank exploration behavior between VPA and control groups, although a significant difference in total time of this behavior was observed between VPA and RSV groups (Fig. 1C). Rats prenatally exposed to VPA also displayed significantly reduced number and time of following behavior when interacting reciprocally with an unfamiliar rat, compared to the control group (Fig. 1D). Animals exposed to VPA presented decreased total pro-social interactions and spent less time interacting with an unfamiliar conspecific rat compared to the control group (Fig. 1E). When VPA animals are treated with RSV, there was a significant increase in both number and time of pro social behaviors compared to VPA animals injected only with the vehicle. Thus, the RSV treatment was able to prevent the combined social deficits of VPA rats, even though analyses of individual behaviors were not statistically significant.

3.1.2. Social transmission of food preference

Fig. 2 represents the ratio of food preference of Wistar rats

28

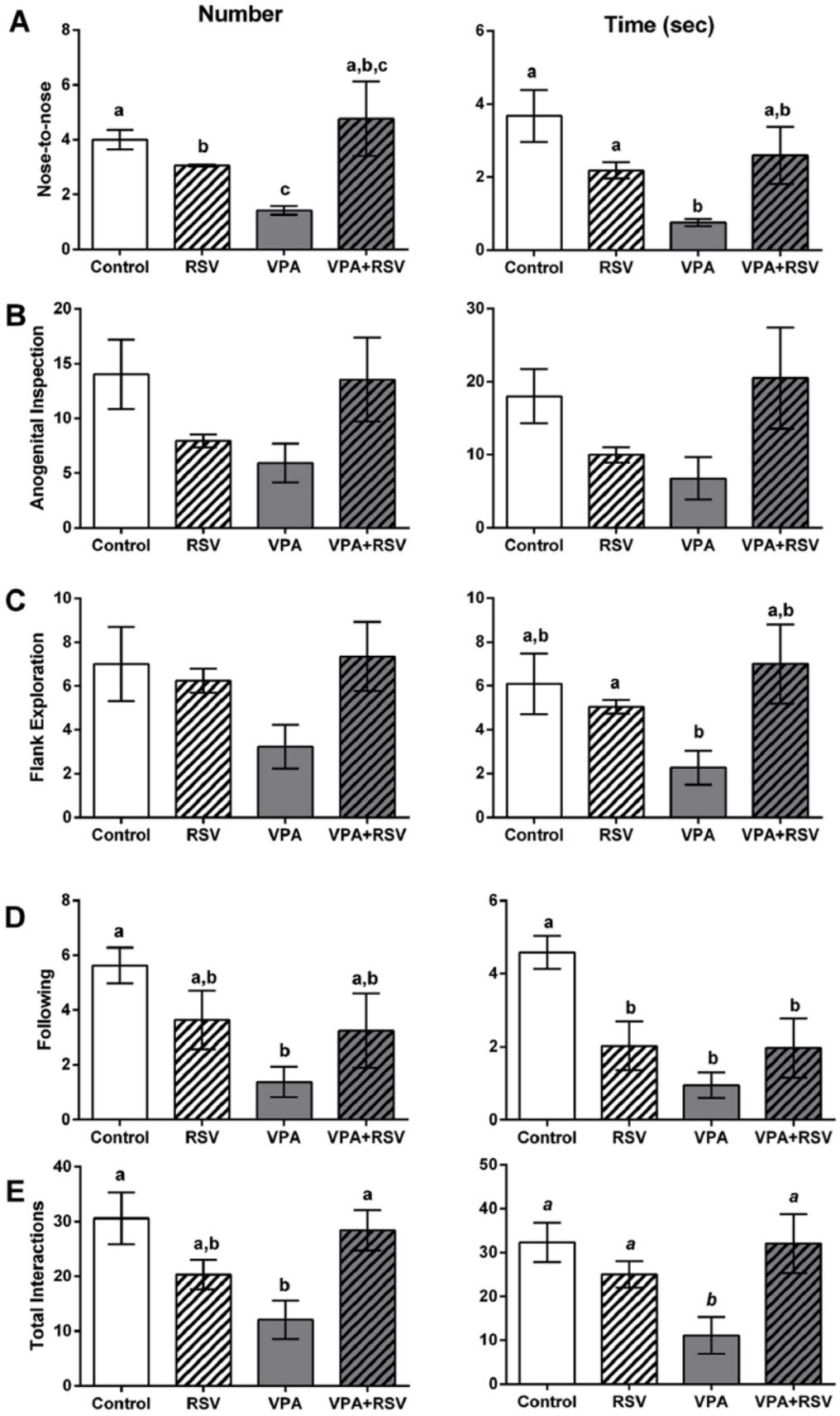


Fig. 1. Resveratrol prevents deficits in reciprocal social interaction in the VPA model of autism. Number and time of nose-to-nose sniffing (A), anogenital inspection (B), flank exploration (C), following (D) and total pro-social interactions (E). Plots show mean ± SEM. Different letters indicate statistical differences with $p < 0.05$. Statistical analysis: Generalized Estimating Equations (GEE) followed by Bonferroni. Control (n = 8), RSV (n = 9), VPA (n = 9), VPA + RSV (n = 6).

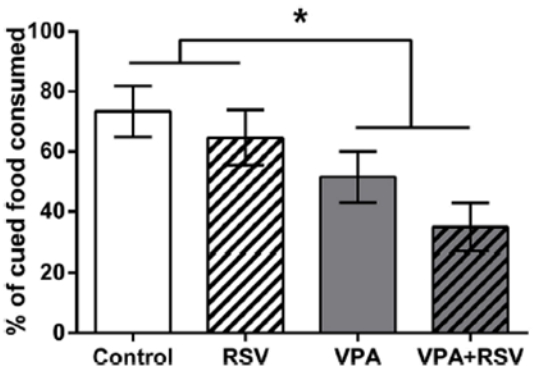


Fig. 2. VPA exposure impairs social transmission of food preference. Percentage of cued food consumed by observer rats. Data expressed as mean ± SEM with *p < 0.05. Statistical analysis: Two-way ANOVA followed by Bonferroni. Control (n = 7), RSV (n = 6), VPA (n = 8), VPA + RSV (n = 7).

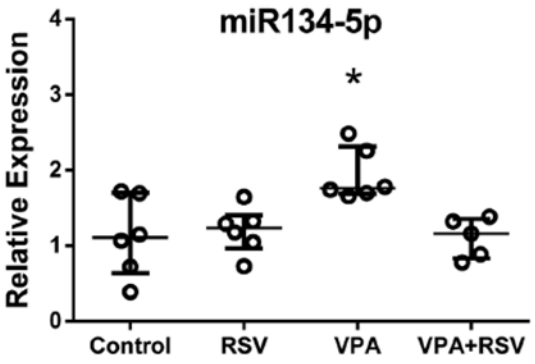


Fig. 4. miR134-5p expression is increased in peripheral blood of the VPA model of autism, which is prevented by RSV treatment. Plots presented as medians ± interquartile intervals with *p < 0.05 considered significant. Statistical analysis: Independent-Samples Kruskal-Wallis followed by Dunn's test. Control (n = 6), RSV (n = 6), VPA (n = 6), VPA + RSV (n = 5).

prenatally exposed to VPA, compared to the control animals, after treatment with RSV or vehicle. There was a significant effect of VPA exposure in food preference ($p = 0.026$), with a decreased ratio of food preference. However, the RSV treatment was not able to prevent this alteration.

Regarding the absolute food consumption, animals exposed to VPA tend to eat less food with the flavor cued by the demonstrator (Data in Brief Fig. 1A, $p = 0.080$). On the other hand, animals presented no differences in consumption of alternative (non-cued) food across interventions (Data in Brief Fig. 1B, $p = 0.493$).

3.1.3. Repetitive self-grooming

The self-grooming behavior was evaluated across two testing periods (0–5 and 5–10 min). During the first period, no significant effect was found, indicating that the time spent self-grooming was the same for all animals tested (Fig. 3B). In the second period of test, Wald chi-square test showed that animals exposed to VPA spent more time self-grooming and this effect was not prevented by RSV (Fig. 3B, $p = 0.005$). A similar result was observed in relation to total test period, with animals exposed to VPA spending more time grooming and no preventive effect of RSV (Fig. 3C, $p = 0.008$). The latency to start a self-grooming behavior was similar across experimental groups (Fig. 3D).

3.2. Analysis of miRNA expression in animal model of autism

We determined the relative expression of miRNA in blood of 30 days-old rats prenatally exposed to VPA or vehicle (saline) and treated with RSV or vehicle (DMSO). The GeNorm algorithm ranked miRNA miR181a-5p and miR181b-5p as most stable and they were used as normalizers to evaluate the relative expression of the remaining miRNA. miR134–5p was significantly increased ($p = 0.030$) in total blood of animals prenatally exposed to VPA, and RSV was able to prevent this alteration (Fig. 4). On the other hand, there were no significant differences in the 13-remaining miRNA (Data in Brief Fig. 2).

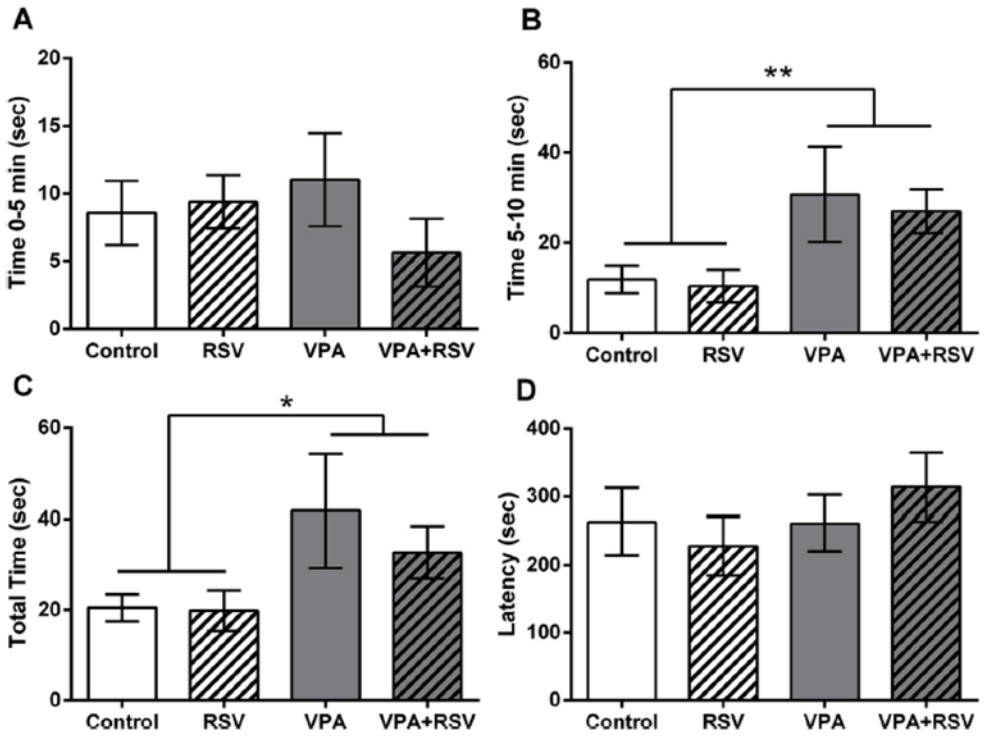


Fig. 3. VPA exposure increases self-grooming behavior. Time of grooming (sec): (A) 0–5 min of test, (B) 5–10 min of test, (C) total time and (D) latency to start grooming. Data expressed as mean ± SEM with *p < 0.05 and **p < 0.01 considered significant. Statistical analysis: Generalized Estimating Equations (GEE) followed by Bonferroni. Control (n = 14), RSV (n = 12), VPA (n = 12), VPA + RSV (n = 13).

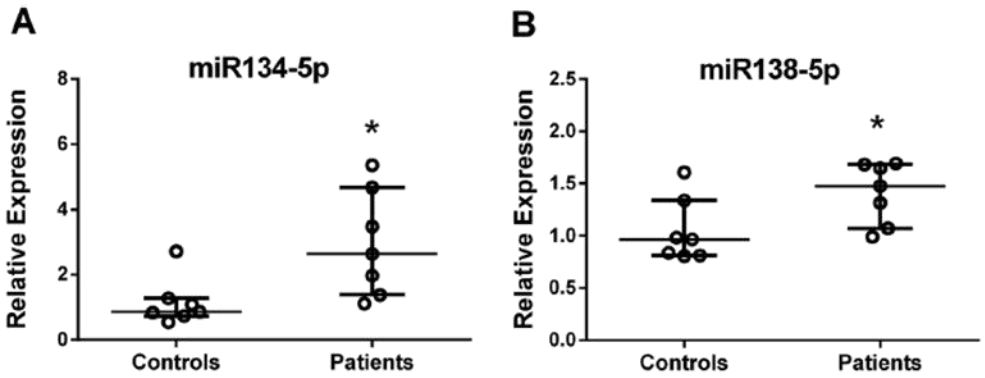


Fig. 5. mi134-5p and miR138-5p are upregulated in peripheral blood of autistic children compared to control group. Results expressed as median ± interquartile intervals with *p < 0.05 considered significant. Statistical analysis: Mann-Whitney *U* test. Control (n = 7) and autistic patients (n = 7).

3.3. Analysis of miRNA expression in autistic subjects

We also determined the relative expression of miRNA in peripheral blood of autistic subjects, comparing with controls in the same age-range (5–15 years). The GeNorm algorithm ranked miR23a-3p, miR146a-5p and miR181a-5p as most stable and they were used as normalizers to evaluate the relative expression of the remaining miRNA. There were no intra-group differences related to age on miRNA levels. The analysis of relative expression revealed significant increases in expression of miR134–5p (Fig. 5A, p = 0.011) and miR138–5p (Fig. 5B, p = 0.026) in peripheral blood of autistic children, compared to control subjects. Levels of the 11-remaining miRNA showed no differences between groups (Data in Brief Fig. 3).

4. Discussion

The prenatal exposure to VPA has been used to mimic the ASD pathogenesis in animals for two decades, providing important insights about the morphological, biochemical and behavioral characteristics of ASD (Bambini-Junior et al., 2011; Rodier et al., 1997; Schneider et al., 2008; Schneider and Przewłocki, 2005). Nevertheless, the exact mechanisms by which VPA acts are still currently unknown.

Social behavior impairments are a key defining feature of ASD (Dover and Le Couteur, 2007; Pascoe, 2014; Rapin and Tuchman, 2008) and are also seen in many animal models of autism, including the VPA animal model (Bambini-Junior et al., 2011; Schneider et al., 2008; Schneider and Przewłocki, 2005). However, the mechanisms that underlie these impairments are also poorly understood. Our group previously demonstrated that prenatal administration of RSV (daily subcutaneous injections from E6.5 to E18.5) prevented ASD-like social deficits induced by VPA in rats (Bambini-Junior et al., 2014). Thus, the investigation of common targets of VPA and RSV in rodents can potentially facilitate the elucidation of the mechanisms by which VPA triggers the ASD-like social impairments. Moreover, it might contribute to the discovery of new clinical markers and the development of novel therapeutic approaches for ASD.

In the present study, we extended these analyses to include other behavioral assessments: reciprocal social behavior, social transmission of food preference and repetitive self-grooming. Here, we confirm the preventive actions of prenatal exposure of RSV on social impairments of the VPA model of autism. VPA rats showed reduced total reciprocal social interaction with a conspecific (anogenital inspection, flank exploration, nose-to-nose sniffing and following behavior), which was prevented by the RSV intervention.

Whereas the reciprocal social interaction test primarily focuses on social behavior of a test animal with an unfamiliar conspecific (Schneider and Przewłocki, 2005), the STFP test allows investigating of social communication by analyzing the transmission of preferences for specific foods between conspecifics (Bessières et al., 2017). Surprisingly, RSV was not able to prevent the effects of VPA in the STFP test. Perhaps, VPA rats might have an impaired ability to detect, process or utilize socially transmitted information. Moreover, the fact that RSV is not able to prevent the deficits triggered by VPA in the SFTP, while being effective in social impairments, suggests that neural processes independent of social interaction might be important for a normal STFP behavior.

Rats exposed to VPA also spent more time self-grooming than the controls, especially in the second half of this 10-min test protocol, which was not prevented by RSV. Hence, RSV can prevent some but not all aspects altered by VPA. The apparent specificity of RSV for social behaviors deserves further investigation and can be used to narrow analysis down to pathways underlying the development of social impairments in ASD.

In addition to these behavioral findings, we identified miR134–5p as a miRNA altered in opposing ways by VPA and RSV. Interestingly, this same miRNA was also elevated in autistic patients compared to controls and we also identified a similar elevation on the circulating levels of miR138–5p in autistic patients. Intriguingly, both miR134–5p (Schratt et al., 2006) and miR 138–5p (Obernosterer et al., 2006) are thought to be brain-specific. miR134–5p is localized at the synaptodendritic compartment of rat neurons and is able to negatively regulate the size of dendritic spines in postsynaptic sites by inhibiting the translation of an mRNA encoding the protein kinase Limk1, which controls spine development (Schratt et al., 2006). On the other hand, miR138–5p may also be implicated in changes in the development of dendritic spines, since it favors the activation of the RhoA-Rock pathway (Fig. 6) (Schratt, 2009; Siegel et al., 2009). Therefore, investigation of this model, especially of the molecular mechanism of VPA action, can shed light in important biomarkers and etiological triggers of ASD.

Overall, our study suggests that evaluation of miRNA expression profile may be used to identify biological pathways altered in ASD. Since miRNA can pass to bloodstream from cells, tissues and organs (Creemers et al., 2012; Ludwig et al., 2016), changes in circulating miRNA levels may reflect changes in other tissues, including nervous system and lymphoid organs. Further studies are necessary to evaluate additional miRNA and assess different developmental stages and tissues to clarify the roles of miRNA in the etiology of ASD. Nevertheless, the translational approach employed in this work could further support the potential use of miRNA as biomarkers and therapeutic targets in ASD.

Future studies exploring the molecular alterations induced by VPA during pregnancy, and how this changes lead to long-term effects in the offspring are necessary. This is particularly important because epigenetic modulation may mediate the onset of ASD phenotypes (Andrews et al., 2017; Gottfried et al., 2015; Perera and Herbstman, 2011). In addition, studies of VPA effects on mother to embryo communication can help to clarify mechanisms involved in the etiology of ASD. The anti-inflammatory and antioxidant properties of RSV might be

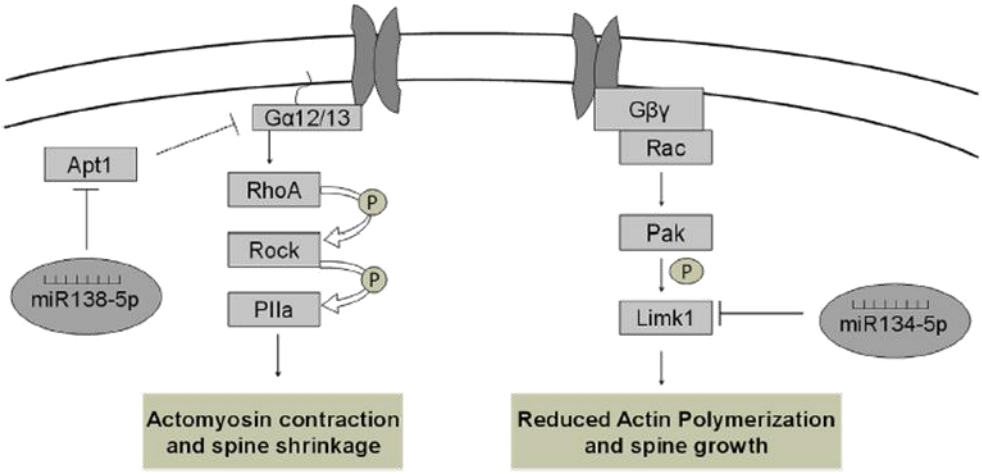


Fig. 6. Modulation of dendritic spines by miR134-5p and miR138-5p. The cytoskeleton within dendrites and spines is dynamically regulated by two antagonistic pathways: RhoA–ROCK cascade and a Rac–LIMK1 signaling. miR138–5p favors activation of the RhoA pathway and spine shrinkage by downregulation of the depalmitoylation enzyme APT1 and the resulting membrane localization and activation of the RhoA stimulatory Gα protein. On the other hand, miR134–5p inhibits LIMK1 production, thereby reducing the polymerization of filamentous actin and spine growth. Adapted from Schratt et al. (Schratt, 2009).

protective for this mother-embryo relationship in animals of the VPA model of autism.

In summary, our findings revealed important preventive actions of RSV in the VPA model of autism. These effects ranged from behavior, (as seen in the evaluation of reciprocal social interaction), to molecular alterations (miR134–5p expression). We also demonstrate for the first time that animals exposed prenatally to VPA had no preference in consuming the food presented by the demonstrator, indicating that social communication could be impaired in this model. This study reinforces the validity of the VPA model of autism and its utility to study the ASD pathophysiology.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

Brazilian National Institute of Science and Technology on Neuroimmunomodulation (INCT-NIM #465489/2014-1), Rio de Janeiro, Brazil National Council of Technological and Scientific Development (CNPq); Coordination for the Improvement of Higher Education Personnel (CAPES) and Clinical Hospital of Porto Alegre (FIPE-HCPA #140679). We would also like to thank Fluxome (Stenløse, Denmark) for the generous gift of resveratrol.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2018.02.061.

References

Ameis, S.H., Catani, M., 2015. Altered white matter connectivity as a neural substrate for social impairment in Autism spectrum disorder. Cortex 62, 158–181. http://dx.doi.org/10.1016/j.cortex.2014.10.014.

American Psychiatric Association, 2013. Diagnostic and Statistical Manual of Mental Disorders. American Psychiatric Associationhttp://dx.doi.org/10.1176/appi.books.9780890425596.

Andrews, S.V., Ellis, S.E., Bakulski, K.M., Sheppard, B., Croen, L.A., Hertz-Picciotto, I., Newschaffer, C.J., Feinberg, A.P., Arking, D.E., Ladd-Acosta, C., Fallin, M.D., 2017. Cross-tissue integration of genetic and epigenetic data offers insight into autism spectrum disorder. Nat. Commun. 8, 1011. http://dx.doi.org/10.1038/s41467-017-00868-y.

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

Bambini-Junior, V., Zanatta, G., Della Flora Nunes, G., Mueller de Melo, G., Michels, M., Fontes-Dutra, M., Nogueira Freire, V., Riesgo, R., Gottfried, C., 2014. Resveratrol prevents social deficits in animal model of autism induced by valproic acid. Neurosci. Lett. 583, 176–181. http://dx.doi.org/10.1016/j.neulet.2014.09.039.

Bessières, B., Nicole, O., Bontempi, B., 2017. Assessing recent and remote associative olfactory memory in rats using the social transmission of food preference paradigm. Nat. Protoc. 12, 1415–1436. http://dx.doi.org/10.1038/nprot.2017.050.

Chandra, S., Vimal, D., Sharma, D., Rai, V., Gupta, S.C., Chowdhuri, D.K., 2017. Role of miRNAs in development and disease: lessons learnt from small organisms. Life Sci. 185, 8–14. http://dx.doi.org/10.1016/j.lfs.2017.07.017.

Chen, C., Ridzon, D.A., Broomer, A.J., Zhou, Z., Lee, D.H., Nguyen, J.T., Barbisin, M., Xu, N.L., Mahuvakar, V.R., Andersen, M.R., Lao, K.Q., Livak, K.J., Guegler, K.J., 2005. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 33, e179. http://dx.doi.org/10.1093/nar/gni178.

Chomczynski, P., 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 15 (532–4), 536–537.

Christianson, A.L., Chesler, N., Kromberg, J.G., 1994. Fetal valproate syndrome: clinical and neuro-developmental features in two sibling pairs. Dev. Med. Child Neurol. 36, 361–369.

Creemers, E.E., Tijsen, A.J., Pinto, Y.M., 2012. Circulating MicroRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? Circ. Res. 110, 483–495. http://dx.doi.org/10.1161/CIRCRESAHA.111.247452.

Dendrinos, G., Hemelt, M., Keller, A., 2011. Prenatal VPA exposure and changes in sensory processing by the superior colliculus. Front. Integr. Neurosci. 5, 68. http://dx.doi.org/10.3389/fnint.2011.00068.

Dover, C.J., Le Couteur, A., 2007. How to diagnose autism. Arch. Dis. Child. 92, 540–545. http://dx.doi.org/10.1136/adc.2005.086280.

Galef-Jr, B.G., Wigmore, S.W., 1983. Transfer of information concerning distant foods: a laboratory investigation of the "information-centre" hypothesis. Anim. Behav. 31, 748–758. http://dx.doi.org/10.1016/S0003-3472(83)80232-2.

Geschwind, D.H., 2009. Advances in autism. Annu. Rev. Med. 60, 367–380. http://dx.doi.org/10.1146/annurev.med.60.053107.121225.

Gottfried, C., Bambini-Junior, V., Francis, F., Riesgo, R., Savino, W., 2015. The impact of neuroimmune alterations in autism spectrum disorder. Front. Psychiatr. 6, 121. http://dx.doi.org/10.3389/fpsyt.2015.00121.

Grandin, T., 2009. Visual abilities and sensory differences in a person with autism. Biol. Psychiatr. 65, 15–16. http://dx.doi.org/10.1016/j.biopsych.2008.11.005.

Hu, Y., Ehli, E.A., Boomsma, D.I., 2017. MicroRNAs as biomarkers for psychiatric disorders with a focus on autism spectrum disorder: current progress in genetic association studies, expression profiling, and translational research. Autism Res. 10, 1184–1203. http://dx.doi.org/10.1002/aur.1789.

Hutsler, J.J., Casanova, M.F., 2016. Review: cortical construction in autism spectrum disorder: columns, connectivity and the subplate. Neuropathol. Appl. Neurobiol. 42, 115–134. http://dx.doi.org/10.1111/nan.12227.

Klintwall, L., Holm, A., Eriksson, M., Carlsson, L.H., Olsson, M.B., Hedvall, Å., Gillberg, C., Fernell, E., 2011. Sensory abnormalities in autism. Res. Dev. Disabil. 32, 795–800. http://dx.doi.org/10.1016/j.ridd.2010.10.021.

Ludwig, N., Leidinger, P., Becker, K., Backes, C., Fehlmann, T., Pallasch, C., Rheinheimer, S., Meder, B., Stähler, C., Meese, E., Keller, A., 2016. Distribution of miRNA expression across human tissues. Nucleic Acids Res. 44, 3865–3877. http://dx.doi.org/10.1093/nar/gkw116.

Mabunga, D.F.N., Gonzales, E.L.T., Kim, J.-W., Kim, K.C., Shin, C.Y., 2015. Exploring the validity of valproic acid animal model of autism. Exp. Neurobiol. 24, 285–300. http://dx.doi.org/10.5607/en.2015.24.4.285.

McFarlane, H.G., Kusek, G.K., Yang, M., Phoenix, J.L., Bolivar, V.J., Crawley, J.N., 2008. Autism-like behavioral phenotypes in BTBR T+tf/J mice. Gene Brain Behav. 7, 152–163. http://dx.doi.org/10.1111/j.1601-183X.2007.00330.x.

Moore, S.J., Turnpenny, P., Quinn, A., Glover, S., Lloyd, D.J., Montgomery, T., Dean, J.C., 2000. A clinical study of 57 children with fetal anticonvulsant syndromes. J. Med. Genet. 37, 489–497. http://dx.doi.org/10.1136/JMG.37.7.489.

Nienborg, H., Cumming, B., 2010. Correlations between the activity of sensory neurons and behavior: how much do they tell us about a neuron's causality? Curr. Opin. Neurobiol. 20, 376–381. http://dx.doi.org/10.1016/j.conb.2010.05.002.

Obernosterer, G., Leuschner, P.J.F., Alenius, M., Martinez, J., 2006. Post-transcriptional

regulation of microRNA expression. RNA 12, 1161–1167. http://dx.doi.org/10.1261/ma.2322506.
Onaolapo, O.J., Paul, T.B., Onaolapo, A.Y., 2017. Comparative effects of sertraline, haloperidol or olanzapine treatments on ketamine-induced changes in mouse behaviours. Metab. Brain Dis. http://dx.doi.org/10.1007/s11011-017-0031-3.
Pascoe, J.M., 2014. Foreword. Curr. Probl. Pediatr. Adolesc. Health Care 44, 73. http://dx.doi.org/10.1016/j.cppeds.2013.12.006.
Peltier, H.J., Latham, G.J., 2008. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA 14, 844–852. http://dx.doi.org/10.1261/ma.939908.
Perera, F., Herbstman, J., 2011. Prenatal environmental exposures, epigenetics, and disease. Reprod. Toxicol. 31, 363–373. http://dx.doi.org/10.1016/j.reprotox.2010.12.055.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.
Ramakers, C., Ruijter, J.M., Deprez, R.H.L., Moorman, A.F.M., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62–66.
Rapin, I., Tuchman, R.F., 2008. Autism: definition, neurobiology, screening. Diagnosis. Pediatr. Clin. North Am. 55, 1129–1146. http://dx.doi.org/10.1016/j.pcl.2008.07.005.
Rodier, P.M., Ingram, J.L., Tisdale, B., Croog, V.J., 1997. Linking etiologies in humans and animal models: studies of autism. In: Reproductive Toxicology, pp. 417–422. http://dx.doi.org/10.1016/S0890-6238(97)80001-U.
Ryan, B.C., Young, N.B., Moy, S.S., Crawley, J.N., 2008. Olfactory cues are sufficient to elicit social approach behaviors but not social transmission of food preference in C57BL/6J mice. Behav. Brain Res. 193, 235–242. http://dx.doi.org/10.1016/j.bbr.2008.06.002.
Schneider, M., Koch, M., 2005. Deficient social and play behavior in juvenile and Adult rats after neonatal cortical lesion: effects of chronic pubertal cannabinoid treatment. Neuropsychopharmacology 30, 944–957. http://dx.doi.org/10.1038/sj.npp.1300634.
Schneider, T., Przewłocki, R., 2005. Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacology 30, 80–89. http://dx.doi.org/10.1038/sj.npp.1300518.
Schneider, T., Roman, A., Basta-Kaim, A., Kubera, M., Budziszewska, B., Schneider, K., Przewłocki, R., 2008. Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. Psychoneuroendocrinology 33, 728–740. http://dx.doi.org/10.1016/j.psyneuen.2008.02.011.
Schratt, G., 2009. microRNAs at the synapse. Nat. Rev. Neurosci. 10, 842–849. http://dx.doi.org/10.1038/nrn2763.
Schratt, G.M., Tuebing, F., Nigh, E.A., Kane, C.G., Sabatini, M.E., Kiebler, M., Greenberg, M.E., 2006. A brain-specific microRNA regulates dendritic spine development. Nature 439, 283–289. http://dx.doi.org/10.1038/nature04367.
Siegel, G., Obernosterer, G., Fiore, R., Oehmen, M., Bicker, S., Christensen, M., Khudayberdiev, S., Leuschner, P.F., Busch, C.J.L., Kane, C., Hübel, K., Dekker, F., Hedberg, C., Rengarajan, B., Drepper, C., Waldmann, H., Kauppinen, S., Greenberg, M.E., Draguhn, A., Rehmsmeier, M., Martinez, J., Schratt, G.M., 2009. A functional screen implicates microRNA-138-dependent regulation of the depalmitoylation enzyme APT1 in dendritic spine morphogenesis. Nat. Cell Biol. 11, 705–716. http://dx.doi.org/10.1038/ncb1876.
Sungur, A.Ö., Vörckel, K.J., Schwarting, R.K.W., Wöhr, M., 2014. Repetitive behaviors in the Shank1 knockout mouse model for autism spectrum disorder: developmental aspects and effects of social context. J. Neurosci. Meth. 234, 92–100. http://dx.doi.org/10.1016/j.jneumeth.2014.05.003.
Tyzio, R., Nardou, R., Ferrari, D.C., Tsintsadze, T., Shahrokhi, A., Eftekhari, S., Khalilov, I., Tsintsadze, V., Brouchoud, C., Chazal, G., Lemonnier, E., Lozovaya, N., Burnashev, N., Ben-Ari, Y., 2014. Oxytocin-mediated GABA inhibition during delivery Attenuates autism pathogenesis in rodent offspring. Science (80-.) 343, 675–679. http://dx.doi.org/10.1126/science.1247190.
Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 RESEARCH0034.
Wahid, F., Shehzad, A., Khan, T., Kim, Y.Y., 2010. MicroRNAs: synthesis, mechanism, function, and recent clinical trials. Biochim. Biophys. Acta Mol. Cell Res. 1803, 1231–1243. http://dx.doi.org/10.1016/j.bbamcr.2010.06.013.
Williams, G., King, J., Cunningham, M., Stephan, M., Kerr, B., Hersh, J.H., 2001. Fetal valproate syndrome and autism: additional evidence of an association. Dev. Med. Child Neurol. 43, 202–206.
Williams, P.G., Hersh, J.H., 1997. A male with fetal valproate syndrome and autism. Dev. Med. Child Neurol. 39, 632–634.
Wrenn, C.C., Wrenn, C., C, 2004. Social transmission of food preference in mice. In: Current Protocols in Neuroscience. John Wiley & Sons, Inc., Hoboken, NJ, USA. http://dx.doi.org/10.1002/0471142301.ns0805gs28. 8.5G.1-8.5G.7.
Wu, Y.E., Parikshak, N.N., Belgard, T.G., Geschwind, D.H., 2016. Genome-wide, integrative analysis implicates microRNA dysregulation in autism spectrum disorder. Nat. Neurosci. http://dx.doi.org/10.1038/nn.4373.

Capítulo IB:

Artigo Publicado:

“Data on social transmission of food preference in a model of autism induced by valproic acid and translational analysis of circulating microRNA”

Data in Brief 18 (2018) 1433–1440.

Data in Brief 18 (2018) 1433–1440

Contents lists available at ScienceDirect

Data in Brief

journal homepage: www.elsevier.com/locate/dib

Data Article

Data on social transmission of food preference in a model of autism induced by valproic acid and translational analysis of circulating microRNA

Mauro Mozael Hirsch [a,b,c,*], Iohanna Deckmann [a,b,c], Mellanie Fontes-Dutra [a,b,c], Guilherme Bauer-Negrini [a,b,c], Gustavo Della-Flora Nunes [a,b,c], Walquiria Nunes [a,b,c], Bruna Rabelo [a,b], Rudimar Riesgo [a,c,d], Rogerio Margis [c,e], Victorio Bambini-Junior [a,c,f], Carmem Gottfried [a,b,c,*]

[a] Translational Group in Autism Spectrum Disorder - GETTEA, Clinical Hospital of Porto Alegre, RS, Brazil
[b] Department of Biochemistry, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, RS, Brazil
[c] Brazilian National Institute of Science and Technology on Neuroimmunomodulation (INCT-NIM), Rio de Janeiro, Brazil
[d] Child Neurology Unit, Clinical Hospital of Porto Alegre, Federal University of Rio Grande do Sul, Porto Alegre, Brazil
[e] Center of Biotechnology and PPGBCM, Laboratory of Genomes and Plant Populations, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, RS, Brazil
[f] School of Pharmacy and Biomedical Sciences, University of Central Lancashire, Preston, UK

ARTICLE INFO

Article history:
Received 2 March 2018
Received in revised form
12 April 2018
Accepted 13 April 2018
Available online 21 April 2018

Keywords:
Autism
Social transmission of food preference
microRNA
Resveratrol
Translational research

ABSTRACT

This article contains data of Social Transmission of Food Preference in an animal model of autism and the evaluation of a set of microRNA analyzed in autistic patients and animal model of autism. The analyses of the absolute consumption of two flavored food by male rats prenatally exposed to valproic acid (VPA) and treated with resveratrol (RSV), showed that VPA animals show a trend to eat less of the flavored food presented by a demonstrator rat. We also identified 13 microRNA with similar levels among rodents' experimental groups, as well as 11 microRNA with no alterations between autistic and control subjects. Further evaluation of mechanisms of VPA and RSV actions on behavioral and molecular alterations can shed light

DOI of original article: https://doi.org/10.1016/j.fct.2018.02.061
* Corresponding authors at: Translational Group in Autism Spectrum Disorder - GETTEA, Clinical Hospital of Porto Alegre, RS, Brazil.
E-mail addresses: mauromhirsch@gmail.com (M.M. Hirsch), cgottfried@ufrgs.br (C. Gottfried).

https://doi.org/10.1016/j.dib.2018.04.047

Preclinical models
Valproate

in important biomarkers and etiological triggers of autistic spectrum disorders.

Specifications Table

Subject area	*Pharmacology, Toxicology and Pharmaceutical Science*
More specific subject area	*Autism Spectrum Disorder* *Toxicology* *Natural products*
Type of data	*Table, Figures*
How data was acquired	*Animal behavior analysis and Reverse Transcription followed by Quantitative Polymerase Chain Reaction (RT-qPCR)*
Data format	*Analyzed*
Experimental factors	*The animal model of autism was induced by a single intraperitoneal injection of 600 mg/kg VPA or saline solution on embryonic day 12.5 (E12.5). RSV treatments were achieved by daily subcutaneous injections of RSV (3.6 mg/kg) or DMSO from E6.5 to E18.5. Blood samples from these animals were obtained by cardiac puncture 30 days after birth. Peripheral blood samples from autistic male individuals and from the control group (5–15 years-old range) were obtained at Clinical Hospital of Porto Alegre (HCPA).*
Experimental features	*Social Transmission of Food Preference (STFP) test was performed in male rats after food habituation, consumption of one of flavored food by demonstrator rat and interaction between demonstrator and observer rats. The amount of cued and non-cued food eaten by observers from each litter was weighed and recorded. After homogenization of blood samples, we performed RNA extraction and the mature miRNA expression was evaluated by reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR), using fluorescence of SYBR Green to detect amplification, estimate Ct values and to determine specificity after melting curve analysis.*
Data source location	*Department of Biochemistry, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, RS, Brazil. Clinical Hospital of Porto Alegre, Porto Alegre, RS, Brazil.*
Data accessibility	*Hirsch, M. M. et al. Behavioral alterations in autism model induced by valproic acid and translational analysis of circulating microRNA.* Food Chem. Toxicology 115 (2018): 336–343. doi: 10.1016/j.fct.2018.02.061.[1]

Value of the data

- Social transmission by absolute consumption of two flavored food in VPA-induced animal model of autism.
- MicroRNA analysis in blood samples from male rats prenatally exposed to VPA and treated with RSV, compared to control animals.
- MicroRNA analysis in blood samples from 5 to 15 years old autistic subjects.

1. Data

Fig. 1 presents the absolute food consumption of Wistar rats prenatally exposed to VPA, compared to the control animals, after treatment with RSV or vehicle. The animals exposed to VPA have a tendency to eat less of the flavored-food cued by demonstrator (Fig. 1A, $p=0.080$). On the other hand,

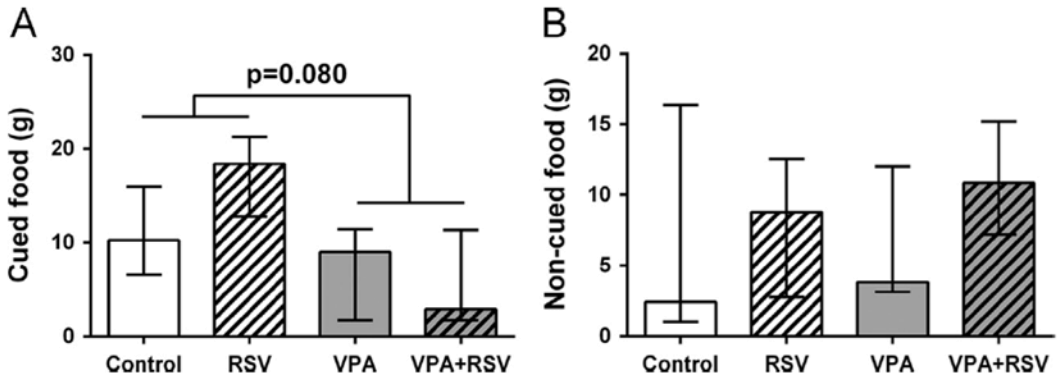


Fig. 1. Absolute weights of consumed food in VPA autism model. Cued (A) and non-cued (B) food. Plots show medians ± interquartile intervals. Statistical analysis: Independent-Samples Kruskal–Wallis test. Control ($n=8$), RSV ($n=9$), VPA ($n=9$), VPA+RSV ($n=6$).

animals presented no differences in consumption of alternative (non-cued) food across interventions (Fig. 1B, $p=0.493$).

We evaluated the relative expression of miRNA in blood of 30 days rats prenatally exposed to VPA or vehicle (saline) and treated with RSV or vehicle (DMSO). The GeNorm algorithm ranked miRNA miR181a-5p and miR181b-5p as the most stable ones and they were used as normalizers to evaluate the relative expression of the remaining miRNA. We observed no significant differences in levels of 13 miRNA (Fig. 2).

We also determined the relative expression of a set of miRNA in peripheral blood of autistic subjects, compared with controls in the same age-range (5–15 years). The GeNorm algorithm ranked miR23a-3p, miR146a-5p and miR181a-5p as the most stable ones and they were used as normalizers to evaluate the relative expression of the remaining miRNA. There were no intra-group differences related to age on miRNA levels. The analysis of relative expression revealed no significant differences in 11 miRNA in peripheral blood of autistic children, compared to control subjects (Fig. 3).

2. Experimental design, materials and methods

2.1. Animal model of autism induced by VPA and resveratrol treatment

The animal model of autism was induced as previously described [2,3]. Briefly, female Wistar rats (UFRGS-Biochemistry Department CREAL), with controlled fertility cycles, were mated overnight. The first day of gestation was determined by the presence of spermatozoa in the vaginal smear (embryonic day 0.5). Pregnant females received a single intraperitoneal injection of 600 mg/kg VPA (sodium valproate, Sigma-Aldrich, USA) or saline solution on embryonic day 12.5 (E12.5), and daily subcutaneous injections of RSV (trans-resveratrol, Fluxome, Stenløse, Denmark, 3.6 mg/kg) or DMSO from E6.5 to E18.5 [4]. Only males of the offspring were used in this study. Blood samples from these animals were obtained by cardiac puncture 30 days after birth. This project was approved by the local animal ethics committee (CEUA-UFRGS 140367/140431) and all animals were handled in accordance with established guidelines (National Council for the Control of Animal Experimentation (CONCEA)).

2.2. Evaluation of food consume in STFP test

This experiment was adapted from Wrenn et al. protocol [5]. Rats at 47 days of age were habituated for 72 h to eat pelleted food made from powdered chow. In the following day, food was removed three hours before the test. Next, one animal from each cage (demonstrator) was housed alone in a separate housing box and was allowed to eat a randomly assigned flavored food for 1 h: either cinnamon (1% w/w) or cocoa (2% w/w). Then the demonstrator rat was housed with their littermates (the observer rats) and allowed free interaction for 30 min. After this interaction period, the demonstrator animal was removed from the housing box and the observer animals were provided

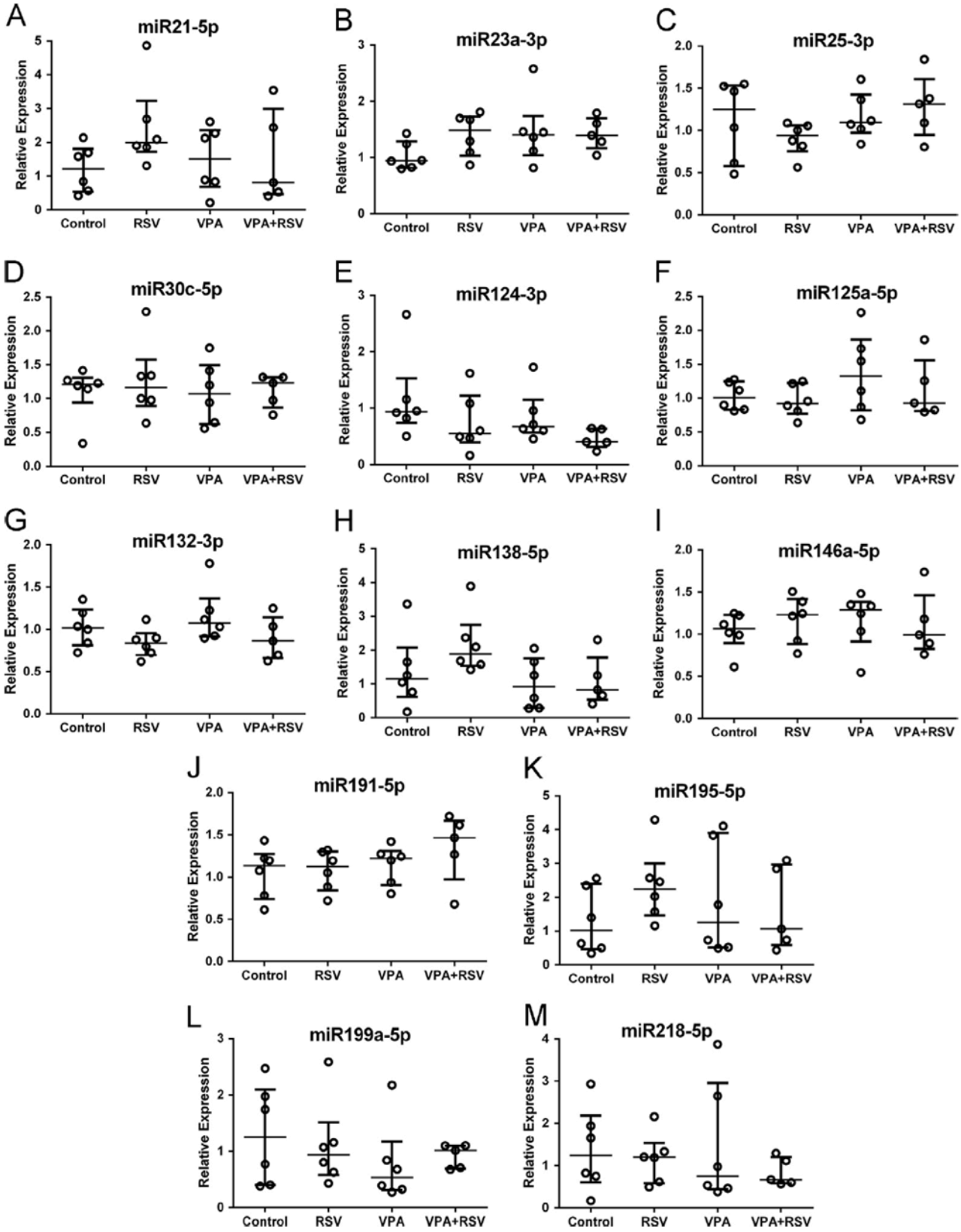


Fig. 2. Relative expressions of unchanged miRNA in peripheral blood of VPA autism model at P30. Plots presented as medians ± interquartile intervals. Statistical analysis: Independent-Samples Kruskal–Wallis test. Control ($n=6$), RSV ($n=6$), VPA ($n=6$), VPA + RSV ($n=5$).

with two choices of powdered food in identical pellets, one with flavor of the cued food presented by demonstrator rat and the other with the alternative food. Rats were allowed to eat for one and a half hours, and the amount of cued and non-cued food eaten from each litter was weighed and recorded.

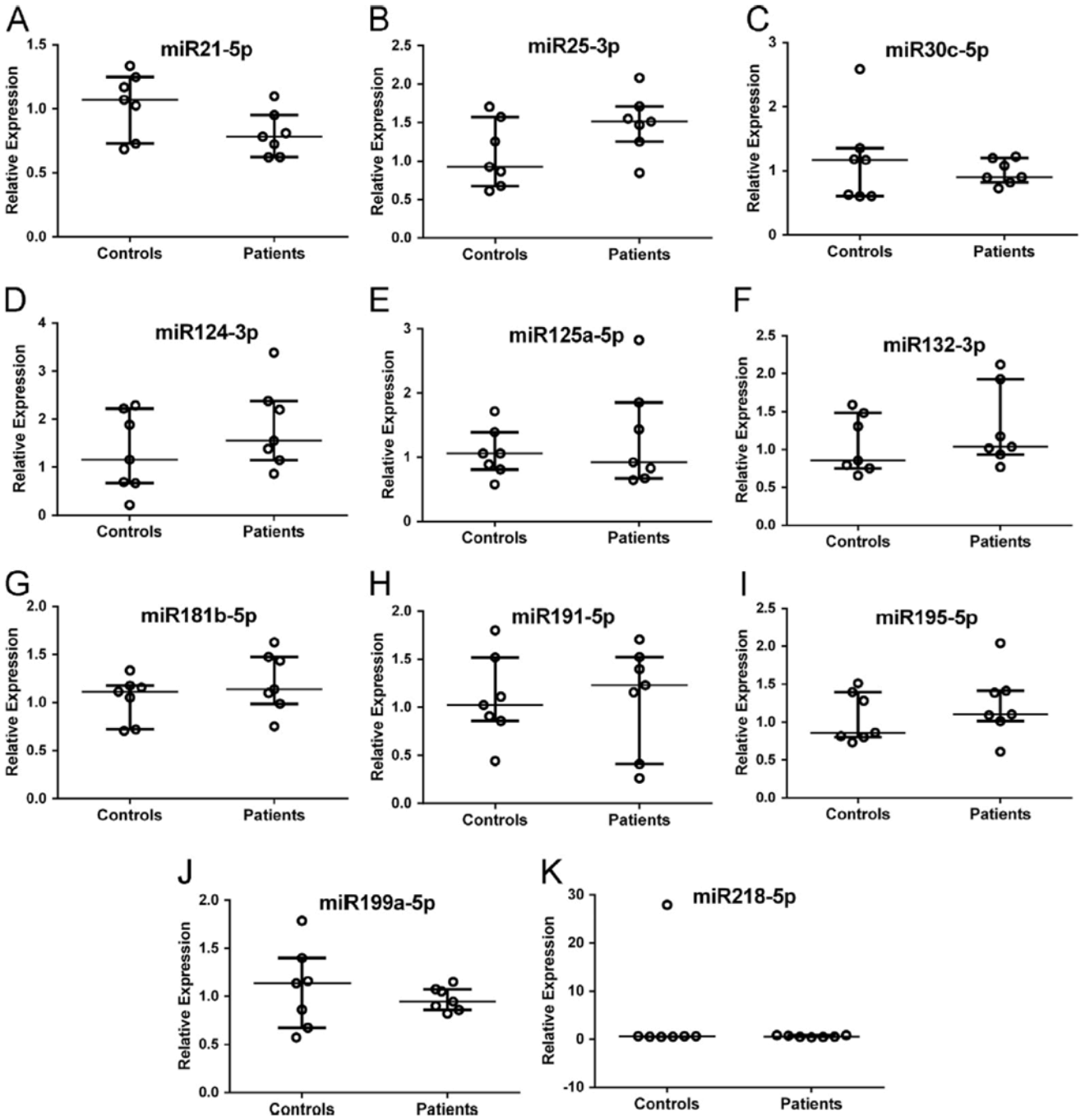


Fig. 3. Relative expressions of uncharged miRNA in peripheral blood of autistic children compared to control group. Results expressed as medians ± interquartile intervals. Statistical analysis: Mann–Whitney U-test. Control ($n=7$) and autistic patients ($n=7$).

2.3. Blood samples from autistic and control subjects

Peripheral blood samples from autistic male individuals and from the control group (5–15 years-old range) were obtained at the Clinical Hospital of Porto Alegre (HCPA). Inclusion criteria were age 5–15 years, have a clinical diagnosis of ASD according to DSM-5 and confirmed using the Autism Diagnostic Observation Schedule. Autistic individuals who presented secondary autism or autism as an associated feature to an identified genetic condition (Fragile X Syndrome, Rett Syndrome, Angelman Syndrome, Prader-Willi Syndrome, Smith-Lemli-Opitz Syndrome and Tuberous Sclerosis) were excluded from the study. This project was approved by the local ethics committee (CEUA-UFRGS 33863).

Table 1
Primer sequences for 16 miRNA evaluated. Forward and RT stem-loop primers and an universal reverse primer.

miRNA ID	miRNA sequence	Primer type	Primer sequences
miR132-3p	uaacagucuacagccauggucg	Forward	TCC GGC TAA CAG TCT ACA GCC A
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC cgacca
miR138-5p	agcugguguugugaaucaggccg	Forward	TCC GGA AGC TGG TGT TGT GAA TC
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC cggcct
miR125a-5p	ucccugagacccuuuaaccuguga	Forward	GTC GCG ATC CCT GAG ACC CTT TA
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC tcacag
miR195-5p	uagcagcacagaaauauuggc	Forward	GGG CGC TAG CAG CAC AGA AAT A
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC gccaat
miR199a-5p	cccaguguucagacuaccuguuc	Forward	GAT GCG CCC AGT GTT CAG ACT
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC gaacag
miR134-5p	ugugacugguugaccagagggg	Forward	GGC TCT TGT GAC TGG TTG ACC A
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC cccctc
miR124-3p	uaaggcacgcggugaaugcc	Forward	CTA GCT TAA GGC ACG CGG TGA
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC ggcatt
miR181a-5p	aacauucaacgcugucggugagu	Forward	GCG CTG AAC ATT CAA CGC TGT C
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC actcac
miR181b-5p	aacauucauugcugucgguggguu	Forward	GCT GCG CAA CAT TCA TTG CTG TC
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC acccac
miR25-3p	cauugcacuugucucggucuga	Forward	TCA GCA CAT TGC ACT TGT CTC GG
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC tcagac
miR21-5p	uagcuuaucagacugauguuga	Forward	CCG GCG CTA GCT TAT CAG ACT GAT
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC tcaaca
miR23a-3p	aucacauugccagggauuucc	Forward	GCT GTC ATC ACA TTG CCA GGG A
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC ggaaat
miR146a-5p	ugagaacugaauuccauggguu	Forward	CGT GGC GTG AGA ACT GAA TTC CA
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC aaccca
miR218-5p	uugugcuugaucuaaccaugu	Forward	GCC GTC CTT GTG CTT GAT CTA ACC
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC acatgg
miR30c-5p	uguaaacauccuacacucucagc	Forward	GCG TCG CTG TAA ACA TCC TAC ACT C
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC gctgag
miR191-5p	caacggaaucccaaaagcagcug	Forward	GGA GCG TCA ACG GAA TCC CAA AAG
		RT stem-loop	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC cagctg
Reverse Universal Primer		Reverse	CCA GTG CAG GGT CCG AGG TA

2.4. RNA extraction and RT-qPCR procedure

After homogenization of blood samples with Trizol® reagent (Invitrogen, USA), chloroform was added to perform phase separation, and RNA was precipitated from the upper aqueous layer using isopropanol. The precipitated RNA was washed with ethanol to remove impurities, resuspended in RNase-free water and stored at −80 °C [6].

Mature miRNA expression was evaluated by reverse transcriptase followed by quantitative polymerase chain reaction (RT-qPCR) [7]. Complementary DNA (cDNA) was synthesized from mature miRNA using reverse transcriptase reaction containing 2 μg of total RNA, 1 μL of 10 mM dNTP mix (Invitrogen, USA), 3 μL of stem loop RT primer mix (Supplementary Table 1), 4 μL M-MLV reverse transcriptase 5× reaction buffer (Invitrogen, USA), 2 μL of 0.1 M DTT (Invitrogen, USA), 1 μL of RNase inhibitor (Invitrogen, USA), 1.0 μL of M-MLV reverse transcriptase (Invitrogen, USA), and sterile distilled water to a final volume of 20 μL. The synthesis of the cDNA was completed after a sequence of three incubations at 65 °C for 5 min, 37 °C for 50 min and 70 °C for 15 min.

The quantitative PCR mix was comprised by 12 μL of cDNA (1:33), 1.0 μL of specific miRNA forward and universal reverse (10 μM) primers (as detailed in Table 1), 0.5 μL of 10 μM dNTP mix, 2.4 μL of 10× PCR buffer (Invitrogen, USA), 0.8 μL of 50 mM $MgCl_2$ (Invitrogen, USA), 2.4 μL of 1× SYBR™ Green (Molecular Probes, USA), 0.1 μL of Platinum Taq DNA Polymerase (Invitrogen, USA) and sterile distilled water to a final volume of 24 μL. The fluorescence of SYBR™ Green was used to detect amplification, estimate Ct values, and to determine specificity after melting curve analysis. PCR cycling conditions were standardized to 95 °C for 5 min followed by 40 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. After the main amplification, sample fluorescence was measured from 60 °C to 95 °C, with an increasing ramp of 0.3 °C each, to obtain the denaturing curve of the amplified products and Tm estimation, to assure their homogeneity after peak detection. Data was obtained from an Applied Biosystems StepOne System (USA). The set of 16 miRNA selected for this study includes miRNA involved in immunological and/or synaptic plasticity modulation, both processes altered in ASD and includes miRNA commonly altered in many neurodevelopmental disorders.

2.5. Calculation of miRNA relative expression

The RT-qPCR results were imported into Microsoft Excel and the geNorm program was used to assess the variance in expression levels of the miRNA analyzed [8,9]. This program scanned the present miRNA two at a time. Then, the expression stabilities of the set of miRNA were evaluated. All miRNA were ranked accordingly to their stability. A pairwise variation analysis was performed by geNorm to determine the number of miRNA required for accurate normalization and to identify the most suitable miRNA to be used as normalizers.

The average value of crossing threshold (Ct) values (in triplicate) was converted to quantities for geNorm analysis by the difference between Ct values from two groups taken in each comparison. PCR efficiency was calculated from the slope of the amplification curve by exponential amplification analysis using the LinRegPCR algorithm [10]. The relative expression was obtained using the $-\Delta\Delta$Ct method where Ct values of a group are subtracted from the average Ct values of the control group. The relative expression of miRNA was calculated considering the PCR efficiency and the $-\Delta\Delta$Ct values for each miRNA [11] and were normalized to the normalizers identified by the geNorm software.

2.6. Statistical analysis

IBM SPSS Statistics 20.0 (IBM SPSS, Armonk, NY, USA) was used to perform the statistical analysis. Kolgomorov–Smirnov and Shapiro–Wilk tests of normality were applied to determine data distribution. The absolute consumption of each food flavor presented non-normal distribution, so that were compared by non-parametric Kruskal–Wallis test and the results expressed as median ± interquartile interval. The miRNA relative expressions of the four animal groups were compared using non-parametric Kruskal–Wallis test and the results were expressed as median ± interquartile interval. For the analysis of the human samples, non-parametric Mann–Whitney U-test was performed and the results were expressed as median ± interquartile interval. All statistical analyses were supervised by the Biostatistics Unit at the Clinical Hospital of Porto Alegre.

Acknowledgements

Brazilian National Institute of Science and Technology on Neuroimmunomodulation (INCT-NIM), Rio de Janeiro, Brazil; National Council of Technological and Scientific Development (CNPq); Coordination for the Improvement of Higher Education Personnel (CAPES) and Clinical Hospital of Porto Alegre (FIPE-HCPA). We would also like to thank Fluxome (Stenløse, Denmark) for the generous gift of resveratrol.

Transparency document. Supporting information

Transparency data associated with this article can be found in the online version at https://doi.org/10.1016/j.dib.2018.04.047.

References

[1] M.M. Hirsch, I. Deckmann, M. Fontes-Dutra, G. Bauer-Negrini, G. Della-Flora Nunes, W. Nunes, B. Rabelo, R. Riesgo, R. Margis, V. Bambini-Junior, C. Gottfried, Behavioral alterations in autism model induced by valproic acid and translational analysis of circulating microRNA, Food Chem. Toxicol. 115 (2018) 336–343.
[2] V. Bambini-Junior, L. Rodrigues, G.A. Behr, J.C.F. Moreira, R. Riesgo, C. Gottfried, Animal model of autism induced by prenatal exposure to valproate: behavioral changes and liver parameters, Brain Res. 1408 (2011) 8–16.
[3] T. Schneider, R. Przewłocki, Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism, Neuropsychopharmacology 30 (2005) 80–89.
[4] V. Bambini-Junior, G. Zanatta, G. Della Flora Nunes, G. Mueller de Melo, M. Michels, M. Fontes-Dutra, V. Nogueira Freire, R. Riesgo, C. Gottfried, Resveratrol prevents social deficits in animal model of autism induced by valproic acid, Neurosci. Lett. 583 (2014) 176–181.
[5] C.C. Wrenn, C.C. Wrenn, Social transmission of food preference in mice, in: Curr. Protoc. Neurosci., John Wiley & Sons, Inc, Hoboken, NJ, USA, 2004 (8.5G.1-8.5G.7).
[6] P. Chomczynski, A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples., Biotechniques 15 (532–4) (1993) 536–537.
[7] C. Chen, D.A. Ridzon, A.J. Broomer, Z. Zhou, D.H. Lee, J.T. Nguyen, M. Barbisin, N.L. Xu, V.R. Mahuvakar, M.R. Andersen, K.Q. Lao, K.J. Livak, K.J. Guegler, Real-time quantification of microRNAs by stem-loop RT-PCR, Nucleic Acids Res. 33 (2005) e179.
[8] H.J. Peltier, G.J. Latham, Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues, RNA 14 (2008) 844–852.
[9] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) 1–11 (RESEARCH0034).
[10] C. Ramakers, J.M. Ruijter, R.H.L. Deprez, A.F.M. Moorman, Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data, Neurosci. Lett. 339 (2003) 62–66.
[11] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) e45.

Capítulo II:

“MicroRNA analysis by polymerase chain reaction: from biological sources to target searching”

A ser submetido no periódico “BMC Molecular Biology”.

MicroRNA analysis by polymerase chain reaction: from biological sources to target searching

Mauro Mozael Hirsch[1,2,3*], Giovanna Carello-Collar [1,2,3], Carmem Gottfried[1,2,3*].

1. Translational Group in Autism Spectrum Disorder - GETTEA, Clinical Hospital of Porto Alegre, RS – Brazil
2. Department of Biochemistry, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, RS, Brazil.
3. Brazilian National Institute of Science and Technology on Neuroimmunomodulation (INCT-NIM), Rio de Janeiro, Brazil.

*CORRESPONDING AUTHORS:
MMH (mauromhirsch@gmail.com) and CG (cgottfried@ufrgs.br)
Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos 2600 – 21111. CEP: 90035-003 Porto Alegre-RS, Brazil.

Abstract

MicroRNA (miRNA) is a group of small non-coding RNA that control protein expression, emerging emerge as important regulators of several biological processes and may exert important roles in triggering and progress of several disorders. The presence of miRNA in different cell types, tissues and even in body fluids has implicated these small RNA as potential biomarkers of several diseases. However, in order to explore miRNA in this feature, it is required the use of expensive methodological sequencing. On the other hand, miRNA may provide important clues about altered pathways and mechanisms involved in several diseases, in addition to provide new possibilities for therapeutic strategies. In this context, RT-qPCR emerges as a more accurate and less expensive method able to supply the biomedical research about this field. Here, we report several key procedural aspects of miRNA quantification in tissues and peripheral compartments. We also highlight some cautions to be taken during the RT-qPCR experiments, in order to obtain more accurate and reliable results about miRNA relative expression in context of several human disorders.

Keywords: biological samples, biomarkers, CNS disorders, mechanistic studies, microRNA, RT-qPCR, therapeutic strategies.

Introduction

Since the discovery of the first miRNA in 1993 [1], the knowledge about these small molecules has been growing up year after year. Once it was discovered that miRNA are widespread in both plant and animal kingdoms and exhibit a complex expression profile [2–4], the study of these small molecules has attracted much importance in the last years.

In physiological conditions, miRNA may regulate cell differentiation, cell proliferation and survival, and metabolism, among many other functions [5]. Once miRNA regulate thousands of genes, it is not surprising that the disruption of their pattern of expression may be related to onset and progression of several diseases, including neuropsychiatric diseases. *Thus,* since miRNA research has made a significant impact on all aspects of biomedical research, their potential role as prognostic and predictive biomarkers in patient management has been described [6–11]. In addition to these prognostic approaches, miRNA can provide a better understanding of pathway regulation in model systems to explain coordinated gene expression changes in diseases and create new possibilities for mechanistic studies in order to investigate new nucleic acid–based therapies.

Biological sources of miRNA

Several studies have been supported the evidence that miRNA play a role in central nervous system (CNS) development and homeostasis, including neurogenesis, neuronal differentiation and survival, glial function,

synaptogenesis an neuroplasticity [12–15]. Post mortem brain analysis has been revealed significant alterations in miRNA levels in several CNS diseases, including schizophrenia [16,17], bipolar diseases [18,19], Alzheimer's disease, Parkinson's disease and Autism Spectrum Disorders [20].

In human CNS disorders, the research is frequently dependent of the reliability on peripheral sources of miRNA to obtain useful data to the better understanding of disease mechanism and therapeutics, since living brain tissue samples are poorly accessible from human subjects, which make more difficult the investigation of miRNA levels in CNS disorders.

The remarkable discovery that stable miRNA could be found in total blood, serum and plasma was soon confirmed and extended by many researchers [21–23]. Recent studies demonstrated that miRNA can pass to bloodstream from cells or tissues and organs [24–26], which makes reasonable to assume that changes in miRNA levels in blood reflect possible changes in other tissues, including CNS. Based on this, several researchers focused on the possibility that changes in levels of circulating miRNA could be adopted as less invasive or even noninvasive biomarkers for a variety of pathological conditions.

Two major sources of circulating miRNA are extracellular miRNA and miRNA within peripheral mononuclear blood cells (PBMC) [27,28]. Extracellular miRNA is most certainly found in conjugated forms, because naked miRNA degraded within seconds due to high levels of nucleases in blood. Several reports have demonstrated that extracellular miRNA obtains its stability through several mechanisms. Serum stability can result from the formation of complexes between circulating miRNA and specific proteins [29–31]. However, although

the extracellular miRNA are stable in purified plasma or serum, it is recommended that the blood be processed within 2-4 hours in EDTA tubes[32], once citrate and heparin can interfere in PCR analysis [33]. In addition to this, cellular components of blood can release miRNA until separation procedure, so that miRNA from blood cells can contributes to extracellular miRNA levels found is plasma or serum [34]. Expression of circulating miRNA may also be determined from isolated peripheral blood mononuclear cells [28,35], which can be very interesting when the studied disorder is associated to immune and inflammatory impairments.

In addition to these blood-derived samples, miRNA expression may be also obtained from other body fluids, including whole urine, saliva and cerebrospinal fluid [36]. Other studies have found miRNA within circulating exosomes and other microvesicles, which ensure their stability even in naked form. However, to attend biomedical research, it is necessary to clarify if the miRNA levels in these compartments are able to resemble biological processes in the brain, arguably the most unique of organs with a distinct composition and cellular milieu. Besides, the miRNA content in these alternative samples should be sufficient to provide accurate and satisfactory results. Unfortunately, the most promising human samples available to biomarkers discovery (those obtained by less invasive manners) usually have very low miRNA content, which requires additional – and frequently more expensive – methods to improve the miRNA quantification.

miRNA profiling techniques

The miRNA analysis from a sample can be accomplished in several ways, each of which has advantages and limitations. The choice of technique to

be used for miRNA analysis depends on its purpose. Today, the major approaches are microarrays, RNAseq, and RT-qPCR.

Screening with microarrays requires relatively large amounts of RNA input and is limited in both sensitivity and dynamic range. The range of miRNA expression in a typical cell is several orders of magnitude larger than the dynamic range of microarray, therefore a large number of expressed sequences will be undetectable. Likewise, it is obvious that only is possible to detect miRNA species that have corresponding assays on the array, meaning that this tool is not a suitable for discovery of new miRNA species. In addition to these disadvantages, microarrays are currently a very expensive assay, due the high costs of available platforms, which should include specific probes for every miRNA to be analyzed.

Next-generation sequencing (NGS) of RNA, often called RNA-seq, is a relatively new technology that takes advantage of massively parallel sequencing on a solid support. This technique is highly suited to discovery, enabling characterization of SNPs, mutations, processing variants, and novel miRNA species. RNA-seq is generally regarded as a screening technology. It characterizes all sequences in a sample in a semi-quantitative, but exhaustively thorough, manner. Thus, RNA-seq technique plays an essential role in search for biomarkers for several diseases, since it is highly suited to discovery novel miRNA species [10,37]. On the other hand, microarray and RT-qPCR techniques require prior knowledge of the sequences of all the miRNA to be investigated.

Although miRNA represent a relatively abundant class of transcripts, their expression levels vary greatly among species, organs, tissues and cell types

[38]. Thus, depending on nature and amount of sample, some less abundant miRNA routinely escape detection with technologies such microarray analysis [39,40].

For miRNA profiling, RT-qPCR is actually the preferred method for accurate and sensitive detection of miRNA and other small RNA. In a single run, miRNA ranging from 10 to 10^7 copies can be accurately quantified. Sample requirements for RT-qPCR are much lower than for RNA-seq or microarrays. Excellent sensitivity can be achieved with less than 1 µg. From serum or plasma, this corresponds to the miRNA content of approximately 100 µl sample. In addition, RT-qPCR allows miRNA profiling from other body fluids that have far less miRNA than serum, such as cerebrospinal fluid, saliva and urine. RT-qPCR is an excellent technique for studies of molecular mechanisms involving one or a few miRNA, whose sequences have already been described and well known. In addition, one must have prior knowledge if this group of miRNA to be analyzed is expressed in the species, organs and tissues or cells of interest.

On the other hand, the RT-qPCR technique alone is not able to supply some research needs. For example, in the search for possible miRNA that could serve as potential biomarkers (including some miRNA whose sequence is still unknown) for certain pathological conditions, the methodology requires a previous screening of all the possible miRNA expressed in the study species, in general human. For this purpose, microarray and RNA-seq techniques are more useful.

Principle of the RT-qPCR for miRNA quantification

Quantification of miRNA with RT-qPCR was first proposed by Chen. Here, we listed the main points to be considered on the investigations of miRNA

relative expression from various biological samples and some methodological procedures about RT-qPCR.

RNA isolation:

The outcomes of miRNA analysis depend on several aspects of the overall process, beginning with the nature of the sample and the way it is collected, preserved and processed. Generally, all procedures must be carefully performed in order to avoid sample contamination. The collection of fresh samples requires their preservation by different methods, such as coagulation prevention, freezing and fixing. The preservation process must be carried out properly in order to avoid degradation of the miRNA in the sample.

For RNA isolation, the most widely used process is based on the use of a reagent containing phenol and guanidine thiocyanate [41]. A biological sample is homogenized in the reagent and next properly stored at low temperatures. The isolation of RNA can be accomplished by three procedure steps: phase separation with chloroform, RNA precipitation using isopropanol and RNA wash with 75% ethanol [42].

There are several studies demonstrating alternative forms of isolation of genetic material from various biological samples. In general, analysis of miRNA expression requires previous stages of isolation of small RNA, which require the acquisition of commercial kits that increase the cost of the procedure.

After RNA isolation, we assessed the RNA quality by running an extract aliquot on a Northern blot assay. Additionally, the quantity of RNA in sample may be measured with a spectrophotometer, which also may provide RNA quality through spectrophotometric indexes A_{260}/A_{230} and A_{260}/A_{280}. RNA

samples with acceptable quality and sufficient amount of total RNA can be used for reverse transcription.

Reverse Transcription:

Unlike mature messenger RNA, which have a polyadenylated tail at their 3 'end, complementary DNA (cDNA) synthesis can not be performed using oligoDT primers. Thus, for this purpose, it is necessary to acquire specific primers, called stem-loop primers. By using these primers, the strand cDNA synthesis lengthens the target from its original approximately 22 nt to >60 nt.

The design of stem-loop primers combines the following 44 nt described by Chen: 5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC -3′ with the complement of the six 3’ nt of the mature miRNA sequence [43,44].

The amount of total RNA extract used in this step is closely dependent of relative abundance of miRNA on sample to be analyzed. Generally, the synthesis of cDNA is accomplished by using 2 µg of total RNA, which can provide sufficient amount of miRNA for our proposed PCR analysis. In order to preserve the miRNA stability in the reverse transcription procedure, we recommend the use of nuclease inhibitors. Besides, several cautions must be taken, like the use of nuclease-free materials and sterile and distilled water.

Real time quantitative PCR:

PCR amplification utilizes a forward primer which includes extra 5’ nts to adjust for an appropriate Tm. First, we take the first 12 to 17 nt of the 5’ end of the mature miRNA, and 3 to 7 additional 5’ nt selected in order to get the Tm approximately 60°C. Finally, by using the same 44 nt portion in the sequence for all stem-loop primers, a universal primer that is complementary to this

sequence can be designed, which is able to annealing to the cDNA during amplification. Chen used the following 16 nt sequence of reverse universal primer: *5'- GTG CAG GGT CCG AGG T -3'* [43]*. However, in order to improve the selectivity, Kramer recommended the following 20 nt sequence* 5'-CCA GTG CAG GGT CCG AGG TA-3' [44].

Optionally, a hydrolysis probe for detection and quantification of PCR products can be designed in conjugation to its fluorophore binder. However, in order to reduce the costs of miRNA expression analysis, SyBr Green-based amplification detections may be used, providing satisfactory results when the necessary precautions are taken prior and during the analysis, including a careful primers design and precaution against sample contaminations.

Normalization for miRNA expression analysis

The accuracy of the miRNA expression analyzes by RT-qPCR is critically dependent on the normalization of the obtained data. The purpose of normalization is to remove variations due to sampling, allowing real differences in miRNA expression profiles as a consequence of pathological condition, treatment or stage of development.

Several studies reported the use of small, non-coding RNAs, such as snRNA and snoRNA, as normalization strategies to determine miRNA expression levels. However, these RNA are not expressed in serum and plasma or may present fluctuations in their expressions between experimental groups. Thus, alternative methods of normalization are necessary in experiments involving these sample types [45]

Some miRNA exhibit expression profiles that do not change under different conditions, and may serve as reference miRNA in the quantifications of

other miRNA that do not exhibit such stability. In our miRNA analysis, we usually performed a scan of the miRNA selected for the study, with the aim of finding the miRNA normalizers for our analyses. We used the geNorm and NormFinder algorithms to investigate the variance of all selected miRNAs and then assess the stability of miRNA expression levels [46].

Calculation of miRNA expression

The level of miR expression was measured using the average of Ct (cycle threshold) values. Frequently, miRNA relative expression can be accomplished by comparative $2^{-\Delta\Delta Ct}$ method, using the most stable miRNA (or alternative non-coding small RNA) as normalizers [46,47]. However, in some cases, lower values of PCR efficiencies require the calculation of exact value of amplification efficiencies for each reaction.

The PCR efficiency may be calculated from the slope of the amplification curve by exponential amplification analysis using the LinRegPCR algorithm [48]. Thus, the relative expression of miRNA may be calculated considering the exact PCR efficiency (Eff) and the –ΔΔCt values for each sample, according to methodology previously described [47].

In order to accurately measure the miRNA relative expression, we recommend the use of multiple normalizers instead of only one. The number of normalizers will depend on the distribution and stability of the data among samples and experimental groups. Finally, for more accurate results, a geometric mean for different normalizers was proposed [49].

Identification of downstream targets of miRNA

The identification of direct targets of altered miRNA can be accomplished in two different ways. Firstly, there are many available target predictions

databases which have been provided important clues about mechanisms of action of several miRNA, although the predictive value is frequently limited. Second, physiological miRNA targets can be identified and validated through different current experimental strategies [50].

Regarding to predicted targets, different predictive platforms are currently available, including TargetScan [51], MiRWalk [52], MiRDB [53] and microRNA.org [54,55]. Some less rigorous programs present lower accurate predictions, whilst others reach higher percentages but they are significantly less sensitive, so failing to predict a large number of miRNA sequences that were found active by experimental methods.

Most of experimental strategies for miRNA targeting are based on over-expression or inhibition of a specific miRNA in a cell line followed by assessing the downstream effects, for example, by protein levels analysis or by assessing a specific cellular function. Additionally, luciferase assays are commonly used in order to validate putative miRNA targets [56,57].

Conclusion

Since miRNA are important regulators of protein expression and involved in many cellular processes, the research on miRNA involvement in several CNS diseases has grown up in the last years, supporting their role in molecular mechanisms involved on triggering and progress of these conditions. Also, they may prove to be valuable diagnostic markers for a number of diseases and play yet promising targets to therapeutic strategies.

The potential of miRNA as biomarkers is, at this point in time, still rather limited, since the discovery process requires the screening for large numbers of miRNA with a wide range of expression levels in multiple replicates, which

raises the costs of biomedical research in this attempt. On the other hand, studies about molecular mechanisms and putative miRNA-based therapeutic strategies can be accomplished by less expensive techniques, including RT-qPCR.

However, some technical issues have to be solved and several cautions have to be taken. In order to reach reliable and reproducible quantification of miRNA, is essential to compare results arising from different studies and, given that many experimental variables can affect miRNA measurement, all the related technical procedures should be carefully optimized and standardized. These actions will undoubtedly help to identify the roles of these small molecules in several diseases and facilitate the practical application of miRNA expression profiles to diagnostics and development of new therapies.

Conflicts of interest: The authors declare that there are no conflicts of interest.

Acknowledgements

Brazilian National Institute of Science and Technology on Neuroimmunomodulation (INCT-NIM), Rio de Janeiro, Brazil; National Council of Technological and Scientific Development (CNPq); Coordination for the Improvement of Higher Education Personnel (CAPES) and Clinical Hospital of Porto Alegre (FIPE-HCPA).

References

1. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell [Internet].

1993 [cited 2018 Apr 19];75:843–54. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8252621

2. SHABALINA S, KOONIN E. Origins and evolution of eukaryotic RNA interference. Trends Ecol Evol [Internet]. 2008 [cited 2018 Apr 26];23:578–87. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18715673

3. Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M, Dunoyer P, Yamamoto YY, Sieburth L, et al. Widespread translational inhibition by plant miRNAs and siRNAs. Science [Internet]. 2008 [cited 2018 Apr 26];320:1185–90. Available from: http://www.sciencemag.org/cgi/doi/10.1126/science.1159151

4. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet [Internet]. 2004 [cited 2018 Apr 26];5:522–31. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15211354

5. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell [Internet]. 2004 [cited 2018 Apr 26];116:281–97. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14744438

6. Kopkova A, Sana J, Fadrus P, Slaby O. Cerebrospinal fluid microRNAs as diagnostic biomarkers in brain tumors. Clin Chem Lab Med [Internet]. 2018 [cited 2018 Apr 26];0. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29451858

7. Chen Z, Wang K, Huang J, Zheng G, Lv Y, Luo N, et al. Upregulated Serum MiR-146b Serves as a Biomarker for Acute Ischemic Stroke. Cell Physiol Biochem [Internet]. 2018 [cited 2018 Apr 26];45:397–405. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29402769

8. Ridolfi B, Abdel-Haq H. Neurodegenerative Disorders Treatment: The MicroRNA Role. Curr Gene Ther [Internet]. 2018 [cited 2018 Apr 26];17:327–63.

Available from: http://www.ncbi.nlm.nih.gov/pubmed/29357791

9. van Vliet EA, Puhakka N, Mills JD, Srivastava PK, Johnson MR, Roncon P, et al. Standardization procedure for plasma biomarker analysis in rat models of epileptogenesis: Focus on circulating microRNAs. Epilepsia [Internet]. 2017 [cited 2018 Apr 26];58:2013–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28960286

10. Tiedt S, Prestel M, Malik R, Schieferdecker N, Duering M, Kautzky V, et al. RNA-Seq Identifies Circulating miR-125a-5p, miR-125b-5p, and miR-143-3p as Potential Biomarkers for Acute Ischemic StrokeNovelty and Significance. Circ Res [Internet]. 2017 [cited 2018 Apr 26];121:970–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28724745

11. Fries GR, Quevedo J. Exosomal MicroRNAs as Potential Biomarkers in Neuropsychiatric Disorders. Methods Mol Biol [Internet]. 2018 [cited 2018 Apr 26]. p. 79–85. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29435924

12. Edbauer D, Neilson JR, Foster KA, Wang C-F, Seeburg DP, Batterton MN, et al. Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132. Neuron [Internet]. 2010 [cited 2016 Oct 9];65:373–84. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20159450

13. Siegel G, Saba R, Schratt G. microRNAs in neurons: manifold regulatory roles at the synapse. Curr Opin Genet Dev [Internet]. 2011 [cited 2018 Apr 26];21:491–7. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0959437X11000761

14. Ziats MN, Rennert OM. Identification of differentially expressed microRNAs across the developing human brain. Mol Psychiatry [Internet]. 2014 [cited 2018 Apr 26];19:848–52. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/23917947

15. Olde Loohuis NFM, Kos A, Martens GJM, Van Bokhoven H, Nadif Kasri N, Aschrafi A. MicroRNA networks direct neuronal development and plasticity. Cell Mol Life Sci [Internet]. 2012 [cited 2018 Apr 26];69:89–102. Available from: http://link.springer.com/10.1007/s00018-011-0788-1

16. Perkins DO, Jeffries CD, Jarskog LF, Thomson JM, Woods K, Newman MA, et al. microRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. Genome Biol [Internet]. 2007 [cited 2018 Apr 26];8:R27. Available from: http://genomebiology.biomedcentral.com/articles/10.1186/gb-2007-8-2-r27

17. Santarelli DM, Beveridge NJ, Tooney PA, Cairns MJ. Upregulation of dicer and microRNA expression in the dorsolateral prefrontal cortex Brodmann area 46 in schizophrenia. Biol Psychiatry [Internet]. 2011 [cited 2018 Apr 26];69:180–7. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0006322310009996

18. Kim AH, Reimers M, Maher B, Williamson V, McMichael O, McClay JL, et al. MicroRNA expression profiling in the prefrontal cortex of individuals affected with schizophrenia and bipolar disorders. Schizophr Res [Internet]. 2010 [cited 2018 Apr 26];124:183–91. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0920996410013812

19. Moreau MP, Bruse SE, David-Rus R, Buyske S, Brzustowicz LM. Altered microRNA expression profiles in postmortem brain samples from individuals with schizophrenia and bipolar disorder. Biol Psychiatry [Internet]. 2011 [cited 2018 Apr 26];69:188–93. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0006322310010085

20. Abu-Elneel K, Liu T, Gazzaniga FS, Nishimura Y, Wall DP, Geschwind DH, et al. Heterogeneous dysregulation of microRNAs across the autism spectrum. Neurogenetics. 2008;9:153–61.

21. Chmielewska N, Szyndler J, Makowska K, Wojtyna D, Maciejak P, Płaźnik A. Looking for novel, brain-derived, peripheral biomarkers of neurological disorders. Neurol Neurochir Pol [Internet]. 2018 [cited 2018 Apr 26]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/29478670

22. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci [Internet]. 2008 [cited 2018 Apr 26];105:10513–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18663219

23. Chim SSC, Shing TKF, Hung ECW, Leung T-Y, Lau T-K, Chiu RWK, et al. Detection and characterization of placental microRNAs in maternal plasma. Clin Chem [Internet]. 2008 [cited 2018 Apr 26];54:482–90. Available from: http://www.clinchem.org/cgi/doi/10.1373/clinchem.2007.097972

24. Creemers EE, Tijsen AJ, Pinto YM. Circulating MicroRNAs: Novel Biomarkers and Extracellular Communicators in Cardiovascular Disease? Circ Res [Internet]. 2012 [cited 2017 Nov 13];110:483–95. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22302755

25. Li JJ, Wang B, Kodali MC, Chen C, Kim E, Patters BJ, et al. In vivo evidence for the contribution of peripheral circulating inflammatory exosomes to neuroinflammation. J Neuroinflammation [Internet]. 2018 [cited 2018 Apr 26];15:8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29310666

26. Ebrahimkhani S, Vafaee F, Young PE, Hur SSJ, Hawke S, Devenney E, et

al. Exosomal microRNA signatures in multiple sclerosis reflect disease status. Sci Rep [Internet]. 2017 [cited 2018 Apr 26];7:14293. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29084979

27. Talebizadeh Z, Butler MG, Theodoro MF. Feasibility and relevance of examining lymphoblastoid cell lines to study role of microRNAs in autism. Autism Res Off J Int Soc Autism Res. 2008;1:240–50.

28. Sarachana T, Zhou R, Chen G, Manji HK, Hu VW. Investigation of post-transcriptional gene regulatory networks associated with autism spectrum disorders by microRNA expression profiling of lymphoblastoid cell lines. Genome Med [Internet]. 2010 [cited 2018 Apr 26];2:23. Available from: http://genomemedicine.biomedcentral.com/articles/10.1186/gm144

29. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A [Internet]. 2011 [cited 2018 Apr 26];108:5003–8. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.1019055108

30. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol [Internet]. 2011 [cited 2018 Apr 26];13:423–33. Available from: http://www.nature.com/doifinder/10.1038/ncb2210

31. Wang K, Zhang S, Weber J, Baxter D, Galas DJ. Export of microRNAs and microRNA-protective protein by mammalian cells. Nucleic Acids Res [Internet]. 2010 [cited 2018 Apr 26];38:7248–59. Available from: https://academic.oup.com/nar/article-lookup/doi/10.1093/nar/gkq601

32. Tsui NBY, Ng EKO, Lo YMD. Stability of endogenous and added RNA in

blood specimens, serum, and plasma. Clin Chem [Internet]. 2002 [cited 2018 Apr 26];48:1647–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12324479

33. García ME, Blanco JL, Caballero J, Gargallo-Viola D. Anticoagulants interfere with PCR used to diagnose invasive aspergillosis. J Clin Microbiol [Internet]. 2002 [cited 2018 Apr 26];40:1567–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11923400

34. Duttagupta R, Jiang R, Gollub J, Getts RC, Jones KW. Impact of Cellular miRNAs on Circulating miRNA Biomarker Signatures. Hill AF, editor. PLoS One [Internet]. 2011 [cited 2018 Apr 26];6:e20769. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21698099

35. Monleau M, Bonnel S, Gostan T, Blanchard D, Courgnaud V, Lecellier C-H. Comparison of different extraction techniques to profile microRNAs from human sera and peripheral blood mononuclear cells. BMC Genomics [Internet]. 2014 [cited 2018 Apr 26];15:395. Available from: http://bmcgenomics.biomedcentral.com/articles/10.1186/1471-2164-15-395

36. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. Clin Chem [Internet]. 2010 [cited 2018 Apr 26];56:1733–41. Available from: http://www.clinchem.org/cgi/doi/10.1373/clinchem.2010.147405

37. Umu SU, Langseth H, Bucher-Johannessen C, Fromm B, Keller A, Meese E, et al. A comprehensive profile of circulating RNAs in human serum. RNA Biol [Internet]. 2018 [cited 2018 Apr 26];15:242–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29219730

38. Kim J, Krichevsky A, Grad Y, Hayes GD, Kosik KS, Church GM, et al.

Identification of many microRNAs that copurify with polyribosomes in mammalian neurons. Proc Natl Acad Sci U S A [Internet]. 2004 [cited 2018 Apr 26];101:360–5. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.2333854100

39. Liu C-G, Calin GA, Meloon B, Gamliel N, Sevignani C, Ferracin M, et al. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. Proc Natl Acad Sci U S A [Internet]. 2004 [cited 2018 Apr 26];101:9740–4. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.0403293101

40. Krichevsky AM, King KS, Donahue CP, Khrapko K, Kosik KS. A microRNA array reveals extensive regulation of microRNAs during brain development. RNA [Internet]. 2003 [cited 2018 Apr 26];9:1274–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/13130141

41. Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques [Internet]. 1993 [cited 2016 Oct 9];15:532–4, 536–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7692896

42. Hummon AB, Lim SR, Difilippantonio MJ, Ried T. Isolation and solubilization of proteins after TRIzol extraction of RNA and DNA from patient material following prolonged storage. Biotechniques [Internet]. 2007 [cited 2018 Apr 24];42:467–72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17489233

43. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res [Internet]. 2005 [cited 2016 Oct 9];33:e179. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16314309

44. Kramer MF. Stem-loop RT-qPCR for miRNAs. Curr Protoc Mol Biol [Internet]. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2011 [cited 2018 Apr 26];Chapter 15:Unit 15.10. Available from: http://doi.wiley.com/10.1002/0471142727.mb1510s95

45. Lin YL, Lai ZX. Evaluation of suitable reference genes for normalization of microRNA expression by real-time reverse transcription PCR analysis during longan somatic embryogenesis. Plant Physiol Biochem PPB [Internet]. 2013 [cited 2018 Apr 26];66:20–5. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0981942813000399

46. Peltier HJ, Latham GJ. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA [Internet]. 2008 [cited 2016 Oct 9];14:844–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18375788

47. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods [Internet]. 2001 [cited 2018 Apr 24];25:402–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11846609

48. Ramakers C, Ruijter JM, Deprez RHL, Moorman AFM. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett [Internet]. 2003 [cited 2016 Oct 9];339:62–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12618301

49. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol [Internet].

2002 [cited 2016 Oct 9];3:RESEARCH0034. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12184808

50. Thomson DW, Bracken CP, Goodall GJ. Experimental strategies for microRNA target identification. Nucleic Acids Res [Internet]. 2011 [cited 2018 Apr 26];39:6845–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21652644

51. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell [Internet]. 2005 [cited 2016 Oct 9];120:15–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15652477

52. Dweep H, Sticht C, Pandey P, Gretz N. miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. J Biomed Inform [Internet]. 2011 [cited 2018 Apr 26];44:839–47. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1532046411000785

53. Wang X, El Naqa IM. Prediction of both conserved and nonconserved microRNA targets in animals. Bioinformatics [Internet]. 2008 [cited 2016 Oct 9];24:325–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18048393

54. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. Nucleic Acids Res [Internet]. 2008 [cited 2018 Apr 26];36:D149-53. Available from: https://academic.oup.com/nar/article-lookup/doi/10.1093/nar/gkm995

55. Betel D, Koppal A, Agius P, Sander C, Leslie C. Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. Genome Biol [Internet]. 2010 [cited 2018 Apr 26];11:R90. Available from: http://genomebiology.biomedcentral.com/articles/10.1186/gb-2010-11-8-r90

56. Clément T, Salone V, Rederstorff M. Dual Luciferase Gene Reporter Assays to Study miRNA Function. Methods Mol Biol [Internet]. 2015 [cited 2018 Apr 26]. p. 187–98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25791601

57. Szostak E, Gebauer F. Translational control by 3'-UTR-binding proteins. Brief Funct Genomics [Internet]. 2013 [cited 2018 Apr 26];12:58–65. Available from: https://academic.oup.com/bfg/article-lookup/doi/10.1093/bfgp/els056

Capítulo III:

"Effects of an antipurinergic therapy on behavioral and molecular alterations in the animal model of autism induced by valproic acid"

A ser submetido no periódico "Brain, Behavior and Immunology"

Effects of an antipurinergic therapy on behavioral and molecular alterations in the animal model of autism induced by valproic acid

Mauro Mozael Hirsch[1,2,3]*, Iohanna Deckmann[1,2,3], Júlio Santos-Terra[1,2,3], Gabriela Zanotto Staevie[1,2,3], Mellanie Fontes-Dutra[1,2,3], Gustavo Brum Schwingel[1,2,3], Guilherme Bauer-Negrini[1,2,3], Bruna Rabelo[1,2,3], Giovanna Carello-Collar[1,2,3], Marília Körbes-Rockenbach[1,2,3], Henning Ulrich[4], Tomasz Schneider[5], Victorio Bambini-Junior[1,3,5], Carmem Gottfried[1,2,3]*

1. Translational Group in Autism Spectrum Disorder - GETTEA, Clinical Hospital of Porto Alegre, RS – Brazil
2. Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.
3. National Institute of Science and Technology on Neuroimmunomodulation - INCT-NIM, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil.
4. Department of Biochemistry, Institute of Chemistry, University of São Paulo, São Paulo, Brazil
5. School of Pharmacy and Biomedical Sciences, Faculty of Clinical and Biomedical Sciences, University of Central Lancashire, Preston, UK.

*CORRESPONDING AUTHORS:
MMH (mauromhirsch@gmail.com) and CG (cgottfried@ufrgs.br)
Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos 2600 – 21111. CEP: 90035-003 Porto Alegre-RS, Brazil.

ABSTRACT

Autism spectrum disorder (ASD) is characterized by deficits in communication and social interaction and restricted interests and stereotypes. It is known that environmental factors, such as prenatal exposure to valproic acid (VPA), may contribute to the increased risk of triggering ASD. Since the purinergic system has been widely associated with the onset of ASD, we analyzed the effects of suramin, a non-selective purinergic antagonist, on behavioral changes by VPA model of autism, as well as the expression of purinergic receptors and proinflammatory cytokines. The VPA-exposed animals showed a reduction in sociability and social novelty in the three-chambered test, in addition to an increase in anxiety-like behavior, which were rescued by postnatal treatment

with suramin. We observed only effect of prenatal exposure to VPA on reciprocal social interaction, latency and number of rearings in open field, self-grooming behavior, whisker nuisance task score and response to withdraw the tail after a thermal stimulus and suramin was not able to rescue these alterations. In addition to behavioral assessments, we quantified gene expression of some purinergic receptor and proinflammatory cytokines in neural tissues from animal model. VPA exposure caused upregulation of P2X4 and P2Y2 in hippocampus, while only P2X4 increased in medial prefrontal cortex. Likewise, interleukin 6 (IL-6) levels were increased in VPA group and suramin was able to rescue this alteration. No differences were observed in locomotor behavior (measured by distance traveled and average speed in open field arena), number of entries in central square in open field arena nor in number of entries in open arm and risk assessments in plus maze test. Taken together, our findings show an important purinergic modulation with suramin treatment rescuing the deficits in social and anxious-like behaviors, in addition to IL-6 levels. We suggest that further studies will be necessary to understand the mechanisms involved in purinergic pathway and to elucidate the etiology of ASD.

Keywords: autism, animal behavior, purinergic system, suramin, valproate.

1. INTRODUCTION

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by a behavioral dyad, which includes impairments in communication and social interaction and repetitive or stereotyped behaviors (American Psychiatric Association, 2013). In the last years, the incidence of ASD had a strong elevation (1:68 live births according to the most recent data

from USA) (Christensen et al., 2016) explained only in part by the changes in diagnostic parameters of DSM-5, demonstrating the necessity to expand studies in order to understand more deeply the pathways and possible risk factors involved in this disorder. Although the etiology is still unclear, it is already known that genetic and environmental factors are determinant for shaping the heterogeneous phenotypes exhibited by individuals with ASD (Chaste and Leboyer, 2012). Several studies demonstrated that the use of valproic acid (VPA) - an anticonvulsant drug widely used in the treatment of epilepsy, migraine and mood instabilities - during pregnancy, especially in the first trimester, can significantly increase the risk of developing autism (Christensen et al., 2013; Williams et al., 2001).

The animal model of autism by prenatal exposure to valproic acid is a consolidated study method (Mabunga et al., 2015), being capable to replicate autistic-like features as classic behavioral dyad (Schneider and Przewłocki, 2005), comorbidities as anxiety, epilepsy and morphofunctional alterations in sensory and social related areas (Fontes-Dutra et al., 2018; Lin et al., 2013). The studies using this model improved the understanding of many components of ASD, including altered neuroimmune pathways (Gottfried et al., 2015), electrophysiological impairments (Gogolla et al., 2009) and cytoarchitecture disruptions (Casanova et al., 2002; Fontes-Dutra et al., 2018; Hutsler and Casanova, 2016). Thus, it became necessary to understand how VPA induces developmental alterations that lead to ASD analyzing, for example, modulation of different components of synaptic transmission, like the purinergic system.

Several studies have demonstrated mitochondrial dysfunctions in ASD (Filipek et al., 2003; Patowary et al., 2017). This impairment of mitochondrial

energetic metabolism and consequent increase in extracellular ATP levels (Faas et al., 2017) leads to the onset of inflammatory processes via purinergic signaling, suggesting that this system may be involved in the etiology of ASD. Previous studies demonstrated that suramin, a non-selective inhibitor of the purinergic system, has therapeutic effects on autistic-like behaviors in the animal model of autism through maternal immune activation (MIA) (Naviaux et al., 2014, 2013). However, it is still unclear how an antipurinergic effect could prevent behavioral changes in this animal model, which seem to be related with elevated levels of interleukin 6 (IL-6) (Smith et al., 2007).

Based on these previous findings, the aim of this work was to evaluate the therapeutic effects of suramin on several behaviors in an animal model of autism induced by prenatal exposure to VPA and elucidate the role of purinergic signaling on inflammatory responses in autism context.

2. METHODS

2.1. Animals and ethics

Wistar rats obtained from the local breeding colony (ICBS-Federal University of Rio Grande do Sul) were maintained under standard laboratory conditions. The animals were mated overnight, and when pregnancy was established, this day was considered the zero embryonic day (E0). The offspring was weaned at postnatal day 21 (PND 21) and only male animals were used in this study (Bambini-Junior et al., 2011). This project was approved by the local animal ethics committee (CEUA-UFRGS 23884) and all procedures were approved by the Institutional Ethics Committee on Animal Use in accordance with Brazilian Law 11794/2008 (Arouca Law) and National Institutes

of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023).

2.2. Treatments

Valproic acid (sodium valproate, Sigma-Aldrich, USA) was dissolved in 0.9% saline for a concentration of 250 mg/mL. Pregnant females received a single intraperitoneal injection of 600 mg/kg VPA or physiological saline on E12.5. Male offspring received a single intraperitoneal injection of suramin (hexasodium salt, Sigma-Aldrich, USA) 20 mg/kg or PBS 0,1M on PND 30. Thus, four experimental groups were formed: Control, Suramin, VPA and VPA + SUR.

2.3. Behavioral tests

Experienced researchers blinded to experimental groups performed all behavioral experiments. In between testing trials, testing apparatuses were entirely cleaned. Behavioral testing was performed in prepubertal phase of offspring, between PND 32-40, two days after the injection of suramin. All behavioral tests were performed under light conditions of 60 LUX. The following behavioral tests were performed: Elevated Plus Maze, Open Field/Grooming, Whisker Nuisance Task (WNT), Three-Chambered Test, Reciprocal Sociability Test and Tail Flick.

2.3.1. Elevated Plus Maze (PND 32): the anxiety-like behavior was assessed in an elevated plus maze apparatus with a 10 cm × 10 cm center, connecting two opposite open arms (length: 50 cm) and two opposite arms closed with 30 cm high walls (length: 50 cm), 1 m above the floor. Rats were placed in the middle of apparatus and its movements were recorded for 5

minutes using a camera connected to a laptop. The time spent in the open arms is considered a measure of non-anxious behavior.

2.3.2. Open Field Test/Self-Grooming (PND 33): adapted from Schneider and colleagues (Schneider and Przewłocki, 2005), the exploratory and locomotor activity and the time and number of stereotyped movements were assessed in an open field, which consists of an wooden box measuring 50x50x50 cm. Rats were placed in the center of the arena and recorded during 30 minutes. Using the Anymaze Software®, we performed a tracking of locomotor activity (travelled distance and average speed) and time spent and number of entries in central square during the 30 minutes of test.

The self-grooming behavior was evaluated considering three different time periods (0-5, 10-15 and 20-25 minutes). We considered grooming as number of body cleaning with paws and face-washing actions and distinguished between complete (cleaning from snout to tail) and incomplete (fast and repetitive movements on snout) self-grooming**.**

2.3.3. Whisker Nuisance Task (PND 34-35): adapted from McNamara et al (McNamara et al., 2010) and described by Fontes-Dutra and collegues (Fontes-Dutra et al., 2018), in this test we observed the animal response to the vibrissae stimulation. One day prior to test, animals were set to the experimenter for 5 minutes, in an empty housing (57.1 × 39.4 x 15.2 cm) coated with an absorbent pad. On the day of test, the vibrissae were stimulated with a wooden toothpick for three consecutive periods of 5 min (15 min in total) with a 30 second interval between them. McNamara et al. (2010) developed a nonparametric scale of 0 to 2 according to the response (0 = absent/typcal, 1 = present/light response and 2 = profound/accentuated response), distributed in 8

categories of behavior: freezing, stance and body position, breathing, whisker position, whisking response, evading stimulation, response to stick presentation and grooming (McNamara et al., 2010). The maximum test score is 16. High scores (8-16) indicate abnormal responses to stimulation, in which the animal freezes, shakes, or is aggressive. The low scores (0-4) indicate normal responses, in which the mouse is calm or indifferent to stimulation.

***2.3.4. Three-Chamber Test (PND 36-37)*:** this test was performed as previously described by Bambini-Junior and colleagues (Bambini-Junior et al., 2011). Briefly, at the beginning, the animal was habituated in the central chamber of the apparatus for 5 minutes. In the Sociability Test, one object was placed in one of the side chambers and in the other an unknown interaction animal (novel rat 1). We measured the time spent in each chamber and the time of exploration of either the rat or the object for 10 minutes.

In the Social Novelty Test, the novel rat 1 (now known rat) remained in this place and an unknown rat (novel rat 2) was placed in the previously empty chamber. The time spent in each chamber and the time of exploration of both the rat known or not was also evaluated for 10 minutes.

***2.3.5. Reciprocal Social Behavior (PND 38)*:** adapted from Schneider et al. (Schneider and Przewłocki, 2005), the test was performed in the same box as the open field test during 15 minutes. The test animal was placed in the apparatus and, after an habituation period of 5 minutes, an unknown and younger interaction animal was placed with the test animal. We evaluated the social behavior of the test animal through some pre-established parameters: nose to nose, anogenital inspection, flank exploration and following.

***2.3.6. Tail Flick (PND 39-40)*:** nociceptive thresholds were evaluated using a tail flick analgesimeter (Insight Equipments, Ribeirão Preto, Brazil). One day prior the test, the animals were gently restrained by hand for 5 minutes in order to habituate to apparatus. Tail flick measurements were taken three times at 30-seconds intervals.

2.4. Dissecation of tissue samples

The animals were deeply anesthetized on PND 41 with Ketamine (300 mg/Kg) and Xilasin (40 mg/Kg). After, the animals were decapitated and the medial prefrontal cortex and hippocampus were dissected. The tissues were immediately homogenized in TRIzol® reagent (Invitrogen, USA) and preserved at ultra-freezer until posterior molecular analysis.

2.5. RNA Extraction and RT-qPCR Procedure

After homogenization of tissues samples, chloroform was added to perform phase separation, and RNA was precipitated from the upper aqueous layer using isopropanol. The precipitated RNA was washed with ethanol to remove impurities, resuspended in RNase-free water and stored at ultra-freezer (Hummon et al., 2007).

The mRNA expressions of purinergic receptors and cytokines were evaluated by reverse transcriptase followed by quantitative polymerase chain reaction (RT-qPCR). Complementary DNA (cDNA) was synthesized from mRNA using reverse transcriptase reaction containing 2 µg of total RNA, 1 µL of 10 mM dNTP mix (Invitrogen, USA), 1 µL of oligodT primer, 4 µL M-MLV reverse transcriptase 5X reaction buffer (Invitrogen, USA), 2 µL of 0.1 M DTT (Invitrogen, USA), 1 µL of RNase inhibitor (Invitrogen, USA), 1.0 µL of M-MLV reverse transcriptase (Invitrogen, USA), and sterile distilled water to a final

volume of 20 µL. The synthesis of the cDNA was completed after a sequence of three incubations at 65ºC for 5 min, 37ºC for 50 min and 70ºC for 15 min.

The quantitative PCR mix was comprised by 12 µL of cDNA (1:40), 0.2 µL of specific forward and universal reverse (10 µM) primers (as detailed in Supplementary Table 1), 0.5 µL of 10 µM dNTP mix, 2.0 µL of 10X PCR buffer (Invitrogen, USA), 0.8 µL of 50 mM $MgCl_2$ (Invitrogen, USA), 2.0 µL of 1X SYBR™ Green (Molecular Probes, USA), 0.1 µL of Platinum Taq DNA Polymerase (Invitrogen, USA) and sterile distilled water to a final volume of 20 µL. The fluorescence of SYBR™ Green was used to detect amplification, estimate Ct values, and to determine specificity after melting curve analysis. PCR cycling conditions were standardized to 95ºC for 5 min followed by 40 cycles at 95ºC for 10 s, 58ºC for 10 s, and 72ºC for 10 s. After the main amplification, sample fluorescence was measured from 60ºC to 95ºC, with an increasing ramp of 0.3ºC each, to obtain the denaturing curve of the amplified products and Tm estimation, to assure their homogeneity after peak detection. Data was obtained from an Applied Biosystems StepOne System (USA).

The RT-qPCR results were imported into Microsoft Excel and the geNorm program was used to assess the variance in expression levels of the mRNA analyzed (Vandesompele et al., 2002). This program scanned all mRNA evaluated and ranked accordingly to their stability. The more stable mRNA were used as housekeeping control.

The average value of crossing threshold (Ct) values (in triplicate) was converted to quantities for geNorm analysis by the difference between Ct value from two groups taken in each comparison. PCR efficiency was calculated from the slope of the amplification curve by exponential amplification analysis using

the LinRegPCR algorithm (Ramakers et al., 2003). The relative expression of mRNA was calculated considering the 100 % PCR efficiency and the –ΔΔCt values for each mRNA (Livak and Schmittgen, 2001) and were normalized to the housekeeping genes identified by the geNorm software.

2.6. Statistical analysis

IBM SPSS Statistics 20.0 (IBM SPSS, Armonk, NY, USA) was used to perform the statistical analysis. Kolgomorov-Smirnov and Shapiro-Wilk tests of normality were applied to determine data distribution. For behavioral tests, we used Generalized Estimating Equations (GEE) to weight both the interventions (VPA exposure and/or suramin treatment) and the litter effect in the behavioral outcome. After a Wald Chi-Square test, we performed pairwise comparisons for the parameters that presented interaction effect between interventions (VPA-by-suramin interaction). If only main effects were observed, the individual effect of VPA or suramin was evaluated. Bonferroni's post hoc test was used as the final evaluation. Data is reported as mean ± standard error of the mean (SEM). The Poisson distribution was used for discrete variables (number), while gamma distribution was used for time variables.

The relative expressions of purinergic receptors and cytokines were compared using one-way ANOVA followed by Bonferroni. The results were expressed as mean ± SEM. All statistical analyzes were supervised by the Biostatistics Unit at the Clinical Hospital of Porto Alegre.

3. RESULTS

3.1. Behavioral tests

3.1.1. Social Behavior

Three-chambered test: Rats from control and suramin group spent significantly more time in the chamber containing a conspecific novel rat than a novel object (CON: p< 0.001; SUR: p=0.025; Figure 1A). In contrast, VPA animals did not present preference between spending time in the chamber with a rat or an object (p=0.550). Interestingly, suramin treatment on VPA-exposed rats was able to reestablish the social feature, as the VPA+SUR group showed preference to stay in chamber with the novel rat (p<0.001). Regarding to social interaction, rats from both control and suramin groups also spent significantly more time exploring the cage containing the conspecific rather than the object (CON: p<0.001 SUR: p<0.001), whilst VPA group showed no preference between rat and object exploration (p=0.131, Figure 1A). Suramin treatment was again able to restore this social behavior (VPA+SUR: p<0.001).

In the test for social novelty, all groups showed no significant difference between the time spent in the novel rat chamber and the known rat chamber (CON: p=0.562; SUR: p=0.760; VPA: p=1.000; VPA+SUR: p=0.235; Figure 2B). However, rats from control and suramin groups spent significantly more time exploring the novel rat than the known rat (CON: p=0.003; SUR: p=0.005, Figure 1B), indicating an interest in social novelty. VPA rats did not show preference in exploration time between novel and known rat (VPA: p=0.13), whilst suramin treatment was able to prevent this social impairment in VPA-exposed animals (VPA+SUR: p=0.016, Figure 1B).

Reciprocal social behavior: Prenatal exposure to VPA significantly reduced every reciprocal social interaction parameter evaluated, except for following behavior (Supplementary Figure S1). We observed only a VPA effect on total reciprocal social behavior, since VPA-exposed animals presented a

decrease in number ($p<0.001$) and time ($p<0.001$) of social approaches and suramin treatment was not able to rescue these impairments (Figure 1C).

3.1.2. Anxiety-like, exploratory and locomotor behavior

Regarding to anxiety-like behavior, rats from VPA group spent significantly less time exploring the open arm of the elevated plus-maze apparatus, compared to animals from control ($p=0.001$) and suramin ($p=0.003$) groups. VPA+SUR group spent more time exploring the open arm of the apparatus compared to the VPA group ($p<0.001$; Figure 2A), indicating that suramin was able to rescue the anxiety-like behaviors in rats exposed to VPA. It is worth to note that no differences were found among experimental groups in open arms entries (all $p>0.570$; Supplementary Figure S2A) and number of risk assessments in the elevated plus-maze apparatus (all $p>0.210$; Supplementary Figure S2B). In the open field arena, animals from VPA group spent significantly less time in central square compared to animals from control ($p=0.007$) and suramin ($p<0.001$) groups. As observed in plus maze evaluation, suramin was able to rescue this alteration, since VPA+SUR group spent more time in central square compared to VPA group ($p=0.004$, Figure 2B).

Regarding the *exploratory behavior*, only the VPA-exposed rats presented lower number of rearing in an open field arena ($p=0.041$, Figure 2C). Finally, when locomotor activity of those rats in the open field arena was evaluated, no significant differences in distance traveled (all $p>0.910$, Figure 2D) and average speed (all $p>0.960$, Supplementary Figure S2D) were found among experimental groups.

3.1.3. Self-grooming behavior

The self-grooming behavior was evaluated across three testing periods (0-5, 10-15 and 20-25 minutes) and distinguished between complete and incomplete self-grooming**.** During the second period, VPA groups spent more time performing complete self-grooming compared to control animals (p=0.039), and suramin was not able to rescue this alteration. Similarly, in the third period, the same pattern was observed in VPA animals (p=0.003, Figure 3A). Taking all periods together, VPA-exposed animals spent more time doing complete self-grooming with no reversion by suramin treatment in this behavior (p=0.002, Figure 3B).

Regarding to time spent doing incomplete self-grooming, no difference was observed among the groups (all p>0.100, Figure 3C or p>0.420, Figure 3D). Regarding the total time performing self-grooming, VPA-exposed animals presented a trend to spend more time self-grooming in 10-15 min (p=0.065), a significant increase in the third period (p= 0.013, Figure 3E), and considering the three periods of test (p=0.002, Figure 3F). In all cases, postnatal treatment with suramin was not able to rescue these alterations.

Regarding the number of self-grooming events, VPA animals presented more events of complete self-grooming only in the third period (p=0.002, Supplementary Figure S3A) with a trend to increased total complete self-grooming (p=0.088, Supplementary Figure S3B). When considered the number of incomplete self-grooming and all grooming events, no differences were observed among groups in the three periods analyzed (all p>0.210, Supplementary Figure S3C) or considering three periods (all p>0.553, Supplementary Figure S3D). Finally, when considered the number of all events of self-grooming, VPA-exposed animals presented an increase only in 20-25

minutes (p=0.005, Supplementary Figure S3E) with no differences in the total period of test (all p>0.130, Supplementary Figure S3F).

3.1.4. Sensorial Behavior

In the whisker nuisance task (WNT), which evaluates the behavioral somatic response from whisker stimulation, the VPA-exposed animals presented a significant increased score when compared to control animals (p=0.001, Figure 4A), indicating higher levels of nuisance when whiskers are stimulated. The postnatal treatment with suramin was not able to rescue this alteration.

Only a VPA effect was observed in the latency to tail withdrawal in *tail flick test*, so that VPA-exposed animals presented higher latencies compared to non-exposed animals (p=0.012, Figure 4B).

3.2. Molecular analysis

3.2.1. Expression of purinergic receptors

Regarding the relative expression of mRNA in the medial prefrontal cortex, GeNorm algorithm ranked P2Y2 and P2Y4 as most stable and they were used as housekeeping to evaluate the relative expression of the remaining receptors. The ionotropic receptor P2X4 was found significantly increased in the cortex of animals prenatally exposed to VPA (p=0.003) and suramin was not able to revert this alteration (Figure 5A). On the other hand, there were no significant differences in levels of remaining receptors (p>0.330, Figure 5A).

When the receptors were evaluated in hippocampus of the young rats, P2X3 and P2Y4 were used as housekeeping. Curiously, the ionotropic receptor P2X4 was found significantly increased in animals from VPA (p=0.041) and VPA+SUR (p=0.003) groups, compared to control group also in this region.

Additionally, animals presented mRNA levels significantly increased of the metabotropic receptor P2Y2 in VPA ($p=0.043$) and VPA+SUR ($p=0.017$) group compared to control group. In both cases, suramin was not able to reverse this alteration (Figure 6B). Nevertheless, levels of remaining purinergic receptors showed no differences among experimental groups ($p>0.200$, Figure 5B).

3.2.2. Expression of cytokine mRNA levels

We also determined the relative expression of four cytokines in medial prefrontal cortex and hippocampus of young rats. In the medial prefrontal cortex, Gapdh and Beta3-tubulin were used as housekeeping to evaluate the relative expression of the IL-1β, IL-6, IFN-γ and TNF-α. The animals from VPA group presented an increase in relative expression of IL-6 mRNA compared to control group ($p=0.007$) and the postnatal treatment with suramin was able to rescue the levels of IL-6 of VPA-exposed rats to control levels (Figure 6A). On the other hand, IL-1β, IFN-γ and TNF-α showed no differences between groups. Regarding to hippocampus and considering the same housekeeping mRNA, no differences were found in cytokine levels among all experimental groups (Figure 6B).

4. DISCUSSION

An important approaching in VPA model is the possibility of developing therapeutic strategies to attenuate several features observed in ASD. For instance, our group demonstrated that resveratrol (RSV), an antioxidant and anti-inflammatory molecule, prevents VPA-induced social impairments in the three-chamber test (Bambini-Junior et al., 2014) and in the number and time of reciprocal social interactions (Hirsch et al., 2018). The present results

corroborate with impairments in sociability and social novelty exploration in the three-chambered test, as previously demonstrated (Bambini-Junior et al., 2014, 2011). As previously shown, the postnatal treatment with a single dose of suramin was able to reverse social impairments (Naviaux et al., 2014, 2013).

Additionally, the decrease in total reciprocal social interaction was not reversed by suramin. In fact, the reciprocal social behavior test involves complex patterns of socialization between two free animals, unlike the three-chambered test, where the conspecific animal remains trapped in a cage. Therefore, this characteristic of the test could be forcing the analyzed animal towards more complex social behavior actions, possibly causing additional impairments that suramin was not able to rescue.

Another main finding of our study was that animals of the VPA model presented a more anxious-like behavior compared to control animals as seen in plus maze apparatus and open field arena. Anxiety behavior is one of most common comorbidities in ASD and has been reported to be present in around 50% of autistic children and adolescents (Simonoff et al., 2008; van Steensel et al., 2011). Our study corroborates previous studies that demonstrated increased anxiety-like behavior in animal models of autism (Patterson, 2011). Interestingly, the treatment with suramin was able to completely rescue this alteration, which was seen by the higher percentage of total time spent exploring the open arm of the apparatus compared to VPA group.

We also observed that VPA-exposed animals do not present significant motor alterations or hyperactivity, but demonstrated a significant reduction in vertical exploratory activity, which could be related to the reduction in social interest of VPA animals described in sociability test. As observed in reciprocal

sociability test, the postnatal treatment with suramin was not able to rescue the impairments observed in this exploratory behavior.

In addition to analyzing social behavior, the present study also assessed another core symptom of autism - the repetitive and stereotyped behavior. In animal models of autism, this feature can be measured by analyzing the repetitive self-grooming behavior. Our work, for the first time in literature, assessed separately the self-grooming behavior as complete and incomplete events. The VPA-exposed animals showed increased time of complete self-grooming, without alteration in both number and time of incomplete groomings. Studies have demonstrated that only complete grooming is initiated by cerebellar midline or locus coeruleus (LC) stimulation (Strazielle et al., 2012) and that ATP can induce depolarization and increase excitability of norepinephrinergic system from LC, possibly mediated by specific modulators of P2 receptors (Masaki et al., 2001; Yao and Lawrence, 2005), suggesting a putative role of purinergic system in grooming outcomes.

Previous studies already demonstrated that grooming behavior could be related to sensory components (Houghton et al., 2018). Corroborating a previous work from our group (Fontes-Dutra et al., 2018), we observed that VPA animals presented hypersensitivity to a non-harmful stimulus in WNT, suggesting a disturbing in sensory gating which could lead to increase in self-grooming behavior. Hyposensitivity to pain is also frequently observed in autistic subjects, although this feature is not a consensus, since different findings were observed depending on how the studies were conducted (Moore, 2015). In our study, VPA-exposed rats presented higher latencies to sense a thermal stimulus, demonstrating a lower nociceptive reactivity in VPA model of

autism, in accordance to previous work (Schneider et al., 2008). As observed in self-grooming, postnatal treatment with suramin was not able to rescue these sensorial impairments in VPA animals.

There are sparse studies in literature indicating the roles of purinergic system in sensory processing. Nevertheless, it is known that purinergic system plays important roles in sensory pathways (Irnich et al., 2002). Our data presented increased expression of cortical heteromeric P2X4 receptor in VPA-exposed animals. Thus, our hypothesis suggests that sensory neurons presenting higher levels of P2X4 receptor, both in periphery and in CNS, might be exacerbating the EPSCs from periphery to cortical areas and, since there is a reduction in inhibitory neurons in deep cortical layers in VPA animal model (Fontes-Dutra et al., 2018), the excitatory/inhibitory balance would be compromised.

Our finding of purinergic receptors' upregulation is a compelling evidence for the involvement of purinergic signaling in the ASD pathophysiology, added to the neuroimmunology alterations already found in patients (Gottfried et al., 2015) and animal models of autism (Wei et al., 2012; Xu et al., 2015). Although this drug is poorly able to across the blood brain barrier (Hawking, 1978; Roboz et al., 1998), previous studies have demonstrated its therapeutic effects on social deficits (Naviaux et al., 2014, 2013). Our hypothesis is that suramin could be acting only at peripheral levels and modulates some characteristics of autism, possibly through a crosstalk between immunological and central nervous systems of these animals.

Our study demonstrated that animals from VPA group showed increased levels of proinflammatory cytokine IL-6 in medial prefrontal cortex. Interestingly,

a remarkable finding in the present work was the restoration IL-6 levels in this area after treatment with suramin. Since the levels of this cytokine are commonly increased in autistic patients (Gottfried et al., 2015) and in animal models of autism (Wei et al., 2012; Xu et al., 2015), the suramin effect on IL-6 levels could play a role on its changes on social and anxiety-like behavior (Xu et al., 2015), which also has been rescued by suramin treatment.

In summary, our findings reinforce the idea of antipurinergic therapy as a novel pharmacological target in disorders associated with inflammatory dysregulation, including autism and provide new insights for the development of effective and safe treatments. Although VPA-exposed animals seem to present higher permeability in blood-brain barrier (Kumar et al., 2015; Kumar and Sharma, 2016a, 2016b), the limited access of suramin to CNS could explain the limitations of suramin-based therapeutic strategies. The present data provide remarkable support for the hypothesis that a drug acting through peripheral immune and inflammatory components can modulate some molecular and behavioral alterations in VPA autism model. Further studies are necessary to elucidate the mechanisms of suramin action. In addition to this, the use of some specific and safer drugs could be more efficient to rescue autistic-related impairments.

CONFLICT OF INTEREST: The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

Brazilian National Institute of Science and Technology on Neuroimmunomodulation (INCT-NIM), Rio de Janeiro, Brazil; National Council of Technological and Scientific Development (CNPq); Coordination for the Improvement of Higher Education Personnel (CAPES) and Clinical Hospital of Porto Alegre (FIPE-HCPA).

REFERENCES

American Psychiatric Association, 2013. Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5), Diagnostic and Statistical Manual of Mental Disorders 4th edition TR. doi:10.1176/appi.books.9780890425596.744053

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

Bambini-Junior, V., Zanatta, G., Della Flora Nunes, G., Mueller de Melo, G., Michels, M., Fontes-Dutra, M., Nogueira Freire, V., Riesgo, R., Gottfried, C., 2014. Resveratrol prevents social deficits in animal model of autism induced by valproic acid. Neurosci. Lett. 583, 176–81. doi:10.1016/j.neulet.2014.09.039

Casanova, M.F., Buxhoeveden, D.P., Switala, A.E., Roy, E., 2002. Minicolumnar pathology in autism. Neurology 58, 428–32.

Chaste, P., Leboyer, M., 2012. Autism risk factors: genes, environment, and gene-environment interactions. Dialogues Clin. Neurosci. 14, 281–92.

Christensen, D.L., Baio, J., Braun, K.V.N., Bilder, D., Charles, J., Constantino, J.N., Daniels, J., Durkin, M.S., Fitzgerald, R.T., Kurzius-Spencer, M., Lee, L.-C., Pettygrove, S., Robinson, C., Schulz, E., Wells, C., Wingate, M.S., Zahorodny, W., Yeargin-Allsopp, M., Centers for Disease Control and Prevention (CDC), 2016. Prevalence and Characteristics of Autism Spectrum Disorder Among Children Aged 8 Years — Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2012. MMWR. Surveill. Summ. 65, 1–23. doi:10.15585/mmwr.ss6503a1

Christensen, J., Grønborg, T.K., Sørensen, M.J., Schendel, D., Parner, E.T., Pedersen, L.H., Vestergaard, M., 2013. Prenatal Valproate Exposure and Risk of Autism Spectrum Disorders and Childhood Autism. JAMA 309, 1696. doi:10.1001/jama.2013.2270

Faas, M.M., Sáez, T., de Vos, P., 2017. Extracellular ATP and adenosine: The Yin and Yang in immune responses? Mol. Aspects Med. doi:10.1016/j.mam.2017.01.002

Filipek, P.A., Juranek, J., Smith, M., Mays, L.Z., Ramos, E.R., Bocian, M., Masser-Frye, D., Laulhere, T.M., Modahl, C., Spence, M.A., Gargus, J.J., 2003. Mitochondrial dysfunction in autistic patients with 15q inverted duplication. Ann. Neurol. 53, 801–4. doi:10.1002/ana.10596

Fontes-Dutra, M., Santos-Terra, J., Deckmann, I., Brum Schwingel, G., Della-Flora Nunes, G., Hirsch, M.M., Bauer-Negrini, G., Hedin-Pereira, C., Bambini-Junior, V., Riesgo, R.S., Gottfried, C., 2018. Resveratrol prevents

cellular and behavioral sensory alterations in the animal model of autism induced by valproic acid. Front. Synaptic Neurosci. 10, 9. doi:10.3389/FNSYN.2018.00009

Gogolla, N., Leblanc, J.J., Quast, K.B., Südhof, T.C., Fagiolini, M., Hensch, T.K., 2009. Common circuit defect of excitatory-inhibitory balance in mouse models of autism. J. Neurodev. Disord. 1, 172–81. doi:10.1007/s11689-009-9023-x

Gottfried, C., Bambini-Junior, V., Francis, F., Riesgo, R., Savino, W., 2015. The Impact of Neuroimmune Alterations in Autism Spectrum Disorder. Front. Psychiatry 6, 121. doi:10.3389/fpsyt.2015.00121

Hawking, F., 1978. Suramin: with special reference to onchocerciasis. Adv. Pharmacol. Chemother. 15, 289–322.

Hirsch, M.M., Deckmann, I., Fontes-Dutra, M., Bauer-Negrini, G., Della-Flora Nunes, G., Nunes, W., Rabelo, B., Riesgo, R., Margis, R., Bambini-Junior, V., Gottfried, C., 2018. Behavioral alterations in autism model induced by valproic acid and translational analysis of circulating microRNA. Food Chem. Toxicol. 115, 336–343. doi:10.1016/J.FCT.2018.02.061

Houghton, D.C., Alexander, J.R., Bauer, C.C., Woods, D.W., 2018. Abnormal perceptual sensitivity in body-focused repetitive behaviors. Compr. Psychiatry 82, 45–52. doi:10.1016/j.comppsych.2017.12.005

Hummon, A.B., Lim, S.R., Difilippantonio, M.J., Ried, T., 2007. Isolation and solubilization of proteins after TRIzol extraction of RNA and DNA from patient material following prolonged storage. Biotechniques 42, 467–472.

Hutsler, J.J., Casanova, M.F., 2016. Review: Cortical construction in autism spectrum disorder: columns, connectivity and the subplate. Neuropathol. Appl. Neurobiol. 42, 115–134. doi:10.1111/nan.12227

Irnich, D., Tracey, D.J., Polten, J., Burgstahler, R., Grafe, P., 2002. ATP stimulates peripheral axons in human, rat and mouse--differential involvement of A(2B) adenosine and P2X purinergic receptors. Neuroscience 110, 123–9.

Kumar, H., Sharma, B., 2016a. Memantine ameliorates autistic behavior, biochemistry & blood brain barrier impairments in rats. Brain Res. Bull. 124, 27–39. doi:10.1016/j.brainresbull.2016.03.013

Kumar, H., Sharma, B., 2016b. Minocycline ameliorates prenatal valproic acid induced autistic behaviour, biochemistry and blood brain barrier impairments in rats. Brain Res. 1630, 83–97. doi:10.1016/j.brainres.2015.10.052

Kumar, H., Sharma, B.M., Sharma, B., 2015. Benefits of agomelatine in behavioral, neurochemical and blood brain barrier alterations in prenatal valproic acid induced autism spectrum disorder. Neurochem. Int. 91, 34–45. doi:10.1016/j.neuint.2015.10.007

Lin, H.-C., Gean, P.-W., Wang, C.-C., Chan, Y.-H., Chen, P.S., 2013. The amygdala excitatory/inhibitory balance in a valproate-induced rat autism model. PLoS One 8, e55248. doi:10.1371/journal.pone.0055248

Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25, 402–408. doi:10.1006/meth.2001.1262

Mabunga, D.F.N., Gonzales, E.L.T., Kim, J.-W., Kim, K.C., Shin, C.Y., 2015. Exploring the Validity of Valproic Acid Animal Model of Autism. Exp. Neurobiol. 24, 285–300. doi:10.5607/en.2015.24.4.285

Masaki, E., Kawamura, M., Kato, F., 2001. Reduction by sevoflurane of adenosine 5'-triphosphate-activated inward current of locus coeruleus neurons in pontine slices of rats. Brain Res. 921, 226–32.

McNamara, K.C.S., Lisembee, A.M., Lifshitz, J., 2010. The whisker nuisance task identifies a late-onset, persistent sensory sensitivity in diffuse brain-injured rats. J. Neurotrauma 27, 695–706. doi:10.1089/neu.2009.1237

Moore, D.J., 2015. Acute pain experience in individuals with autism spectrum disorders: A review. Autism 19, 387–399. doi:10.1177/1362361314527839

Naviaux, J.C., Schuchbauer, M.A., Li, K., Wang, L., Risbrough, V.B., Powell, S.B., Naviaux, R.K., 2014. Reversal of autism-like behaviors and metabolism in adult mice with single-dose antipurinergic therapy. Transl. Psychiatry 4, e400–e400. doi:10.1038/tp.2014.33

Naviaux, R.K., Zolkipli, Z., Wang, L., Nakayama, T., Naviaux, J.C., Le, T.P., Schuchbauer, M.A., Rogac, M., Tang, Q., Dugan, L.L., Powell, S.B., 2013. Antipurinergic therapy corrects the autism-like features in the poly(IC) mouse model. PLoS One 8, e57380. doi:10.1371/journal.pone.0057380

Patowary, A., Nesbitt, R., Archer, M., Bernier, R., Brkanac, Z., 2017. Next Generation Sequencing Mitochondrial DNA Analysis in Autism Spectrum Disorder. Autism Res. doi:10.1002/aur.1792

Patterson, P.H., 2011. Modeling Autistic Features in Animals. Pediatr. Res. 69,

34R–40R. doi:10.1203/PDR.0b013e318212b80f

Ramakers, C., Ruijter, J.M., Deprez, R.H.L., Moorman, A.F.M., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62–6.

Roboz, J., Deng, L., Ma, L., Holland, J.F., 1998. Investigation of suramin-albumin binding by electrospray mass spectrometry. Rapid Commun. Mass Spectrom. 12, 1319–1322. doi:10.1002/(SICI)1097-0231(19981015)12:19<1319::AID-RCM332>3.0.CO;2-J

Schneider, T., Przewłocki, R., 2005. Behavioral Alterations in Rats Prenatally Exposed to Valproic Acid: Animal Model of Autism. Neuropsychopharmacology 30, 80–89. doi:10.1038/sj.npp.1300518

Schneider, T., Roman, A., Basta-Kaim, A., Kubera, M., Budziszewska, B., Schneider, K., Przewłocki, R., 2008. Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. Psychoneuroendocrinology 33, 728–40. doi:10.1016/j.psyneuen.2008.02.011

Simonoff, E., Pickles, A., Charman, T., Chandler, S., Loucas, T., Baird, G., 2008. Psychiatric disorders in children with autism spectrum disorders: prevalence, comorbidity, and associated factors in a population-derived sample. J. Am. Acad. Child Adolesc. Psychiatry 47, 921–9. doi:10.1097/CHI.0b013e318179964f

Smith, S.E.P., Li, J., Garbett, K., Mirnics, K., Patterson, P.H., 2007. Maternal immune activation alters fetal brain development through interleukin-6. J. Neurosci. 27, 10695–702. doi:10.1523/JNEUROSCI.2178-07.2007

Strazielle, C., Lefevre, A., Jacquelin, C., Lalonde, R., 2012. Abnormal grooming activity in Dab1(scm) (scrambler) mutant mice. Behav. Brain Res. 233, 24–8. doi:10.1016/j.bbr.2012.04.038

van Steensel, F.J.A., Bögels, S.M., Perrin, S., 2011. Anxiety Disorders in Children and Adolescents with Autistic Spectrum Disorders: A Meta-Analysis. Clin. Child Fam. Psychol. Rev. 14, 302–317. doi:10.1007/s10567-011-0097-0

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, RESEARCH0034.1-11.

Wei, H., Chadman, K.K., McCloskey, D.P., Sheikh, A.M., Malik, M., Brown, W.T., Li, X., 2012. Brain IL-6 elevation causes neuronal circuitry imbalances and mediates autism-like behaviors. Biochim. Biophys. Acta - Mol. Basis Dis. 1822, 831–842. doi:10.1016/j.bbadis.2012.01.011

Williams, G., King, J., Cunningham, M., Stephan, M., Kerr, B., Hersh, J.H., 2001. Fetal valproate syndrome and autism: additional evidence of an association. Dev. Med. Child Neurol. 43, 202–6.

Xu, N., Li, X., Zhong, Y., 2015. Inflammatory cytokines: potential biomarkers of immunologic dysfunction in autism spectrum disorders. Mediators Inflamm. 2015, 531518. doi:10.1155/2015/531518

Yao, S.T., Lawrence, A.J., 2005. Purinergic modulation of cardiovascular function in the rat locus coeruleus. Br. J. Pharmacol. 145, 342–352. doi:10.1038/sj.bjp.0706179

FIGURES

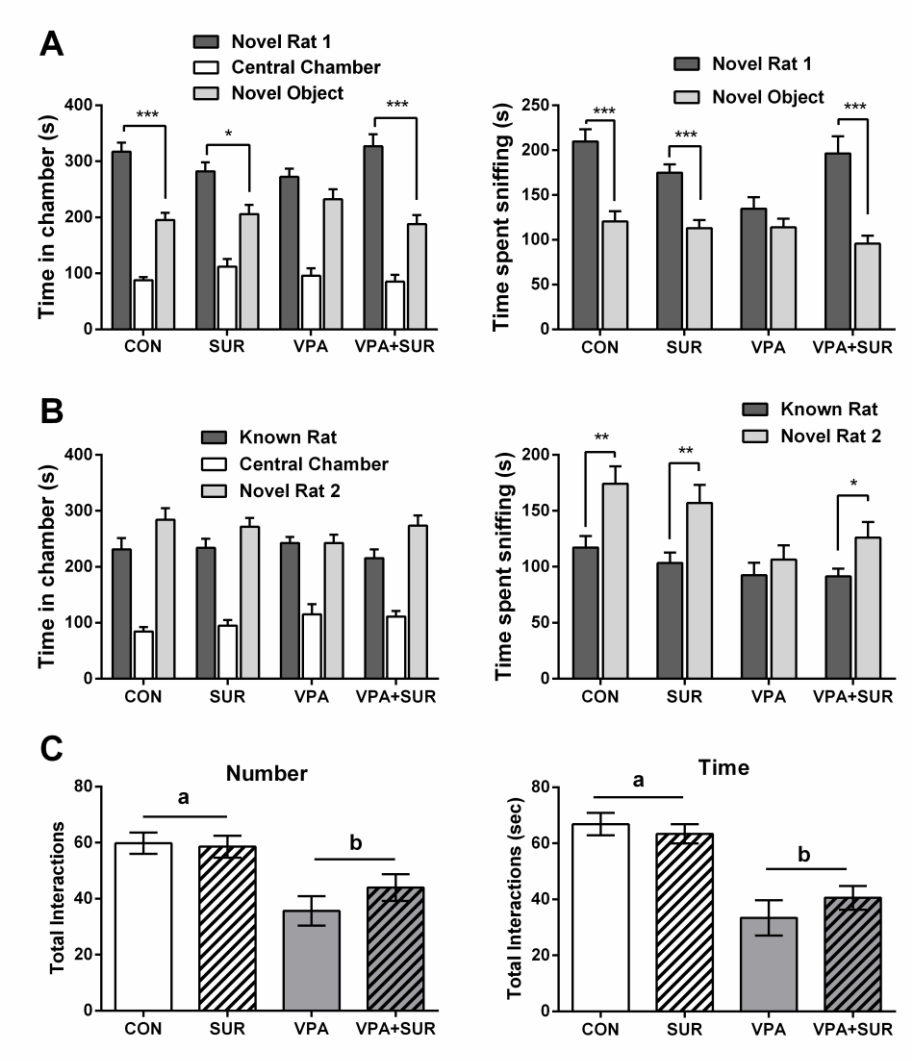


Figure 1. Social behavior in VPA autism model. (A) Time spent in chambers and interaction time in sociability (B), social novelty tests in a three-chambered apparatus and (C) number and time of total pro-social interactions. Data expressed as means ± SEM. Asterisks indicate statistical

differences with *p<0.05, **p<0.01, ***p<0.001. Different letters indicate statistical differences with p<0.05 considered significant. Statistical analysis: Generalized Estimating Equations (GEE) followed by Bonferroni. Three-chambered test: CON (n = 15), SUR (n = 15), VPA (n = 11), VPA+SUR (n = 13). Reciprocal interactions: CON (n = 17), SUR (n = 17), VPA (n = 15), VPA+SUR (n = 13).

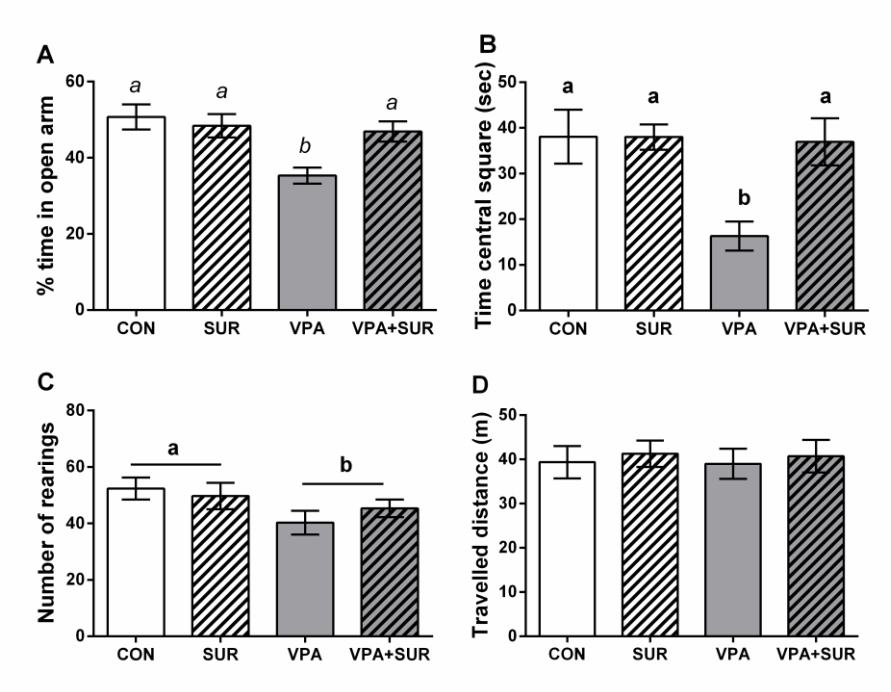


Figure 2. Anxiety, exploratory and locomotor behavior in VPA autism model. (A) Percent of time spent in the open arms in the elevated plus-maze; (B) Time spent in central square; (C) Number of rearings and (D) distance travelled in a 50x50x50 open field arena. Data expressed as means ± SEM. Different letters indicate statistical differences with p<0.05 considered significant. Statistical analysis: Generalized Estimating Equations (GEE) followed by Bonferroni. Plus Maze: CON (n = 17), SUR (n = 17), VPA (n = 16), VPA+SUR (n = 14). Open Field: CON (n = 15), SUR (n = 15), VPA (n = 15), VPA+SUR (n = 15).

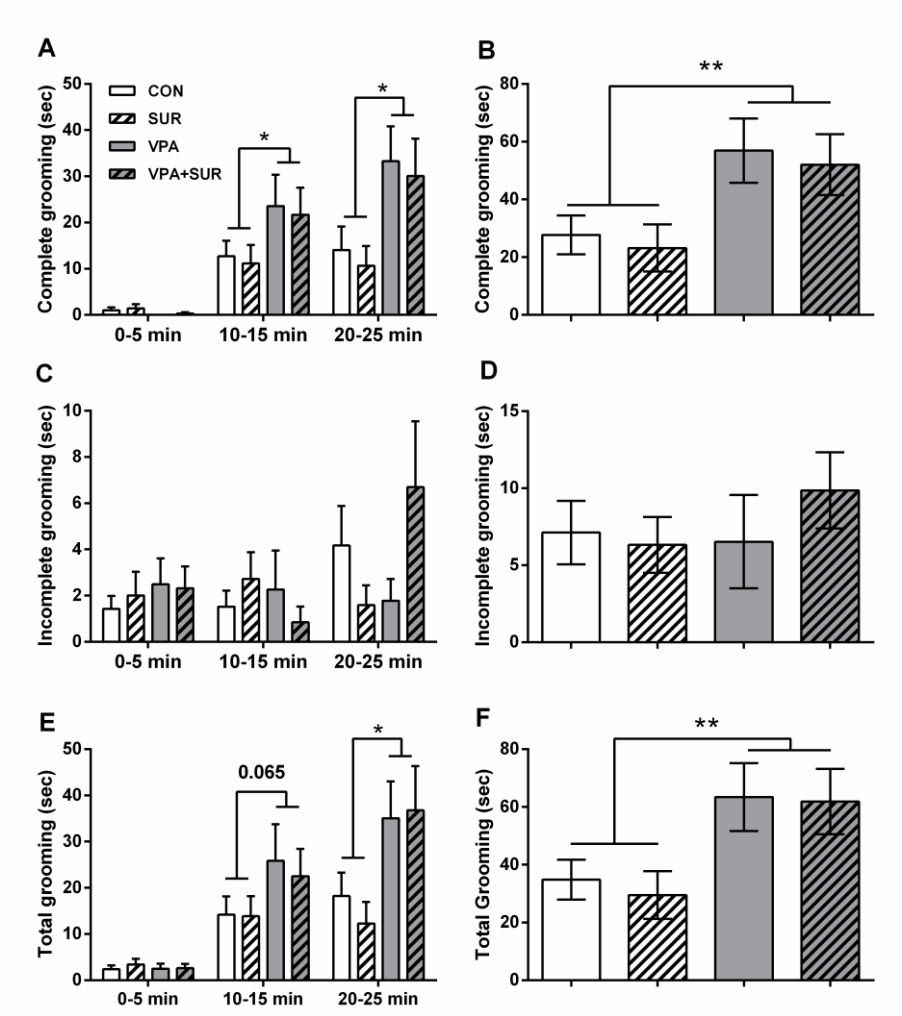


Figure 3. Self-grooming behavior in VPA autism model. (A) time of complete grooming in different time ranges and (B) total time of complete grooming; (C) time of incomplete grooming in different time ranges and (D) total time of incomplete grooming; (E) time of total grooming in three different time ranges and (F) total time of grooming. Data expressed as means ± SEM with *p<0.05 and **p<0.01 considered significant**.** Statistical analysis: Generalized Estimating Equations (GEE) followed by Bonferroni. CON (n = 16), SUR (n = 15), VPA (n = 13), VPA+SUR (n = 13).

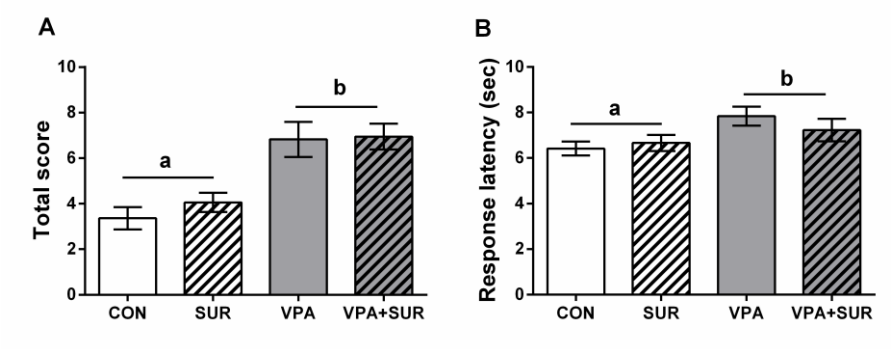


Figure 4. Sensorial behavior in VPA autism model. (A) Total score in Whisker Nuisance Task and (B) latency to respond to a thermal stimuli. Different letters indicate statistically significant differences caused by VPA exposure. Data expressed as means ± SEM with *p<0.05. Statistical analysis: Generalized Estimating Equations (GEE) followed by Bonferroni. WNT: CON (n = 15), SUR (n = 14), VPA (n = 13), VPA+SUR (n = 13). Tail flick: CON (n = 17), SUR (n = 14), VPA (n = 13), VPA+SUR (n = 14).

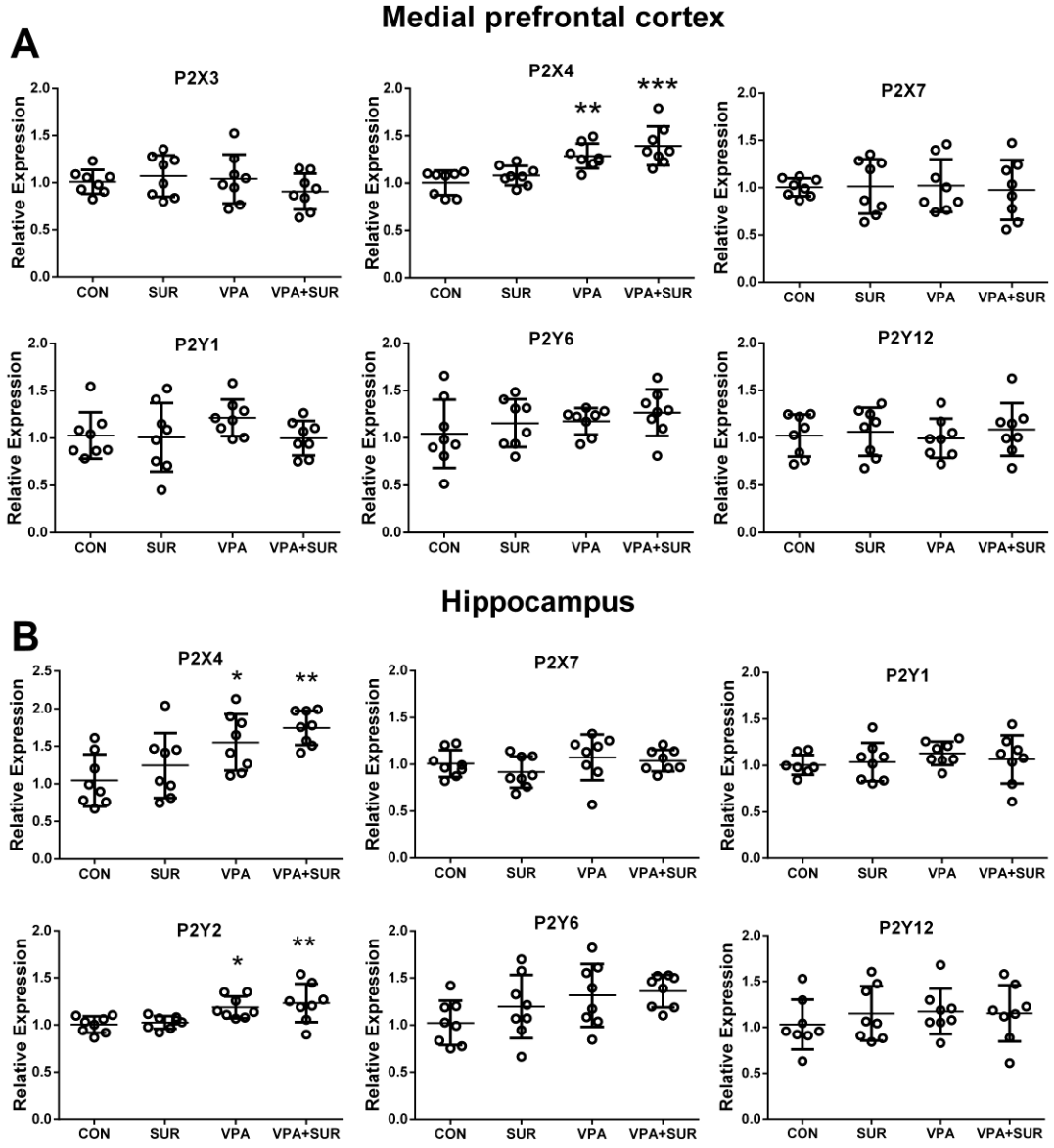


Figure 5. Relative expression of purinergic receptors in medial prefrontal cortex (A) and hippocampus (B) of young rats from VPA autism model. Plots presented as mean ± SEM with *p<0.05, **p<0.01, ***p<0.001. Statistical analysis: One-Way ANOVA followed by Tukey's test. n = 8 for all groups in both tissues.

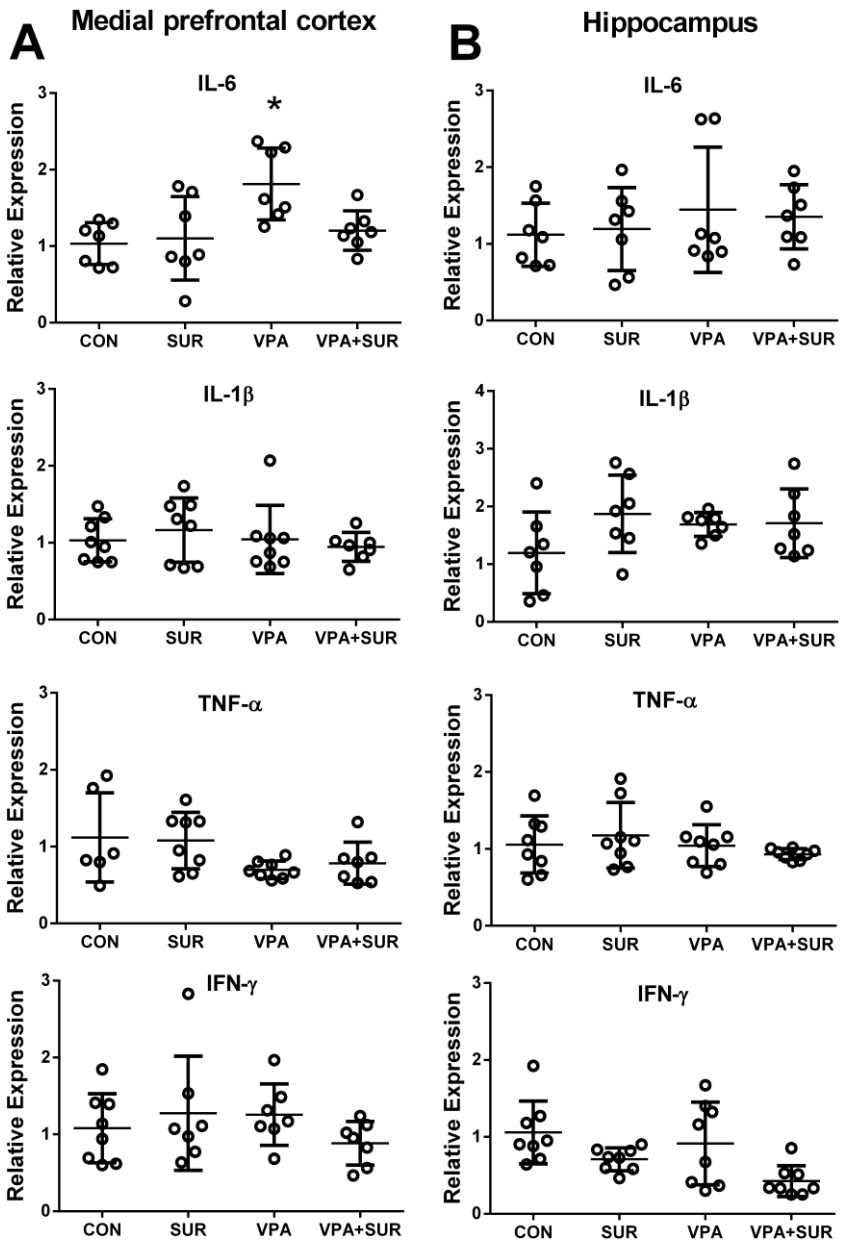
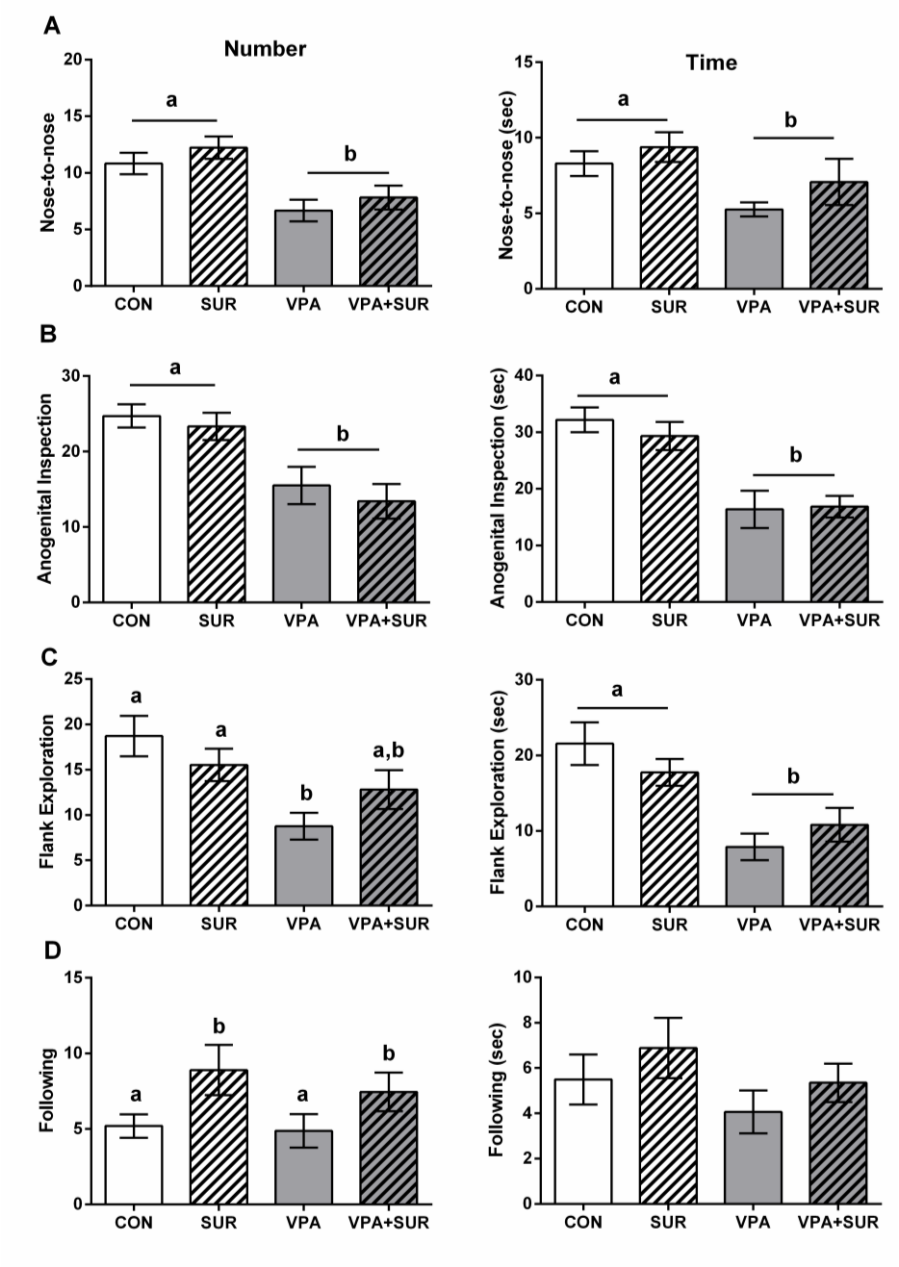
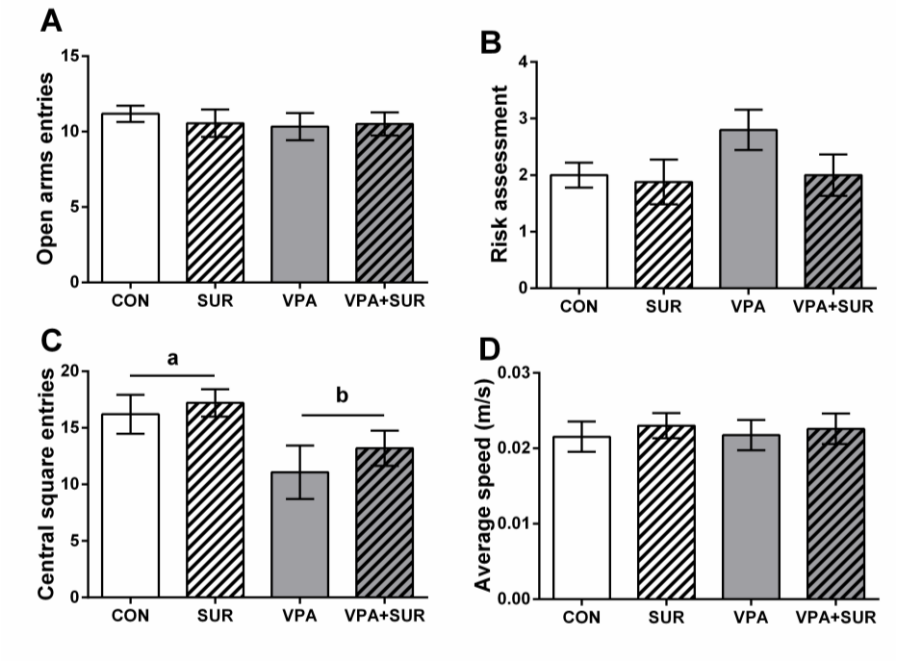


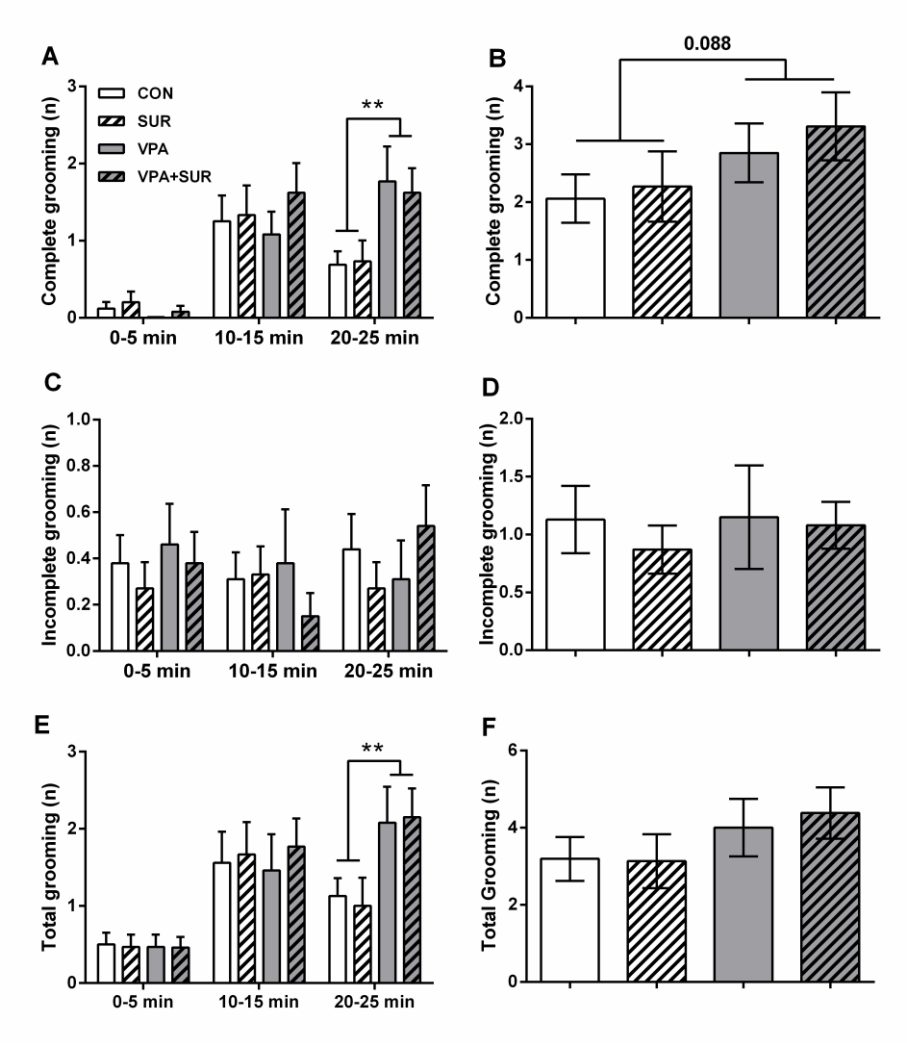
Figure 6. Relative expression of proinflammatory cytokines in medial prefrontal cortex (A) and hippocampus (B) of young rats from VPA autism model. Results expressed as means ± SEM with *p <0.05 considered significant. Statistical analysis: One-Way ANOVA followed by Tukey's test. n_{MPC} = 6-8 and n_{HIP} = 8 for all groups.



Supplementary Figure 1. Number and time of (A) nose-to-nose sniffing, (B) anogenital inspection, (C) flank exploration and (D) following. Plots show means ± SEM. Different letters indicate statistical differences with $p<0.05$. Statistical analysis: Generalized Estimating Equations (GEE) followed by Bonferroni. CON (n = 17), SUR (n = 17), VPA (n = 15), VPA+SUR (n = 13).



Supplementary Figure 2. (A) Number of entries in open arms and (B) risk assessments in elevated plus maze apparatus; (C) number of entries in central square and (D) average speed in open field arena. Data expressed as means ± SEM. Different letters indicate statistical differences with $p<0.05$ considered significant. Statistical analysis: Generalized Estimating Equations (GEE) followed by Bonferroni. Plus Maze: CON (n = 17), SUR (n = 17), VPA (n = 16), VPA+SUR (n = 14). Open Field: CON (n = 15), SUR (n = 15), VPA (n = 15), VPA+SUR (n = 15).



Supplementary Figure 3. (A) Number of complete grooming in different time ranges and (B) total number of complete grooming; (C) number of incomplete grooming in different time ranges and (D) total number of incomplete grooming; (E) number of total grooming in three different time ranges and (F) total number of grooming. Data expressed as means ± SEM with **p<0.01 considered significant**.** Statistical analysis: Generalized Estimating Equations (GEE) followed by Bonferroni. CON (n = 16), SUR (n = 15), VPA (n = 13), VPA+SUR (n = 13).

PARTE III

DISCUSSÃO GERAL

O Grupo de Estudos Translacionais em Transtorno do Espectro Autista (GETTEA) vem utilizando o modelo animal de autismo induzido por exposição pré-natal ao VPA para avaliar diversos aspectos moleculares e comportamentais do TEA, uma vez que ele apresenta diversas características similares àquelas encontradas em pacientes. Entre estas, se destaca a replicação das duas características centrais do diagnóstico do TEA: prejuízos na comunicação e interação social e comportamentos repetitivos e estereotipados (BAMBINI-JUNIOR et al., 2011; SCHNEIDER; KOCH, 2005; SCHNEIDER et al., 2008a). Além disso, é comum estes animais apresentarem diversas comorbidades frequentemente encontradas em indivíduos com TEA, incluído rigidez comportamental, prejuízos sensoriais e comportamentos do tipo ansioso (BAMBINI-JUNIOR et al., 2011; SCHNEIDER et al., 2008b; SCHNEIDER; PRZEWŁOCKI, 2005). Dessa forma, o modelo VPA é extremamente útil no estudo do autismo, uma vez que proporciona importantes descobertas relacionadas a comprometimentos metabólicos que muitas vezes estão subjacentes às alterações comportamentais encontradas no contexto do TEA. Entretanto, apesar das diversas descobertas já realizadas através da utilização de modelos animais de autismo, ainda pouco se sabe sobre a fisiopatologia deste transtorno. Os exatos mecanismos pelos quais o VPA estaria agindo para desencadear as características do tipo autista em roedores ainda permanecem desconhecidos.

A utilização de modelos animais de autismo em roedores possibilita, de uma forma mais aprofundada, o estudo das características do TEA, visando dois objetivos principais. Primeiramente, o modelo animal possibilita o estudo

de mecanismos moleculares que possam estar envolvidos no desencadeamento do transtorno. Além disso, a utilização de modelos animais de autismo pode ser utilizada como potencial ferramenta para o estudo de estratégias terapêuticas que possam prevenir, reverter ou atenuar diversas características comportamentais apresentadas no TEA.

Em um trabalho anterior do nosso grupo de pesquisa, foi demonstrado que o RSV foi capaz de prevenir prejuízos sociais no modelo animal de autismo induzido pelo VPA através do teste Três Câmaras (BAMBINI-JUNIOR et al., 2014). Em um dos trabalhos realizados na presente tese, nós complementamos e corroboramos esse estudo de 2014, mostrando que o RSV também foi capaz de prevenir prejuízos sociais no teste de sociabilidade recíproca. Apesar de envolver padrões de interações sociais mais complexos que aqueles observados no teste de três câmaras, nesse teste de sociabilidade recíproca também foi possível observar que houve prejuízos no comportamento sociais nos ratos cujas mães foram expostas ao VPA e, da mesma forma, o tratamento pré-natal com RSV foi capaz de prevenir essas alterações.

Em outro trabalho desenvolvido, nós também analisamos o comportamento social de ratos prenatalmente expostos ao VPA, porém testando um tratamento pós-natal com suramina, um antagonista purinérgico não-seletivo (DUNN; BLAKELEY, 1988). Ratos do grupo VPA apresentaram prejuízos na sociabilidade e na novidade social no teste de Três Câmaras, corroborando um trabalho anterior do grupo (BAMBINI-JUNIOR et al., 2011, 2014). No presente trabalho, uma única injeção de suramina em ratos de 30 dias foi capaz de reverter estes prejuízos sociais induzidos pelo VPA observados nesse teste. Por outro lado, a suramina não foi capaz de reverter

os prejuízos sociais induzidos pelo VPA, quando observamos o comportamento pelo teste de sociabilidade recíproca.

Como já foi dito acima, o teste de sociabilidade recíproca provavelmente envolve padrões mais complexos de interações sociais entre dois animais livres, diferente do teste de três câmaras, onde o animal de interação permanece dentro de uma gaiola, de forma que a interação depende de um comportamento ativo do animal testado. No teste da sociabilidade recíproca, por sua vez, a interação social pode ocorrer por uma iniciativa do animal que está sendo testado, ou ele pode ser estimulado a interagir após uma iniciativa do seu companheiro de caixa. Assim, a característica peculiar desse teste pode estar envolvendo outros mecanismos de comportamento social, causando alterações adicionais que foram prevenidas pelo RSV, embora nenhum efeito tenha sido observado após tratamento pós-natal com suramina.

Além da análise do comportamento social, os trabalhos realizados nessa tese também envolveram outro aspecto central do TEA: o comportamento repetitivo e estereotipado. Em roedores, essa característica pode ser mensurada de várias formas, dependendo do contexto utilizado. Testes utilizados para isso incluem comportamento de autolimpeza excessivo, cambalhotas pra trás e movimentos corporais rítmicos. Estes comportamentos compartilham características importantes com aquelas observadas no TEA, incluindo a pouca variação na forma de resposta e o fato de não haver nenhum propósito ou função óbvia nesses movimentos (LEWIS et al., 2007). Aqui, utilizamos o comportamento de autolimpeza para avaliar comportamento repetitivo e estereotipado no modelo animal de autismo induzido por VPA, uma vez que esse teste é considerado padrão-ouro para avaliação desse tipo de

comportamento. Nossos resultados mostraram, em conjunto, que ratos do modelo VPA gastam mais tempo fazendo autolimpeza do que os ratos que não foram expostos ao VPA, especialmente em períodos finais dos testes. Em ambos os trabalhos, o tratamento pré-natal com RSV ou pós-natal com suramina não tiveram efeito terapêutico sobre esse comportamento.

Além disso, em um dos trabalhos, nós avaliamos separadamente os eventos de autolimpeza completo e incompleto. Um padrão de autolimpeza completo se caracteriza como uma sequência ordenada de movimentos no sentido cefalocaudal, enquanto na autolimpeza incompleta (ou fragmentada) o animal não realiza todos os movimentos ou os realiza de forma desordenada (BERRIDGE, 1989; CANNON et al., 1992). Em nosso estudo, animais expostos ao VPA apenas apresentaram um maior número de eventos de autolimpeza completos, enquanto nenhuma diferença foi encontrada na autolimpeza incompleta. Uma explicação para essa diferença pode residir no fato de que diferentes regiões encefálicas e conexões neuronais podem estar envolvidas nesses dois tipos de autolimpeza. Por exemplo, um estudo de 2012 demonstrou que apenas eventos de autolimpeza completos são iniciados através de um estímulo na linha média cerebelar (ou *locus coeruleus*) (STRAZIELLE et al., 2012). Uma hipótese é a de que as alterações moleculares e/ou morfológicas causadas pelo VPA possam estar ocorrendo em diferentes regiões encefálicas em graus de intensidade distintas, o que poderia explicar tais discrepâncias em diferentes parâmetros comportamentais.

Um aspecto importante relacionado ao comportamento de autolimpeza é o componente sensorial. Estudos já demonstraram que um aumento na sensitividade sensorial pode ser observado em indivíduos com hábitos de

higiene compulsiva, como puxar o cabelo, pegar a pele e roer as unhas, os quais são coletivamente conhecidos como comportamentos repetitivos focados no corpo (do inglês *body-focused repetitive behaviors*, BFRB) (HOUGHTON et al., 2018). Prejuízos sensoriais são uma das características mais importantes do TEA, afetando mais de 90% dos pacientes (GESCHWIND, 2009). Hiper-responsividades táctil e auditiva são exemplos dessas alterações sensitivas, as quais poderiam impactar em muitos aspectos a qualidade de vida dos indivíduos com TEA (COSKUN et al., 2009; O'CONNOR, 2012; PUTS et al., 2014). Um dos trabalhos realizados mostrou que os ratos expostos ao VPA apresentaram altos escores no teste de estimulação das vibrissas (do inglês *whisker nuisance task, WNT*), corroborando um trabalho recente do grupo (FONTES-DUTRA et al., 2018). Estes resultados podem sugerir um incômodo ou perturbação no controle sensorial, podendo levar ao aumento do comportamento de autolimpeza. Enquanto o RSV foi capaz de prevenir o aumento dos escores no WNT nesse mesmo estudo anterior do grupo, nenhum efeito da suramina foi observado, sugerindo mais uma vez que essas duas moléculas agem em diferentes tecidos, regiões e/ou vias de sinalização.

O teste de transmissão social por preferência alimentar (do inglês *social transmission of food preference, STFP*) é aplicado para avaliação indireta da comunicação social entre os ratos. Isto se dá pela apresentação de uma comida com um novo sabor para um representante de um grupo de ratos (o demonstrador) e pela subsequente avaliação da preferência alimentar dos demais ratos da ninhada (os observadores) entre a comida com este sabor apresentado e uma comida com sabor desconhecido. Quando analisamos a porcentagem de consumo da comida apresentada, os ratos expostos ao VPA

apresentaram uma menor porcentagem em relação aos ratos do grupo controle e RSV, indicando que aqueles não tiveram preferência pelo sabor previamente experimentado pelo seu irmão em relação a estes. Dada essa menor porcentagem de consumo do sabor apresentado, investigamos se esse efeito poderia ser causado por um menor consumo relativo da comida com sabor apresentado ou, alternativamente, a um maior consumo relativo da comida não apresentada. Assim, ao analisarmos o consumo médio absoluto por grupo, vimos que, de uma forma geral, os animais não apresentam diferença significativa no consumo da comida não apresentada. Em contrapartida, os animais expostos ao VPA tendem a consumir menos da comida apresentada.

Uma vez que existe uma alteração sensorial de cunho somático importante mostrada pela primeira vez por nosso grupo (FONTES-DUTRA et al., 2018), a contrapreferência dos ratos expostos ao VPA pode ser um indicativo da alteração do processamento sensorial de cunho olfatório, possivelmente, nesses roedores. Assim, o estímulo olfatório gerado pela apresentação daquele sabor poderia estar gerando uma memória aversiva a esses animais, fazendo com que eles tendam a comer menos daquela comida previamente apresentada, refletindo sobre a menor porcentagem de consumo desta.

Apesar de se saber que a informação sensorial é processada primariamente por regiões corticais com redes especializadas para o tipo de modalidade, estudos vêm demonstrando o papel importante de regiões subcorticais, como a amígdala, nessa atribuição de cunho afetivo em diferentes tipos de informação, como as sensoriais. Tanto em indivíduos com TEA, quanto no modelo animal VPA, alterações da amígdala já foram observadas

(BANERJEE et al., 2014; LIN et al., 2013; MARKRAM et al., 2008). É possível que nossos resultados apontem não só para um processamento sensorial alterado, mas também para a possibilidade de atribuição de valor afetivo do tipo aversivo sobre esta informação por possível hiperativação da amígdala, devido a um hiperprocessamento sensorial no STFP. Apesar de estudos prévios terem demonstrado que alterações de cunho somático são prevenidas pela administração de RSV (FONTES-DUTRA et al., 2018), nenhum efeito preventivo foi observado no STFP.

Sujeitos com TEA frequentemente apresentam hipossensitividade à dor. No entanto, a observação desta característica depende de como os testes são conduzidos (MOORE, 2015), não sendo, portanto, um consenso na literatura. Em nosso estudo, os animais expostos ao VPA apresentam maiores latências para sentir um estímulo térmico, indicando uma menor reatividade nociceptiva no modelo VPA, o que também já foi observado em outros trabalhos utilizando o mesmo modelo (SCHNEIDER et al., 2008a). Assim como observado para o WNT, o tratamento pós-natal com suramina não foi capaz de reverter o prejuízo na sensitividade térmica causado pelo VPA.

Outro achado importante desta tese foi o fato de que os animais expostos ao VPA apresentam comportamento do tipo ansioso mais exacerbado que os animais não expostos, o que foi verificado através do menor tempo explorando os braços abertos no labirinto em cruz elevado. Esse achado corrobora estudos anteriores utilizando modelos animais de autismo (PATTERSON, 2011), além de refletir em modelos animais uma comorbidade frequentemente encontrada em crianças e adolescentes com TEA (SIMONOFF et al., 2008; VAN STEENSEL; BÖGELS; PERRIN, 2011). O tratamento pós-

natal com suramina, além de ter efeito terapêutico sobre o comportamento social em nossos dados e em trabalhos anteriores (NAVIAUX et al., 2014, 2015, 2013), foi capaz de reverter totalmente o aumento do comportamento do tipo ansioso em animais expostos ao VPA.

Apesar de um tratamento antipurinérgico ter um efeito ansiolítico no modelo animal de autismo induzido por VPA, um estudo anterior demonstrou que outro antagonista não-específico de receptores purinérgicos, o piridoxalfosfato-6-azofenil-2′,4′-ácido dissulfônico (PPADS) possui efeitos ansiogênicos (KITTNER et al., 2003), o que vai de encontro aos nossos achados. Talvez os efeitos ansiolíticos da suramina (o qual também é um antagonista não específico de receptors purinérgicos do tipo P2) possa estar envolvendo rotas alternativas que não sejam seus efeitos sobre o sistema purinérgico, uma vez que essa molécula é capaz de bloquear diversos outros receptores e enzimas que não necessariamente estejam envolvidas na sinalização purinérgica (BUTLER et al., 1988; CALCATERRA; VICARIO; ROVERI, 1988; FORTES; ELLORY; LEW, 1973; MAHONEY; AZZI; HUANG, 1990; MORIYAMA; NELSON, 1988; ONO; NAKANE; FUKUSHIMA, 1988; WILLS; WORMALL, 1950).

As atividades locomotoras e exploratórias do modelo animal de autismo também foram avaliadas em um dos trabalhos desenvolvidos nessa tese, utilizando um aparato de campo aberto. Através de programa de monitoramento automatizado da atividade (Anymaze, Stoelting, U.S.A.), nós mensuramos a distância percorrida e a velocidade média dos animais no aparato durante 30 minutos. Nenhuma diferença foi encontrada em nenhum desses parâmetros entre os grupos experimentais, indicando que a exposição

ao VPA não causa alterações motoras a ponto de comprometer a locomoção destes animais, conforme já foi observado em estudos anteriores (BANERJEE et al., 2014). No entanto, os animais do grupo VPA ficaram menos tempo no quadrante central no campo aberto, corroborando o resultado de um maior comportamento do tipo ansioso nesses animais, uma vez que os animais mais ansiosos tendem a ir para os cantos do aparato. Igualmente ao observado no teste no labirinto em cruz elevado, suramina foi capaz de reverter essa alteração.

Em relação à atividade exploratória, nós observamos uma redução significativa no número de explorações verticais nos animais expostos ao VPA, comparado aos controles. Esse achado pode ser relacionado com a redução do interesse social, também observado nestes animais nos testes de sociabilidade. Assim como observado em outros parâmetros avaliados, o tratamento com suramina não foi capaz de reverter essa alteração na atividade exploratória desses animais.

Além de análises comportamentais, os trabalhos desenvolvidos nessa tese também abrangeram algumas análises moleculares, com o objetivo de tentar elucidar alguns mecanismos da ação do VPA e dos tratamentos realizados. Nós utilizamos a técnica de transcrição reversa seguida da reação em cadeia de polimerase quantitativa (do inglês *reverse transcription followed by quantitative polymerase chain reaction*, RT-qPCR) para analisar a expressão relativa de um conjunto de miRNA em amostras de sangue de pacientes com TEA e no modelo animal de autismo induzido pela exposição ao VPA. Nós encontramos um aumento nos níveis de miR138-5p e miR134-5p no sangue de pacientes, comparado aos controles. Curiosamente, o miR134-5p também

apresentou um aumento em sangue de animais do grupo VPA. Estes achados sugerem que a avaliação do perfil de expressão de miRNA pode ser utilizada para identificar as vias biológicas potencialmente alteradas na TEA. Uma vez que miRNA podem passar para a corrente sanguínea a partir de células, tecidos e órgãos (CREEMERS; TIJSEN; PINTO, 2012; LUDWIG et al., 2016), alterações nos níveis de miRNA circulante podem refletir alterações em outros tecidos, incluindo os sistemas nervoso e imunitário.

Esse trabalho traz novas perspectivas para a avaliação dos níveis de miRNA em diferentes estágios de desenvolvimento e diferentes tecidos, para podermos explorar as funções dessas pequenas moléculas na etiologia do TEA. Para isso, muitos estudos ainda são necessários e a técnica de RT-qPCR ganha destacada importância nesse aspecto. Diferente da análise da expressão de RNA mensageiros, que é comumente realizada por PCR, a quantificação de miRNA é mais frequentemente realizada pela técnica de microarranjo. No entanto, o tamanho reduzido e as quantidades limitadas de miRNA extraídas de tecidos animais trazem algumas dificuldades para as análises de miRNA utilizando essa técnica (LIN; LAI, 2013). Experimentos de microarranjo permitem o monitoramento do perfil de expressão simultâneo de diversos miRNA, mas seu uso apresenta algumas desvantagens, como a baixa sensibilidade e reprodutibilidade.

A técnica de RT-qPCR, utilizada neste trabalho para avaliação da expressão de um conjunto de miRNA, apresenta-se como uma metodologia mais sensível e confiável que nos permitiu identificar tênues diferenças na expressão relativa dessas moléculas em diferentes grupos experimentais, o que provavelmente não seria possível se fosse utilizada a técnica de

microarranjo. No entanto, para que os resultados obtidos pela técnica de RT-qPCR pudessem ser alcançados, foi necessária uma extensa padronização do protocolo, principalmente devido à carência de informações na literatura a respeito do uso dessa técnica para avaliar expressão de miRNA. Nesse sentido, escrevemos um manuscrito onde informamos passo a passo o protocolo de análise de miRNA por RT-qPCR, com o objetivo de facilitar estudos posteriores do grupo e de outros pesquisadores.

Um dos principais aspectos a serem levados em conta é a utilização de uma normalização adequada para as análises de miRNA. O propósito da normalização é remover as variações entre grupos devido à amostragem, permitindo identificar reais diferenças nos perfis de expressão de miRNA como consequência do estado patológico, tratamento ou estágio do desenvolvimento. Alguns miRNA exibem perfis de expressão que não se alteram sob diferentes condições, podendo servir como miRNA de referência nas quantificações de outros miRNA de interesse. Neste trabalho, nós realizamos uma varredura de 16 miRNA selecionados para este trabalho, com o objetivo de encontrar os miRNA normalizadores para nossas análises. Nós utilizamos o algoritmo geNorm para investigarmos a variância de todos os miRNA selecionados e então avaliar a estabilidade nos níveis de expressão dos miRNA (PELTIER; LATHAM, 2008).

A técnica de RT-qPCR é frequentemente utilizada para análises de expressão de RNA mensageiros. No entanto, quando utilizada para análise de miRNA, algumas diferenças devem ser destacadas. Uma delas é a necessidade de desenhar e sintetizar iniciadores específicos para transcrição reversa de cada um dos miRNA analisados. Isso ocorre porque, diferente dos

RNA mensageiros maduros, que possuem uma cauda poliadenilada em sua extremidade 3', a síntese de DNA complementar aos miRNA não pode ser realizada utilizando iniciadores complementares oligoDT. Assim, para tal, é necessária a aquisição desses iniciadores específicos, chamados de iniciadores em grampo (do inglês, *stem-loop primers*). Em contrapartida, para as análises de PCR quantitativo, apenas um iniciador reverso é necessário para a quantificação de todos os miRNA analisados, uma vez que todos os iniciadores em grampo utilizados para a transcrição reversa possuem uma porção comum para todos os miRNA, na qual esse iniciador reverso universal é capaz de se ligar na etapa de anelamento ao DNA complementar durante a amplificação.

Outra diferença da análise de miRNA em relação às análises de RNA mensageiros por RT-qPCR são as quantidades de molde utilizado. Uma vez que, dependendo do tecido analisado e do RNA em questão, as quantidades de miRNA são mais limitadas que as de RNA mensageiros, maiores quantidades de material de partida são necessárias para sua quantificação. Assim, partindo de um mesmo DNA complementar (o qual foi sintetizado a partir de um mesmo extrato de RNA), é possível realizar diferentes diluições, a fim de se encontrar as melhores condições para análise de diferentes miRNA em diferentes tecidos. Atenção especial também deve ser dada à quantidade e à qualidade dos iniciadores utilizados para as análises de miRNA por PCR. Para isso, além de um bom desenho dos iniciadores e escolha de uma empresa que os sintetize de forma adequada, é necessário realizar testes para verificar a especificidade dos iniciadores em relação aos produtos desejados, além de otimizar suas concentrações. Uma vez que as amostras periféricas

podem ser mais facilmente obtidas de pacientes em relação a outros tecidos, a abordagem translacional que empregamos neste trabalho é capaz de sustentar o potencial uso de miRNA como biomarcadores no TEA. Não obstante, outros tipos de materiais biológicos podem futuramente ser utilizados com este objetivo, como as amostras de saliva de pacientes. Além de potenciais biomarcadores, a análise de miRNA pode permitir a realização de diversos estudos mecanísticos envolvendo rotas de sinalização possivelmente alteradas no TEA, bem como a investigação de possíveis estratégias terapêuticas envolvendo essas moléculas.

Trabalhos do grupo demonstraram um efeito preventivo do RSV sobre a diminuição do comportamento social e alterações sensoriais em ratos expostos prenatalmente ao VPA (BAMBINI-JUNIOR et al., 2014; FONTES-DUTRA et al., 2018). Além disso, já foi demonstrado que essa molécula foi capaz de prevenir alterações na organização cortical na área somatossensorial primária desses animais (FONTES-DUTRA et al., 2018), o que poderia explicar seu efeito sobre as alterações sensoriais encontradas. Aqui, nós demonstramos que o RSV também foi capaz de prevenir um aumento dos níveis do miR134-5p, um miRNA de grande importância na maturação de espinhos dendríticos e no desenvolvimento de sinapses (SCHRATT et al., 2006; TAI; SCHUMAN, 2006). Dada suas propriedades anti-inflamatórias e antioxidantes e seus efeitos preventivos em modelos animais de autismo, o RSV surge como uma potencial molécula para futuras estratégias terapêuticas sobre sintomas e comorbidades do TEA, o que já está sendo buscado com testes pré-clínicos desenvolvidos pelo nosso grupo de pesquisa.

Por outro lado, os efeitos terapêuticos da suramina sobre alguns parâmetros comportamentais observados no modelo animal de autismo induzido por exposição ao VPA nos levaram a investigar um possível papel do sistema purinérgico no desencadeamento das características do tipo autista em ratos. Para responder a essa questão, nós quantificamos a expressão relativa de RNA mensageiros de alguns receptores purinérgicos do tipo P2 nos grupos experimentais. Nossos resultados demonstraram um aumento nos níveis corticais do receptor ionotrópico P2X4, enquanto no hipocampo houve um aumento de P2X4 e do receptor metabotrópico P2Y2 nos animais expostos prenatalmente ao VPA. Esses achados fortalecem a hipótese do envolvimento do sistema purinérgico no contexto do TEA, trazendo assim novas possibilidades de manipulações farmacológicas que explorem essa via.

Embora nosso trabalho e outros estudos anteriores tenham demonstrado efeitos terapêuticos da suramina sobre prejuízos sociais em modelos animais de autismo (NAVIAUX et al., 2014, 2013), é sabido que esse fármaco dificilmente passa pela barreira hematoencefálica (BHE) em condições fisiológicas (HAWKING, 1940; RASEROKA; ORMEROD, 1986; SANDERSON; KHAN; THOMAS, 2007). Sendo assim, existem duas possibilidades que poderiam explicar tais resultados apesar dessa peculiaridade dessa molécula. Primeiramente, sabe-se que a suramina é capaz de se ligar a diversas proteínas plasmáticas como a albumina (HAWKING, 1978; ROBOZ et al., 1998), de forma que poderia então estar atravessando a BHE através de transportadores proteicos específicos. No entanto, mesmo que isso esteja ocorrendo, a suramina não é capaz de alcançar concentrações suficientemente

altas no SNC para que possa exercer seus efeitos (SANDERSON; KHAN; THOMAS, 2007).

Uma segunda explicação pode estar relacionada com a ação da suramina em tecidos periféricos. Uma das hipóteses mais investigadas no nosso grupo de pesquisa é que o VPA possa estar desencadeando alterações no sistema imunitário de roedores prenhes, as quais poderiam causar alterações imunológicas e inflamatórias na prole. Assim, uma vez que durante o desenvolvimento fetal existe uma interação entre sistema imunitário e SNC, possivelmente as alterações causadas pelo VPA possam estar indiretamente causando alterações relacionadas à fisiopatologia do TEA. Por consequência, uma possível ação da suramina a nível periférico poderia modular algumas características do tipo autista a nível imunológico, mesmo não agindo diretamente sobre o SNC.

Guiados por essa segunda hipótese, quantificamos os níveis de RNA mensageiro de algumas citocinas pró-inflamatórias em duas regiões encefálicas de ratos expostos prenatalmente ao VPA e tratados ou não com suramina. Corroborando estudos anteriores com tecidos *post mortem* de indivíduos com TEA e em modelos animais (WEI et al., 2012; XU; LI; ZHONG, 2015), encontramos níveis aumentados de IL-6 no córtex medial pré-frontal de animais expostos ao VPA, de forma que o tratamento com suramina foi capaz de reverter essa alteração. Visto que os níveis de IL-6 podem estar relacionados com o desencadeamento de algumas características do tipo autista, uma vez que já foram demonstrados níveis aumentados dessa citocina em pacientes e em modelos animais (WEI et al., 2012; XU; LI; ZHONG, 2015), nós acreditamos que o efeito positivo da suramina sobre o comportamento

social de ratos do modelo VPA possa ter alguma relação com essa restauração dos níveis de IL-6.

De uma forma geral, nossos dados reforçam a possibilidade do uso de moléculas com diferentes propriedades como estratégias terapêuticas para alguns sintomas e comorbidades encontrados no TEA, apresentando para a comunidade científica novas possibilidades para o desenvolvimento de tratamentos seguros e eficazes. No entanto, muitos desafios ainda precisam ser contornados a fim de aperfeiçoar a utilização dessas moléculas em estudos clínicos. Somado a isso, nossos resultados trazem novas pistas sobre rotas de sinalização que possam ter grande importância no contexto do TEA, trazendo assim a possibilidade de investigação de novas moléculas para potenciais intervenções farmacológicas que possam atenuar ou prevenir certas alterações, com o objetivo de melhorar a qualidade de vida dos pacientes com TEA e de suas famílias.

CONCLUSÕES

No presente trabalho, utilizamos um modelo animal de autismo obtido pela exposição pré-natal ao VPA para avaliar o comportamento social e comportamentos repetitivos e estereotipados, que são os sintomas centrais do diagnóstico do TEA, além de alguns parâmetros comportamentais que aparecem como sintomas associados, como comportamento do tipo ansioso e alterações sensoriais.

Primeiramente, em relação ao comportamento social, os animais do modelo VPA apresentaram prejuízo social tanto no teste três câmaras quanto no teste de sociabilidade recíproca, corroborando dados anteriores do grupo e da literatura. O tratamento pré-natal com RSV foi capaz de prevenir as alterações sociais observados no teste de sociabilidade recíproca. O tratamento com suramina, por sua vez, foi capaz de reverter alterações sociais no teste de três câmaras, mas não no teste de sociabilidade recíproca.

Em relação ao comportamento repetitivo, animais expostos ao VPA gastaram mais tempo fazendo autolimpeza quando comparados aos animais dos grupos controle, e os tratamentos com RSV e suramina não foram capazes de prevenir ou reverter essas alterações. Por outro lado, o tratamento com suramina foi capaz de reverter o aumento do comportamento do tipo ansioso apresentado pelos animais do grupo VPA.

Quando avaliamos parâmetros sensoriais, foi observado um prejuízo na memória olfativa dos animais expostos ao VPA, uma vez que estes não tiveram preferência por consumir um alimento com um sabor previamente apresentado por um animal da sua ninhada. O tratamento com RSV, no entanto, não foi capaz de prevenir essa alteração. Adicionalmente, a exposição ao VPA causou

uma hiper-responsividade a estímulos não nocivos (avaliada pelo teste WNT) e uma hiporresponsividade a um estímulo nocivo (avaliada pelo teste de retirada da cauda). O tratamento com suramina não foi capaz de reverter nenhuma dessas alterações sensoriais encontradas.

Animais do grupo VPA também apresentaram um maior comportamento do tipo ansioso em relação aos animais do grupo controle, e curiosamente o tratamento com suramina foi capaz de reverter essa alteração. Nenhuma alteração significativa foi encontrada em relação à atividade locomotora e exploratória no modelo VPA.

Ao avaliarmos a expressão relativa de um grupo de miRNA em sangue pacientes com TEA, foi observado um aumento na expressão de dois deles, miR138-5p e miR134-5p, sendo que esse último também teve sua expressão aumentada no modelo animal de autismo induzido por VPA, mas essa alteração foi totalmente prevenida pelo tratamento com RSV. Relacionado às análises de expressão de miRNA, concluímos que a técnica de RT-qPCR foi adequada para os propósitos deste estudo, após tomadas as devidas precauções e adequações ao longo do protocolo.

A expressão gênica de dois receptores purinérgicos foi encontrada aumentada em estruturas em regiões encefálicas de animais VPA, sem nenhum efeito do tratamento com suramina. Por fim, os níveis da citocina IL-6 foram encontrados aumentados no córtex medial pré-frontal desses animais, mas o tratamento com suramina foi capaz de reverter totalmente essa alteração.

Como conclusão final, observamos que o RSV preveniu um prejuízo social e aumento dos níveis de miRNA no modelo de autismo induzido pelo

VPA, enquanto a suramina reverteu prejuízo no comportamento social e o comportamento do tipo ansioso, além do aumento de moléculas pró-inflamatórias no encéfalo de animais do modelo VPA. Os efeitos do RSV reforçam a relevância dessa estratégia como ferramenta para estudos voltados para aspectos etiológicos durante o período embrionário, entre o momento da indução e o final da gestação. Os efeitos da suramina colocam o sistema purinérgico como um importante alvo de estudos na busca por estratégias terapêuticas no TEA. A análise de miRNA traz grandes perspectivas para análises de mecanismos moleculares envolvidos no desencadeamento e dos sintomas e também pode ter importância no desenvolvimento de novas estratégias terapêuticas, de forma que a técnica de RT-qPCR aparece com papel fundamental nesses aspectos.

PERSPECTIVAS

Considerando os resultados obtidos na presente tese e evidências de que os comportamentos do tipo autista e alguns parâmetros moleculares estão alterados no modelo animal, e que estratégias terapêuticas foram capazes de prevenir ou reverter tais alterações, algumas perspectivas emergem para o seguimento da pesquisa relacionada ao TEA no grupo, incluindo o estudo em embriões:

- Avaliar o efeito de outros tratamentos antipurinérgicos sobre os comportamentos alterados, através do uso de inibidores seletivos de alguns receptores que apresentaram expressão aumentada em animais VPA;
- Analisar os níveis de miRNA em estruturas encefálicas no modelo animal, bem como o efeito de estratégias terapêuticas;
- Quantificar os níveis proteicos de alvos de miRNA alterados no modelo VPA e buscar mecanismos moleculares que possam estar envolvidos no desencadeamento de características do tipo autistas nesses animais;
- Avaliar a influência dos níveis de miRNA sobre parâmetros específicos no modelo VPA, como alterações na morfologia de espinhos dendríticos, migração e diferenciação neuronal;
- Avaliar a influência de miRNA sobre parâmetros imunitários e inflamatórios no modelo VPA;
- Utilizar dados obtidos de técnicas mais abrangentes (como RNA-seq) para estudo de miRNA possivelmente alterados no contexto do TEA, tanto em humanos quanto no modelo animal, a fim de se encontrar possíveis biomarcadores que possam servir como ferramenta para diagnóstico precoce do TEA.

- Utilizar os conhecimentos obtidos sobre alterações nos níveis de miRNA no contexto do TEA para desenvolvimento de possíveis estratégias terapêuticas baseadas nessas pequenas moléculas, como por exemplo através da utilização de antagomirs.

REFERÊNCIAS BIBLIOGRÁFICAS

ABRAHAMS, Brett S.; GESCHWIND, Daniel H. Advances in autism genetics: on the threshold of a new neurobiology. **Nature reviews. Genetics**, [s. l.], v. 9, n. 5, p. 341–55, 2008. Disponível em: <http://www.nature.com/articles/nrg2346>. Acesso em: 19 abr. 2018.

ABU-ELNEEL, Kawther et al. Heterogeneous dysregulation of microRNAs across the autism spectrum. **Neurogenetics**, [s. l.], v. 9, n. 3, p. 153–161, 2008.

AMBROS, Victor. The functions of animal microRNAs. **Nature**, [s. l.], v. 431, n. 7006, p. 350–5, 2004. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/15372042>. Acesso em: 9 out. 2016.

AMEIS, Stephanie H.; SZATMARI, Peter. Imaging-genetics in autism spectrum disorder: advances, translational impact, and future directions. **Frontiers in psychiatry**, [s. l.], v. 3, p. 46, 2012. Disponível em: <http://journal.frontiersin.org/article/10.3389/fpsyt.2012.00046/abstract>. Acesso em: 19 abr. 2018.

AMERICAN PSYCHIATRIC ASSOCIATION (APA). Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition - DSM IV. [s. l.], 1994.

AMERICAN PSYCHIATRY ASSOCIATION (APA). **Diagnostic and Statistical Manual of Mental Disorders, 5th Edition**. 5th Ed. ed. Washington, DC: American Psychiatric Publishing, Inc, 2013.

APA, American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition Text Revision - DSM IV-TR. **DSM IV-TR**, [s. l.], 2000.

ASHWOOD, Paul et al. Intestinal lymphocyte populations in children with regressive autism: evidence for extensive mucosal immunopathology. **Journal of clinical immunology**, [s. l.], v. 23, n. 6, p. 504–17, 2003.

ASHWOOD, Paul et al. Associations of impaired behaviors with elevated plasma chemokines in autism spectrum disorders. **Journal of Neuroimmunology**, [s. l.], v. 232, n. 1–2, p. 196–199, 2011. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0165572810004753>. Acesso em: 26 abr. 2017.

ASHWOOD, Paul; WAKEFIELD, Andrew J. Immune activation of peripheral blood and mucosal CD3+ lymphocyte cytokine profiles in children with autism and gastrointestinal symptoms. **Journal of neuroimmunology**, [s. l.], v. 173, n. 1–2, p. 126–34, 2006.

ATLADÓTTIR, Hjördis O. et al. Maternal infection requiring hospitalization during pregnancy and autism spectrum disorders. **Journal of autism and developmental disorders**, [s. l.], v. 40, n. 12, p. 1423–30, 2010. Disponível em: <http://link.springer.com/10.1007/s10803-010-1006-y>. Acesso em: 26 abr. 2017.

BAMBINI-JUNIOR, Victorio et al. Animal model of autism induced by prenatal exposure to valproate: behavioral changes and liver parameters. **Brain research**, [s. l.], v. 1408, p. 8–16, 2011. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0006899311010973>. Acesso em: 26 abr. 2017.

BAMBINI-JUNIOR, Victorio et al. Resveratrol prevents social deficits in animal model of autism induced by valproic acid. **Neuroscience letters**, [s. l.], v. 583, p. 176–81, 2014.

BANERJEE, Anwesha et al. Abnormal emotional learning in a rat model of autism exposed to valproic acid in utero. **Frontiers in Behavioral Neuroscience**, [s. l.], v. 8, p. 387, 2014. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/25429264>. Acesso em: 19 abr. 2018.

BARBATO, Christian et al. Thinking about RNA? MicroRNAs in the brain. **Mammalian genome : official journal of the International Mammalian Genome Society**, [s. l.], v. 19, n. 7–8, p. 541–51, 2008. Disponível em: <http://link.springer.com/10.1007/s00335-008-9129-6>. Acesso em: 19 abr. 2018.

BARICORDI, O. R. et al. Increased proliferation rate of lymphoid cells transfected with the P2X(7) ATP receptor. **The Journal of biological chemistry**, [s. l.], v. 274, n. 47, p. 33206–8, 1999. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/10559192>. Acesso em: 28 abr. 2017.

BARTEL, David P. MicroRNAs: target recognition and regulatory functions. **Cell**, [s. l.], v. 136, n. 2, p. 215–33, 2009. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/19167326>. Acesso em: 9 out. 2016.

BAUM, Sarah H.; STEVENSON, Ryan A.; WALLACE, Mark T. Behavioral, perceptual, and neural alterations in sensory and multisensory function in autism spectrum disorder. **Progress in Neurobiology**, [s. l.], v. 134, p. 140–160, 2015. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/26455789>. Acesso em: 28 abr. 2017.

BAUMAN, Margaret L. Medical comorbidities in autism: Challenges to diagnosis and treatment. **Neurotherapeutics**, [s. l.], v. 7, n. 3, p. 320–327, 2010. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/20643385>. Acesso em: 19 abr. 2018.

BAUMAN, Melissa D. et al. Activation of the Maternal Immune System During Pregnancy Alters Behavioral Development of Rhesus Monkey Offspring. **Biological Psychiatry**, [s. l.], v. 75, n. 4, p. 332–341, 2014. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0006322313006732>. Acesso em: 26 abr. 2017.

BAUR, Joseph A. et al. Resveratrol improves health and survival of mice on a high-calorie diet. **Nature**, [s. l.], v. 444, n. 7117, p. 337–42, 2006. Disponível em: <http://www.nature.com/articles/nature05354>. Acesso em: 19 abr. 2018.

BAXTER, Andrew W. et al. Role of P2X4 receptors in synaptic strengthening in mouse CA1 hippocampal neurons. **European Journal of Neuroscience**, [s. l.], v. 34, n. 2, p. 213–220, 2011. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/21749490>. Acesso em: 28 abr. 2017.

BELMONTE, M. et al. NMR intensity of corpus callosum differs with age but not with diagnosis of autism. **Neuroreport**, [s. l.], v. 6, n. 9, p. 1253–6, 1995. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/7669980>. Acesso em: 26 abr. 2017.

BEN-SASSON, Ayelet et al. A meta-analysis of sensory modulation symptoms in individuals with autism spectrum disorders. **Journal of autism and**

developmental disorders, [s. l.], v. 39, n. 1, p. 1–11, 2009. Disponível em: <http://link.springer.com/10.1007/s10803-008-0593-3>. Acesso em: 19 abr. 2018.

BERRIDGE, K. C. Progressive degradation of serial grooming chains by descending decerebration. **Behavioural brain research**, [s. l.], v. 33, n. 3, p. 241–53, 1989. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/2757783>. Acesso em: 25 abr. 2018.

BOLITHO, Christine et al. The anti-apoptotic activity of albumin for endothelium is mediated by a partially cryptic protein domain and reduced by inhibitors of G-coupled protein and PI-3 kinase, but is independent of radical scavenging or bound lipid. **Journal of vascular research**, [s. l.], v. 44, n. 4, p. 313–24, 2007. Disponível em: <http://www.karger.com/?doi=10.1159/000101777>. Acesso em: 28 abr. 2017.

BORCHERT, Glen M.; LANIER, William; DAVIDSON, Beverly L. RNA polymerase III transcribes human microRNAs. **Nature Structural & Molecular Biology**, [s. l.], v. 13, n. 12, p. 1097–1101, 2006. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/17099701>. Acesso em: 19 abr. 2018.

BORTOLATO, Marco et al. Pharmacological insights into the role of P2X4 receptors in behavioural regulation: lessons from ivermectin. **The international journal of neuropsychopharmacology**, [s. l.], v. 16, n. 5, p. 1059–70, 2013. Disponível em: <https://academic.oup.com/ijnp/article-lookup/doi/10.1017/S1461145712000909>. Acesso em: 28 abr. 2017.

BRISTOT SILVESTRIN, Roberta et al. Animal model of autism induced by prenatal exposure to valproate: altered glutamate metabolism in the hippocampus. **Brain research**, [s. l.], v. 1495, p. 52–60, 2013. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0006899312018422>. Acesso em: 26 abr. 2017.

BRODERICK, J. A.; ZAMORE, P. D. MicroRNA therapeutics. **Gene therapy**, [s. l.], v. 18, n. 12, p. 1104–10, 2011. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/21525952>. Acesso em: 9 out. 2016.

BROWN, W. T. et al. Autism is associated with the fragile-X syndrome. **Journal of autism and developmental disorders**, [s. l.], v. 12, n. 3, p. 303–8, 1982. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/7153204>. Acesso em: 26 abr. 2017.

BURNSTOCK, G. Physiology and Pathophysiology of Purinergic Neurotransmission. **Physiological Reviews**, [s. l.], v. 87, n. 2, p. 659–797, 2007. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/17429044>. Acesso em: 28 abr. 2017.

BUTLER, S. J. et al. Differential effects of suramin on the coupling of receptors to individual species of pertussis-toxin-sensitive guanine-nucleotide-binding proteins. **The Biochemical journal**, [s. l.], v. 251, n. 1, p. 201–5, 1988. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/2839158>. Acesso em: 12 mar. 2018.

CALCATERRA, N. B.; VICARIO, L. R.; ROVERI, O. A. Inhibition by suramin of mitochondrial ATP synthesis. **Biochemical pharmacology**, [s. l.], v. 37, n. 13, p. 2521–7, 1988. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/2968800>. Acesso em: 12 mar. 2018.

CANNON, Richard L. et al. Alterations in self-grooming sequences in the rat as a consequence of hippocampal damage. **Psychobiology**, [s. l.], v. 20, n. 3, p. 205–218, 1992. Disponível em: <https://link.springer.com/article/10.3758%2FBF03332049>. Acesso em: 25 abr. 2018.

CAO, Xinwei et al. Nonconding RNAs in the mammalian central nervous system. **Annual Review of Neuroscience**, [s. l.], v. 29, n. 1, p. 77–103, 2006. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/16776580>. Acesso em: 19 abr. 2018.

CHAN, A.; GOLD, R.; VON AHSEN, N. ATP-binding cassette transporters in inflammatory brain disease. **Current pharmaceutical design**, [s. l.], v. 17, n. 26, p. 2803–7, 2011. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/21827405>. Acesso em: 28 abr. 2017.

CHOI, Gloria B. et al. The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. **Science (New York, N.Y.)**, [s. l.], v. 351, n. 6276, p. 933–9, 2016. Disponível em: <http://www.sciencemag.org/cgi/doi/10.1126/science.aad0314>. Acesso em: 28 abr. 2017.

CHRISTENSEN, Jakob et al. Prenatal Valproate Exposure and Risk of Autism Spectrum Disorders and Childhood Autism. **JAMA**, [s. l.], v. 309, n. 16, p. 1696, 2013. Disponível em: <http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.2013.2270>. Acesso em: 26 abr. 2017.

CHRISTIANSON, A. L.; CHESLER, N.; KROMBERG, J. G. Fetal valproate syndrome: clinical and neuro-developmental features in two sibling pairs. **Developmental medicine and child neurology**, [s. l.], v. 36, n. 4, p. 361–9, 1994. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/7512516>. Acesso em: 13 nov. 2017.

CORRIDEN, Ross; INSEL, Paul A. Basal release of ATP: an autocrine-paracrine mechanism for cell regulation. **Science signaling**, [s. l.], v. 3, n. 104, p. re1, 2010. Disponível em: <http://stke.sciencemag.org/cgi/doi/10.1126/scisignal.3104re1>. Acesso em: 28 abr. 2017.

COSKUN, Mehmet Akif et al. How somatic cortical maps differ in autistic and typical brains. **NeuroReport**, [s. l.], v. 20, n. 2, p. 175–179, 2009. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/19057419>. Acesso em: 12 mar. 2018.

CREEMERS, E. E.; TIJSEN, A. J.; PINTO, Y. M. Circulating MicroRNAs: Novel Biomarkers and Extracellular Communicators in Cardiovascular Disease? **Circulation Research**, [s. l.], v. 110, n. 3, p. 483–495, 2012. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/22302755>. Acesso em: 13 nov. 2017.

CROEN, Lisa A. et al. Maternal autoimmune diseases, asthma and allergies, and childhood autism spectrum disorders: a case-control study. **Archives of pediatrics & adolescent medicine**, [s. l.], v. 159, n. 2, p. 151–7, 2005. Disponível em: <http://archpedi.jamanetwork.com/article.aspx?doi=10.1001/archpedi.159.2.151>. Acesso em: 26 abr. 2017.

DAHLGREN, Jovanna et al. Interleukin-6 in the maternal circulation reaches the rat fetus in mid-gestation. **Pediatric research**, [s. l.], v. 60, n. 2, p. 147–51, 2006.

DAVIS-DUSENBERY, Brandi N.; HATA, Akiko. Mechanisms of control of microRNA biogenesis. **Journal of biochemistry**, [s. l.], v. 148, n. 4, p. 381–92, 2010. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/20833630>. Acesso em: 27 out. 2016.

DE SMAELE, Enrico; FERRETTI, Elisabetta; GULINO, Alberto. MicroRNAs as biomarkers for CNS cancer and other disorders. **Brain Research**, [s. l.], v. 1338, p. 100–111, 2010. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/20380821>. Acesso em: 19 abr. 2018.

DOENCH, John G.; SHARP, Phillip A. Specificity of microRNA target selection in translational repression. **Genes & development**, [s. l.], v. 18, n. 5, p. 504–11, 2004. Disponível em: <http://www.genesdev.org/cgi/doi/10.1101/gad.1184404>. Acesso em: 19 abr. 2018.

DOVER, Clare J.; LE COUTEUR, Ann. How to diagnose autism. **Archives of disease in childhood**, [s. l.], v. 92, n. 6, p. 540–5, 2007. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/17515625>. Acesso em: 9 nov. 2017.

DUBYAK, George R. Signal Transduction by P $_2$ -Purinergic Receptors for Extracellular ATP. **American Journal of Respiratory Cell and Molecular Biology**, [s. l.], v. 4, n. 4, p. 295–300, 1991. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/1707633>. Acesso em: 19 abr. 2018.

DUCHAN, Erin; PATEL, Dilip R. Epidemiology of autism spectrum disorders. **Pediatric clinics of North America**, [s. l.], v. 59, n. 1, p. 27–43, ix–x, 2012. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0031395511001386>. Acesso em: 19 abr. 2018.

DUNN, P. M.; BLAKELEY, A. G. Suramin: a reversible P2-purinoceptor antagonist in the mouse vas deferens. **British journal of pharmacology**, [s. l.], v. 93, n. 2, p. 243–5, 1988. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/3359103>. Acesso em: 12 mar. 2018.

DURKIN, Maureen S. et al. Advanced parental age and the risk of autism spectrum disorder. **American journal of epidemiology**, [s. l.], v. 168, n. 11, p. 1268–1276, 2008.

FAAS, M. M.; SÁEZ, T.; DE VOS, P. Extracellular ATP and adenosine: The Yin and Yang in immune responses? **Molecular Aspects of Medicine**, [s. l.], 2017. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/28093236>. Acesso em: 28 abr. 2017.

FAVRE, Mônica R. et al. General developmental health in the VPA-rat model of autism. **Frontiers in behavioral neuroscience**, [s. l.], v. 7, p. 88, 2013. Disponível em: <http://journal.frontiersin.org/article/10.3389/fnbeh.2013.00088/abstract>. Acesso em: 26 abr. 2017.

FELICIANO, David M. et al. A circuitry and biochemical basis for tuberous sclerosis symptoms: from epilepsy to neurocognitive deficits. **International journal of developmental neuroscience : the official journal of the**

International Society for Developmental Neuroscience, [s. l.], v. 31, n. 7, p. 667–78, 2013. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S073657481300035X>. Acesso em: 26 abr. 2017.

FILIPEK, Pauline A. et al. Mitochondrial dysfunction in autistic patients with 15q inverted duplication. **Annals of neurology**, [s. l.], v. 53, n. 6, p. 801–4, 2003. Disponível em: <http://doi.wiley.com/10.1002/ana.10596>. Acesso em: 26 abr. 2017.

FILIPOWICZ, Witold; BHATTACHARYYA, Suvendra N.; SONENBERG, Nahum. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? **Nature reviews. Genetics**, [s. l.], v. 9, n. 2, p. 102–14, 2008. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/18197166>. Acesso em: 9 out. 2016.

FONTES-DUTRA, Mellanie et al. Resveratrol prevents cellular and behavioral sensory alterations in the animal model of autism induced by valproic acid. **Frontiers in Synaptic Neuroscience**, [s. l.], v. 10, p. 9, 2018. Disponível em: <https://www.frontiersin.org/articles/10.3389/fnsyn.2018.00009/abstract>. Acesso em: 4 maio. 2018.

FORTES, P. A. G.; ELLORY, J. C.; LEW, V. L. Suramin: A potent ATPase inhibitor which acts on the inside surface of the sodium pump. **Biochimica et Biophysica Acta (BBA) - Biomembranes**, [s. l.], v. 318, n. 2, p. 262–272, 1973. Disponível em: <https://www.sciencedirect.com/science/article/pii/0005273673901193>. Acesso em: 12 mar. 2018.

FREDHOLM, B. B. et al. Nomenclature and classification of purinoceptors. **Pharmacological reviews**, [s. l.], v. 46, n. 2, p. 143–56, 1994. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/7938164>. Acesso em: 28 abr. 2017.

FREITAG, Christine M. et al. Genetics of autistic disorders: review and clinical implications. **European Child & Adolescent Psychiatry**, [s. l.], v. 19, n. 3, p. 169–178, 2010. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/19941018>. Acesso em: 19 abr. 2018.

FRÉMONT, Lucie. Biological effects of resveratrol. **Life Sciences**, [s. l.], v. 66, n. 8, p. 663–673, 2000.

FRIEDMAN, R. C. et al. Most mammalian mRNAs are conserved targets of microRNAs. **Genome Research**, [s. l.], v. 19, n. 1, p. 92–105, 2008. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/18955434>. Acesso em: 19 abr. 2018.

FUKUCHI, Mamoru et al. Valproic acid induces up- or down-regulation of gene expression responsible for the neuronal excitation and inhibition in rat cortical neurons through its epigenetic actions. **Neuroscience Research**, [s. l.], v. 65, n. 1, p. 35–43, 2009. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/19463867>. Acesso em: 26 abr. 2017.

GADIA, Carlos A.; TUCHMAN, Roberto; ROTTA, Newra T. [Autism and pervasive developmental disorders]. **Jornal de pediatria**, [s. l.], v. 80, n. 2 Suppl, p. S83-94, 2004. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/15154076>. Acesso em: 26 abr. 2017.

GESCHWIND, Daniel H. Advances in autism. **Medicine**, [s. l.], v. 60, p. 367–380, 2009.

GESUNDHEIT, Benjamin et al. Immunological and autoimmune considerations of Autism Spectrum Disorders. **Journal of autoimmunity**, [s. l.], v. 44, p. 1–7, 2013.

GLASER, Talita et al. Modulation of mouse embryonic stem cell proliferation and neural differentiation by the P2X7 receptor. **PloS one**, [s. l.], v. 9, n. 5, p. e96281, 2014. Disponível em: <http://dx.plos.org/10.1371/journal.pone.0096281>. Acesso em: 28 abr. 2017.

GLASER, Talita; RESENDE, Rodrigo R.; ULRICH, Henning. Implications of purinergic receptor-mediated intracellular calcium transients in neural differentiation. **Cell Communication and Signaling**, [s. l.], v. 11, n. 1, p. 12, 2013. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/23414261>. Acesso em: 28 abr. 2017.

GOINES, Paula E.; ASHWOOD, Paul. Cytokine dysregulation in autism spectrum disorders (ASD): possible role of the environment. **Neurotoxicology and teratology**, [s. l.], v. 36, p. 67–81, 2013. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0892036212001407>. Acesso em: 26 abr. 2017.

GOTTFRIED, Carmem et al. Valproic Acid in Autism Spectrum Disorder: From an Environmental Risk Factor to a Reliable Animal Model. In: **Recent Advances in Autism Spectrum Disorders - Volume I**. [s.l.] : InTech, 2013.

GRANDIN, Temple. Visual abilities and sensory differences in a person with autism. **Biological psychiatry**, [s. l.], v. 65, n. 1, p. 15–6, 2009. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0006322308013991>. Acesso em: 19 abr. 2018.

GREENBERG, D. A. et al. Excess of twins among affected sibling pairs with autism: implications for the etiology of autism. **American journal of human genetics**, [s. l.], v. 69, n. 5, p. 1062–7, 2001. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0002929707613226>. Acesso em: 19 abr. 2018.

GRIGORENKO, Elena L. Pathogenesis of autism: a patchwork of genetic causes. **Future neurology**, [s. l.], v. 4, n. 5, p. 591–599, 2009. Disponível em: <http://www.futuremedicine.com/doi/10.2217/fnl.09.29>. Acesso em: 19 abr. 2018.

GRIMSON, Andrew et al. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. **Molecular cell**, [s. l.], v. 27, n. 1, p. 91–105, 2007. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S1097276507004078>. Acesso em: 19 abr. 2018.

GUPTA, Subash C. et al. Chemosensitization of tumors by resveratrol. **Annals of the New York Academy of Sciences**, [s. l.], v. 1215, p. 150–60, 2011.

GUZMAN, Segundo J.; GEREVICH, Zoltan. P2Y Receptors in Synaptic Transmission and Plasticity: Therapeutic Potential in Cognitive Dysfunction. **Neural Plasticity**, [s. l.], v. 2016, p. 1–12, 2016. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/27069691>. Acesso em: 28 abr. 2017.

HARDEN, Cynthia L. In utero valproate exposure and autism: long suspected, finally proven. **Epilepsy currents**, [s. l.], v. 13, n. 6, p. 282–4, 2013. Disponível em: <http://epilepsycurrents.org/doi/abs/10.5698/1535-7597-13.6.282>. Acesso em: 26 abr. 2017.

HARDEN, T. K. et al. Signalling and pharmacological properties of the P2Y14 receptor. **Acta Physiologica**, [s. l.], v. 199, n. 2, p. 149–160, 2010. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/20345417>. Acesso em: 28 abr. 2017.

HAWKING, F. Suramin: with special reference to onchocerciasis. **Advances in pharmacology and chemotherapy**, [s. l.], v. 15, p. 289–322, 1978. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/358805>. Acesso em: 13 mar. 2018.

HAWKING, Frank. Concentration of Bayer 205 (Germanin) in human blood and cerebrospinal fluid after treatment. **Transactions of the Royal Society of Tropical Medicine and Hygiene**, [s. l.], v. 34, n. 1, p. 37–52, 1940. Disponível em: <https://academic.oup.com/trstmh/article-lookup/doi/10.1016/S0035-9203(40)90088-8>. Acesso em: 13 mar. 2018.

HOUGHTON, David C. et al. Abnormal perceptual sensitivity in body-focused repetitive behaviors. **Comprehensive psychiatry**, [s. l.], v. 82, p. 45–52, 2018. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0010440X17302675>. Acesso em: 12 mar. 2018.

HOWITZ, Konrad T. et al. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. **Nature**, [s. l.], v. 425, n. 6954, p. 191–6, 2003. Disponível em: <http://www.nature.com/articles/nature01960>. Acesso em: 19 abr. 2018.

HSU, Sheng-Da et al. miRTarBase: a database curates experimentally validated microRNA-target interactions. **Nucleic acids research**, [s. l.], v. 39, n. Database issue, p. D163-9, 2011. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/21071411>. Acesso em: 9 out. 2016.

HUANG, Jiung-Pang et al. Insulin and resveratrol act synergistically, preventing cardiac dysfunction in diabetes, but the advantage of resveratrol in diabetics with acute heart attack is antagonized by insulin. **Free Radical Biology and Medicine**, [s. l.], v. 49, n. 11, p. 1710–1721, 2010.

ICHIYAMA, T. et al. Sodium valproate inhibits production of TNF-alpha and IL-6 and activation of NF-kappaB. **Brain research**, [s. l.], v. 857, n. 1–2, p. 246–51, 2000.

JAFFAR, Z. H.; PEARCE, F. L. Histamine secretion from mast cells stimulated with ATP. **Agents and actions**, [s. l.], v. 30, n. 1–2, p. 64–6, 1990. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/1695463>. Acesso em: 28 abr. 2017.

JANG, M. et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. **Science (New York, N.Y.)**, [s. l.], v. 275, n. 5297, p. 218–20, 1997.

JO, Young-Hwan et al. Cross-talk between P2X4 and gamma-aminobutyric acid, type A receptors determines synaptic efficacy at a central synapse. **The Journal of biological chemistry**, [s. l.], v. 286, n. 22, p. 19993–20004, 2011. Disponível em: <http://www.jbc.org/cgi/doi/10.1074/jbc.M111.231324>. Acesso

em: 28 abr. 2017.

JUMAH, Fareed et al. Neuroanatomical variation in autism spectrum disorder: A comprehensive review. **Clinical Anatomy**, [s. l.], v. 29, n. 4, p. 454–465, 2016. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/27004599>. Acesso em: 28 abr. 2017.

KAMAL, Mohammad A.; MUSHTAQ, Gohar; GREIG, Nigel H. Current Update on Synopsis of miRNA Dysregulation in Neurological Disorders. **CNS & neurological disorders drug targets**, [s. l.], v. 14, n. 4, p. 492–501, 2015.

KANNER, L. Autistic disturbances of affective contact. **Acta paedopsychiatrica**, [s. l.], v. 35, n. 4, p. 100–36, 1968. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/4880460>. Acesso em: 26 abr. 2017.

KAPLAN, Gabriel; MCCRACKEN, James T. Psychopharmacology of Autism Spectrum Disorders. **Pediatric Clinics of North America**, [s. l.], v. 59, n. 1, p. 175–187, 2012. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/22284801>. Acesso em: 19 abr. 2018.

KATAOKA, S. et al. Autism-like behaviours with transient histone hyperacetylation in mice treated prenatally with valproic acid. **Int J Neuropsychopharmacol**, [s. l.], v. 16, n. 1, p. 91–103, 2013.

KEDDE, Martijn et al. RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. **Cell**, [s. l.], v. 131, n. 7, p. 1273–86, 2007. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0092867407015371>. Acesso em: 19 abr. 2018.

KERN, Janet K. et al. Sensory correlations in autism. **Autism : the international journal of research and practice**, [s. l.], v. 11, n. 2, p. 123–34, 2007. Disponível em: <http://journals.sagepub.com/doi/10.1177/1362361307075702>. Acesso em: 19 abr. 2018.

KITTNER, Holger et al. Stimulation of P2Y1 Receptors Causes Anxiolytic-like Effects in the Rat Elevated Plus-maze: Implications for the Involvement of P2Y1 Receptor-Mediated Nitric Oxide Production. **Neuropsychopharmacology**, [s. l.], v. 28, n. 3, p. 435–444, 2003. Disponível em: <http://www.nature.com/articles/1300043>. Acesso em: 12 mar. 2018.

KLINTWALL, Lars et al. Sensory abnormalities in autism. **Research in Developmental Disabilities**, [s. l.], v. 32, n. 2, p. 795–800, 2011. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/21111574>. Acesso em: 19 abr. 2018.

KORVATSKA, Elena et al. Genetic and immunologic considerations in autism. **Neurobiology of disease**, [s. l.], v. 9, n. 2, p. 107–25, 2002. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0969996102904791>. Acesso em: 26 abr. 2017.

KOSIK, Kenneth S. The neuronal microRNA system. **Nature Reviews Neuroscience**, [s. l.], v. 7, n. 12, p. 911–920, 2006. Disponível em: <http://www.nature.com/doifinder/10.1038/nrn2037>. Acesso em: 19 abr. 2018.

KRISHTAL, O. A.; MARCHENKO, S. M.; PIDOPLICHKO, V. I. Receptor for ATP in the membrane of mammalian sensory neurones. **Neuroscience letters**, [s. l.], v. 35, n. 1, p. 41–5, 1983. Disponível em:

<http://www.ncbi.nlm.nih.gov/pubmed/6302606>. Acesso em: 19 abr. 2018.

KUGELBERG, Elisabeth. Neuroimmunology: IL-17A mediates a path to autism. **Nature Reviews Immunology**, [s. l.], v. 16, n. 4, p. 205–205, 2016. Disponível em: <http://www.nature.com/doifinder/10.1038/nri.2016.35>. Acesso em: 28 abr. 2017.

LEE, Jin-Ah et al. Resveratrol as a Bioenhancer to Improve Anti-Inflammatory Activities of Apigenin. **Nutrients**, [s. l.], v. 7, n. 11, p. 9650–61, 2015.

LEE, R. C.; FEINBAUM, R. L.; AMBROS, V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. **Cell**, [s. l.], v. 75, n. 5, p. 843–54, 1993. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/8252621>. Acesso em: 19 abr. 2018.

LEWIS, C. et al. Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. **Nature**, [s. l.], v. 377, n. 6548, p. 432–5, 1995. Disponível em: <http://www.nature.com/doifinder/10.1038/377432a0>. Acesso em: 28 abr. 2017.

LEWIS, M. et al. Animal models of restricted repetitive behavior in autism. **Behavioural Brain Research**, [s. l.], v. 176, n. 1, p. 66–74, 2007. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/16997392>. Acesso em: 19 abr. 2018.

LIN, Hui-Ching et al. The amygdala excitatory/inhibitory balance in a valproate-induced rat autism model. **PloS one**, [s. l.], v. 8, n. 1, p. e55248, 2013. Disponível em: <http://dx.plos.org/10.1371/journal.pone.0055248>. Acesso em: 19 abr. 2018.

LIN, Yu Ling; LAI, Zhong Xiong. Evaluation of suitable reference genes for normalization of microRNA expression by real-time reverse transcription PCR analysis during longan somatic embryogenesis. **Plant Physiology and Biochemistry**, [s. l.], v. 66, p. 20–25, 2013. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0981942813000399>. Acesso em: 19 abr. 2018.

LINTAS, C. et al. Lack of infection with XMRV or other MLV-related viruses in blood, post-mortem brains and paternal gametes of autistic individuals. **PLoS One**, [s. l.], 2011.

LIU, Jidong. Control of protein synthesis and mRNA degradation by microRNAs. **Current Opinion in Cell Biology**, [s. l.], v. 20, n. 2, p. 214–221, 2008. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/18329869>. Acesso em: 19 abr. 2018.

LOMBARD, J. Autism: a mitochondrial disorder? **Medical hypotheses**, [s. l.], v. 50, n. 6, p. 497–500, 1998. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/9710323>. Acesso em: 28 abr. 2017.

LUDWIG, Nicole et al. Distribution of miRNA expression across human tissues. **Nucleic acids research**, [s. l.], v. 44, n. 8, p. 3865–77, 2016. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/26921406>. Acesso em: 13 nov. 2017.

MABUNGA, Darine Froy N. et al. Exploring the Validity of Valproic Acid Animal Model of Autism. **Experimental neurobiology**, [s. l.], v. 24, n. 4, p. 285–300, 2015.

MAHONEY, C. W.; AZZI, A.; HUANG, K. P. Effects of suramin, an anti-human immunodeficiency virus reverse transcriptase agent, on protein kinase C. Differential activation and inhibition of protein kinase C isozymes. **The Journal of biological chemistry**, [s. l.], v. 265, n. 10, p. 5424–8, 1990. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/1690710>. Acesso em: 12 mar. 2018.

MALKOVA, Natalia V et al. Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. **Brain, behavior, and immunity**, [s. l.], v. 26, n. 4, p. 607–16, 2012. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S088915911200013X>. Acesso em: 26 abr. 2017.

MARKRAM, Kamila et al. Abnormal fear conditioning and amygdala processing in an animal model of autism. **Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology**, [s. l.], v. 33, n. 4, p. 901–12, 2008. Disponível em: <http://www.nature.com/doifinder/10.1038/sj.npp.1301453>. Acesso em: 26 abr. 2017.

MEFFORD, Heather C.; BATSHAW, Mark L.; HOFFMAN, Eric P. Genomics, Intellectual Disability, and Autism. **New England Journal of Medicine**, [s. l.], v. 366, n. 8, p. 733–743, 2012. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/22356326>. Acesso em: 19 abr. 2018.

MEYER, Urs et al. Adult brain and behavioral pathological markers of prenatal immune challenge during early/middle and late fetal development in mice. **Brain, behavior, and immunity**, [s. l.], v. 22, n. 4, p. 469–86, 2008.

MOHAMMADSHAHI, Majid; HAIDARI, Fatemeh; SOUFI, Farhad Ghadiri. Chronic resveratrol administration improves diabetic cardiomyopathy in part by reducing oxidative stress. **Cardiology Journal**, [s. l.], v. 21, n. 1, p. 39–46, 2014.

MOORE, David J. Acute pain experience in individuals with autism spectrum disorders: A review. **Autism**, [s. l.], v. 19, n. 4, p. 387–399, 2015. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/24687688>. Acesso em: 12 mar. 2018.

MOORE, S. J. et al. A clinical study of 57 children with fetal anticonvulsant syndromes. **Journal of medical genetics**, [s. l.], v. 37, n. 7, p. 489–97, 2000. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/10882750>. Acesso em: 13 nov. 2017.

MORIYAMA, Y.; NELSON, N. Inhibition of vacuolar H+-ATPases by fusidic acid and suramin. **FEBS letters**, [s. l.], v. 234, n. 2, p. 383–6, 1988. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/2899033>. Acesso em: 12 mar. 2018.

MUHLE, R.; TRENTACOSTE, S. V; RAPIN, I. The genetics of autism. **Pediatrics**, [s. l.], v. 113, n. 5, p. e472-86, 2004.

NAVIAUX, J. C. et al. Reversal of autism-like behaviors and metabolism in adult mice with single-dose antipurinergic therapy. **Translational Psychiatry**, [s. l.], v. 4, n. 6, p. e400–e400, 2014. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/24937094>. Acesso em: 12 mar. 2018.

NAVIAUX, Jane C. et al. Antipurinergic therapy corrects the autism-like features in the Fragile X (Fmr1 knockout) mouse model. **Molecular autism**, [s. l.], v. 6,

n. 1, p. 1, 2015. Disponível em: <http://www.molecularautism.com/content/6/1/1>. Acesso em: 28 abr. 2017.

NAVIAUX, Robert K. et al. Antipurinergic therapy corrects the autism-like features in the poly(IC) mouse model. **PloS one**, [s. l.], v. 8, n. 3, p. e57380, 2013. Disponível em: <http://dx.plos.org/10.1371/journal.pone.0057380>. Acesso em: 28 abr. 2017.

NAZEER, Ahsan; GHAZIUDDIN, Mohammad. Autism Spectrum Disorders: Clinical Features and Diagnosis. **Pediatric Clinics of North America**, [s. l.], v. 59, n. 1, p. 19–25, 2012. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/22284790>. Acesso em: 19 abr. 2018.

NOVALES-LI, P. Suramin exerts in vivo cytokine modulatory properties on splenocytes from experimental allergic encephalomyelitis-induced SJL mice: implications for autoimmune disease therapy. **Immunopharmacology**, [s. l.], v. 35, n. 2, p. 155–62, 1996. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/8956979>. Acesso em: 28 abr. 2017.

NUMIS, A. L. et al. Identification of risk factors for autism spectrum disorders in tuberous sclerosis complex. **Neurology**, [s. l.], v. 76, n. 11, p. 981–987, 2011. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/21403110>. Acesso em: 26 abr. 2017.

NUTAKUL, Wasamon et al. Inhibitory effects of resveratrol and pterostilbene on human colon cancer cells: a side-by-side comparison. **Journal of agricultural and food chemistry**, [s. l.], v. 59, n. 20, p. 10964–70, 2011.

O'CONNOR, K. Auditory processing in autism spectrum disorder: A review. **Neuroscience & Biobehavioral Reviews**, [s. l.], v. 36, n. 2, p. 836–854, 2012. Disponível em: <https://www.sciencedirect.com/science/article/pii/S0149763411002065?via%3Dihub>. Acesso em: 12 mar. 2018.

OKAMURA, Katsutomo et al. The regulatory activity of microRNA* species has substantial influence on microRNA and 3' UTR evolution. **Nature structural & molecular biology**, [s. l.], v. 15, n. 4, p. 354–63, 2008. Disponível em: <http://www.nature.com/articles/nsmb.1409>. Acesso em: 19 abr. 2018.

OLSEN, J.; ZHU, J. L. RE: "ADVANCED PARENTAL AGE AND THE RISK OF AUTISM SPECTRUM DISORDER" **American Journal of Epidemiology**, [s. l.], v. 169, n. 11, p. 1406–1406, 2009. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/19395694>. Acesso em: 26 abr. 2017.

ONO, K.; NAKANE, H.; FUKUSHIMA, M. Differential inhibition of various deoxyribonucleic and ribonucleic acid polymerases by suramin. **European journal of biochemistry**, [s. l.], v. 172, n. 2, p. 349–53, 1988. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/2450743>. Acesso em: 12 mar. 2018.

PANGENI, Rudra et al. Resveratrol: review on therapeutic potential and recent advances in drug delivery. **Expert Opinion on Drug Delivery**, [s. l.], v. 11, n. 8, p. 1285–1298, 2014. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/24830814>. Acesso em: 19 abr. 2018.

PASTURAL, Elodie et al. Novel plasma phospholipid biomarkers of autism: mitochondrial dysfunction as a putative causative mechanism. **Prostaglandins, leukotrienes, and essential fatty acids**, [s. l.], v. 81, n. 4, p. 253–64, 2009.

Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0952327809001112>. Acesso em: 28 abr. 2017.

PATOWARY, Ashok et al. Next Generation Sequencing Mitochondrial DNA Analysis in Autism Spectrum Disorder. **Autism Research**, [s. l.], 2017. Disponível em: <http://doi.wiley.com/10.1002/aur.1792>. Acesso em: 28 abr. 2017.

PATTERSON, Paul H. Immune involvement in schizophrenia and autism: Etiology, pathology and animal models. **Behavioural Brain Research**, [s. l.], v. 204, n. 2, p. 313–321, 2009. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/19136031>. Acesso em: 26 abr. 2017.

PATTERSON, Paul H. Modeling Autistic Features in Animals. **Pediatric Research**, [s. l.], v. 69, n. 5 Part 2, p. 34R–40R, 2011. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/21289542>. Acesso em: 12 mar. 2018.

PELTIER, Heidi J.; LATHAM, Gary J. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. **RNA (New York, N.Y.)**, [s. l.], v. 14, n. 5, p. 844–52, 2008. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/18375788>. Acesso em: 9 out. 2016.

PETERSON, Troy S. et al. P2Y2 nucleotide receptor-mediated responses in brain cells. **Molecular neurobiology**, [s. l.], v. 41, n. 2–3, p. 356–66, 2010. Disponível em: <http://link.springer.com/10.1007/s12035-010-8115-7>. Acesso em: 28 abr. 2017.

PHILIPPE JEANDET, *,† et al. Phytoalexins from the Vitaceae: Biosynthesis, Phytoalexin Gene Expression in Transgenic Plants, Antifungal Activity, and Metabolism. [s. l.], 2002.

PICARD, Martin et al. Progressive increase in mtDNA 3243A>G heteroplasmy causes abrupt transcriptional reprogramming. **Proceedings of the National Academy of Sciences of the United States of America**, [s. l.], v. 111, n. 38, p. E4033-42, 2014. Disponível em: <http://www.pnas.org/cgi/doi/10.1073/pnas.1414028111>. Acesso em: 26 abr. 2017.

PUCHAŁOWICZ, Kamila et al. Purinergic signaling and the functioning of the nervous system cells. **Cellular and Molecular Biology Letters**, [s. l.], v. 20, n. 5, p. 867–918, 2015. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/26618572>. Acesso em: 28 abr. 2017.

PUTS, Nicolaas A. J. et al. Impaired tactile processing in children with autism spectrum disorder. **Journal of neurophysiology**, [s. l.], v. 111, n. 9, p. 1803–11, 2014. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/24523518>. Acesso em: 12 mar. 2018.

QUINCOZES-SANTOS, André; GOTTFRIED, Carmem. Resveratrol modulates astroglial functions: neuroprotective hypothesis. **Annals of the New York Academy of Sciences**, [s. l.], v. 1215, n. 1, p. 72–78, 2011.

RALEVIC, V.; BURNSTOCK, G. Receptors for purines and pyrimidines. **Pharmacological reviews**, [s. l.], v. 50, n. 3, p. 413–92, 1998. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/9755289>. Acesso em: 28 abr. 2017.

RALEVIC, Vera; DUNN, William R. Purinergic transmission in blood vessels. **Autonomic Neuroscience**, [s. l.], v. 191, p. 48–66, 2015. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/26004513>. Acesso em: 28 abr. 2017.

RAPIN, I.; TUCHMAN, R. F. What is new in autism? **Curr Opin Neurol**, [s. l.], v. 21, n. 2, p. 143–149, 2008.

RASEROKA, B. H.; ORMEROD, W. E. The trypanocidal effect of drugs in different parts of the brain. **Transactions of the Royal Society of Tropical Medicine and Hygiene**, [s. l.], v. 80, n. 4, p. 634–41, 1986. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/3810797>. Acesso em: 13 mar. 2018.

RATAJCZAK, Helen V. Theoretical aspects of autism: biomarkers--a review. **Journal of immunotoxicology**, [s. l.], v. 8, n. 1, p. 80–94, 2011.

ROBOZ, John et al. Investigation of suramin-albumin binding by electrospray mass spectrometry. **Rapid Communications in Mass Spectrometry**, [s. l.], v. 12, n. 19, p. 1319–1322, 1998. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/9773522>. Acesso em: 13 mar. 2018.

RODIER, P. M. et al. Linking etiologies in humans and animal models: studies of autism. **Reproductive toxicology (Elmsford, N.Y.)**, [s. l.], v. 11, n. 2–3, p. 417–22, [s.d.]. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/9100317>. Acesso em: 26 abr. 2017.

ROILIDES, E. et al. Suppression of polymorphonuclear leukocyte bactericidal activity by suramin. **Antimicrobial agents and chemotherapy**, [s. l.], v. 37, n. 3, p. 495–500, 1993. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/7681657>. Acesso em: 4 maio. 2017.

RONALD, Angelica; HOEKSTRA, Rosa A. Autism spectrum disorders and autistic traits: a decade of new twin studies. **American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics**, [s. l.], v. 156B, n. 3, p. 255–74, 2011. Disponível em: <http://doi.wiley.com/10.1002/ajmg.b.31159>. Acesso em: 19 abr. 2018.

ROSE, Destanie; ASHWOOD, Paul. Potential cytokine biomarkers in autism spectrum disorders. **Biomarkers in medicine**, [s. l.], v. 8, n. 9, p. 1171–1181, 2014.

ROSSI, Lucas E. et al. Histone deacetylase inhibitors impair NK cell viability and effector functions through inhibition of activation and receptor expression. **Journal of leukocyte biology**, [s. l.], v. 91, n. 2, p. 321–31, 2012.

ROULLET, F. I. et al. Behavioral and molecular changes in the mouse in response to prenatal exposure to the anti-epileptic drug valproic acid. **Neuroscience**, [s. l.], v. 170, n. 2, p. 514–22, 2010. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0306452210009413>. Acesso em: 26 abr. 2017.

ROULLET, F. I.; LAI, J. K.; FOSTER, J. A. In utero exposure to valproic acid and autism--a current review of clinical and animal studies. **Neurotoxicol Teratol**, [s. l.], v. 36, p. 47–56, 2013.

RUBIO, M. E.; SOTO, F. Distinct Localization of P2X receptors at excitatory postsynaptic specializations. **The Journal of neuroscience : the official journal of the Society for Neuroscience**, [s. l.], v. 21, n. 2, p. 641–53, 2001.

Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/11160443>. Acesso em: 28 abr. 2017.

SÁNCHEZ-FIDALGO, Susana et al. Dietary supplementation of resveratrol attenuates chronic colonic inflammation in mice. **European Journal of Pharmacology**, [s. l.], v. 633, n. 1–3, p. 78–84, 2010.

SANDERSON, Lisa; KHAN, Adil; THOMAS, Sarah. Distribution of suramin, an antitrypanosomal drug, across the blood-brain and blood-cerebrospinal fluid interfaces in wild-type and P-glycoprotein transporter-deficient mice. **Antimicrobial agents and chemotherapy**, [s. l.], v. 51, n. 9, p. 3136–46, 2007. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/17576845>. Acesso em: 13 mar. 2018.

SAUGSTAD, Julie A. MicroRNAs as effectors of brain function with roles in ischemia and injury, neuroprotection, and neurodegeneration. **Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism**, [s. l.], v. 30, n. 9, p. 1564–76, 2010. Disponível em: <http://journals.sagepub.com/doi/10.1038/jcbfm.2010.101>. Acesso em: 19 abr. 2018.

SCHNEIDER, Miriam; KOCH, Michael. Deficient Social and Play Behavior in Juvenile and Adult Rats after Neonatal Cortical Lesion: Effects of Chronic Pubertal Cannabinoid Treatment. **Neuropsychopharmacology**, [s. l.], v. 30, n. 5, p. 944–957, 2005. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/15592349>. Acesso em: 5 dez. 2017.

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

SCHNEIDER, Tomasz et al. Prenatal exposure to valproic acid disturbs the enkephalinergic system functioning, basal hedonic tone, and emotional responses in an animal model of autism. **Psychopharmacology**, [s. l.], v. 193, n. 4, p. 547–55, 2007. Disponível em: <http://link.springer.com/10.1007/s00213-007-0795-y>. Acesso em: 26 abr. 2017.

SCHNEIDER, Tomasz et al. Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. **Psychoneuroendocrinology**, [s. l.], v. 33, n. 6, p. 728–40, 2008. b. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0306453008000619>. Acesso em: 26 abr. 2017.

SCHNEIDER, Tomasz; PRZEWŁOCKI, Ryszard. Behavioral Alterations in Rats Prenatally Exposed to Valproic Acid: Animal Model of Autism. **Neuropsychopharmacology**, [s. l.], v. 30, n. 1, p. 80–89, 2005. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/15238991>. Acesso em: 26 abr. 2017.

SCHOUTEN, Alexander et al. Resveratrol acts as a natural profungicide and induces self-intoxication by a specific laccase. **Molecular Microbiology**, [s. l.], v. 43, n. 4, p. 883–894, 2002.

SCHRATT, Gerhard M. et al. A brain-specific microRNA regulates dendritic spine development. **Nature**, [s. l.], v. 439, n. 7074, p. 283–289, 2006.

Disponível em: <http://www.nature.com/doifinder/10.1038/nature04367>. Acesso em: 10 nov. 2017.

SIEGEL, Gabriele et al. A functional screen implicates microRNA-138-dependent regulation of the depalmitoylation enzyme APT1 in dendritic spine morphogenesis. **Nature cell biology**, [s. l.], v. 11, n. 6, p. 705–16, 2009. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/19465924>. Acesso em: 9 out. 2016.

SILVER, Wendy G.; RAPIN, Isabelle. Neurobiological basis of autism. **Pediatric clinics of North America**, [s. l.], v. 59, n. 1, p. 45–61, x, 2012. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0031395511001453>. Acesso em: 19 abr. 2018.

SIMONOFF, Emily et al. Psychiatric disorders in children with autism spectrum disorders: prevalence, comorbidity, and associated factors in a population-derived sample. **Journal of the American Academy of Child and Adolescent Psychiatry**, [s. l.], v. 47, n. 8, p. 921–9, 2008. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0890856708600593>. Acesso em: 12 mar. 2018.

SMITH, Stephen E. P. et al. Maternal immune activation alters fetal brain development through interleukin-6. **The Journal of neuroscience : the official journal of the Society for Neuroscience**, [s. l.], v. 27, n. 40, p. 10695–702, 2007. Disponível em: <http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.2178-07.2007>. Acesso em: 26 abr. 2017.

SMITH, Victoria; BROWN, Nick. Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. **Archives of disease in childhood - Education & practice edition**, [s. l.], v. 99, n. 5, p. 198–198, 2014. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/24692263>. Acesso em: 26 abr. 2017.

STRAZIELLE, C. et al. Abnormal grooming activity in Dab1(scm) (scrambler) mutant mice. **Behavioural brain research**, [s. l.], v. 233, n. 1, p. 24–8, 2012. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0166432812002999>. Acesso em: 12 mar. 2018.

SWEETEN, Thayne L. et al. Increased prevalence of familial autoimmunity in probands with pervasive developmental disorders. **Pediatrics**, [s. l.], v. 112, n. 5, p. e420, 2003.

TAI, Hwan-Ching; SCHUMAN, Erin M. MicroRNA: microRNAs reach out into dendrites. **Current biology : CB**, [s. l.], v. 16, n. 4, p. R121-3, 2006. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S096098220601116X>. Acesso em: 19 abr. 2018.

TALEBIZADEH, Zohreh; BUTLER, Merlin G.; THEODORO, Mariana F. Feasibility and relevance of examining lymphoblastoid cell lines to study role of microRNAs in autism. **Autism Research: Official Journal of the International Society for Autism Research**, [s. l.], v. 1, n. 4, p. 240–250, 2008.

TANG, Bor Luen. Resveratrol is neuroprotective because it is not a direct activator of Sirt1—A hypothesis. **Brain Research Bulletin**, [s. l.], v. 81, n. 4–5, p. 359–361, 2010.

THEOHARIDES, Theoharis C.; ASADI, Shahrzad; PATEL, Arti B. Focal brain inflammation and autism. **Journal of Neuroinflammation**, [s. l.], v. 10, n. 1, p. 815, 2013. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/23570274>. Acesso em: 28 abr. 2017.

TOMCHEK, Scott D.; DUNN, Winnie. Sensory processing in children with and without autism: a comparative study using the short sensory profile. **American Journal of Occupational Therapy**, [s. l.], v. 61, n. 2, p. 190–200, 2007. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/17436841>. Acesso em: 19 abr. 2018.

TOMÉ-CARNEIRO, Joao et al. Resveratrol and clinical trials: the crossroad from in vitro studies to human evidence. **Current pharmaceutical design**, [s. l.], v. 19, n. 34, p. 6064–93, 2013. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/23448440>. Acesso em: 19 abr. 2018.

TSAO, Chang-Yong; MENDELL, Jerry R. Autistic Disorder in 2 Children With Mitochondrial Disorders. **Journal of Child Neurology**, [s. l.], v. 22, n. 9, p. 1121–1123, 2007. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/17890412>. Acesso em: 26 abr. 2017.

TSUJINO, Naohisa et al. Abnormality of circadian rhythm accompanied by an increase in frontal cortex serotonin in animal model of autism. **Neuroscience research**, [s. l.], v. 57, n. 2, p. 289–95, 2007. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0168010206002914>. Acesso em: 26 abr. 2017.

TUCHMAN, R.; MOSHE, S. L.; RAPIN, I. Convulsing toward the pathophysiology of autism. **Brain Dev**, [s. l.], v. 31, n. 2, p. 95–103, 2009.

TURK, Jeremy; GRAHAM, Philip. Fragile X Syndrome, Autism and Autistic Features. **Autism**, [s. l.], v. 1, n. 2, p. 175–197, 1997.

TYZIO, R. et al. Oxytocin-Mediated GABA Inhibition During Delivery Attenuates Autism Pathogenesis in Rodent Offspring. **Science**, [s. l.], v. 343, n. 6171, p. 675–679, 2014. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/24503856>. Acesso em: 4 dez. 2017.

VAN STEENSEL, Francisca J. A.; BÖGELS, Susan M.; PERRIN, Sean. Anxiety Disorders in Children and Adolescents with Autistic Spectrum Disorders: A Meta-Analysis. **Clinical Child and Family Psychology Review**, [s. l.], v. 14, n. 3, p. 302–317, 2011. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/21735077>. Acesso em: 12 mar. 2018.

VANG, Ole et al. What Is New for an Old Molecule? Systematic Review and Recommendations on the Use of Resveratrol. **PLoS ONE**, [s. l.], v. 6, n. 6, p. e19881, 2011.

VON KÜGELGEN, Ivar; HOFFMANN, Kristina. Pharmacology and structure of P2Y receptors. **Neuropharmacology**, [s. l.], v. 104, p. 50–61, 2016. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/26519900>. Acesso em: 28 abr. 2017.

WANG, Li et al. Altered nocifensive behavior in animal models of autism spectrum disorder: The role of the nicotinic cholinergic system. **Neuropharmacology**, [s. l.], v. 111, p. 323–334, 2016. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/27638450>. Acesso em: 28 abr. 2017.

WAYMAN, Gary A. et al. An activity-regulated microRNA controls dendritic plasticity by down-regulating p250GAP. **Proceedings of the National Academy of Sciences**, [s. l.], v. 105, n. 26, p. 9093–9098, 2008. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/18577589>. Acesso em: 12 nov. 2017.

WEI, Hongen et al. Brain IL-6 elevation causes neuronal circuitry imbalances and mediates autism-like behaviors. **Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease**, [s. l.], v. 1822, n. 6, p. 831–842, 2012. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/22326556>. Acesso em: 13 mar. 2018.

WEISMAN, Gary A. et al. Neuroprotective roles of the P2Y2 receptor. **Purinergic Signalling**, [s. l.], v. 8, n. 3, p. 559–578, 2012. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/22528682>. Acesso em: 28 abr. 2017.

WILLIAMS, G. et al. Fetal valproate syndrome and autism: additional evidence of an association. **Developmental medicine and child neurology**, [s. l.], v. 43, n. 3, p. 202–6, 2001. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/11263692>. Acesso em: 13 nov. 2017.

WILLIAMS, P. G.; HERSH, J. H. A male with fetal valproate syndrome and autism. **Developmental medicine and child neurology**, [s. l.], v. 39, n. 9, p. 632–4, 1997. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/9344057>. Acesso em: 13 nov. 2017.

WILLS, E. D.; WORMALL, A. Studies on suramin; the action of the drug on some enzymes. **The Biochemical journal**, [s. l.], v. 47, n. 2, p. 158–70, 1950. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/14791338>. Acesso em: 12 mar. 2018.

WOOD, Jason G. et al. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. **Nature**, [s. l.], v. 430, n. 7000, p. 686–9, 2004. Disponível em: <http://www.nature.com/articles/nature02789>. Acesso em: 19 abr. 2018.

XU, Ningan; LI, Xiaohong; ZHONG, Yan. Inflammatory cytokines: potential biomarkers of immunologic dysfunction in autism spectrum disorders. **Mediators of inflammation**, [s. l.], v. 2015, p. 531518, 2015. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/25729218>. Acesso em: 13 mar. 2018.

YANG, Eun-Jeong et al. Early Behavioral Abnormalities and Perinatal Alterations of PTEN/AKT Pathway in Valproic Acid Autism Model Mice. **PLOS ONE**, [s. l.], v. 11, n. 4, p. e0153298, 2016. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/27071011>. Acesso em: 28 abr. 2017.

YANG, Weidong et al. Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. **Nature structural & molecular biology**, [s. l.], v. 13, n. 1, p. 13–21, 2006. Disponível em: <http://www.nature.com/articles/nsmb1041>. Acesso em: 19 abr. 2018.

YAO, Liangqi et al. Resveratrol relieves gestational diabetes mellitus in mice through activating AMPK. **Reproductive biology and endocrinology : RB&E**, [s. l.], v. 13, p. 118, 2015.

YI, Rui et al. Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. **RNA (New York, N.Y.)**, [s. l.], v. 11, n. 2, p. 220–6, 2005. Disponível em:

<http://www.rnajournal.org/cgi/doi/10.1261/rna.7233305>. Acesso em: 19 abr. 2018.

YOCHUM, Carrie L. et al. VPA-induced apoptosis and behavioral deficits in neonatal mice. **Brain research**, [s. l.], v. 1203, p. 126–32, 2008. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/S0006899308000966>. Acesso em: 26 abr. 2017.

YONK, L. J. et al. CD4+ helper T cell depression in autism. **Immunology letters**, [s. l.], v. 25, n. 4, p. 341–5, 1990.

ZEMKOVA, Hana et al. Characterization of purinergic P2X4 receptor channels expressed in anterior pituitary cells. **American journal of physiology. Endocrinology and metabolism**, [s. l.], v. 298, n. 3, p. E644-51, 2010. Disponível em: <http://ajpendo.physiology.org/cgi/doi/10.1152/ajpendo.00558.2009>. Acesso em: 28 abr. 2017.

ZHANG, Bodi et al. Stimulated human mast cells secrete mitochondrial components that have autocrine and paracrine inflammatory actions. **PloS one**, [s. l.], v. 7, n. 12, p. e49767, 2012. Disponível em: <http://dx.plos.org/10.1371/journal.pone.0049767>. Acesso em: 28 abr. 2017.

ANEXOS

ANEXO 1 - Carta de aprovação da Comissão de Ética no Uso de Animais (CEUA) Projeto 14-0679

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

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

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

Projeto: 140679
Data da Versão do Projeto: 10/12/2014

Pesquisadores:
RUDIMAR DOS SANTOS RIESGO
MELLANIE FONTES DUTRA DA SILVA
GUSTAVO DELLA FLORA NUNES
CARMEM GOTTFRIED

Título: Modelo animal de autismo por exposição pré-natal ao ácido valpróico: análise de um conjunto de microRNA e sua influência sobre a patofisiologia do autismo

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

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

Porto Alegre, 09 de janeiro de 2015.

Biol. Michael Everton Andrades
Coordenador CEUA/HCPA

ANEXO 2 –Carta de aprovação da Comissão de Ética no Uso de Animais (CEUA) Projeto 14-0367

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

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

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

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

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

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

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

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

Porto Alegre, 07 de outubro de 2014.

Profª Iraci Lucena da Silva Torres
Coordenadora CEUA/HCPA

ANEXO 3 –Carta de aprovação da Comissão de Ética no Uso de Animais (CEUA) Projeto 26384

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 26384
Título: Análise de microRNA em modelo animal de autismo e em pacientes com autismo clássico

Pesquisadores:

Equipe UFRGS:

CARMEM JURACY SILVEIRA GOTTFRIED - coordenador desde 03/03/2013
Gustavo Endres Cuccarolo - Aluno de Mestrado desde 03/03/2013

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 13/10/2014 - Sala I do Gabinete do Reitor - Prédio da Reitoria - Campus do Centro - Porto Alegre, em seus aspectos éticos e metodológicos, para a utilização de 33 ratos Wistar machos, de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Segunda-Feira, 10 de Novembro de 2014

STELA MARIS KUZE RATES
Coordenador da comissão de ética

ANEXO 4 –Carta de aprovação da Comissão de Ética no Uso de Animais (CEUA) Projeto 23884

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

PROPESQ

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 23884
Título: MODELO DE AUTISMO INDUZIDO POR ACIDO VALPROICO EM RATOS WISTAR: ESTUDO DA ETIOLOGIA, TRATAMENTO E MARCADORES CLINICOS

Pesquisadores:

Equipe UFRGS:

CARMEM JURACY SILVEIRA GOTTFRIED - coordenador desde 22/09/2012
RUDIMAR DOS SANTOS RIESGO - pesquisador desde 22/09/2012
Victorio Bambini Junior - Aluno de Doutorado desde 22/09/2012

Comissão De Ética No Uso De Animais aprovou o mesmo em seus aspectos éticos e metolodológicos, para a utilização de 80 ratos Wistar (fêmeas e machos), de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Sexta-Feira, 28 de Junho de 2013

STELA MARIS KUZE RATES
Coordenador da comissão de ética

ANEXO 5 - Artigo publicado em colaboração durante o período de doutorado

NEUROSCIENCE

RESEARCH ARTICLE

M. M. Hirsch et al. / Neuroscience 379 (2018) 405–414

Sex Differences and Estrous Cycle Changes in Synaptic Plasticity-related microRNA in the Rat Medial Amygdala

Mauro Mozael Hirsch,[a,b] Janaína Brusco,[c,d] Tamara Vaccaro,[a] Rogerio Margis,[b,e] Jorge E. Moreira,[c,d] Carmem Gottfried[a,b*] and Alberto A. Rasia-Filho[f*]

[a] *Research Group in Neuroglial Plasticity, Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS 90035-003, Brazil*

[b] *National Institute of Science and Technology on Neuroimmunomodulation, INCT-NIM/CNPq. Oswaldo Cruz Foundation, Rio de Janeiro, Brazil*

[c] *Department of Pathology and Forensic Medicine, Ribeirão Preto School of Medicine, University of São Paulo, Ribeirão Preto, SP 14049-900, Brazil*

[d] *Department of Neurology, Neuroscience and Behavior, Ribeirão Preto School of Medicine, University of São Paulo, Ribeirão Preto, SP 14049-900, Brazil*

[e] *Center of Biotechnology and PPGBCM, Laboratory of Genomes and Plant Populations, Federal University of Rio Grande do Sul – UFRGS, Porto Alegre, RS, Brazil*

[f] *Department of Basic Sciences/Physiology, Federal University of Health Sciences, Porto Alegre, RS 90050-170, Brazil*

Abstract—The posterodorsal medial amygdala (MePD) is a sex steroid-sensitive and sexually dimorphic subcortical area that dynamically modulates social behaviors in rats. As different microRNA (miRNA) can act as post-transcriptional regulators of synaptic processing, we addressed changes that occur in miRNA expression in the MePD of males and females along the estrous cycle. The expression of miR25-3p, miR132-3p, miR138-5p, miR181a-5p, miR195-5p, and miR199a-5p, involved in neuronal cytoskeleton remodeling and synaptic plasticity, were evaluated by RT-qPCR. We found that the expression of miR138-5p was higher in males than in females along the different phases of the estrous cycle. Males also showed higher levels of miR-181a when compared to females in diestrus and estrus. On the other hand, when compared to females in proestrus, males presented lower levels of miR132-3p and miR199a-5p. The expression of miR25-3p was higher in diestrus females than in proestrus females. In addition, diestrus females showed higher values of miR25-3p, miR181a-5p, and miR195-5p when compared to estrus females. These miRNA expression profiles indicate a variable and fine-tuned protein regulation in the adult MePD. It is likely that these miRNA can be involved in structural and functional synaptic features and plasticity characteristic of males and cycling females and for the MePD regulation of mammalian reproduction.

Key words: extended amygdala, neural plasticity, sexual dimorphism, estrous cycle, noncoding RNA.