UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM SAÚDE DA CRIANÇA E DO ADOLESCENTE

AVALIAÇÃO DO SISTEMA HISTAMINÉRGICO NO MODELO ANIMAL DE AUTISMO INDUZIDO POR EXPOSIÇÃO PRÉ-NATAL AO ÁCIDO VALPRÓICO

TESE DE DOUTORADO

DIEGO MOURA BARONIO

Porto Alegre, Brasil

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“Acima de tudo, não tema momentos difíceis.
O melhor surge deles. ”

Rita Levi-Montalcini

RESUMO

Introdução: O Transtorno do Espectro do Autismo (TEA) é caracterizado por prejuízos na interação social e comunicação, e por comportamentos repetitivos e repertório restrito de interesses. Alterações em diferentes sistemas de neurotransmissores, como o serotoninérgico, GABAérgico e glutamatérgico já foram estudadas e relatadas no TEA. Surpreendentemente, o sistema histaminérgico recebeu pouca atenção e poucos estudos sobre histamina (HA) e TEA estão disponíveis na literatura. Além disso, ligantes do receptor histaminérgico 3 (H3R) são considerados agentes terapêuticos promissores para o tratamento de diferentes desordens neurológicas e prejuízos cognitivos, mas seus efeitos em características do tipo autista ainda não foram determinados.

Objetivos: Avaliar o efeito do ciproxifan (CPX), um agonista inverso do H3R, sobre o modelo animal de autismo induzido por exposição pré-natal ao ácido valpróico (VPA, do inglês: *valproic acid*) e medir a concentração de HA e os níveis do mRNA de H3R e H4R em diferentes estruturas encefálicas desse modelo. Além disso, investigar o papel do H3R no comportamento social e estereotipado.

Métodos: Camundongos *Swiss* sofreram exposição pré-natal ao VPA no dia embrionário 11 e, aos 50 dias de vida pós-natal foram submetidos à avaliação de comportamento social, limiar de nocicepção e comportamento repetitivo, com ou sem tratamento com CPX. Após as avaliações comportamentais, os animais foram eutanasiados por exsanguinação depois de terem sido anestesiados com isoflurano. Córtex cerebral, estriado e hipocampo foram removidos, congelados em nitrogênio líquido e armazenados a -80°C para quantificação de mRNA do H3R e H4R por qPCR e de conteúdo de HA por cromatografia líquida de alta eficiência (HPLC, do inglês: *High Performance Liquide Chromatography*). Camundongos H3R KO e WT C57BL/6J, aos 50 dias de vida, foram

usados nos testes com o aparato de três câmaras e *marble burying* para determinação do papel do H3R na sociabilidade e comportamento estereotipado.

Resultados: O grupo VPA apresentou menor índice se sociabilidade quando comparado aos animais do grupo Controle, e o tratamento com CPX foi capaz de reverter completamente este efeito. Entretanto, o aumento no limiar de nocicepção observado no grupo VPA não foi revertido pelo tratamento. No teste *marble burying*, o grupo VPA enterrou um maior número de bolinhas de gude em relação ao grupo Controle, indicando comportamento repetitivo, um efeito também prevenido pelo CPX. Além disso, a análise molecular entre os grupos VPA e Controle mostrou um aumento significativo nos níveis de mRNA do H3R, bem como uma maior concentração de HA no estriado de animais VPA. O H3R pode ter um papel no interesse por novidade social, característica prejudicada no TEA, já que camundongos H3R KO demonstraram comportamento anormal quando testados para este parâmetro no teste com aparato de três câmaras.

Conclusões: Uma dose aguda de CPX foi capaz de reverter prejuízo na sociabilidade, bem como comportamento repetitivo apresentados pelo modelo VPA de autismo. Além disso, este é o primeiro relato sobre anormalidades no sistema histaminérgico nesse modelo. Estes achados contribuem para investigações futuras sobre a fisiopatologia do TEA, bem como apontam para um possível novo tratamento para características do tipo autista. Mais estudos são necessários para corroborar e expandir esses dados iniciais.

Palavras-chave: Transtorno do Espectro do Autismo, Histamina, Ácido Valpróico, Ciproxifan

ABSTRACT

Introduction: Autism spectrum disorder (ASD) is primarily characterized by impaired social interaction and communication, and by restricted repetitive behaviors and interests. Alterations in different neurotransmitter systems, such as serotonergic, GABAergic and glutamatergic have been studied and reported in ASD. Surprisingly, the histaminergic system has received less attention and few studies are available on the literature about histamine and ASD. In addition, ligands of histamine receptor 3 (H3R) are considered potential therapeutic agents for the treatment of different brain disorders and cognitive impairments, but its effects on autistic-like features have not been evaluated.

Objectives: The objective of this study was to evaluate the actions of ciproxifan (CPX), an H3R inverse agonist, on the animal model of autism induced by prenatal exposure to valproic acid (VPA) and to measure the concentration of histamine and the H3R and H4R mRNA levels in different brain structures of this model. In addition, to investigate the role of H3R in sociability and stereotypic behavior.

Methods: Swiss mice were prenatally exposed to VPA on embryonic day 11 and assessed for social behavior, nociceptive threshold and repetitive behavior at 50 days of life after treatment with CPX. After the behavioral tests, animals were killed by exsanguination after being anesthetized with isoflurane. Cerebral cortex, striatum and hippocampus were removed, frozen in liquid nitrogen and stored at -80°C for quantification of H3R and H4R mRNA by qPCR and histamine content by HPLC analysis. H3R KO and WT C57BL/6J mice, at 50 days of life, were used in the three-chamber test and marble burying test to determine the role of H3R in sociability stereotypic behavior.

Results: The VPA group presented lower sociability index compared to VPA animals that were treated with CPX. Compared to the Control group, VPA animals presented a significantly higher nociceptive threshold, and treatment with CPX was not able to

modify this parameter. In the marble burying test, the number of marbles buried by VPA animals was consistent with markedly repetitive behavior. VPA animals that received CPX buried a reduced amount of marbles. A significant increase in H3R mRNA, as well as higher concentration of histamine were detected in striatal samples from the VPA mice. H3R might influence the search for social novelty, a feature that is impaired in ASD, as H3R KO mice displayed abnormal behavior when tested for this parameter in the three chambers test.

Conclusions: In summary, an acute dose of CPX is able to revert sociability deficits and stereotypies present in the VPA model of autism. In addition, this is the first report of abnormalities in the histaminergic system of this model. These findings have the potential to help the investigations of both the molecular underpinnings of ASD and of possible treatments to ameliorate the ASD symptomatology, although more research is still necessary to corroborate and expand this initial data.

Keywords: Autism Spectrum Disorder, Histamine, Valproic Acid, Ciproxifan

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Ach: Acetilcolina

AMPA: alfa-amino-3-hidroxi-metil-5-4-isoxazolpropinóico

cAMP: Monofosfato de adenosina cíclico

BHE: Barreira Hematoencefálica

CPX: Ciproxifan

DAG: Descarboxilase do ácido glutâmico

DSM-V: Manual Diagnóstico e Estatístico de Transtornos Mentais, do inglês: *Diagnostic and Statistical Manual of Mental Disorders*

GABA: Ácido gama-aminobutírico

GPCR: Receptores acoplados à proteína G

H1R: Receptor histaminérgico H1

H2R: Receptor histaminérgico H2

H3R: Receptor histaminérgico H3

H4R: Receptor histaminérgico H4

HA: Histamina

HDC: Histidina descarboxilase

HNMT: histamina-N-metiltransferase

HPLC: cromatografia líquida de alta eficiência, do inglês: *High Performance Liquide Chromatography*

IV: Intravenosa

LCR: Líquido cefalorraquidiano

MAO-B: monoaminoxidase B

MAPK: proteína-cinases ativadas por mitógenos

mGlur: Receptor metabotrópico de glutamato

NMDA: N-metil-d-aspartato

NTM: Núcleo Tuberomamilar

PKA: Proteína cinase A

PLA2: fosfolipase A2

REM: movimento rápido dos olhos, do inglês: *rapid eye movement*

mRNA: Ácido ribonucléico mensageiro, do inglês: *Messenger RiboNucleic Acid*

SFV: Síndrome Fetal do Valproato

SNC: Sistema Nervoso Central

SNP: Sistema Nervoso Periférico

TEA: Transtorno do Espectro do Autismo

t-MHA: tele-metil-histamina

t-mIAA: ácido tele-metil-imidazolacético

VMAT: Transportador vesicular de monoaminas

VPA: Ácido valpróico, do inglês: *valproic acid*

WT: Wild Type

SUMÁRIO

1. INTRODUÇÃO

O termo "autista" foi cunhado pelo psiquiatra suíço Eugen Bleuler em 1911 para descrever características de indivíduos com esquizofrenia (Bleuler, 1911). O termo foi novamente utilizado em 1943 por Leo Kanner em seu artigo "*Autistic disturbances of affective contact*", no qual ele descreve 11 crianças com dificuldades de comunicação e não sociáveis (Kanner, 1943). No mesmo ano, Hans Asperger submete sua tese de doutorado intitulada *"Die 'Autistischen psychopathen' im kindesalter"* [*Autistic psychopathy in childhood*] e posteriormente publicada em 1944 (Asperger, 1944).

Em sua tese, Asperger afirma ter adotado o termo criado por Bleuler enquanto no artigo de Kanner não se encontram referências a esse respeito. Na verdade, Asperger já havia usado o termo "autista" antes de ambas as publicações, em uma palestra no Hospital Universitário de Viena no dia 03 de outubro de 1938, na qual ele descreveu crianças "autistas psicopatas". O conteúdo da palestra foi publicado com o título de *"Das psychisch abnormale Kind"* [*The psychically abnormal child*] (Asperger, 1938).

De acordo com a 5ª edição do Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-V, do inglês: *Diagnostic and Statistical Manual of Mental Disorders*), os diferentes subgrupos de autismo estão englobados sob o termo Transtorno do Espectro do Autismo (TEA). Este transtorno é definido como um conjunto de desordens do desenvolvimento cujos pacientes apresentam: 1) prejuízos na capacidade de se comunicar e de se relacionar socialmente com outros indivíduos e 2) repertório restrito de atividades e comportamentos repetitivos (DSM-V, 2013). A inexistência de uma etiologia definida, de biomarcadores que possam auxiliar no diagnóstico e de tratamentos eficazes, faz com que o TEA seja um transtorno amplamente estudado.

Uma das ferramentas mais utilizadas na pesquisa do TEA é o modelo animal de autismo induzido por exposição pré-natal ao ácido valpróico (VPA, do inglês: *valproic*

acid) (Bambini-Junior et al., 2014a). Desenvolvido em 1996 (Rodier et al., 1996), este modelo foi baseado em informações epidemiológicas que apontavam para a alta incidência de TEA em crianças cujas mães fizeram uso de VPA, um fármaco para o tratamento de epilepsia e controle do humor, durante o período gestacional (Strömland et al., 1994).

O modelo reproduz alterações anatômicas, neuroquímicas e comportamentais equivalentes às encontradas em pacientes com TEA. Entre as alterações neuroquímicas, podemos citar anormalidades em sistemas de neurotransmissores (Pardo and Eberhart, 2007), como o GABAérgico (Pizzarelli and Cherubini, 2011), glutamatérgico (Carlson, 2012; Bristot Silvestrin et al., 2013) e serotoninérgico (Gabriele et al., 2014). Surpreendentemente, o estudo do sistema histaminérgico no TEA foi praticamente ignorado ao longo dos mais de 70 anos que se passaram após as primeiras descrições.

O sistema histaminérgico está envolvido na modulação de diversas funções fisiológicas importantes no sistema nervoso central (SNC), como o ciclo sono-vigília, termorregulação, memória e aprendizagem (Nuutinen and Panula, 2010). Alterações nesse sistema estão associadas à fisiopatologia de diversas desordens neurológicas, como a esquizofrenia (Prell et al., 1995) e a síndrome de Tourette (Fernandez et al., 2012), condições que compartilham alguns sintomas com o TEA. Além disso, existem relatos de alterações histaminérgicas na doença de Parkinson (Rinne et al., 2002), doença de Alzheimer (Kim et al., 2001) e narcolepsia (Kanbayashi et al., 2009). Sendo assim, os receptores de histamina (H1R, H2R, H3R e H4R), tornaram-se alvos terapêuticos promissores para o tratamento de alterações cognitivas, distúrbios do sono e neuroproteção (Tiligada et al., 2011).

O estudo do sistema histaminérgico no modelo VPA, bem como a investigação dos efeitos de um agonista inverso do H3R são os temas da presente tese de doutorado.

Os resultados aqui apresentados trazem uma importante contribuição para compreensão da fisiopatologia do TEA e abrem novas perspectivas para estudos subsequentes.

1. REVISÃO DA LITERATURA

2.1 Transtorno do Espectro do Autismo (TEA)

O Transtorno do Espectro de Autismo (TEA) é um conjunto de desordens do desenvolvimento, geralmente diagnosticado na infância e com sintomas que, na maioria dos casos, perduram ao longo da vida do paciente. As características principais do TEA incluem prejuízos na capacidade de se comunicar e de se relacionar socialmente com outros indivíduos. Os indivíduos com TEA também podem apresentar um repertório restrito de atividades e comportamentos repetitivos, como por exemplo, a organização obsessiva de objetos ou seguirem rotinas extremamente específicas (DSM-V, 2013).

Existem diversos instrumentos utilizados para diagnosticar o TEA, entre eles podemos citar: *Autism Diagnostic Interview Revised* - ADI-R (Entrevista diagnóstica para autismo revisada); *Autism Diagnostic Observation Schedule* – ADOS (Protocolo de observação para diagnóstico de autismo); *Childhood Autism Rating Scale-CARS* (Escala de avaliação para autismo infantil); *Autism Behavioral Checklist* – ABC (lista de checagem de comportamento autístico); e *Autism Spectrum Screening Questionnaire* – ASSQ (Questionário de triagem para autismo) (Rapin and Tuchman, 2008).

Atualmente o TEA afeta cerca de 1% da população mundial (Investigators and Prevention, 2012) e juntamente com suas morbidades representa elevado custo à saúde pública (Buescher et al., 2014). Estima-se que o gasto com um indivíduo com TEA e retardo mental ao longo de sua vida possa ser aproximadamente U$ 2,4 milhões pelos Estados Unidos e Reino Unido. Já as despesas ao longo da vida de um indivíduo com TEA e sem retardo mental podem atingir a marca de U$1,4 milhão nos Estados Unidos e

Reino Unido. Durante a infância os maiores custos estão em serviços de educação, além da perda de produtividade dos pais no mercado de trabalho.

A etiologia do TEA não é totalmente compreendida, mas sabe-se que fatores genéticos e ambientais corroboram para o desenvolvimento deste transtorno. Do ponto de vista genético, a literatura mostra que existe uma alta taxa (73-95%) de TEA em gêmeos monozigóticos, elevada herdabilidade (>90%) e risco de recorrência entre irmãos (Muhle et al., 2004; Persico and Bourgeron, 2006). Além disso o TEA encontra-se associado à diferentes síndromes genéticas, como a Esclerose Tuberosa, Síndrome do X Frágil, Síndrome de Marinesco-Sjogren e Síndrome de Moebius (Moss and Howlin, 2009).

Do ponto de vista ambiental, infecções maternas de oriegens bacterianas e virais durante a gestação são fatores de risco para desordens do neurodesenvolvimento, incluindo o TEA (Ornoy et al., 2015). Um estudo que comparou 183 pacientes com TEA e 209 controles, relatos feitos pelas mães possibilitaram a constatação de que a infecção viral materna, além de outros fatores ambientais, demonstra uma importante associação entre infecção e incidência de TEA (Wilkerson et al., 2002). Outro estudo demonstrou que infecções genito-urinárias também está associada com elevado risco de TEA. A maioria das mulheres tiveram essas infecções diagnosticadas no terceiro trimestre gestacional (Zerbo et al., 2013).

A exposição pré-natal à determinadas drogas também têm sido considerada como um fator de risco. Um estudo sueco demonstrou que a incidência de TEA é 50 vezes maior em indivíduos que foram expostos à talidomida no período pré-natal (Strömland et al., 1994). Conforme citado anteriormente, o uso do VPA durante a gestação tem sido considerado como um fator ambiental de risco para o desenvolvimento do TEA. Desta maneira, esta droga passa de um fator de risco para o desenvolvimento de um importante

modelo animal de autismo (Rodier et al., 1996; Schneider and Przewłocki, 2005; Bambini-Junior et al., 2014a).

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

O VPA é um ácido graxo de cadeia curta sintetizado pela primeira vez em 1882 por B.S Burton (Burton, 1882). Na década de 1960 foi descrito como tratamento para epilepsia e transtorno bipolar (Meunier et al., 1963; Lambert et al., 1966). Dependendo da severidade do quadro do paciente a dose diária de VPA pode variar entre 200 mg e 3000 mg (Perucca, 2002). Em ratos, após administração intravenosa (IV), o VPA cruza rapidamente a barreira hematoencefálica (BHE) atingindo sua concentração máxima após 10 – 15 minutos (Lücke et al., 1994).

O VPA é um inibidor não seletivo das enzimas histona desacetilases (HDAC). As HDAC de classe I, que incluem HDAC1 e HDAC2, são expressas no SNC e o VPA inibe suas atividades através de diferentes mecanismos. A inibição da HDAC1 ocorre através da interação entre o VPA e o sítio catalítico da enzima (Göttlicher et al., 2001), enquanto que a inibição da HDAC2 através da indução de degradação proteossomal (Krämer et al., 2003). A inibição de HDAC1 e HDAC2 pode levar a um aumento no número de sinapses e facilitação da maturação de sinapses excitatórias (Akhtar et al., 2009).

Exposição ao VPA durante o primeiro trimestre gestacional tem sido associada com diversas malformações congênitas, como espinha bífida, defeito no septo atrial, fenda palatina e polidactilia, que constituem a síndrome fetal do valproato (SFV) (DiLiberti et al., 1984). Após relatos de casos de pacientes com SFV que demonstravam características do tipo autista, a exposição pré-natal ao VPA passou a ser considerada fator de risco para o desenvolvimento de TEA. O período de exposição ao VPA que está mais associado ao desenvolvimento de TEA em humanos permeia o período da

neurulação, entre o 18 (invaginação da placa neural) e os 28-30 dias de gestação (fechamento dos neuroporos) (Rice and Barone Jr, 2000). Esses achados levaram ao desenvolvimento do modelo animal de autismo baseado na exposição pré-natal a este teratógeno (Rodier et al., 1996). Além de facilitar o estudo da fisiopatologia deste transtorno, esta ferramenta possibilita a investigação de novas estratégias para o tratamento de sintomas do TEA. Várias intervenções terapêuticas já foram propostas e testadas neste modelo visando atenuar e, até mesmo, prevenir prejuízos comportamentais, neuroquímicos ou anatômicos (Sandhya et al., 2012; Kim et al., 2013; Bambini-Junior et al., 2014b).

O desenvolvimento do modelo ocorreu em 1996 (Rodier et al., 1996). Fêmeas prenhes receberam 350 mg/kg de VPA nos dias gestacionais 11,5, 12 ou 12,5. A exposição ao VPA reduziu o número de neurônios motores, sendo que as alterações mais exacerbadas ocorreram nos animais induzidos no dia 12,5. Nesse primeiro estudo o comportamento dos animais não foi avaliado, mas os autores salientaram que a severidade das alterações morfológicas provavelmente levaria às alterações comportamentais.

O primeiro estudo que avaliou o comportamento de roedores expostos ao VPA durante a gestação foi realizado apenas em 2005 (Schneider and Przewłocki, 2005). Os autores descreveram diversas alterações comportamentais exibidas pelos ratos, como estereotipias e comportamentos exploratórios e sociais reduzidos. Posteriormente, Schneider mostrou que as alterações no modelo VPA são gênero-especificas (Schneider et al., 2008), o que vai ao encontro da desproporção existente entre o número de meninos e meninas diagnosticados com TEA (5:1) (Investigators and Prevention, 2012). A **Tabela 1** apresenta diferenças metodológicas em estudos que fizeram uso do modelo VPA.

Além das alterações morfológicas e comportamentais similares às encontradas em pacientes com TEA, os roedores do modelo VPA também apresentam diversas alterações

neuroquímicas. Isso facilita a investigação sobre o papel das alterações em diferentes sistemas de neurotransmissores na fisiopatologia do TEA.

Tabela 1- Diferentes métodos empregados no modelo VPA

Roedor	Dia da indução	Dose VPA (mg/kg)	Via	Referência
Camundongo	9	200-800	Injeção (I.P)	(Downing et al., 2010)
	13	600	Injeção (SC)	(Gandal et al., 2010)
	11	800	Oral	(Kolozsi et al., 2009)
	13	600	Injeção (SC)	(Mehta et al., 2011)
	9, 12,5 ou 14,5	500	Injeção	(Kataoka et al., 2011)
	12,5	500	Injeção	(Hara et al., 2012)
	12, 13 ou 14	100	Injeção (I.P)	(Fucic et al., 2010)
Rato	9	600	Injeção (I.P)	(Dufour-Rainfray et al., 2010)
	12	400	Injeção	(Go et al., 2011)
	9 ou 11	800	Oral	(Kuwagata et al., 2009)
	9	800	Oral	(Miyazaki et al., 2005)
	9	800	Oral	(Narita et al., 2002)
	7, 9,5, 12 ou 15	400	Injeção	(Kim et al., 2011)
	11,5, 12 ou 12,5	350	Injeção (I.P)	(Rodier et al., 1996)
	11,5	500	Injeção (I.P)	(Favre et al., 2015)
	11,5	500	Injeção (I.P)	(Rinaldi et al., 2007)
	12,5	600	Injeção (I.P)	(Bambini-Junior et al., 2014b)
	12,5	600	Injeção (I.P)	(Bambini-Junior et al., 2011)
	12,5	400-600	Injeção (I.P)	(Dendrinos et al., 2011)
	12,5	600	Injeção (I.P)	(Felix-Ortiz and Febo, 2012)
	12,5	600	Injeção (I.P)	(Foley et al., 2012)
	12,5	600	Injeção (I.P)	(Ingram et al., 2000)
	12,5	600	Injeção	(Lukose et al., 2011)

2.1.3 Sistemas de neurotransmissores no Transtorno do Espectro do Autismo

Existem relatos de diferentes alterações em sistemas de neurotransmissores no TEA, principalmente nos sistemas GABAérgico, glutamatérgico e serotoninérgico (Pardo and Eberhart, 2007). No SNC a neurotransmissão, além de estar diretamente ligada às interações sinápticas, possui papel importante na maturação do encéfalo e organização cortical (Levitt, 2003). Os neurotransmissores e seus receptores agem como moléculas de sinalização parácrinas no encéfalo imaturo para regular mecanismos envolvidos na migração neuronal (Ben-Ari, 2002; Dayer, 2014; Panula et al., 2014). Tendo em vista essas funções importantes, é possível afirmar que, quando alterados, neurotransmissores e seus receptores exercem papéis importantes na fisiopatologia do TEA.

O Ácido gama-aminobutírico (GABA) é o principal neurotransmissor inibitório no encéfalo. Ele inibe o disparo neuronal através da ativação de duas classes de receptores, $GABA_A$ e $GABA_B$. Os receptores $GABA_A$ são canais ionotrópicos, enquanto os receptores $GABA_B$ são metabotrópicos. A abertura dos receptores $GABA_A$ leva ao influxo de íons de cloro e, consequentemente à hiperpolarização da membrana (Huang, 2006).

Análises *post mortem* de tecidos encefálicos de pacientes com TEA, bem como estudos genéticos e *in vivo*, contribuíram significativamente para revelar o impacto de alterações do sistema GABAérgico nesses transtornos. A quantidade de enzimas de síntese do neurotransmissor inibitório GABA, descarboxilase do ácido glutâmico (DAG) dos tipos 65 e 67 (DAG 65 e DAG 67), está diminuída em amostras cerebelares *post mortem* de pacientes com TEA (Yip et al., 2007, 2009). Os níveis de DAG 65 e 67 se encontraram reduzidos também no córtex de ratos que sofreram exposição pré-natal ao VPA (Cusmano and Mong, 2014). O número de receptores GABAérgicos também é

menor, além de apresentarem reduzida densidade e expressão proteica, no cerebelo e em áreas corticais específicas (Blatt and Fatemi, 2011). Usando espectroscopia de prótons por ressonância magnética, foi detectada redução na concentração de GABA no lobo frontal de crianças com TEA, quando comparadas a controles (Yip et al., 2009).

O glutamato é o principal neurotransmissor excitatório do encéfalo e age através de receptores metabotrópico e ionotrópicos. Existem 3 grupos de receptores metabotrópicos de glutamato (mGluR) e são classificados da seguinte maneira: Grupo 1 (mGluR 1 e 5), Grupo 2 (mGluR 2 e 3) e Grupo 3 (mGluR 4 e 6-8). Os receptores ionotrópicos de glutamato são canais iônicos ativados por ligantes e são nomeados de acordo com seus agonistas: NMDA (N-metil-d-aspartato), AMPA (alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico) e cainato (Nedergaard et al., 2002).

A ativação do mGluR8 inibe a liberação de glutamato. Desta maneira, este receptor possui papel importante na prevenção da hiperexcitabilidade e manutenção da homeostase neural (Cartmell and Schoepp, 2000). A região q31 do cromossomo 7 contém o gene do mGlur8 (GRM8), cuja mutação leva a um aumento da hiperatividade glutamatérgica e excitotoxicidade, condicionando suscetibilidade ao TEA ((IMGSAC), 2001).

Pacientes com TEA apresentam maiores níveis plasmáticos de glutamato quando comparados a controles, além da severidade do transtorno ter sido correlacionada com esses níveis elevados (Aldred et al., 2003). O uso de espectroscopia de prótons por ressonância magnética demonstrou hiperglutamatergia no córtex cingulado anterior pregenual em crianças e adolescentes com TEA (Bejjani et al., 2012). Contrastando com esse fato, os efeitos terapêuticos do piracetam, um modulador positivo do receptor AMPA, podem refletir em um estado hipoglutamatérgico no TEA (Akhondzadeh et al., 2008).

A serotonina é um dos neurotransmissores mais importantes para a saúde mental. A maior parte da serotonina está distribuída fora do sistema nervoso central (SNC) e modula diversos processos fisiológicos em diferentes órgãos. Contudo, a serotonina presente no SNC pode ter papel importante na fisiopatologia de diversas desordens neurológicas.

A hiperserotonemia detectada em pacientes com TEA (Gabriele et al., 2014) sugere um papel para o sistema histaminérgico na fisiopatologia dessa desordem. Além dos elevados níveis de serotonina no sangue, sabe-se que a exposição às drogas que aumentam os níveis deste neurotransmissor, incluindo cocaína e álcool, está relacionada com alta incidência de TEA em crianças (Ornoy et al., 2015). Contudo, através de neuroimagem, foi possível constatar que a síntese de serotonina no encéfalo de crianças com TEA é significativamente menor quando comparada à de crianças com desenvolvimento típico (Chugani et al., 1999). Além disso, outro estudo demonstrou que a ligação ao transportador de serotonina é menor em estruturas encefálicas específicas destes pacientes (Makkonen et al., 2008).

A literatura apresenta poucos estudos envolvendo o sistema histaminérgico e o TEA, que serão abordados no item 2.4.6 desta tese. O próximo tópico desta tese abordará de maneira mais completa os papéis fisiológicos e fisiopatológicos do sistema histaminérgico no SNC.

2.2 O Sistema histaminérgico

Em 1940 a histamina (HA) foi observada pela primeira vez no encéfalo (Kwiatkowski, 1941), mas seu papel como neurotransmissor foi proposto somente nos anos 1970 (Schwartz et al., 1976). O sistema histaminérgico foi considerado um sistema de neurotransmissão apenas em 1984, quando foi demonstrada a existência de neurônios

histaminérgicos no hipotálamo posterior e suas projeções através do encéfalo (Panula et al., 1984; Watanabe et al., 1984). O núcleo tuberomamilar (NTM) é a única fonte de corpos celulares histaminérgicos.

Os neurônios histaminérgicos são células relativamente grandes (~25-30 μM de diâmetro) (Panula et al., 1984; Watanabe et al., 1984). Além de HA, subpopulações de neurônios do NTM também expressam substância P, galanina, GABA e sua enzima de síntese, a GAD (Staines et al., 1986; Ericson et al., 1991; Airaksinen et al., 1992).

Os neurônios do NTM projetam-se através de quase todo o encéfalo e algumas partes da medula espinhal (Panula et al., 1984; Watanabe et al., 1984). Existem duas vias ascendentes e uma descendente de neurônios histaminérgicos inervando o encéfalo (Köhler et al., 1985). A via ascendente ventral inerva a parte ventral do encéfalo incluindo o hipotálamo e septo, enquanto a via a ascendente dorsal projeta para o tálamo, hipocampo, amígdala e estruturas rostrais do prosencéfalo. A via descendente projeta para cerebelo, tronco encefálico e medula espinhal **(Figura 1)**.

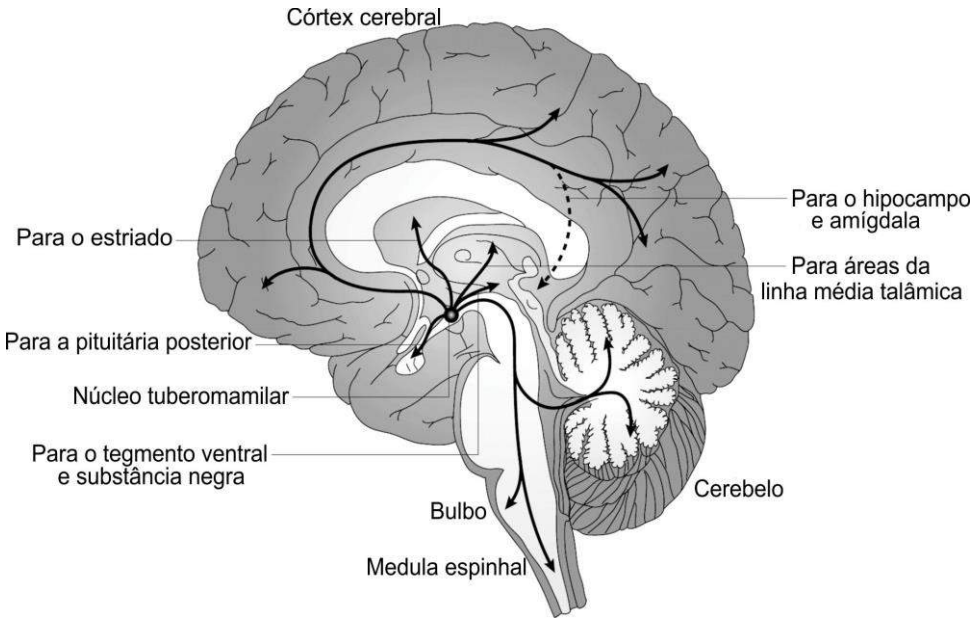

Figura 1 **– O sistema histaminérgico no encéfalo humano.** As fibras histaminérgicas se originam do Núcleo Tuberomamilar e se projetam para todo o sistema nervoso central. Modificado de (Haas et al., 2008).

O NMT do rato foi subdividido em cinco subdivisões, mas existem poucas evidências de que estes cinco subnúcleos sejam funcionalmente diferentes, sendo possível

considerá-los um grupo funcional (Inagaki et al., 1990; Wada et al., 1991). Um único neurônio, proveniente de um dos subnúcleos do NTM, pode enviar projeções para diferentes partes do encéfalo, inclusive para ambas as vias ascendente e descendente (Köhler et al., 1985). Contudo, evidências recentes mostram que os neurônios histaminérgicos são funcionalmente heterogêneos e organizados em circuitos distintos, cada um deles responsável por respostas fisiológicas específicas (Giannoni et al., 2010). Esta diversidade pode ser reflexo da funcionalidade dos receptores histaminérgicos expressos nestes neurônios, em especial as diferentes isoformas do H3R.

No encéfalo, a HA é produzida por mastócitos e neurônios (Garbarg et al., 1974; Martres et al., 1975). A quantidade desta molécula que penetra o encéfalo através do sangue é insignificante (Nuutinen and Panula, 2010). A L-histidina, é o fator limitante para a síntese da HA e é fornecido aos neurônios através de um sistema de transporte de aminoácidos. A enzima de síntese de HA, a histidina descarboxilase (HDC) transforma a L-histidina em HA (Garbarg et al., 1974). A HA é então empacotada em vesículas intracelulares pelo transportador de monoaminas vesicular (VMAT) (Merickel and Edwards, 1995). A entrada de um potencial de ação na porção terminal do axônio induz a liberação da HA na fenda sináptica de uma maneira cálcio dependente. Após esta liberação, a HA se liga a seus receptores alvo e a fração que não se liga é inativada e transformada por metilação em tele-metil-histamina (t-mHA), através da histamina-N-metiltransferase (HNMT) (BROWN et al., 1959). A monoaminoxidase B (MAO-B) converte a t-mHA em ácido tele-metil-imidazolacético (t-mIAA) por desaminação oxidativa (Hough and Domino, 1979).

Entre os quatro receptores histaminérgicos existentes (H1R, H2R, H3R e H4R), o H1R é o alvo da maioria das drogas aprovadas (Overington et al., 2006) e é encontrado em diferentes tecidos e tipos celulares (Chang et al., 1979)(Chang et al., 1979)(Chang et

al., 1979). Assim como todos os receptores histaminérgicos, pertence à família de receptores acoplados à proteína G (GPCR). A transdução de sinal do H1R inclui a ativação da fosfolipase C, que promove a liberação de cálcio e ativação da proteína cinase C (Smit et al., 1999). O H1R está envolvido na modulação de diferentes processos, como o ciclo sono-vigília (Huang et al., 2006).

O H2R é abundante no estômago, sendo responsável pela secreção de ácido gástrico. Seu papel no SNC não é totalmente compreendido, mas evidências apontam para um envolvimento em processos de aprendizado e memória, controle motor e termorregulação (Dai et al., 2007b; Tabarean et al., 2012). A sua ativação estimula a adenil cliclase, levando à produção de monofosfato de adenosina cíclico (cAMP) (Hegstrand et al., 1976).

O receptor H4R está relacionado principalmente com o sistema imunológico e regula diferentes processos, como a atividade de células dendríticas e secreção de interleucinas . A expressão e função do H4R no SNC e sistema nervoso periférico (SNP) é motivo de debate há mais de uma década. Existem algumas publicações que relatam a presença de H4R funcional em neurônios do SNC e SNP, e um número maior de relatos que apontam para a ausência desse receptor (Schneider et al., 2015).

2.3 O Receptor H3R

2.3.1 Função e Localização no encéfalo humano

Caracterizado inicialmente como um autoreceptor que regula a secreção e síntese de HA (Arrang et al., 1983), o H3R foi posteriormente classificado como sendo também um heteroreceptor que regula a liberação de diversos outros neurotransmissores, incluindo noradrenalina (Schlicker et al., 1994), acetilcolina (Ach) (Blandina et al., 1996)

e glutamato (Molina-Hernández et al., 2001). A **Figura 2** demonstra o efeito regulatório do H3R sobre a liberação de HA e outros neurotransmissores.

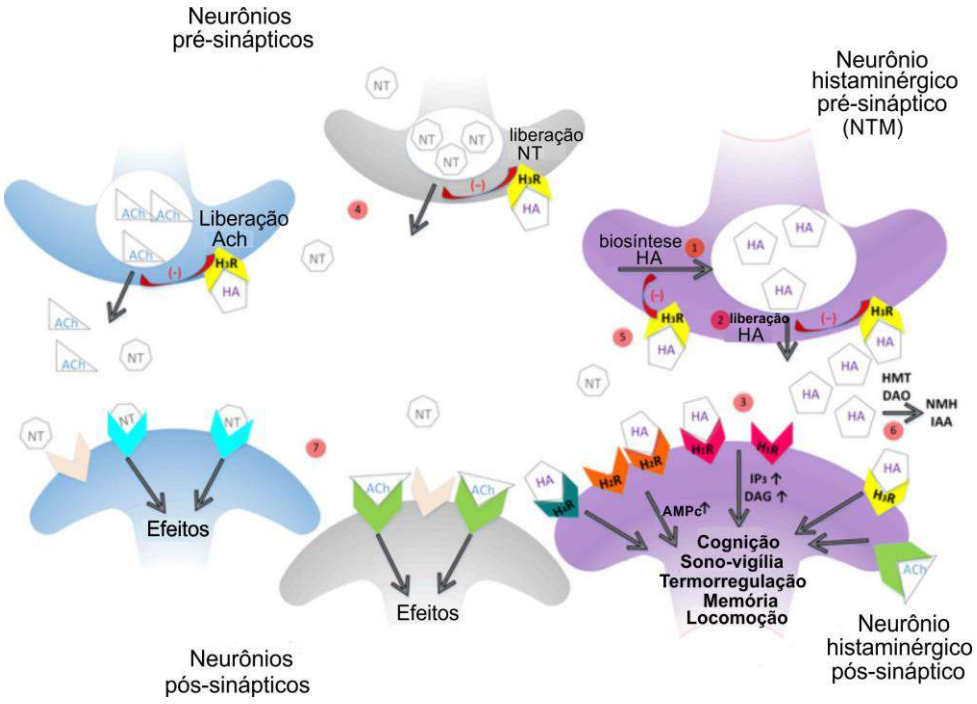

Figura 2 – **Papel fisiológico do H3R no SNC.** Efeito regulatório do H3R sobre a liberação de HA e outros neurotransmissores. Modificado de (Nikolic et al., 2014)

No córtex pré-frontal, a expressão do mRNA do H3R é detectada na substância cinzenta com maior nível de expressão na camada cortical 5 e menor nível de expressão na camada 1. Esse padrão de distribuição é similar em diferentes áreas corticais pré-frontais do encéfalo humano, como as ínsulas granular e agranular (Jin and Panula, 2005). No córtex temporal sítios de ligação do H3R detectados por [^{3}H]N$^{\alpha}$-metilhistamina são vistos principalmente na substância cinzenta. A maior densidade dos sítios de ligação é vista em camadas corticais mediais (camadas 3 e 4) enquanto a menor densidade é vista na camada 6.

O mRNA do H3R é expresso também em alta intensidade no tálamo dorsal humano, a única parte do tálamo que projeta para o córtex. De modo geral, de alto a moderado nível de expressão é visto na maioria dos principais núcleos relé (núcleos

anterior, lateral e medial; núcleo geniculado lateral), enquanto que o nível de expressão é baixo no núcleo geniculado medial, região talâmica posterior, e tálamo ventral (Jin et al., 2002). A maior densidade de sítios de ligação foi vista nos núcleos da linha média, núcleo dorsomedial e núcleos pulvinares (Jin et al., 2002).

No hipocampo, a ligação do H3R é mais acentuada na região CA1 e, por ordem decrescente, em CA3, CA4 e CA2. De forma similar ao que acontece em outras áreas corticais, uma preferência por camada também é observada no complexo subicular e no córtex entorrinal. No complexo subicular, a densidade de ligação é maior na camada profunda do subículo e camadas superficiais do pré-subículo e parassubículo. No córtex entorrinal, a densidade de ligação é maior nas camadas superficiais e profundas que nas camadas médias.

O H3R é expresso abundantemente nos núcleos da base, onde diversos circuitos importantes são regulados por esse receptor. A expressão de mRNA do H3R é alta no núcleo caudado e putâmen, baixa no globo pálido e muito baixa ou ausente na substância negra. De forma contrária, o nível de ligação de radioligantes do H3R é alto na substância negra e globo pálido, mas moderado no putâmen e núcleo caudado (Anichtchik et al., 2001).

2.3.2 O gene H3R e isoformas do receptor

O gene *H3R* está localizado no cromossomo 20 e possui 3 éxons e dois íntrons ou quatro éxons e três íntrons, facilitando os rearranjos de sequências de cDNA e, por fim, as proteínas do receptor (Tardivel-Lacombe et al., 2001). O gene pode sofrer *splicing* alternativo que resultará em variações descritas em diferentes tecidos humanos (Tardivel-Lacombe et al., 2001) e isoformas presentes em roedores (Drutel et al., 2001) e primatas

(Yao et al., 2003). As diferentes isoformas variam de espécie para espécie e se distinguem entre si por suas propriedades de sinalização e padrões de expressão.

Enquanto o receptor H3R em sua forma integral é descrito como consistindo de 445 aminoácidos, *splicing* alternativo do gene do receptor resulta em pelo menos 20 isoformas do mRNA do H3R identificadas por RT-PCR (Bongers et al., 2007b). Estas isoformas exibem variações nos comprimentos de terminações carboxi- e amino-terminal e na terceira alça intracelular, bem como deleções de domínios transmembrana. Até o momento, 8 das 20 isoformas (H3(445), H3(453), H3(415), H3(413), H3(409), H3(373), H3(365) e H3(329)) foram descritas como funcionais (Esbenshade et al., 2006). As 12 isoformas restantes não são funcionais ou suas atividades biológicas ainda precisam ser identificadas.

2.3.3 Sinalização

Já se sabe que a ativação do H3R permite a modulação de diversas vias de sinalização intracelular **(Figura 3)**. A inibição da adenilato ciclase pela ativação do H3R leva a uma redução do cAMP intracelular e subsequente diminuição da atividade da proteína cinase A (PKA) (Lovenberg et al., 1999). A PKA participa em diversas vias de sinalização responsáveis por diferentes respostas biológicas, incluindo expressão gênica, plasticidade sináptica e comportamento (Brandon et al., 1997).

Outra via de sinalização modulada pelo H3R, através da proteína Gαi/o, é a ativação da fosfolipase A2 (PLA2), que leva à liberação de ácido araquidônico (Burgaud and Oudart, 1993). Além disso, pode haver também a liberação de ácido docosahexaenóico e lisofosfolipídeos. Estes metabólitos, além de possuírem efeitos fisiológicos intrínsecos, são substratos para síntese de mediadores lipídicos como fator de ativação plaquetária, eicosanóides e 4-hidroxinonenal. Este último é um metabólito

citotóxico, associado com apoptose neural e está aumentado em doenças neurológicas, como doença de Alzheimer e de Parkinson (Farooqui and Horrocks, 2006). Além da proteína Gαi/o, subunidades GβϒΥ também são capazes de mediar a ativação de certas vias, como as das proteína-cinases ativadas por mitógenos (MAPK) (Levi et al., 2007). As MAPK são conhecidas por terem efeitos no crescimento celular, plasticidade neuronal e memória (Thomas and Huganir, 2004).

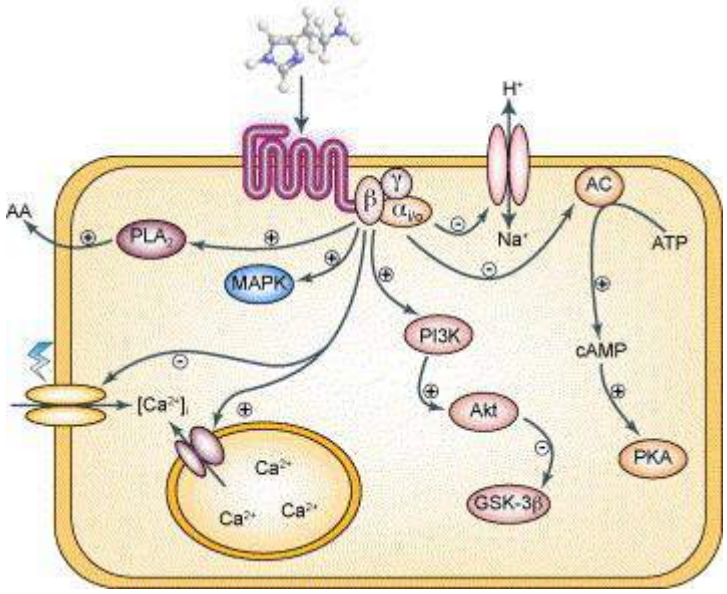

Figura 3 - **Representação das vias de sinalização moduladas pela ativação do H3R.** O H3R é capaz de modular diversas vias de sinalização, incluindo a inibição da adenil ciclase (AC) proteína-cinases ativadas por mitógenos (MAPK), ativação da fosfolipase A_2 (PLA_2), mobilização de cálcio intracelular, ativação do eixo Akt/GSK-3β e inibição do trocador Na^+/H^+ (Bongers et al., 2007a)

O eixo Akt/GSK-3β também pode ser ativado pelo H3R em uma linhagem celular de neuroblastoma, cultura primária de neurônios corticais e fatias de estriado de ratos *Spraque–Dawley* (Bongers et al., 2007c). No SNC, o eixo Akt/GSK-3β tem papel importante na migração neuronal, proteção contra apoptose neuronal e está alterado na doença de Alzheimer e esquizofrenia (Brazil et al., 2004; Rickle et al., 2004).

Em células humanas de neuroblastoma (SH-SY5Y), foi mostrado que a ativação do H3R reduziu a mobilização intracelular de cálcio. Este mecanismo foi ligado ao efeito inibitório do H3R sobre a exocitose da norepinefrina nessas células e sinaptossomas obtidos de tecido cardíaco (Silver et al., 2002).

O trocador de Na^+/H^+ é essencial pra a restauração do pH fisiológico intracelular, através da troca de um H+ intracelular por um Na+ extracelular (Karmazyn, 1999). A ativação do H3R diminui a atividade do trocador, e esta via foi proposta como o mecanismo pelo qual o H3R inibe a liberação excessiva de norepinefrina durante o processo de isquemia cardíaca (Silver et al., 2001).

2.3.4 Atividade constitutiva

O H3R possui atividade constitutiva, sendo capaz de se tornar ativo independente da presença de um agonista. O nível de atividade constitutiva varia de acordo com a espécie, isoforma e tipos celulares (Morisset et al., 2000). A descoberta da atividade constitutiva do H3R permitiu uma caracterização mais detalhada dos ligantes farmacológicos, além de apontar que a maioria dos ligantes inicialmente classificados como antagonistas do H3R, são na realidade agonistas inversos (Arrang et al., 2007).

2.3.5 Agonistas inversos do H3R

O primeiro agonista inverso do H3R a ser sintetizado foi o composto à base de imidazol, tioperamida (Stark et al., 2004). Outros compostos à base de imidazol foram desenvolvidos, mas sua capacidade de interferir na atividade de enzimas citocromo p450 (Yang et al., 2002), além de sua baixa capacidade de penetração no SNC (Vacondio et al., 2004), levaram ao desenvolvimento de outras drogas que não fossem à base de imidazol. O pitolisant foi o primeiro antagonista não-imidazólico do H3R a ser utilizado em estudos clínicos (Schwartz, 2011).

2.4 Desordens neurológicas e sistema histaminérgico

A HA regula diversas funções fisiológicas no encéfalo e alterações no sistema histaminérgico já foram relatadas na fisiopatologia de diferentes desordens neurológicas

(Baronio et al., 2014). Considerando este fato, não é surpreendente a existência de estudos farmacológicos que visam explorar a potencialidade de agonistas inversos de receptores histaminérgicos como tratamento para estas desordens (Passani and Blandina, 2011). A **Tabela 2** apresenta diferentes ligantes de receptores histaminérgicos e estudos que investigaram seus efeitos em diferentes em diferentes desordens neurológicas.

A HA possui um importante papel no controle do ciclo sono-vigília. Foi demonstrado que os neurônios histaminérgicos do NTM de gatos, durante a vigília, disparam de forma lenta e regular. Durante o sono REM (movimento rápido dos olhos, do inglês: *rapid eye movement*) há uma ausência de disparo por parte desses neurônios (Vanni-Mercier et al., 1984). Camundongos HDC KO mostraram que a síntese de HA alterada impede que o animal permaneça em um estado de vigília (Parmentier et al., 2002).

Em modelos animais, os agonistas inversos do H3R melhoram o desempenho cognitivo provavelmente por aumentarem a liberação de HA e a atenção e vigilância mediada por esse composto. Além disso, esta melhora também pode ser resultado do efeito regulatório que o H3R exerce sobre a liberação de outros neurotransmissores importantes em processos cognitivos. A seguir será discutido o papel do sistema histaminérgico na fisiopatologia de desordens neurológicas. Também serão relatadas abordagens terapêuticas (experimentais e clínicas) baseadas neste sistema.

Tabela 2 - Agonistas inversos dos receptores histaminérgicos

Receptor	Droga	Desordem	Estudo	Resultado	Referência
H1R	Clorfeniramina	Modelo de estresse por imobilização/ distúrbio do sono	Pré-clínico	Redução no sono REM.	(Rojas-Zamorano et al., 2009)
	Dimebon	Doença de Alzheimer	Clínico	Sem melhora significativa na fase III do estudo	(Bezprozvanny, 2010)
	Doxepina	Insônia	Clínico	Melhoras na manutenção do sono em um estudo com pacientes idosos.	(Lankford et al., 2012)
H2R	Dimebon	Doença de Alzheimer	Clínico	Sem melhora significativa na fase III do estudo.	(Bezprozvanny, 2010)
	Famotidina	TEA	Clínico	Atenuou sintomas como irritabilidade, hiperatividade e contato visual atípico em crianças com TEA.	(Linday et al., 2001)
		Esquizofrenia	Clínico	Reduziu os escores em BPRS, CGI e SANS.	(Deutsch et al., 1993)
H3R	GSK239512	Doença de Alzheimer	Clínico	Sem melhoras na memória/função executora em estudo randomizado, duplo-cego, placebo-controlado.	(Nathan et al., 2013)
	ABT-288	Doença de Alzheimer	Clínico	Sem melhora significativa em um estudo randomizado	(Haig et al., 2014b)
		Esquizofrenia	Clínico	Sem melhoras cognitivas nos pacientes	(Haig et al., 2014a)
	JNJ-10181457	Modelo de Doença de Alzheimer	Pré-clínico	Reverteu déficits cognitivos induzidos pela escopolamina e normalizou a neurotransmissão de ACh.	(Galici et al., 2009)
	Pitolisant	Narcolepsia	Clínico	Reduziu sonolência diurna excessiva	(Lin et al., 2008)
	ABT-239	Modelo de Esquizofrenia	Pré-clínico	Atenuou déficits cognitivos causados pela cetamina e MK-801.	(Brown et al., 2013)
	A-431404	Modelo de Esquizofrenia	Pré-clínico	Atenuou déficits cognitivos causados pela cetamina e MK-801.	(Brown et al., 2013)
	JNJ-39220675	Modelo de alcoolismo	Pré-clínico	Reduziu o consumo de álcool após período de abstinência.	(Galici et al., 2011)
	Tioperamida	Modelo de Doença de Parkinson	Pré-clínico	Diminuiu a hiperatividade.	(Nowak et al., 2009)
	GSK189254	Modelo de dor neuropática	Pré-clínico	Efeito analgésico.	(Medhurst et al., 2008)
	GSK334429	Modelo de dor neuropática	Pré-clínico	Efeito analgésico.	(Hsieh et al., 2010)
	SAR110894	Modelo de Esquizofrenia	Pré-clínico	Normalizou o comportamento social alterado.	(Griebel et al., 2012)
	Ciproxifan	Modelo de Esquizofrenia	Pré-clínico	Normalizou déficits cognitivos.	(Browman et al., 2004)
		Modelo de Doença de Alzheimer	Pré-clínico	Melhorou a hiperatividade e memória.	(Bardgett et al., 2011)

2.4.1 Narcolepsia

A narcolepsia é caracterizada por sonolência diurna excessiva e sono noturno prejudicado. Além disso, pode ser acompanhada pela cataplexia, atonia muscular súbita (Dauvilliers et al., 2007), uma desordem causada por alteração na neurotransmissão de orexina (Peyron et al., 2000). A orexina é um peptídeo excitatório originado do hipotálamo lateral, com projeções ao longo do encéfalo. O sistema orexinérgico projeta para neurônios aminérgicos, incluindo os histaminérgicos. Existem relatos que indicam que a ativação da neurotransmissão histaminérgica é necessária para o processo de vigília estimulado pela orexina (Huang et al., 2001).

Níveis reduzidos de HA foram encontrados no líquido cefalorraquidiano (LCR) de pacientes com insônia idiopática ou narcolepsia (Kanbayashi et al., 2009). Estudos experimentais foram realizados com agonistas inversos do H3R e modelos de narcolepsia, o que levou ao desenvolvimento de estudos clínicos com essas drogas. O pitolisant é o agonista inverso do H3R que mostrou maior sucesso até o momento em estudo clínico de fase III com pacientes afetados por sonolência diurna excessiva (Lin et al., 2008; Dauvilliers et al., 2013).

2.4.2 Doença de Alzheimer

Apesar da existência de informações conflitantes sobre o papel do sistema histaminérgico na doença de Alzheimer, algumas anormalidades já foram relatadas. Um estudo *post mortem* mostrou redução nos níveis de HA no hipotálamo, hipocampo e córtex temporal de pacientes com essa doença (Shan et al., 2012b). Outro relato mostra que pacientes com a doença de Alzheimer apresentam 57% menos neurônios histaminérgicos do que controles no NMT. Apesar desta perda celular, supõe-se que a produção de HA não esteja afetada, já que os níveis de mRNA da HDC permanecem

inalterados (Shan et al., 2012b). Contudo, já foi demonstrado que o fator de liberação de HA encontra-se diminuído no córtex temporal de pacientes com doença de Alzheimer (Kim et al., 2001), o que pode indicar também uma redução nos níveis de HA nesta doença.

Em um estudo preliminar, a tolerabilidade e efeitos cognitivos do GSK239512, um agonista inverso do H3R, foram testados em pacientes com doença de Alzheimer (Nathan et al., 2013). Esses indivíduos não demonstraram alterações hematológicas, bioquímicas ou cardiovasculares. Melhoras cognitivas foram detectadas, com indivíduos mostrando efeitos positivos sobre memória e atenção. Em um estudo maior, o GSK239512 foi considerado seguro e capaz de induzir melhoras na memória episódica, porém o mesmo não foi detectado quando a melhora de trabalho foi avaliada (Grove et al., 2014).

O agonista inverso do H3R, o ciproxifan (CPX) foi testado em um modelo genético da doença de Alzheimer (Bardgett et al., 2011). Parâmetros como hiperatividade e déficits de memória foram reduzidos após administração desta droga. Ratos expostos à escopolamina, um antagonista muscarínico que leva a prejuízos comportamentais e redução na secreção de Ach, anormalidades similares às encontradas em pacientes com doença de Alzheimer, foram tratados com JNJ-101811457 (Galici et al., 2009), um agonista inverso do H3R. Este tratamento, além de reverter os déficits cognitivos, normalizou a secreção de Ach.

2.4.3 Esquizofrenia

A esquizofrenia é uma desordem heterogênea, com grande influência genética e que afeta 1,1 % da população dos Estados Unidos (Jouan et al., 2013). O envolvimento do sistema dopaminérgico na fisiopatologia desta desordem é bem conhecido, com

antipsicóticos agindo principalmente no receptor D2 de dopamina (Seeman, 2010), contudo um papel para o sistema histaminérgico é levado em consideração.

Os níveis de t-MHA no LCR, representando a HA liberada e metabolizada, se encontram elevados em pacientes com esquizofrenia (Prell et al., 1995). Além disso, o H3R apresentou maior potencial de ligação no córtex pré-frontal e essa alteração foi correlacionada com sintomas psicóticos, indicando um papel para o H3R na modulação de processos cognitivos (Jin et al., 2009). Em um ensaio clínico randomizado o ABT-288, agonista inverso do H3R, foi testado em pacientes com esquizofrenia (Haig et al., 2014a). Apesar de ter sido melhor tolerado em altas doses por esses indivíduos do que por voluntários saudáveis, este composto não foi capaz de promover melhoras cognitivas. Na verdade, a administração do ABT-288 foi associada à distúrbios do sono e psicose.

2.4.4 Síndrome de Tourette

A síndrome de Tourette é uma desordem neurológica caracterizada por movimentos repetitivos, estereotipados, e involuntários, além de ecolalia (Serajee and Mahbubul Huq, 2015). Pacientes com essa desordem podem apresentar disfunções no sistema histaminérgico. A ocorrência de síndrome de Tourette em duas gerações de uma família foi associada com uma mutação rara, mas funcional, do gene *HDC* (Ercan-Sencicek et al., 2010). Estudos posteriores com pacientes e camundongos HDC KO apontaram para um possível papel do sistema histaminérgico na fisiopatologia da síndrome de Tourette (Fernandez et al., 2012; Karagiannidis et al., 2013; Castellan Baldan et al., 2014).

2.4.5 Doença de Parkinson

Existem relatos de altos níveis de HA no sangue de pacientes com doença de Parkinson e estudos postmortem indicam um possível papel do sistema histaminérgico na

fisiopatologia desta desordem neurológica (Coelho et al., 1991). A inervação histaminérgica na porção média da substância negra, pars compacta e reticulata, está aumentada no encéfalo de pacientes com doença de Parkinson, o que pode ser um efeito compensatório resultante da deficiência de dopamina (Anichtchik et al., 2000). Quando comparadas às amostras postmortem de indivíduos saudáveis, amostras postmortem de encéfalos de pacientes com doença de Parkinson apresentavam conteúdo elevado de HA no putâmen, substância negra pars compacta, globo pálido interno e globo pálido interno (Rinne et al., 2002). Outro estudo demonstra uma diminuição significativa na expressão do mRNA do H3R e um aumento significativo na expressão do mRNA do HNMT na substância negra de pacientes com doença de Parkinson. A duração da doença (anos entre diagnóstico e óbito) foi negativamente correlacionada com a expressão de mRNA de HNMT (Shan et al., 2012a).

Altos níveis de HA são detectados em um modelo animal de doença de Parkinson (Nowak et al., 2009). Características do modelo, como atividade locomotora exacerbada e comportamentos repetitivos, foram atenuados pela administração de tioperamida, um antagonista do H3R.

2.4.6 Transtorno do Espectro do Autismo

Evidências envolvendo o TEA e sistema histaminérgico são escassas. Na década de 1970 foram relatados níveis elevados de histidina no plasma e níveis reduzidos de histidase na pele de crianças com TEA (Neville et al., 1972), uma condição previamente associada ao retardo mental. Um relato mais recente aponta para níveis reduzidos de histidina e outros aminoácidos na urina de pacientes com TEA (Ming et al., 2012). Contudo, quando os níveis de HA na urina e sangue total foram comparados aos de

pacientes controles, não foi encontrada diferença estatisticamente significativa entre os grupos (Launay et al., 1988).

Sintomas como irritabilidade, hiperatividade e contato visual atípico foram atenuados em pacientes com TEA após a administração do agonista inverso do H2R, a famotidina (Linday et al., 2001). Resultados similares já haviam sido obtidos quando a niaprazina, antagonista do H1R foi utilizada como tratamento para indivíduos com a mesma desordem (Rossi et al., 1999). É provável que estes resultados sejam consequência do efeito sedativo promovido pela niaprazina.

3. JUSTIFICATIVA

Ainda não haviam sido realizados estudos envolvendo o sistema histaminérgico no TEA, nem agonistas inversos do H3R haviam sido cogitados ou testados como potenciais tratamentos para características do tipo autista. Esse é um fato surpreendente, considerando o importante papel que o sistema histaminérgico desempenha em processos cognitivos e outras funções fisiológicas. Além disso, existe um número significativo de desordens neurológicas que apresentam alterações histaminérgicas em suas fisiopatologias e estudos experimentais que demonstraram efeitos benéficos de ligantes dos receptores histaminérgicos em modelos animais destas desordens.

Este trabalho justifica-se pela falta de biomarcadores específicos, bem como a ausência de avanços no desenvolvimento de terapias inovadoras para o tratamento de sintomas do TEA. Os resultados obtidos proporcionam evidências sobre um sistema de neurotransmissão que ainda não havia sido investigado em um modelo experimental de uma das desordens do neurodesenvolvimento mais prevalentes da atualidade.

4. OBJETIVOS

4.1 Geral

Testar um agonista inverso do H3R como tratamento para alterações comportamentais exibidas no modelo animal de autismo induzido por exposição pré-natal ao VPA. Além disso, investigar possíveis alterações do sistema histaminérgico por este modelo experimental e o papel do H3R em parâmetros comportamentais relevantes para o TEA.

4.2 Específicos

Para camundongos do modelo VPA com 50 dias de vida:

- Realizar um levantamento sobre o papel do sistema histaminérgico na fisiopatologia de diferentes desordens neurológicas, incluindo o TEA, bem como sobre o uso de ligantes dos receptores histaminérgicos como medidas terapêuticas para tais desordens
- Avaliar o efeito de um agonista inverso do H3R (CPX) administrado 30 minutos antes de cada avaliação de parâmetros comportamentais relevantes ao TEA (sociabilidade, estereotipia e nocicepção).
- Quantificar a expressão do mRNA dos receptores histaminérgicos H3R e H4R em diferentes estruturas encefálicas (córtex, estriado e hipocampo).
- Quantificar os níveis de HA em diferentes estruturas encefálicas (córtex, estriado e hipocampo) através da técnica de HPLC.

Para camundongos H3R KO

- Avaliar o papel do H3R na sociabilidade e estereotipia através dos testes de três câmaras e *marble burying*.

5. METODOLOGIA

5.1 Locais de execução do trabalho

As análises comportamentais envolvendo o modelo VPA (teste com aparato de três câmaras, *marble burying* e *tail-flick*) e o tratamento com CPX, bem como a eutanásia destes animais, foram realizadas na Unidade de Experimentação Animal do Hospital de Clínicas de Porto Alegre (UEA-HCPA). As análises dos tecidos obtidos do modelo VPA, bem como testes comportamentais envolvendo os animais H3R KO (teste com aparato de três câmaras e *marble burying*), foram realizados na Universidade de Helsinque. Todos os experimentos foram previamente aprovados pelos comitês de ética das duas instituições.

5.2 Modelo VPA

Trinta e seis fêmeas e 9 machos de camundongo da linhagem *Swiss* foram obtidos com 7-8 semanas de vida através da Universidade Federal de Pelotas (Pelotas, Brasil). Fêmeas e machos eram mantidos separados, com 4-5 animais por caixa e água e comida *ad libitum* na UEA-HCPA. No momento do acasalamento, 2 fêmeas eram acomodadas em uma caixa com um macho das 19:00 às 7:00. No dia seguinte, as fêmeas eram separadas e a prenhez confirmada pela presença de tampão vaginal, sendo este considerado o dia embrionário 0 (E0). No E11, 18 fêmeas prenhes receberam uma injeção intraperitoneal 500 mg/kg de VPA (Sigma-Aldrich, St. Louis, MO, USA) dissolvido em salina. Dezoito fêmeas controle receberam o mesmo volume de salina. Quatro fêmeas morreram após a injeção de VPA. Dezesseis filhotes de fêmeas tratadas (VPA) e 16

filhotes de fêmeas que receberam apenas salina (Controle) foram usados no presente trabalho.

Metade de cada grupo recebeu uma injeção de 3mg/kg de CPX antes de cada teste comportamental, enquanto que o restante recebeu uma injeção de salina (SAL). No último dia de testes comportamentais, os animais foram eutanasiados. Após injeção de cetamina e xilasina foi realizada punção cardíaca, decapitação do animal e retirada das seguintes estruturas encefálicas: Córtex, hipocampo, estriado e cerebelo. O soro foi separado e armazenados a -80ºC após centrifugação do sangue durante 5 minutos, a 3.550 rpm. As estruturas encefálicas passaram por congelamento rápido com nitrogênio líquido e também foram armazenadas a -80ºC. Estas amostras foram enviadas para o laboratório do professor Pertti Panula na Universidade de Helsinque para serem analisadas durante o meu período lá como aluno de doutorado sanduíche (PDSE-CAPES).

5.3 Animais geneticamente modificados

Camundongos adultos H3R KO foram adquiridos através da *Johnson and Johnson Pharmaceutical Research and Development* (La Jolla, CA), acasalados e mantidos na unidade de experimentação animal da Universidade de Helsinque. Os H3R KO derivam das linhagens de camundongo 129/Ola e C57BL/6J.

5.4 Tratamento com ciproxifan

O CPX (Sigma-Aldrich, St. Louis, MO, USA) foi dissolvida em salina 0,9% antes dos testes comportamentais. Uma injeção de CPX (3 mg/kg) ou SAL no mesmo volume eram administrados 30 minutos antes de cada teste comportamental envolvendo o modelo VPA e camundongos *Swiss* controles. A dose escolhida para o CPX foi baseada em outros estudos com camundongos (Vanhanen et al., 2013).

5.5 Testes comportamentais

5.5.1 Teste com aparato de três câmaras

A mesma metodologia foi empregada para os animais do modelo VPA no Brasil e para os animais H3R KO na Finlândia. O teste com o aparato de três câmaras para avaliação de sociabilidade e preferência por novidade é uma ferramenta que já foi utilizada com diversas linhagens de camundongos. O desenho experimental deste teste permite a avaliação de dois aspectos críticos, mas identificáveis, do. Neste teste foi utilizado um aparato com um tamanho total de 628 × 456 × 220 (comprimento, largura e

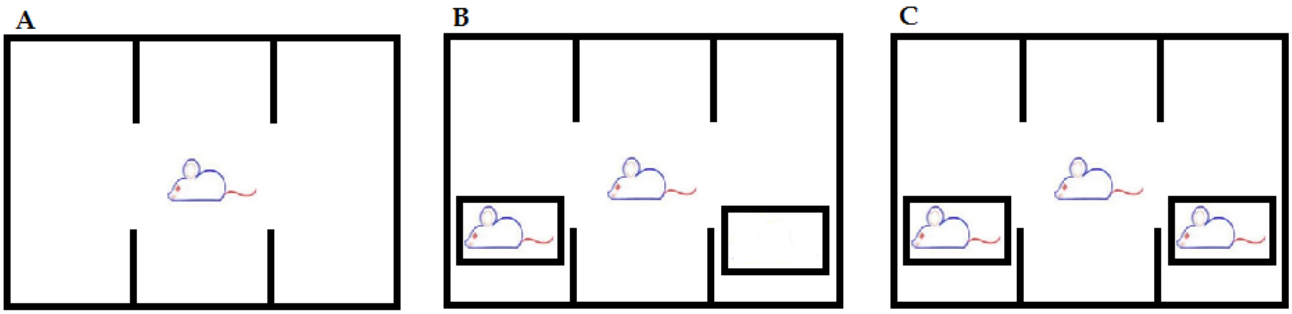

Figura 4 - **Teste de três câmaras. (A)** Antes do início do teste os animais são ambientados no aparato por cinco minutos. **(B)** Após o período de ambientação, o sociabilidade é avaliada durante 10 minutos. **(C)** Imediatamente após a avaliação da sociabilidade, se inicia a avaliação do interesse por novidade social.

altura em milímetros) dividido em três compartimentos para avaliação de sociabilidade e interesse por novidade social **(Figura 4)**. As aberturas entre os compartimentos possibilitam que os animais os explorem. Primeiramente, os animais foram ambientados por 5 minutos no aparato **(Figura 4-A)**. Após a ambientação, o teste de sociabilidade foi iniciado com uma gaiola vazia (objeto) posicionada em uma das câmaras laterais e uma gaiola com um camundongo *Swiss* (animal desconhecido 1) na câmara lateral oposta **(Figura 4-B)**. O animal desconhecido 1 não teve nenhum tipo de contato prévio com o animal avaliado pelo teste. Durante 10 minutos foi cronometrado o tempo que o animal testado gastou explorando cada câmara, bem como interagindo com o objeto ou com o animal desconhecido 1. A avaliação do interesse por novidade social começou

imediatamente após o final do teste de sociabilidade. O animal desconhecido 1 permaneceu em sua gaiola (a partir de agora é denominado como animal conhecido) e um novo animal desconhecido (animal desconhecido 2) foi colocado na gaiola da câmara oposta (que estava vazia anteriormente) **(Figura 4-C)**. O tempo que o animal testado gastou explorando cada câmara, bem como interagindo com o animal conhecido ou com o animal desconhecido 2 foi cronometrado por 10 minutos.

5.2.2 Teste marble burying

A mesma metodologia foi empregada para os animais do modelo VPA no Brasil e para os animais H3R KO na Finlândia. Em seu habitat natural, roedores geralmente tendem a cavar e construir tocas por diversos motivos, incluindo a necessidade de se proteger contra predadores e temperaturas adversas, armazenar alimentos e fazer ninhos. O comportamento de cavar no teste *marble burying* não é correlacionado com atividade exploratória ou outras medidas de ansiedade, não é afetado pelo fato de que o objeto a ser enterrado é uma novidade ao animal e também não se altera após repetidos testes. Isso mostra que este teste é uma ferramenta útil na quantificação de comportamentos repetitivos de camundongos (Thomas et al., 2009).

Os Camundongos foram ambientados individualmente, por dez minutos, em uma caixa com o fundo coberto por aproximadamente 4 cm de maravalha. Após a ambientação, os animais foram retirados da caixa e 20 bolinhas de gude foram acomodadas em um arranjo 4x5 em distâncias iguais **(Figura 5)**. Após os 10 minutos de duração do teste, dois observadores contatam o número de bolinhas de gude que estavam cobertas em mais de 50% pela maravalha. Após cada teste, as bolinhas eram limpas com álcool 70%.

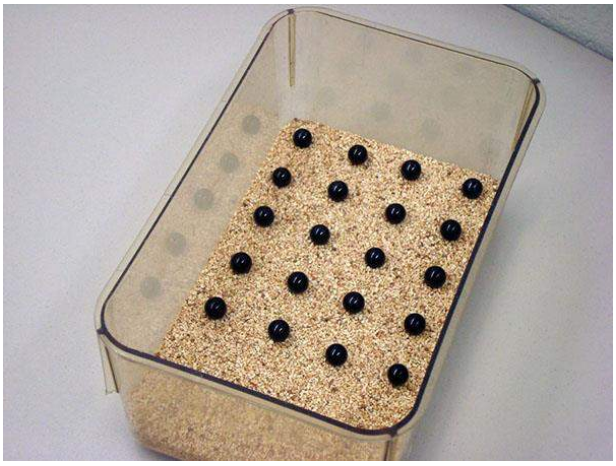

Figura 5 - **Teste *marble burying*.** Avaliação comportamental utilizada para medir comportamento repetitivo.

5.2.3 Teste tail-flick

Este teste foi realizado apenas com os animais do modelo VPA. Os animais foram colocados em um aparelho que possui um aparato com uma fonte térmica e um suporte para o encaixe da cauda (EFF 300L-Light, Insight, Brasil). Quando a cauda do animal era encaixada no suporte, a fonte térmica era acionada e o tempo de retirada da cauda pelo roedor cronometrado e considerado o limiar de nocicepção. Esse teste foi realizado 3 vezes em um intervalo de 30 segundos entre cada teste.

5.3 PCR em Tempo Real

O RNA total das estruturas encefálicas do modelo VPA foi extraído de acordo com as orientações do fabricante do RNeasy mini kit (Qiagen, Hilden, Germany). As amostras de RNA foram armazenadas a -20ºC até o momento do uso. A síntese do cDNA foi realizada a partir de 2 µg das amostras encefálidas dos camundongos do modelo VPA, utilizando o kit *Superscript III Reverse Transcriptase* (Invirogen).

A técnica de PCR em tempo real foi realizada com o sistema de PCR em Tempo Real *LightCycler 480* e *o Master kit LightCycler 480 SYBR green I* (Roche Applied

Science). Os *primers* para amplificação foram desenhados através do *Primer-BLAST* (NCBI) e as sequências são mostradas na **Tabela 3**. A peptidil prolil isomerase A (PPIA) foi usada como controle. Os ciclos utilizados foram os seguintes: 95 °C por 30 s e 45 ciclos de 95 °C por 10 s e 62 °C por 45 s. A análise da curva de dissociação foi realizada (aumento de 0,2 °C por segundo, de 60 °C a 95 °C com leituras de fluorescência contínuas) ao fim dos ciclos para assegurar que apenas um *amplicon* foi obtido. Todas as reações foram realizadas em duplicata. Os dados foram avaliados com o software *LightCycler 480*, versão 1.5. Os resultados foram calculados pelo método comparativo usando os valores Ct do PPIA como controle de referência.

Tabela 3 - Primers utilizados para PCR em tempo real

GENE	**PRIMER *FORWARD* (5'- 3')**	**PRIMER *REVERSE* (5'- 3')**
mus musculus peptidilprolil isomerase a	AGCATACAGGTCCTGGCATC	TTCACCTTCCCAAAGACCAC
mus musculus receptor h3 de histamina	TCAGCTATGACCGATTCCTG	TACTCCCAACTCAGGATGGC
mus musculus receptor h4 de histamina	GGAATCTGCATGTTTTGGCT	ATGATGCCAGTGTGTTGAGC

5.4 HPLC

O conteúdo de histamina das amostras de córtex cerebral, estriado e hipocampo foi analisado por HPLC seguindo protocolo descrito anteriormente (Rozov et al., 2014). As amostras foram homogeneizadas por sonicação com ácido perclórico 2%, centrifugadas por 30 minutos a 15,000 *g* numa temperatura de 4°C e filtradas através de um filtro PVDF de 0,45-µm (Pall Life Sciences, Ann Arbor, MI, USA) antes de serem

avaliadas no sistema de HPLC. O sistema utilizado foi o Shimadzu LC20AD e o software de análise foi o LCSOLUTION 1.21 (Shimadzu, Kyoto, Japan). Para a normalização dos resultados da análise de HPLC, foi utilizado o kit BCA (Thermo Fisher Scientific Inc., Rockford, IL, USA) para medir a concentração proteica.

5.5 Análise estatística

As análises estatísticas estão descritas detalhadamente no **ARTIGO 2** e **ARTIGO 1** desta tese. Resumidamente, os resultados do teste três câmaras foram analisados com o teste GEE (Equações de Estimação Generalizadas) para comparar variáveis independentes múltiplas e compensar a falta de normalidade e homocedasticidade. Duas análises distintas foram realizadas, uma para o tempo nas câmaras e outra para o tempo de interação. Grupo e câmara serão consideradas variáveis independentes.

Os resultados provenientes dos testes *marble burying* e *tail-flick*, e das análises de HPLC e qPCR foram testados com ANOVA duas vias (**ARTIGO2**) - grupo (Controle, VPA) x droga (SAL, CPX) - ou com o teste t de *Student* (**ARTIGO3**).

Todas as análises serão realizadas utilizando o programa SPPSS, versão 20.0 (SPSS, Chicago, IL). Os valores *p* menores que 0,05 foram considerados estatisticamente significativos.

6. ARTIGO 1

ANNALS OF GENERAL PSYCHIATRY

REVIEW Open Access

Histaminergic system in brain disorders: lessons from the translational approach and future perspectives

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Abstract

Histamine and its receptors were first described as part of immune and gastrointestinal systems, but their presence in the central nervous system and importance in behavior are gaining more attention. The histaminergic system modulates different processes including wakefulness, feeding, and learning and memory consolidation. Histamine receptors (H1R, H2R, H3R, and H4R) belong to the rhodopsin-like family of G protein-coupled receptors, present constitutive activity, and are subjected to inverse agonist action. The involvement of the histaminergic system in brain disorders, such as Alzheimer's disease, schizophrenia, sleep disorders, drug dependence, and Parkinson's disease, is largely studied. Data obtained from preclinical studies point antagonists of histamine receptors as promising alternatives to treat brain disorders. Thus, clinical trials are currently ongoing to assess the effects of these drugs on humans. This review summarizes the role of histaminergic system in brain disorders, as well as the effects of different histamine antagonists on animal models and humans.

Keywords: Antagonist, Autism, Brain disorders, Histamine, Histaminergic system

Introduction

Described for the first time in 1910 as a promoter of contraction of smooth muscles and vasodilatation, histamine acts as a transmitter in the central nervous system (CNS) and modulates several other physiological processes, like gastrointestinal and circulatory functions, innate and acquired immunity, cell proliferation and hematopoiesis. Today, its presence in the CNS and importance in behavior are largely studied [1,2].

Histamine synthesis and release are regulated by H3R, an autoreceptor present in the somata and axon terminals of histaminergic neurons. Histamine is synthesized from L-histidine by histidine decarboxylase, and it is metabolized by diamine oxidase and histamine *N*-methyltransferase (HNMT) [3]. Other receptors, such as muscarinic, opioid, and galanin, regulate histamine release in specific brain regions [4-6].

Histaminergic neurons are located in the tuberomamillary nucleus (TMN) of the hypothalamus, with widespread projections innervating most brain areas. Postmortem studies indicate that the number of histaminergic neurons in humans is about 64,000 [7]. There are four histamine receptors, all part of the rhodopsin-like family of G protein-coupled receptors (GPCR). Through these receptors, histamine regulates several basic body functions, such as wakefulness, feeding, and learning and memory [8-10].

Histamine receptors

In 1966, Ash and Schild discovered the H1R while studying the effect of antihistamine drugs in the rat uterus and stomach [11]. After that, three other receptors (H2R, H3R, and H4R) were identified. The four receptors are part of the GPCR superfamily, and they all present constitutive activity [12-15].

The GPCR superfamily modulates several physiological processes and is divided into families and subfamilies, and single subtypes can present different isoforms. The

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discovery of constitutively active mutant receptors proved that these receptors could be activated without the presence of an agonist [16]. Binding of a ligand to a receptor may initiate activity (agonist with positive intrinsic activity) or prevent the effect of an agonist (antagonist with zero intrinsic activity). While the agonists stabilize the receptor in active conformation, the inverse agonists stabilize the receptor in inactive conformation and thus reduce the activity (negative intrinsic activity) [17]. Two isoforms of the H3R are highly constitutively active: the wild type and an isoform with a deletion in the third intracellular loop [18].

From the four histaminergic receptors, H1R is the main target of most of the approved drugs [19] and is found in different tissues and cells, including the smooth muscle, brain, and lymphocytes [20,21]. This receptor is the only member of the family of histamine receptors, of which a co-crystal was obtained, by using the first-generation antagonist doxepin (PDB ID 3RZE) [22]. As for all members of this family, this receptor comprises seven transmembrane helices (TMH), three intracellular loops, and three extracellular loops. The binding pocket of H1R has a conserved hydrophobic nature, which contributes to the low selectivity of doxepin and the first-generation of H1R antagonists, and is associated to an anion-binding region which has been related to the binding of second-generation H1R antagonists. Such crystallographic structure can be used as a model in further development of new blockers as well as in the understanding of the activation-inactivation mechanisms of this receptor family at molecular level. The signal transduction of H1R includes activation of phospholipase C, which promotes the inositol triphosphate-dependent release of Ca^{2+} from intracellular stores and diacylglycerol-sensitive activation of protein kinase C [2,23].

H1R is involved in the modulation of important processes and mice lacking this receptor present different impairments, for example, in spatial memory and in sleep-wake characteristics [24,25]. Injection of a H1R agonist in the median preoptic nucleus, a region involved in basal thermoregulation, induces persistent hyperthermia [26]. Other study with mutant mice lacking H1R suggested a role for this receptor in somatic and visceral pain perceptions. These animals showed fewer nociceptive responses to the hot-plate, tail-flick, tail-pressure, paw-withdrawal, formalin, capsaicin, and abdominal constriction tests [27].

The pioneer study by Ash and Schild indicated, at that time, the existence of at least two classes of histamine receptors, since the antagonists utilized in that experiment did not stop gastric acid secretion [11]. Later, compounds that blocked the gastric acid secretion in guinea pig, named H2R antagonists, were developed by Black and colleagues [28]. Like H1R, H2R presents typical GPCR receptor characteristics. Its activation stimulates adenylyl cyclase leading to cyclic adenosine monophosphate (cAMP) production, a second messenger that has different roles in the cell [29]. The role of H2R in the CNS is not fully understood, but basic research showed that it is related to the processes of learning and memory, motor control, and thermoregulation [30,31]. In some areas of the brain, colocalization of H1R and H2R suggests synergistic interactions between these two receptors [32]. In an animal model of multiple sclerosis, it was demonstrated that both H1R and H2R are propathogenic, mediating immune deviation and blood brain barrier (BBB) disruption [33].

Mainly found in the brain, H3R regulates food intake, memory, acetylcholine (ACh) release, and consolidation of fear memories [34,35]. Activation of H3R inhibits cAMP synthesis and activates MAP kinases and the AKT/GSK3β axis [36-38]. When activated, the receptor inhibits cell firing and decreases the release of histamine by histaminergic neurons, as well as inhibits secretion of norepinephrine, serotonin, and other neurotransmitters [13,39,40]. Recently, several alterations were reported in mice lacking H3R. They presented enhanced histaminergic neurotransmission, which led to changes in the mice phenotype, indicating a possible metabolic disorder as a consequence. The sleep was also altered, in a condition similar to sleep restriction in humans, which matches with obesity tendency presented by these animals [41].

Almost 15 years ago, H4R was the last histamine receptor to be identified. It is mainly related with immune functions, but its presence in the brain is known, as well as in the bone marrow, peripheral blood, spleen, thymus, small intestine, colon, heart, and lung [42-44]. It modulates different processes such as dendritic cells activity, interleukins release, and likely regulation of BBB permeability [45-47].

In humans, the presence of H4R messenger RNA (mRNA) was detected in the spinal cord, hippocampus, cerebral cortex, thalamus, and amygdala, with levels in the spinal cord overcoming the levels found in the spleen and liver. The authors also verified the presence of the receptor in the dorsal root ganglia, which might indicate a nociceptive role for the receptor. In rats, the cerebellum and hypothalamus presented the highest amounts of H4R mRNA [48].

Histaminergic system and brain disorders

Alterations in the histaminergic system have been reported in several brain disorders and might have a significant role in their pathophysiology [49-52]. Considering this, it is not surprising that pharmacological studies are in development to explore the potentialities of histamine antagonists/inverse agonists in the treatment of these disorders. Table 1 shows the uses and outcomes of histamine receptors antagonists cited in this review.

Table 1 Histamine receptors antagonists: therapeutic applications and outcomes

Receptor	Drug	Disorder	Study	Outcome	Reference
H1R	Chlorpheniramine	Model of stress by immobilization/sleep disturb	Preclinical	Reduction in REM sleep.	[53]
	Dimebon	AD	Clinical	No significant improvement in a phase III trial.	[54]
	Doxepin	Insomnia	Clinical	Improvements in sleep maintenance and duration in a 4-week outpatient trial of elderly adults.	[55,56]
H2R	Dimebon	AD	Clinical	No significant improvement in a phase III trial.	[54]
	Famotidine	Autism	Clinical	Attenuated symptoms like irritability, hyperactivity and atypical pattern of eye contact in children with autism.	[57]
		SCH	Clinical	Reduced scores in BPRS, CGI, and SANS.	[58,59]
H3R	GSK239512	AD	Clinical	Failed on improving executive function/working memory in a randomized, double-blind, placebo-controlled.	[60]
	ABT-288	AD	Clinical	No significant improvements in a randomized study.	[61]
		SCH	Clinical	Failed on providing cognitive improvements to patients.	[62]
	JNJ-10181457	Model of AD	Preclinical	Reversed cognitive deficits induced by scopolamine and normalized ACh neurotransmission.	[63]
	Pitolisant	Narcolepsy	Clinical	Reduced excessive daytime sleepiness	[64]
	ABT-239	Model of SCH	Preclinical	Attenuated cognitive deficits caused by ketamine and MK-801.	[50]
	A-431404	Model of SCH	Preclinical	Attenuated cognitive deficits caused by ketamine and MK-801.	[50]
	JNJ-31001074	ADHD	Clinical	No significant improvements in adult patients.	[65]
	Betahistine	SCH	Clinical	Reduced weight gain by patients with SCH treated with olanzapine.	[66]
	JNJ-39220675	Model of alcoholism	Preclinical	Reduced intake of alcohol after a period of abstinence.	[67]
	Thioperamide	Model of SCH	Preclinical	Enhancement of prepulse inhibition.	[68]
		Model of PD	Preclinical	Decreased hyperactivity.	[69]
	GSK189254	Model of neuropathic pain	Preclinical	Antinociceptive effect.	[70,71]
	GSK334429	Model of neuropathic pain	Preclinical	Antinociceptive effect.	[71]
	SAR110894	Model of SCH	Preclinical	Normalized impaired social behavior.	[72]
	Ciproxifan	Model of SCH	Preclinical	Enhancement of prepulse inhibition.	[68]
		Model of AD	Preclinical	Improvements in hyperactivity and memory deficits.	[73]
H4R	JNJ7777120	Model of neuropathic pain	Preclinical	Antinociceptive effect.	[74,75]
	ZPL3893787	Tested in healthy volunteers. Potential treatment for asthma, allergic rhinitis, pain, and other inflammatory diseases.	Clinical	Completed phase I trial. Safe and well tolerated.	[76]
	UR63325	Allergic rhinitis	Clinical	Currently on phase II clinical trial. No data available.	[76]
	KD1157	Tested in healthy volunteers. Potential treatment for allergic rhinitis.	Clinical	Safe and well tolerated.	[76]
	JNJ38518168	Asthma	Clinical	Currently on phase II clinical trial. No data available.	[76]
	JNJ39758979	Asthma	Clinical	Completed phase II clinical trial. No data available.	[76]

Alzheimer's disease

Alzheimer's disease (AD) affects approximately 5.4 million US citizens and the costs with patients, including health care, long-term care, and hospitalization, make it a social problem [77]. Even though there is conflicting information about the role of histaminergic system in AD, abnormalities have been reported. A postmortem study showed reduced histamine content in the hypothalamus, hippocampus, and temporal cortex of patients with AD [7]. Other report showed 57% less histaminergic

neurons in the TMN of AD patients and females had increased prefrontal cortex expression of H3R. Despite the severe cell loss, it is suggested that histamine production is not affected, since the levels of histidine decarboxylase mRNA are unaltered [78]. On the other hand, Kim and colleagues reported decreased levels of the histamine-releasing factor in the temporal cortex of patients with AD, as well as in the temporal cortex, thalamus, and caudate nucleus of patients with Down syndrome, indicating low levels of brain histamine in these disorders [79].

The development of new treatments for AD is a permanent issue. Positive results after a double-blind, placebo-controlled, phase II trial led to great expectations about the possibility of dimebon becoming a drug to treat AD, with patients displaying significant improvement over baseline for Alzheimer's disease Assessment Scale [80]. Dimebon acts as an antagonist of H1R and H2R, and it also binds to adrenergic, serotonergic, and dopaminergic receptors. Unfortunately, a phase III trial with 598 patients found no significant improvement in patients with AD treated with this drug [54].

In a preliminary investigation GSK239512, an H3R antagonist/inverse agonist was tested in order to assess its tolerability and cognitive effects in patients with AD. The patients displayed no abnormalities in hematology, clinical chemistry, urinalysis parameters, and cardiovascular parameters. Enhancements in cognition were assessed, with subjects showing positive effects on attention and memory [60]. In a larger trial, GSK239512 was considered safe and able to improve episodic memory in patients with AD, but it failed on improving executive function/working memory [81]. Other H3R antagonists, such as ABT-288, have also failed in clinical trials [61].

On the other hand, interesting findings were reported when ciproxifan, also H3R antagonist, was tested in a transgenic mouse model of AD. Improvements in some impairments featured in this disorder, such as hyperactivity and memory deficits were detected after administration of this drug [73]. This was also performed with rats that received scopolamine, a non-selective muscarinic antagonist that induces cognitive impairments and reduces ACh release, similar to what is found in AD. Treatment with JNJ-10181457, a selective non-imidazole H3R antagonist, not only reversed cognitive deficits induced by scopolamine but also normalized ACh neurotransmission [63].

Sleep disturbs

Histamine has an important role in the control of sleep-wake regulation. Studying histaminergic TMN neurons of cats in different states, Vanni-Mercier reported a slow, but regular spontaneous firing during wake state, diminished in slow-wave sleep and absent firing during rapid eye movement [82]. Histidine decarboxylase gene knockout mice showed that impaired histamine synthesis prevents remaining in a wake state [83]. In agreement with that, patients with narcolepsy and idiopathic hypersomnia present reduced level of histamine in the cerebrospinal fluid (CSF) [84].

In rats, inverse agonism of H1R with ketotifen increased non-rapid eye movement (REM) sleep and decreased REM sleep, coinciding with significant prolongation of sleep duration and longer slow-wave sleep, suggesting a restful sleep [85]. In a model of stress by immobilization, the percentage for REM sleep is increased but is abolished in rats after administration of an H1R antagonist/inverse agonist, chlorpheniramine. The reduction of REM sleep caused by chlorpheniramine administration was significant even when compared to non-stressed control rats [53].

Recently, doxepin efficacy and tolerability were tested in elderly patients with chronic primary insomnia. During 4 weeks, 130 patients received 6 mg of doxepin nightly while 124 patients received placebo. According to patient self-report instruments and clinician ratings, the treatment was well tolerated and led to significant improvements in sleep maintenance and duration [55]. Another recent trial investigated the effects of doxepin in Parkinson's disease (PD) patients with insomnia. Non-pharmacological treatment, 10 mg of doxepin nightly and placebo were compared during 6 weeks. Sleep variables were improved in the doxepin-treated and in the non-pharmacological groups, with the doxepin treatment appearing to have more substantial clinical benefits. The authors do not claim for a superiority of pharmacological treatment and point to advantages of the non-pharmacological, such as absence of pharmacological side effects and potential long-lasting benefits after treatment [56].

Beneficial effects of histamine antagonists are also seen in narcoleptic patients that received 40 mg of tripolisant daily for a week. The H3R inverse agonist was considered safe, and no serious adverse effects were reported during the study. Despite the small population studied, the authors highlight that after treatment, patients presented a reduction in somnolence, compared to baseline, equivalent to results after several months of modafinil treatment [64]. The same drug was tested in four teenagers with narcolepsy-catalepsy that presented severe daytime sleepiness and were refractory to available treatments. Pitolisant decreased sleepiness with few minor adverse effects [86]. In positron emission tomography (PET) study, after calculating H3R occupancy, it was verified that AZD5213 is able to exert its wake-promoting action during daytime and does not disrupt sleep during the night. This is consequence of its pharmacokinetics that allows circadian fluctuations of H3R occupancy. Thus, patients would benefit from the procognitive effects of H3R without presenting sleep disruption [87].

Schizophrenia

Schizophrenia (SCH) is a heterogeneous disorder with strong genetic influence, highly prevalent, affecting 1.1% of the US population [88]. The involvement of the dopaminergic system in the pathophysiology of this disorder is well known, with antipsychotics acting mainly on D_2 receptor [89]. However, a role for the histaminergic system has been proposed, and several novel pharmaceutical targets are acting on both dopaminergic and histaminergic systems [90]. Iwabuchi and colleagues evaluated the distribution of H1R in the brains of medicated schizophrenic patients and normal human subjects but found no discrepancy between the groups. They also calculated the binding potential of the receptor by PET and doxepin, a radioligand for H1R, and noted that the value of the binding potential was particularly lower in the frontal and prefrontal cortices and the cingulate gyrus of patients with SCH [51]. Analyzing postmortem brain samples of patients with SCH, Jin and colleagues found that medicated patients displayed lower H3R binding level in the hippocampal CA2 region. The prefrontal cortices of the same patients presented higher H3R radioligand binding than the controls', and this alteration was correlated with psychotic symptoms, indicating a role of H3R in modulation of cognition [91]. Recently, it was reported that a H4R polymorphism may be a molecular marker for the prediction of risperidone efficacy [92].

In the 1990s, the effect of an H2R antagonist, commonly used in the treatment of peptic ulcer, was evaluated in patients with SCH. During 3 weeks, in an open-label trial, ten patients received 20 mg twice a day of famotidine without interrupting their treatment with conventional antipsychotics. Famotidine led to significant reduction in the scores of Brief Psychiatric Rating Scale (BPRS) and Clinical Global Impression (CGI), suggesting its administration as a useful alternative for SCH treatment [58]. Later, another open-label trial was performed with 18 patients receiving 100 mg of famotidine daily, during 3 weeks. Significant improvements were found after measurements with BPRS, CGI, and the Schedule for the Assessment of Negative Symptoms (SANS) [93]. Recently, a randomized clinical trial for famotidine was performed with 30 patients with SCH, 16 patients received 100 mg of famotidine twice daily and 14 received placebo. Famotidine caused no significant adverse effects, and it led to great reduction in symptoms for both the Positive and Negative Syndrome Scale and CGI [59].

In an animal model of SCH, impaired prepulse inhibition was enhanced in the DBA/2 mouse after inverse agonism of H3R with thioperamide and ciproxifan [68]. The use of ABT-239 and A-431404, also non-imidazole H3R antagonists, attenuated cognitive deficits caused by ketamine and MK-801 in rats, showing better results than antipsychotics, olanzapine, and risperidone, also used in the study, to treat these deficits [50]. Antagonists/inverse agonists of H3R have also shown to possess antioxidant activity, which could supplement antioxidant needs of SCH [94]. Despite the promising results obtained in preclinical models, when ABT-288 was tested in a randomized trial, it failed in providing cognitive improvements to patients with SCH. In addition, this treatment was associated with sleep disruption [62]. Other reports of disappointing results regarding the use of H3R antagonists in the treatment of disorders, such as attention-deficit hyperactivity disorder (ADHD), raise questions about the practicability of these drugs on the translational level [65].

Weight gain is a common side effect associated with some antipsychotic agents that may affect adherence to treatment [95]. Recently, the use of H3R antagonists was investigated as an alternative to attenuate weight gain caused by olanzapine in patients with SCH. A combination of reboxetine, a selective norepinephrine reuptake inhibitor, and betahistine, a potent antagonist of H3R was tested in a double-blind placebo-controlled study. The combination of 4 mg of reboxetine and 48 mg of betahistine was given daily, for 6 weeks, to 29 patients treated with olanzapine. Placebo was given to 14 patients also treated with olanzapine. The combination of reboxetine/betahistine resulted in significantly less weight gain compared to the placebo group [66].

Addiction

It is known that alterations in different neurotransmission systems, such as glutamate system dysfunction, interaction between serotonin transporter and serotonin receptor 1B genes polymorphisms, and dopamine-beta hydroxylase polymorphism have been associated with drug dependence [96-98]. The histaminergic system might as well be involved in modulation of behaviors associated with addiction. A polymorphism of HNMT gene was found in abundance in alcoholics from two different populations. Higher levels of the enzyme HNMT may lead to decreased levels of histamine and the low levels of this amine could be linked to an anxious behavior, since the patients with the polymorphism also displayed higher harm avoidance, a dimensional rating of anxious personality. Vulnerability to alcohol dependence is commonly associated with anxious behavior. In animals, it was demonstrated that stimulation of H1R modulates an anxiogenic effect, while H2R does the opposite. The authors speculate that the carriers of the polymorphism may present low levels of histamine in the amygdaloid nuclei, a structure associated with anxiety and with high density of H2R [99].

Nuutinen and colleagues showed that when alcohol solution and plain water were offered to mice lacking H3R in a two-bottle choice procedure, these animals would drink less alcohol solution, compared to control animals. Also, in animals without the H3R, alcohol did not generate

a rewarding effect, as well as not impaired motor coordination [100]. Similar results were obtained when rats were treated with JNJ-39220675. After 3 days of deprivation, rats were exposed to alcohol again 15 min after receiving a subcutaneous injection of JNJ-39220675. The animals displayed reduced intake of alcohol after a period of abstinence, just when the urge for drinking is enhanced [67]. Clinical studies should be performed to assess potential benefits and risks of these drugs to treat alcoholic patients.

Parkinson's disease

Reports of higher levels of histamine in the blood of patients with PD and in postmortem studies indicate a role for the histaminergic system on this disease [101]. The histaminergic innervation in the middle portion of substantia nigra pars compacta and reticulata was increased in the brains of patients with PD, which may be due to a compensatory event caused by deficiency of dopamine or a putative fiber growth inhibitory factor [102]. In postmortem brain samples of patients with PD, histamine concentrations were significantly increased in the putamen, substantia nigra pars compacta, internal globus pallidus, and external globus pallidus when compared to age-matched controls. This is probably a consequence of reduced metabolism, since the concentrations of tele-methylhistamine, a histamine metabolite, were unchanged [103]. Other study reported a significant decrease in the expression of H3R mRNA and a significant increase of HNMT mRNA expression, both in the substantia nigra of patients with PD. The disease duration (years between diagnosis and death) was negatively correlated with HNMT mRNA expression [104].

In a rat model of PD, obtained by brain lesion after bilateral *icv* administration of 6-hydroxydopamine, high levels of histamine were identified. Increased locomotor activity caused by the lesion and stereotyped behavior promoted by injection of apomorphine, were attenuated by administration of thioperamide, a H3R antagonist. Antagonists of H1R and H2R were also tested, but the symptoms caused by the lesion were not attenuated [69].

Pain

The histaminergic system has a role in nociception, with histaminergic neurons projecting to the dorsal raphe nucleus and dorsal horn of the spinal cord. The H3R antagonists GSK189254 and GSK334429 demonstrated to be promising therapies for the treatment of neuropathic pain, since animals treated with these drugs presented a decrease in paw withdrawal threshold in the chronic constriction injury and varicella-zoster virus models [70]. In addition, GSK189254 produced antinociceptive effects in the model of monoiodoacetate induced osteoarthritic pain and in a spinal nerve ligation model of neuropathic pain [71].

Antinociceptive properties of H4R antagonists were also detected. JNJ7777120 was tested in four different strains of mice in order to verify its efficacy in a model of croton oil-induced ear inflammation. Reduction in ear edema and significant anti-inflammatory effects were detected in the animals treated with this drug [74]. In other study, after repeated administration, JNJ7777120 demonstrated an anti-hyperalgesic action in a model of neuropathic pain. It is not clear how this effect is produced, although JNJ7777120 possesses anti-inflammatory properties, it is also possible that a central effect is produced due to its capacity to cross the BBB [75]. Clinical studies are currently ongoing in order to evaluate the therapeutic potential of H4R antagonist in other inflammatory diseases, due to the involvement of H4R in immune regulatory functions, including chemotaxis and cytokine secretion [76].

Perspectives

Over the last years, the role of histaminergic system has been studied in the pathophysiology of different brain disorders. Progress in this field of study has been made, making it possible to investigate different pharmacological approaches in order to treat or ameliorate symptoms. Autism, a neurodevelopmental disorder, affects 1 in 68 children in the US and has not a clear etiology or specific biomarkers [105]. Literature presents scarce data about the histaminergic system in autism (Table 2), but the use of an H2R antagonist has been already proposed and tested in patients. Symptoms like irritability, hyperactivity, and atypical pattern of eye contact were attenuated after treatment with famotidine [57,106].

There is evidence that H3R is downregulated in Fragile X syndrome patients, a condition that is strongly associated with autism [111]. In addition, animals exposed to phencyclidine (PCP) develop behavioral impairments, including low interest in social novelty, which is a feature present also in autism. In this experiment, animals exposed to PCP spent 3.5 less time investigating a novel subject than the control group. The administration of a H3R antagonist/inverse agonist, SAR110894, normalized this impairment [72]. Recent data points to an involvement of the histaminergic system in the pathophysiology of Tourette's syndrome, a condition common among patients with autism. A premature termination codon (W317X) in the histidine decarboxylase gene was detected in patients, implying that diminished histaminergic neurotransmission could be related to the outcomes of this syndrome [114].

Since it is likely that the histaminergic system may play a role in SCH and Tourette's syndrome, disorders that have substantial symptomatic overlap with autism,

Table 2 Studies involving autism and histamine

Year	Author	Number of patients	Number of controls	Main results
1972	Neville et al.	7	-	Elevated plasma histidine and low skin histidase levels [107].
1979	Kotsopoulos and Kutty	1	-	Patient with autism presented histidine blood levels seven times higher than the upper normal values [108].
1988	Launay et al.	22	22	Histamine levels in urine and whole blood or plasma of patients with autism did not differ from age- and sex-matched controls [109].
1999	Rossi et al.	25	-	Niaprazine (H1R antagonist) showed a positive effect on hyperkinesias, unstable attention, resistance to change and frustration, mild anxiety signs, hetero-aggressiveness, and sleep disorders [110].
2001	Linday et al.	9	-	Behavioral improvement in children treated with Famotidine (H2R antagonist) [57].
2010	Rosales-Reynoso et al.	10	10	Downregulation of H3R in patients with Fragile X syndrome, subjects that usually meet diagnostic criteria for autism [111].
2012	Ming et al.	48	53	Reduced urinary levels of histidine and other amino acids [112].
2013	Naushad et al.	138	138	When compared to normal controls, autistic children showed elevated levels of histidine (58 +/− 15 vs. 45 +/− 21 micromol/L) [113].

we think that further investigation should be made to characterize this system in autism. The animal model of autism based on prenatal exposure to valproic acid shows neuroanatomical, behavioral, and biochemical alterations that recapitulates the core characteristics of autism [115], which makes it a reliable tool for studying a likely involvement of the histaminergic system in this disorder.

Concluding remarks

Initially described as part of immune and gastrointestinal systems, the presence of histamine and its four described receptors in the CNS have been related to normal and/or abnormal behavior. As a result, a growing amount of information regarding the relationship between histamine and brain is continuously arising from both experimental and clinical fields of research.

Based on preclinical data, different antagonists from histamine receptors have been considered promising therapies for brain disorders. On the other hand, more clinical studies are still required to verify practicability of these drugs. We believe that in-depth investigation involving the histaminergic system and its potential therapeutic targets in other disorders, such as autism, should be performed. Efforts in both preclinical and clinical research will lead to reaching clinically useful and safe treatments.

Abbreviations

H1R: histamine H1 receptor; H2R: histamine H2 receptor; H3R: histamine H3 receptor; H4R: histamine H4 receptor; CNS: central nervous system; HNMT: histamine *N*-methyltransferase; TMH: transmembrane helices; TMN: tuberomamillary nucleus; GPCR: G protein-coupled receptors; BBB: blood brain barrier; AD: Alzheimer's disease; SCH: schizophrenia; PD: Parkinson's disease; ACh: acetylcholine; PCP: phencyclidine; PET: positron emission tomography; BPRS: brief psychiatric rating scale; ADHD: attention-deficit hyperactivity disorder; CGI: Clinical Global Impression.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DB, TG, KC, and GZ wrote and edited the manuscript. CG and RR provided the conceptual input and edited the manuscript. All authors participated in the discussion and approved the final submitted version.

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7. ARTIGO 2

PLOS ONE

RESEARCH ARTICLE

Effects of an H3R Antagonist on the Animal Model of Autism Induced by Prenatal Exposure to Valproic Acid

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Abstract

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders primarily characterized by impaired social interaction and communication, and by restricted repetitive behaviors and interests. Ligands of histamine receptor 3 (H3R) are considered potential therapeutic agents for the treatment of different brain disorders and cognitive impairments. Considering this, the aim of the present study is to evaluate the actions of ciproxifan (CPX), an H3R antagonist, on the animal model of autism induced by prenatal exposure to valproic acid (VPA). Swiss mice were prenatally exposed to VPA on embryonic day 11 and assessed for social behavior, nociceptive threshold and repetitive behavior at 50 days of life. The treatment with CPX (3 mg/kg) or saline was administered 30 minutes before each behavioral test. The VPA group presented lower sociability index compared to VPA animals that were treated with CPX. Compared to the Control group, VPA animals presented a significantly higher nociceptive threshold, and treatment with CPX was not able to modify this parameter. In the marble burying test, the number of marbles buried by VPA animals was consistent with markedly repetitive behavior. VPA animals that received CPX buried a reduced amount of marbles. In summary, we report that an acute dose of CPX is able to attenuate sociability deficits and stereotypies present in the VPA model of autism. Our findings have the potential to help the investigations of both the molecular underpinnings of ASD and of possible treatments to ameliorate the ASD symptomatology, although more research is still necessary to corroborate and expand this initial data.

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Introduction

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders featured by impaired social interaction and communication, and by restricted repetitive behaviors and interests [1]. The pathophysiology of ASD is poorly understood, but evidence indicates that a strong genetic component and the environment act in concert as triggering factors [2,3].

The use of valproic acid (VPA) during pregnancy is an environmental risk factor highly associated with increased incidence of ASD in children [4]. Based on this observation, an animal model of this disorder was proposed, which consists of prenatally exposing rodents to this teratogen [5,6]. The VPA animal model of autism shows neuroanatomical, behavioral, and biochemical alterations that replicate the core characteristics and main comorbidities of patients with ASD [7].

Histamine acts as a transmitter in the central nervous system (CNS) and modulates distinct physiological processes like circulatory functions, innate and acquired immunity, cell proliferation and hematopoiesis [8]. In the last years, there has been a growing interest in the study of histamine in the CNS and its influence on behavior in both physiological conditions and brain disorders [9–11]. Thus, the histaminergic system is an interesting pharmacological target for therapeutic purposes and many efforts have been made to develop drugs that could act on different histamine receptors (H1R, H2R, H3R and H4R) [12].

There are few studies in the literature related to the use of histamine receptor antagonists to treat autistic behavior. In 1997, it was proposed that famotidine, an antagonist of H2R, would be a potential treatment for children with ASD [13]. This proposal was based on a report of a patient with schizophrenia (SCH), a disorder that shares symptoms and genetic factors with ASD [14,15], that showed improved sociability after this treatment [16]. Later, famotidine was tested in a group of children with ASD and 44% of them presented evidence of behavioral improvement. It is noteworthy that children with marked stereotypy did not respond to the treatment [17]. In addition, niaprazine, a H1R antagonist, was also tested in patients with ASD and led to amelioration of symptoms such as unstable attention, resistance to change and frustration [18].

Ligands of H3R are also considered potential therapeutic agents for the treatment of brain disorders, such as Alzheimer's disease, SCH, and narcolepsy [19–21]. In an animal model of SCH, the use of an H3R antagonist ameliorates behavioral impairments [22], including spatial working memory deficit, an abnormality also found in patients with ASD [23]. Recently, a study revealed that antagonism of H3R attenuates impaired social behavior in rodents exposed to phencyclidine (PCP), a finding that may also have implications for ASD [24].

Considering this evidence, we aimed to evaluate the actions of ciproxifan (CPX), an H3R antagonist, on the animal model of autism induced by prenatal exposure to VPA. Sociability and social novelty preference, nociceptive threshold and repetitive behavior were assessed to verify the behavioral outcomes triggered by CPX treatment.

Methods

Animals

Thirty-six female Swiss mice were obtained at 7–8 weeks of age from Federal University of Pelotas (Pelotas, Brazil) and were mated, with pregnancy confirmed by the presence of a vaginal plug on embryonic day 0 (E0). On E11, 18 pregnant females received a single intraperitoneal injection of 500 mg/kg VPA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline. Eighteen control females received an equal volume of saline. Four females died after injection of VPA. Sixteen pups from VPA-treated dams (VPA) and 16 pups from dams that received saline

(Control) were used in this study. Half of each group received an injection of CPX prior to each assay, while the rest received an injection of saline (SAL). One pup was randomly selected from each litter in order to avoid litter effects. The other animals from the litters that were not used in this work were killed and tissues were stored for future analysis. Animals were maintained in a standard 12-hour light/dark cycle, with controlled temperature (22±2°C) and free access to food and water.

Behavioral testing was performed between 9:00 am and 2:00 pm, in an order randomized by group, in the following sequence once the animals were 50 days old: three chambers test, tail-flick, and marble burying. All protocols were approved by the Animal Ethics Committee at the Clinical Hospital of Porto Alegre (HCPA) and were conducted in accordance with National Institutes of Health guidelines.

Treatment

CPX (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile 0.9% saline before the behavioral tests. Intraperitoneal injections of SAL or CPX (3 mg/kg) were given 30 minutes before onset of behavioral tests. Dosage was based on previous publications demonstrating efficacy in mice [25].

Behavioral tests

Three Chambers test

This sociability and social novelty test apparatus consists of an acrylic box with a total size of 628 × 456 × 220 (length, width, height, in millimeters) partitioned into three chambers. The openings between the compartments allow the animals to explore the three chambers. During an initial moment, an object was positioned in one of the lateral chambers, and a set animal+ object was placed in the opposite lateral chamber. This animal (novel mouse 1) was an experimentally naive male Swiss mouse with no previous contact with the testing animal. The object was an empty cage identical to the one used to enclose the novel mouse 1. Time spent in each chamber, as well as the time spent exploring the novel mouse 1 or the novel object, was analyzed by two observers during 10 min.

The social novelty test began immediately after the end of the sociability test. In this test, the novel mouse 1 remained in its wire cage (now it is called the known mouse) and a new unfamiliar mouse (novel mouse 2) was placed in the wire cage in the opposite side (which was previously empty). Time spent in each chamber and time spent exploring each wire cage was recorded during 10 min.

We evaluated a Sociability Index (SI), a mathematical equation designed to allow the direct comparison of social behavior of the groups. In an analogous manner, it is possible to evaluate preference for social novelty by calculating the Social Novelty Preference Index (SNI). The SI and SNI are calculated as showed below:

$$\frac{SI = (timeexploringnovelmouse\ 1 - timeexploringnovelobject)}{(timeexploringnovelmouse\ 1 + timeexploringnovelobject)}$$

$$\frac{SNI = (timeexploringnovelmouse\ 2 - timeexploringknownmouse)}{(timeexploringnovelmouse\ 2 + timeexploringknownmouse)}$$

Tail-flick test

Animals were gently restrained by hand, and radiant heat was directed onto its tail. Measurements were taken three times, with 30-second intervals, using a tail-flick analgesia meter (EFF 300L-Light, Insight, Brazil).

Marble burying test

Mice were acclimated individually in a cage filled with 4 cm of fresh bedding for 10 minutes. To minimize neophobia and novelty-induced anxiety, mice had been previously exposed to the testing room during the three chambers test and tail-flick test. Following acclimation, mice were removed from the cage, and 20 black marbles were placed equidistant in a 4×5 arrangement. Mice were then returned to the same cage for 10 minutes. Following the 10-minute testing period, two observers counted the number of marbles that were more than 50% covered with bedding. After each testing period, the marbles were cleaned with 70% ethanol.

Statistics

For the SI and SNI, marble burying and tail-flick tests statistical significance was assessed with a group (Control, VPA) × drug (SAL, CPX) ANOVA. Bonferroni post-tests were used to determine difference between individual groups.

The values measured for the animals in the three chambers test were integrated in a multivariate linear model to predict the impact of the treatment in the behavioral outcome. We used the Generalized Estimation Equations (GEE) in order to enable the comparison between multiple interdependent variables and overcome the necessity of normality and homoscedasticity. Two distinct analyses were performed, one for time in chambers and another for the interaction time. Group and chamber were considered independent variables and their influence over the dependent variable time was determined. Bonferroni post-test was used as the final evaluation.

All analyses were performed using the SPSS program, Version 20.0 (SPSS, Chicago, IL). The p values less than 0.05 were considered as statistically significant.

Results

Three chambers test

Sociability

The Control mice spent significantly more time exploring the novel mouse 1 than the novel object ($p<0.01$). In contrast, VPA mice showed no preference for the two stimuli, which could reflect decreased sociability. Interestingly, VPA animals treated with CPX spent significantly more time exploring the novel mouse 1 than the novel object ($p<0.01$) (Fig. 1A).

With exception of VPA animals treated with CPX, all the other animals spent less time in the central chamber than in the other chambers ($p<0.01$). The VPA mice treated with CPX spent significantly less time in the central chamber when compared to the novel mouse 1 chamber ($p<0.01$), but no difference was detected when the time spent in the central chamber was compared to the time spent in the novel object chamber ($p>0.05$). Control animals that received SAL and CPX spent significantly more time in the novel mouse 1 than in the novel object chamber (SAL: $p<0.01$; CPX: $p<0.01$), while the time spent by the VPA group in either chamber was not significantly different. After CPX treatment, VPA-exposed animals spent significantly more time in the novel mouse 1 chamber than in the novel object chamber ($p<0.05$) (Fig. 1B).

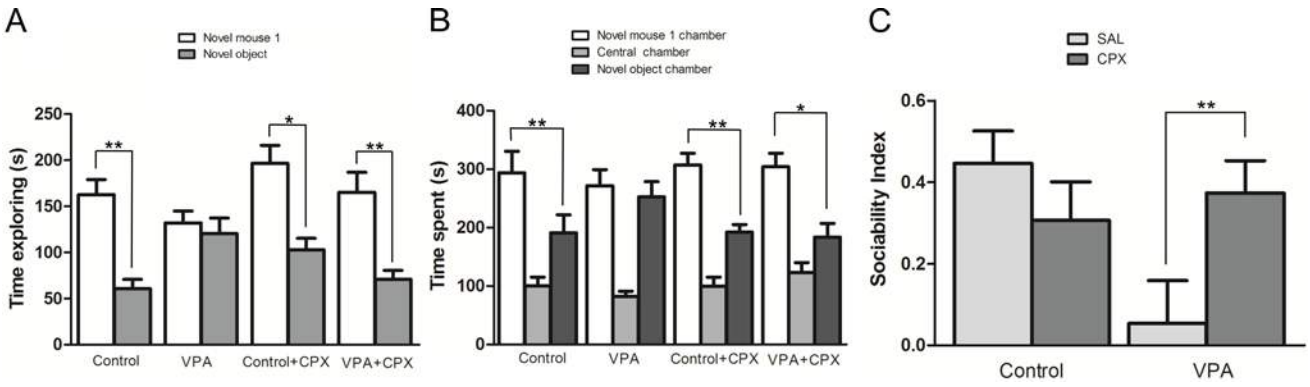

Figure 1. Sociability assessed by the three chambers test. After 5 minutes of acclimatization, male subjects were allowed to explore all chambers for 10 min. With the results obtained in the sociability assessment, the sociability index was calculated as the ratio of the difference to the sum of time spent exploring the novel mouse 1 and the time spent exploring the novel object. **(A)** Time spent exploring novel mouse 1 or novel object. **(B)** Time spent in chambers. **(C)** Sociability Index. Figures show mean ± SEM. (*p<0.05, **p<0.01). Control (n = 8), VPA (n = 8), Control +CPX (n = 8), VPA+CPX (n = 8).

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Regarding the SI, there was a significant group by drug interaction ($F(1, 28) = 6.55$, $p = 0.016$). No significant difference was detected between VPA and Control groups ($F(1, 28) = 3.25$, $p = 0.082$). However, when the SI of VPA animals that received only the SAL was compared to Control animals in the same conditions, a significant difference was detected ($p<0.01$, Bonferroni post-test). The SI of VPA mice treated with CPX was significantly higher than the SI of VPA animals that received only SAL ($p<0.05$, Bonferroni post-test). Treatment with CPX had no effect on Control animals (Fig. 1C).

Social novelty preference

Control mice spent significantly more time exploring the novel mouse 2 than the known mouse ($p<0.01$). In contrast, VPA animals spent similar time exploring the two stimuli. Unlike what happened in the sociability assessment, Control and VPA animals treated with CPX spent similar time exploring the known and novel mouse 2 (Fig. 2A).

Control and VPA animals spent significantly less time in the central chamber than in the other chambers ($p<0.01$). Control mice treated with CPX spent less time in the central chamber than in the novel mouse 2 chamber ($p<0.01$), but it was not statistically different from the

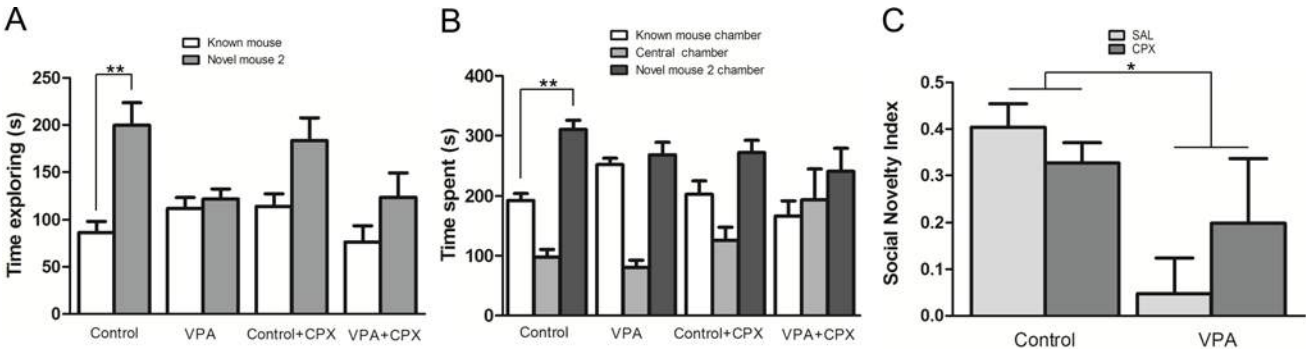

Figure 2. Social Novelty Preference assessed by the three chambers test. Right after the sociability assessment, male subjects were allowed to explore all chambers for 10 min. With the results obtained in the social novelty assessment, the social novelty index was calculated as the ratio of the difference to the sum of time spent exploring the novel mouse 2 and the time spent exploring the known mouse. **(A)** Time spent exploring known mouse or novel mouse 2 **(B)** Time spent in chambers. **(C)** Social Novelty Index. Figures show mean ± SEM. (*p<0.05, **p<0.01). Control (n = 8), VPA (n = 8), Control +CPX (n = 8), VPA+CPX (n = 8).

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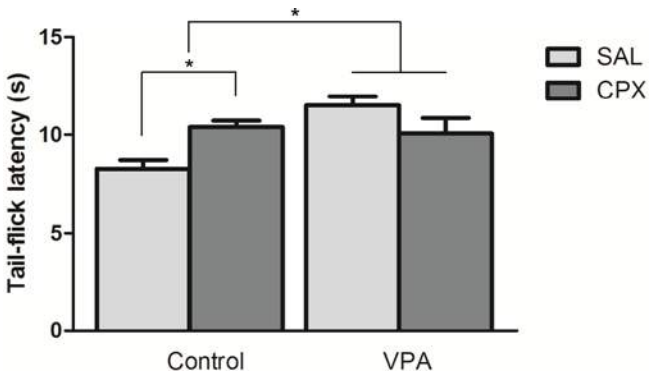

Figure 3. The effect of H3R antagonist CPX on thermal nociceptive threshold. Animals were restrained by hand and the nociceptive threshold was measured three times with intervals of 30 s, in order to calculate mean values. Figure shows mean ± SEM. (*p<0.05). Control (n = 8), VPA (n = 8), Control +CPX (n = 8), VPA+CPX (n = 8).

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time spent in the known mouse chamber. The time spent by VPA-exposed mice treated with CPX in the central chamber was not statistically different from the time spent in the other two chambers. The Control group spent significantly more time exploring in the novel mouse 2 than in the known mouse chamber ($p<0.01$). As expected, the time spent in both chambers by VPA mice did not differ statistically. Interestingly, CPX had no effect on the time spent by Control mice in the chamber with novel mouse 2 and in the one with the known mouse (Fig. 3B). In the SNI, Control mice presented a higher score than VPA animals ($F(1, 28) = 6.01$, $p = 0.021$). There was no significant group by drug interaction ($F(1, 28) = 2.20$, $p = 0.148$). Treatment with CPX had no effect on VPA-exposed animals ($p<0.05$, Bonferroni post-test) (Fig. 2C).

Tail-flick test

Animals from the VPA group presented a higher nociceptive threshold than the Control group ($F(1, 28) = 7.25$, $p = 0.012$). There was a significant group by drug interaction ($F(1, 28) = 11.19$, $p = 0.002$). Treatment with CPX enhanced the nociceptive threshold of Control mice ($p<0.05$, Bonferroni post-test). No effect was detected in VPA animals after treatment with CPX (Fig. 3).

Marble Burying test

We assessed the effect of CPX on marble burying activity (Fig. 4). The VPA group buried significantly more marbles than the Control ($F(1, 28) = 6.12$, $p = 0.020$), consistent with an increase in repetitive behavior. There was a significant group by drug interaction ($F(1, 28) = 9.73$, $p = 0.004$). Marble burying was significantly reduced in VPA-exposed mice treated with CPX compared to VPA animals that received only SAL ($p<0.01$, Bonferroni post-test). However, no difference was detected between Control animals treated with SAL and treated with CPX.

Discussion

The H3R antagonists are considered promising alternative treatments for different brain disorders, such as SCH, Alzheimer's disease and narcolepsy [19]. The present study investigated, for the first time, the effects of the H3R antagonist CPX on a mouse model of autism based on

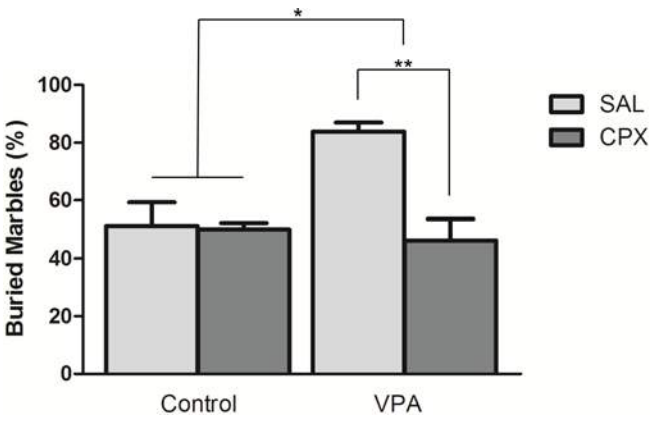

Figure 4. The H3R antagonist CPX attenuates elevated repetitive behavior in mice exposed to VPA in utero. Repetitive marble burying behavior was measured after a 10 minute testing session. VPA mice demonstrated elevated stereotyped, repetitive behaviors that were significantly reduced by CPX. Figure shows mean ± SEM. (*p<0.05, **p<0.01). Control (n = 8), VPA (n = 8), Control +CPX (n = 8), VPA+CPX (n = 8).

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prenatal exposure to VPA. We demonstrated CPX efficacy in attenuating impaired social behavior and stereotypies in VPA mice.

In the three chambers test, sociability is the propensity to spend time exploring an unfamiliar animal, as compared to time spent exploring an object. Treatment with CPX normalized the impaired sociability displayed by animals from VPA model, since these animals presented a SI similar to Control group. Most of the studies about H3R antagonist and social behavior are focused on social memory [26], a parameter that is also altered in ASD [27], and this experiment is the first one to assess the effects on sociability. The mechanism by which this improvement was acquired is not clear, but it might be involved with the capacity of H3R antagonists to mediate the release of different neurotransmitters besides histamine, such as dopamine, serotonin and acetylcholine, in specific brain areas [28]. Assessing the levels of different neurotransmitters in brain structures of the VPA model, as well as in VPA animals treated with H3R antagonists, would help to understand which neural circuits could be involved in this behavioral improvement.

No effect of CPX was detected in the social novelty assessment. That was unexpected, since CPX is able to improve object recognition in chronically stressed rats, abolishing the memory deficits [29]. In an opposite manner, it was demonstrated that when H3R is activated by the agonist imetit, consolidation of object recognition memory is impaired [30]. In addition, H3R antagonist treated rats, of an animal model of SCH induced by PCP exposure, tend to pursue social novelty. In this particular study, control rats presented a novelty discrimination index (ratio of the time spent investigating an unfamiliar subject divided by the time spent investigating the familiar one) 3.5 times greater than adult rats neonatally pretreated with PCP. Treatment with SAR110894, a H3R antagonist, dose-dependently normalized this altered behavior [24].

The difference between Griebel et al. results and ours, regarding social novelty, could be explained by different reasons. *1*) the discordance in the method used to evaluate the preference for social novelty (Griebel et al. used a sequential presentation of known and novel animal, instead of the three chambers test); *2*) the differences in rodent species (Griebel et al. tested rats); *3*) differences between the actions of SAR110894 and CPX at the given doses and *4*) divergence between the physiological mechanisms underlying the social novelty discrimination deficits in these two models. In this regard, it is interesting to note that the social novelty

discrimination impairments in VPA animals could be mediated by factors other than cognitive deficits. For example, VPA-exposed animals seem to present olfactory deficits at postnatal day 10 that could impair olfactory learning, which is important for social recognition [31].

The fact that histamine reduces nociceptive transmission when injected into the brain has been known for some time [32]. This was a reason to believe that enhanced histamine release mediated by H3R antagonists could lead to an analgesic effect. Factors like the dose utilized and how the drug is delivered play a determinant role in the efficacy of H3R antagonists on modulating nociceptive threshold. Central application of H3R antagonist thioperamide increased nociceptive threshold in a partial nerve-ligation model, while systemic application reduced it [33]. In addition, low systemic doses (1–5mg/kg) of thioperamide were not able to produce an analgesic effect in mice on the hot plate test [34,35]. On the other hand, higher doses (5–30 mg/kg) of the same drug were able to increase the nociceptive threshold [36,37].

Recently, CPX and pitolisant, another H3R antagonist, were tested in mice to evaluate thermal nociceptive thresholds. A high dose of pitolisant increased the thermal pain threshold, while CPX (10 mg/kg) produced no effect. The authors suggested that this effect of pitolisant on heat responses was independent of H3R [38]. In the present study, we tested a lower dose of CPX (3 mg/kg), and different effects were detected on the tail-flick test in Control and VPA groups. Contradictory results are found in the literature about pain sensitivity and ASD, therefore, it is a consensus that not all children with ASD react the same way to pain. Generally, the VPA model of autism shows less sensitivity to pain [6], similar to what was found in this study.

The widespread distribution of H3R on fibers throughout the brain and spinal cord, mediating different physiological processes, and the different structures and pharmacokinetic properties of the H3R antagonists, might explain the contradictory findings regarding H3R antagonism and nociception.

In the 80's, the use of L-histidine, a precursor of brain histamine, modified the methamphetamine (MET)-induced stereotypy in mice [39]. Later, it was verified that treatment with an inhibitor of histamine synthesis, α-fluoromethylhistidine, would cause a contrary effect, enhancing stereotyped behavior [40]. Recently, a likely involvement of the histaminergic system in the pathophysiology of Tourette syndrome, a condition common among patients with ASD and featured by stereotypies, has been hypothesized. A premature termination codon (W317X) in the L-histidine decarboxylase (HDC) gene, the rate-limiting enzyme in histamine biosynthesis, was detected in patients with this syndrome, implying that diminished histaminergic neurotransmission could be related to the outcomes of this syndrome [41].

In the present study, VPA mice treated with CPX displayed a reduced repetitive behavior in the marble burying test. Stereotypy and behavior rigidity are widely known as core and defining features of ASD. There are contradictory reports about the efficacy of H3R antagonists on suppressing these impairments. It was reported that CPX (3 mg/kg) was able to suppress locomotor sensitization induced in mice by MET. In addition, sensitization by MET also led to a decrease of N-methyl-D-aspartate (NMDA)-receptor subunit 1 (NR1) mRNA in the cerebral cortex, hippocampus and striatum. The treatment with CPX restored the normal levels of NR1 mRNA [42]. Interestingly, knockout of NMDA receptors, including NR1, in parvalbumin interneurons generates autistic-like phenotypes [43]. On the other hand, no improvements or even exacerbation of hyperactivity were reported after treatment with H3R antagonists in stereotypies of rats [44].

Since it is clear that VPA negatively affects the glial and neuronal development in this model [45,46], and CPX was administered just prior to the behavioral testing, it is unlike that we detected a total reversal in the autistic-like behaviors. At this time point, many of the changes that occurred during the CNS development (such as the deficits in migration,

proliferation and network-establishment) reached a state of functional equilibrium and theoretically could not be easily modified. Nevertheless, a single application of CPX is already enough to improve behavioral deficits. This fact supports the hypotheses that at least some of the main clinical alterations present in ASD could be attenuated even in a late time stage.

In summary, we report that an acute dose of CPX is able to attenuate at least some sociability deficits and stereotypies present in the animal model of autism induced by VPA. More research is still necessary to corroborate and expand this initial data, and to contribute to generate a better understanding of ASD pathophysiology and management.

Author Contributions

Conceived and designed the experiments: DB CG RR. Performed the experiments: DB KC TG GMM. Analyzed the data: DB KC GDFN VB-J. Wrote the paper: DB GDFN VB-J CG RR.

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8. ARTIGO 3

Altered histamine content and H3R mRNA expression in the striatum of the VPA animal model of autism

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Abstract: Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder featured by deficits in sociability and communication, as well as by limited repertoire of interests and behaviors. Alterations in different neurotransmitter systems, such as serotonergic, dopaminergic, GABAergic and glutamatergic ones have been studied and reported in ASD. Surprisingly, the histaminergic system has received less attention and few studies are available on the literature about histamine and ASD. Our objective was to measure the concentration of histamine and the H3R and H4R mRNA levels in the brains of mice from the VPA model of autism. In addition, we used the H3R KO mice in the three chambers and marble burying tests in order to evaluate the role of this receptor in sociability and stereotypic behavior. A significant increase in H3R mRNA, as well as higher concentration of histamine were detected in striatal samples from the VPA mice. We also verified that H3R might influence the search for social novelty, a feature that is impaired in autism, as H3R KO mice displayed abnormal behavior when tested for this parameter. This is the first report of abnormalities in the histaminergic system of an animal model of autism. Further investigation is required to corroborate this initial data.

Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder featured by deficits in sociability and communication, as well as by limited repertoire of interests and behaviors. The pathophysiology of ASD is poorly understood, but prenatal exposure to teratogens, alcohol or viruses, associated with genetic factors, is related to high incidence of ASD in children [14, 15]. Prenatal exposure to valproic acid (VPA) has been used as a reliable model of autism in order to better understand the pathophysiology of this disorder [2]. It is estimated that ASD affects 1% of global population and its core impairment and associated morbidities represent elevated costs to public health [8, 20]. Despite a variety of abnormalities described in patients with ASD, including neuroanatomical, neurochemical and molecular alterations, no specific biomarkers are available for the diagnosis of the disorder.

Alterations in different neurotransmitter systems, such as serotonergic, dopaminergic, GABAergic and glutamatergic ones have been studied and reported in ASD. The roles of these systems in the central nervous system (CNS), besides synaptic interactions, include control of mechanisms of neural cell migration and positioning in the immature brain [9]. Surprisingly, the histaminergic system has received less attention and few studies are available on the literature about histamine and ASD [6].

Different brain disorders are featured by alterations in the histaminergic system [23]. Patients with Alzheimer's disease present reduced numbers of histaminergic neurons and decline in histamine levels, while increased levels of histamine are found in different brain structures of postmortem samples from patients with Parkinson's disease and altered distribution of histamine receptors was detected in schizophrenic patients [16, 28, 32]. In addition, after preclinical studies, the use of histamine receptor (H1R, H2R,

H3R and H4R) ligands have been proposed as new therapeutic options for these disorders [24].

Recently, it was reported that the use of ciproxifan, an inverse agonist of H3R, was able to attenuate behavioral impairments caused by prenatal exposure to VPA in mice [5]. However, no data is available regarding histamine concentration or expression of histamine receptors in the brain of the animal model or in autistic patients' postmortem samples. Evaluating a possible involvement of the histaminergic system on the pathophysiology of ASD could contribute to a better understanding of this disorder and give a hint of how histamine receptor inverse agonists may improve behavioral autistic-like features.

One of the hypotheses trying to explain the mechanisms ASD development is the involvement of immune components in the pathophysiology of this disorder [13]. Microglial activation in postmortem brain samples [34], family history of autoimmune diseases [33] and abnormal levels of inflammatory cytokines [27] are some of the immune disruption evidence found among patients with ASD. Although H4R brain expression is still discussed, the link between this receptor and many immune functions, such as chemotaxis and cell recruitment [11], upregulation of adhesion molecule expression [4] and modulation of cytokine and chemokine release [22], validates its study on the VPA model of autism, a disorder highly associated with alterations of the immune system.

Our objective was to measure the concentration of histamine and the H3R and H4R mRNA levels in the brains of mice prenatally exposed to VPA. Cortical, striatal, and hippocampal samples were used for these analyses. In addition, we used the H3R KO mice in the three chambers and marble burying tests in order to evaluate the role of this receptor in sociability and stereotypic behavior.

Methods

Experimental animals

-VPA model experiments

Swiss mice were obtained from Federal University of Pelotas (Pelotas, Brazil) and were mated, with pregnancy confirmed by the presence of a vaginal plug on embryonic day 0 (E0). On E11, pregnant females received a single intraperitoneal injection of 500 mg/kg VPA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline. Control females received an equal volume of saline. After birth, only one animal from each litter was utilized, in order to avoid litter effect. Pups were separated from the dams at 21 days of life. They were maintained in a standard 12-hour light/dark cycle, with controlled temperature (22±2 °C) and free access to food and water. Animals were killed by exsanguination after being anesthetized with isoflurane at 50 days of life. Cerebral cortex, striatum and hippocampus were removed, frozen in liquid nitrogen and stored at -80°C for qPCR and HPLC analysis. The Animal Ethics Committee at the Clinical Hospital of Porto Alegre (HCPA), under the protocol number 120433, approved this study.

-H3R KO mice

Adult male H3R KO mice were initially supplied by Johnson and Johnson Pharmaceutical Research and Development (La Jolla, CA), bred and maintained by the animal facility at the University of Helsinki. The H3R KO mice were generated on a background of 129/Ola and C57BL/6J mice. Selective backcrossing (10 times) followed by microsatellite marker analysis was conducted to generate a strain, which has at least 99.5% genetic identity to C57BL/6J mice. Males H3R KO (n= 7) and WT C57BL/6J (n= 7), at 50 days of life, were used in the three-chamber test and marble burying test. The Ethics Committee for Animal Experiments at the University of Helsinki approved these

experiments, which were performed in accordance with the laws of Finland and the European Union.

RNA isolation and cDNA synthesis

Total RNA was extracted using RNeasy mini Kit (Qiagen Inc., Valencia, CA) according to the instructions of the manufacturer. For tissue-specific expression, cerebral cortex, striatum and hippocampus from VPA and control mice were utilized. The amount of 0,5 microgram of total RNA was reverse-transcribed using SuperScriptTM III reverse transcriptase (Invitrogen, USA) according to the manufacturer's instruction. We utilized cortical, striatal and hippocampal samples from the VPA model (n =5) and from control Swiss mice (n= 5).

Quantitative real-time PCR (qPCR)

Real-time PCR was performed with a LightCycler 480 real-time PCR system and a LightCycler 480 SYBR green I master kit (Roche Applied Science). Primers for amplification were designed by Primer-BLAST (NCBI) and sequences are shown in Table 1. The peptidylprolyl isomerase A (PPIA) was used as reference control. Cycling parameters were as follows: 95 °C for 30 s and 45 cycles of the following, 95 °C for 10 s and 62 °C for 45 s. Fluorescence changes were monitored with SYBR Green after every cycle. Dissociation curve analysis was performed (0.2 °C per s increase from 60 °C to 95 °C with continuous fluorescence readings) at the end of cycles to ensure that only single amplicon was obtained. All reactions were performed in duplicates. Results were evaluated with the LightCycler 480 Software version 1.5. The data were calculated by the comparative method using Ct values of PPIA as the reference control. Cortical, striatal and hippocampal samples from the VPA model (n= 5) and from control Swiss mice (n= 5) were utilized.

Table 1 – Primers used for quantitative real-time PCR

GENE	FORWARD PRIMER (5'- 3')	REVERSE PRIMER (5'- 3')
mus musculus peptidylprolyl isomerase a	AGCATACAGGTCCTGGCATC	TTCACCTTCCCAAAGACCAC
mus musculus histamine receptor h3	TCAGCTATGACCGATTCCTG	TACTCCCAACTCAGGATGGC
mus musculus histamine receptor h4	GGAATCTGCATGTTTTGGCT	ATGATGCCAGTGTGTTGAGC

HPLC

Histamine was assayed by HPLC as described previously [29, 35]. Briefly, cerebral cortex, striatum and hippocampus were collected from Control and VPA mice. These samples were frozen on liquid nitrogen and stored at −80°C until further processing for HPLC. Samples were then homogenized by sonication in 2% perchloric acid, centrifuged for 30 min at 15,000 *g* at 4°C, and filtered through a 0.45-μm PVDF filter (Pall Life Sciences, Ann Arbor, MI, USA) before loading onto the HPLC system. The analytical HPLC system consisted of four Shimadzu LC20AD pumps, a Shimadzu SIL-20AC autosampler, a Shimadzu RF-10Axl fluorescence detector, a Shimazdu CBM-20A controller, and LCSOLUTION 1.21 software (Shimadzu, Kyoto, Japan). In order to normalize the results from HPLC measurement, we utilized the BCA kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) to measure protein concentration.

Behavioral tests

Three Chambers test

Males H3R KO (n= 7) and WT C57BL/6J (n= 7), at 50 days of life, were used in the three chambers test. As described previously [3], sociability and social novelty are measured with the test apparatus that consists of an acrylic box with a total size of 628 x

456 x 220 (length, width, height, in millimeters), partitioned into three chambers. The openings between the compartments allow the animals to explore the three chambers. During an initial moment, an object was positioned in one of the lateral chambers, and a set animal+object was placed in the opposite lateral chamber. This animal (novel mouse 1) was an experimentally naive male Swiss mouse with no previous contact with the testing animal. The object was an empty cage identical to the one used to enclose the novel mouse 1. The Ethovision software (Noldus Information Technology, Wageningen, the Netherlands) was used to measure time spent in each of the three chambers, as well as the time exploring the novel mouse 1 or the object during 10 minutes. After that, the social novelty test began. In this test, the novel mouse 1 remained in its wire cage (now it is called the known mouse) and a new unfamiliar mouse (novel mouse 2) was placed in the wire cage in the opposite side (which was previously empty). Time spent in each chamber and time spent exploring each wire cage was recorded during 10 minutes and analyzed with Ethovision. We evaluated a Sociability Index (SI), a mathematical transformation designed to allow the direct comparison of social behavior of the groups. In an analogous manner, it is possible to evaluate preference for social novelty by calculating the Social Novelty Preference Index (SNI). The SI and SNI are calculated as showed:

$$\frac{SI \;=\; (time\ exploring\ novel\ mouse\ 1 - time\ exploring\ novel\ object)}{(time\ exploring\ novel\ mouse\ 1 \;+\; time\ exploring\ novel\ object)}$$

$$\frac{SNI \;=\; (time\ exploring\ novel\ mouse\ 2 \;-\; time\ exploring\ known\ mouse)}{(time\ exploring\ novel\ mouse\ 2 \;+\; time\ exploring\ known\ mouse)}$$

Marble burying

The same animals utilized in the three chambers test were submitted to the marble burying test. Mice were acclimated individually in a cage filled with 4 cm of fresh

bedding for 10 minutes. Following acclimation, mice were removed from the cage, and 20 black marbles were placed equidistant in a 4X5 arrangement. Mice were then returned to the same cage for 10 minutes. Following the 10-minute testing period, it was counted the number of marbles that were more than 50% covered with bedding. After each testing period, the marbles were cleaned with 70% ethanol [5].

Statistics

For the qPCR, HPLC, SI and SNI, and marble burying test results statistical significance was assessed using the Student's t test. For the three-chamber test data, we used the Generalized Estimation Equations (GEE) in order to enable the comparison between multiple interdependent variables and overcome the necessity of normality and homoscedasticity. Two distinct analyses were performed, one for time that animals would spend in chambers and another for the time spend interacting with other animals or object. Group and chamber were considered independent variables and their influence over the dependent variable time was determined. Bonferroni post-test was used as the final evaluation. All analyses were performed using the SPSS program, Version 20.0 (SPSS, Chicago, IL). The *p* values less than 0.05 were considered as statistically significant.

Results

H3R, H4R and Histamine

H3R mRNA levels of VPA mice in the cerebral cortex and hippocampus were not different from those of Control mice ($p>0.05$). However, a significant increase in H3R mRNA levels was detected in the striatum of VPA mice ($p<0.05$) (Figure 1). No statistically significant differences between groups were detected in cerebral cortex when H4R mRNA was measured (Control: 0.001777 ± 0.0006385, VPA: 0.0006227 ± 0.0002518; $p>0.05$), striatum (Control: 0.00605 ± 0.003882, VPA: 0.003244 ± 0.001463; $p>0.05$) and hippocampus (Control: 0.002532 ± 0.001498, VPA: 0.000298 ± 0.0001325;

p>0.05). Histamine levels were assayed in homogenates of cortical, striatal, and hippocampal samples of Control and VPA mice. Compared to Control group, VPA animals presented higher concentration of histamine in the striatum ($p<0.05$). No difference between groups was detected in the other brain structures (Figure 2).

Three chamber test

Sociability

WT C57BL/6J and H3R KO stayed in the central chamber for only short periods of time, frequently less than 100 s. Thus, the animals were engaged in the exploration of the new environment and its content. The WT C57BL/6J mice spent significantly more time in the chamber with the novel mouse 1 than in the chamber with the object (316 ± 20s and 182 ± 12s, respectively; $p<0.05$). In addition, these animals explored more the novel mouse 1 than the object (192 ± 23s and 70 ± 8s, respectively; $p<0.05$). H3R KO displayed similar behavior, spending more time in the novel mouse 1 chamber than in the chamber with the object (335 ± 16s and 161 ± 20s, respectively; $p<0.05$) and interacting more with the novel mouse 1 than the object (252 ± 8s and 99 ± 12s, respectively; $p<0.05$). When the SI was calculated, no difference between WT C57BL/6J and H3R KO mice was detected (Figure 3).

Social Novelty

The WT C57BL/6J mice preferred the chamber with the novel mouse 2 to the chamber with the known mouse, indicating an interest in social novelty and/or the formation of social memory (306 ± 14s and 205 ± 15s, respectively; $p<0.05$). Accordingly, these animals spent more time exploring the novel mouse 2 than the known mouse (185 ± 22s and 97 ± 16s, respectively; $p<0.05$). The H3R KO mice did not show difference in the time spend in chambers with novel mouse 2 or known mouse (242 ± 17s and 238 ± 27s, respectively; $p=0.072$). In addition, no difference was detected when the

times exploring novel mouse 2 and known mouse were compared (143 ± 25s and 141 ± 21s, respectively; p=1.000). When the SNI was calculated, WT C57BL/6J mice achieved a higher score than H3R KO (Figure 4).

Marble burying test

In order to evaluate repetitive behavior, animals were submitted to the marble burying test. No significant difference was detected between H3R KO and WT C57BL/6J ($p>0.05$), both groups buried approximately 50% of the marbles (Figure 5).

Discussion

Considering the role of histamine in modulating several important physiological processes, and the reports of involvement of the histaminergic system in the pathophysiology of different brain disorders, including Alzheimer's disease, Parkinson's disease and schizophrenia, it makes sense to hypothesize a disturbance in this system in ASD [23]. Here we report abnormal H3R mRNA and histamine levels in the striatum of the VPA model of autism. We also verified that H3R might influence the search for social novelty, a feature that is impaired in autism, as H3R KO mice displayed abnormal behavior when tested for this parameter.

Histamine is responsible for modulating different behaviors, such as cognition, sleep and wakefulness, pain and emotional behavior. The release of this biogenic amine, as well as other neurotransmitters, is negatively regulated by activation of H3R, a presynaptic auto- and heteroreceptor [23]. In the present study, we detected a higher content of histamine in the striatum of VPA mice, but we also verified that H3R mRNA levels were higher in this structure. These were surprising findings, since H3R blockade, or its deficiency, increase histamine release; one could expect a reduced content of histamine in a structure that H3R mRNA is upregulated. However, this upregulation could be a compensatory change due to VPA effects at protein level. Additionally, it is

noteworthy that we cannot claim the exact source of the histamine detected in the brain structures of VPA mice. There is increasing evidence of immune involvement in ASD pathophysiology [13] and a role for histamine-containing mast cells cannot be discarded. Higher levels of the peptide neurotensin, a trigger of mast cell activation, and macrophage chemoattractant protein-1, a potent chemoattractant for mast cells, were detected in the serum of patients with ASD [1].

Superfusion of the ventral striatum with histamine enhances the release rate of acetylcholine in this structure [26]. In addition, the administration of H3R antagonists, which subsequently led to increased histamine release, produced the same effect on cholinergic activity. Histamine can also modulate striatal GABAergic activity. In fact, high densities of H3R are found in the GABAergic medium spiny neurons of the striatum. Activation of this receptor decreases GABA release by these cells. This would go in agreement with the several reports of deficient GABAergic activity in ASD [25]. Surprisingly, a liquid chromatography-mass spectrometry analysis (LC-MS) showed that BTBR T+ Itpr3tf/J (BTBR) mice, a model of autism, present lower levels of acetylcholine and do not differ from controls regarding histamine content in the prefrontal cortex [21].

Pharmacological studies with H3R inverse agonists, as well as the use of H3R KO animals, allowed a better understanding of the role carried out by this receptor in the CNS [24]. Spatial learning and memory, nociception and regulation of the sleep-wake cycle are some of the processes that involves H3R [31]. In the present study, VPA mice presented higher level of H3R mRNA in the striatum. The upregulation of H3R mRNA in different brain areas was associated with a neuroprotective effect after induction of ischemia and kainic acid-induced seizures in rats, probably caused by a subsequent constitutive signaling through the Akt/GSK-3B pathway [18, 19]. Hypoxic–ischemic necrosis of the striatum has been already described in association with ASD [7], and this

could lead to a postischemic regulation of H3R. Although the literature points to several alterations in different brain areas of patients with ASD, striatal abnormalities are particularly relevant due to their likely connection with one of the core symptoms of ASD. An increase in the growth rate of striatal structures for individuals with ASD is related to the severity of repetitive behaviors displayed by the same subjects [17].

Here we described that H3R KO mice presented normal sociability, but impaired search for social novelty. The use of Ciproxifan, an H3R inverse agonist, improved sociability deficits in VPA mice, but this treatment did not increase preference for social novelty [5]. An increase in histamine release is expected in both situations. Here we did not detect downregulation of H3R mRNA in any of the structures from VPA mice. As mentioned above, it is noteworthy that a discrepancy can exist between mRNA levels and protein expression. We also submitted these animals to the marble burying test, in order to evaluate repetitive behaviors, and no difference was detected in comparison to WT C57BL/6J.

Recently, it was reported that cultured microglia are able to express H4R, as well as the other three histamine receptors, and that the presence of histamine can dose-dependently upregulate its expression [12]. The low specificity presented by the available histamine receptor antibodies renders it difficult to detect H4R on the protein level [30]. Nevertheless, the literature presents some reports indicating the presence of mRNA of this receptor in human cerebellum and hippocampus [10], as well as in the hippocampus of CD1 mice [36]. No differences between groups were detected when H4R mRNA was measured in cerebral cortex, hippocampus and striatum.

In summary, we described higher levels of histamine in the striatum of VPA mice, as well as an upregulation of H3R mRNA in the same structure. A possible role of H3R on sociability can be hypothesized, since H3R KO mice displayed no interest for social

novelty during the three-chamber test. This is the first report of abnormalities in the histaminergic system of an animal model of autism. Further investigation is required to corroborate this initial data.

Figures

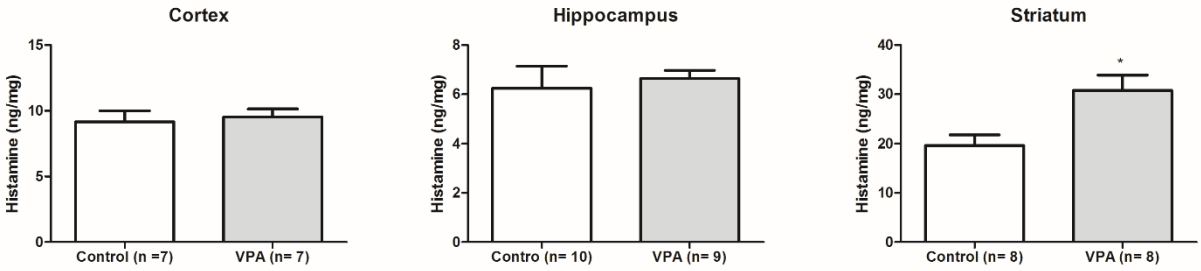

Figure 1 –Histamine content in different brain structures measured by HPLC. Cortical, hippocampal and striatal homogenates from Control and VPA mice were used as samples. VPA mice presented higher levels of histamine in the striatum than the Control animals (*p<0.05). Figures show mean ± SEM.

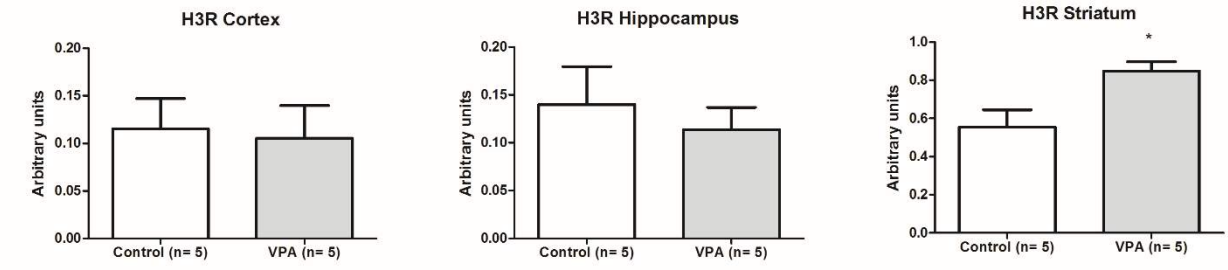

Figure 2 – H3R mRNA levels in in different brain structures measured by qPCR. An upregulation of H3R mRNA was detected in the striatum of VPA mice (*p<0.05). No difference s between groups were detected when cortical and hippocampal samples were analyzed. Figures show mean ± SEM.

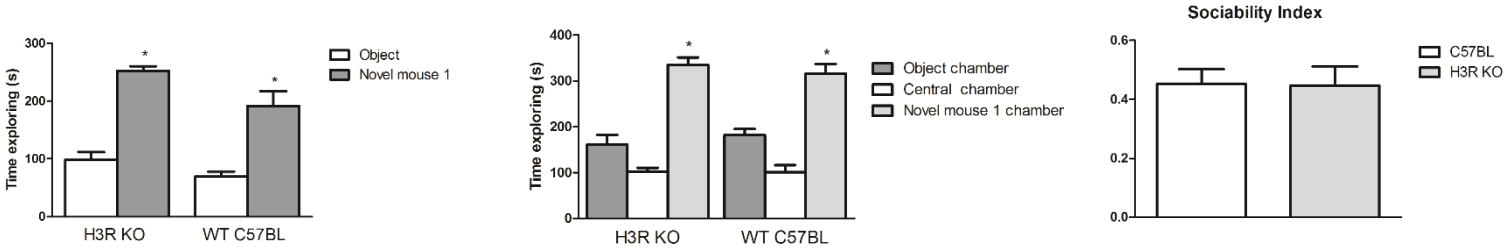

Figure 3 – Sociability was evaluated by the three-chamber test and Sociability Index. After 5 minutes of acclimatization, mice were allowed to explore all chambers for 10 min. With the results obtained in the sociability assessment, the sociability index was calculated as the ratio of the difference to the sum of time spent exploring the novel mouse 1 and the time spent exploring the novel object. (A) Time spent in chambers. (B) Time spent exploring novel mouse 1 or novel object. (C) Sociability Index. Figures show mean ± SEM. (*p<0.05, **p<0.01). H3R KO (n = 7), WT C57BL (n =7).

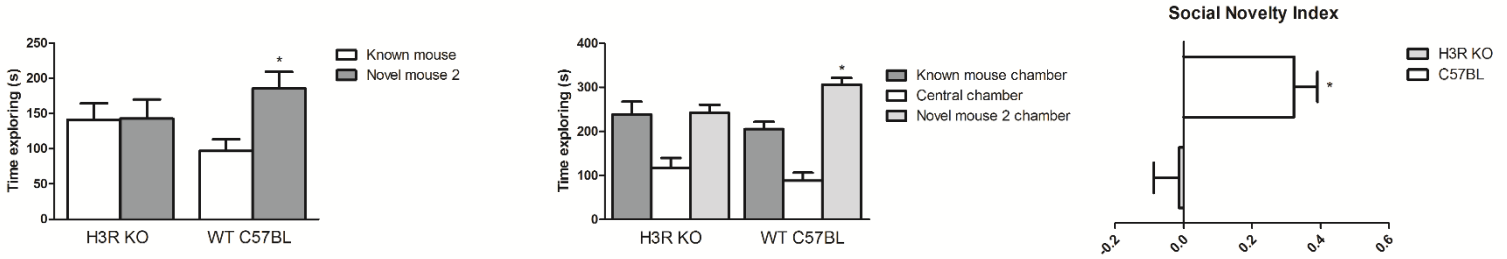

Figure 4 – Preference for Social Novelty was evaluated by the three-chamber test and Social Novelty Index. Right after the sociability assessment, mice were allowed to explore all chambers for 10 min. With the results obtained in the social novelty assessment, the social novelty index was calculated as the ratio of the difference to the sum of time spent exploring the novel mouse 2 and the time spent exploring the known mouse. (A) Time spent in chambers (B) Time spent exploring known mouse or novel mouse 2. (C) Social Novelty Index. Figures show mean ± SEM. (*p<0.05, **p<0.01). H3R KO (n = 7), WT C57BL (n =7).

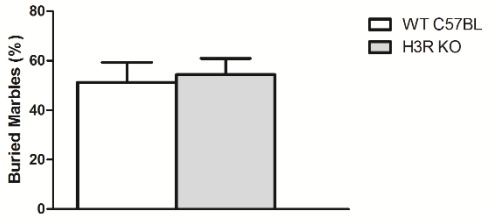

Figure 5 – Repetitive marble burying behavior was measured after a 10 minute testing session. H3R KO and WT C57BL mice demonstrated similar behavior, burying approximately 50% of the marbles. Figure shows mean ± SEM. H3R KO (n = 7), WT C57BL (n =7)

Níveis alterados de hitamina e RNAm do receptor H3R no estriado do modelo animal de autismo induzido por exposição pré-natal ao VPA

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Resumo: O transtorno do espectro do autismo é uma desordem do neurodesenvolvimento (TEA) é uma desordem do movimento caracterizada por déficits de sociabilidade e comunicação, bem como por um repertório limitado de interesses e comportamentos. Alterações em diferentes sistemas de neurotransmissores, como o serotoninérgico, dopaminérgico, GABAérgico e glutamatérgico foram estudadas e relatadas no TEA. Surpreendentemente, o sistema histaminérgico recebeu pouca atenção e poucos estudos sobre TEA e histamina estão disponíveis na literatura. Nosso objetivo foi mensurar a concentração de histamina e do RNAm dos receptores H3R e H4R em diferentes estruturas encefálicas do modelo VPA de autismo. Além disso, nós usamos camundongos H3R KO nos testes com aparato de três câmaras e *marble burying* para avaliar o papel desse receptor na sociabilidade e comportamento estereotipado. Um aumento significativo nos níveis de RNAm do H3R, bem como uma maior concentração de histamina, foi detectado no estriado dos camundongos VPA. Foi também verificado que o H3R pode influenciar na busca por novidade social, um comportamento que está prejudicado no TEA, já que camundongos H3R KO demonstraram comportamento anormal quando foram testados para este parâmetro. Esse é o primeiro relato de anormalidades no sistema histaminérgico de um modelo animal de autismo. Mais investigações são necessárias para corroborar esses dados iniciais.

Introdução

O transtorno do espectro do autismo é uma desordem do neurodesenvolvimento (TEA) é uma desordem do movimento caracterizada por déficits de sociabilidade e comunicação, bem como por um repertório limitado de interesses e comportamentos. A fisiopatologia do TEA é pouco compreendida, mas exposição pré-natal a teratógenos, álcool ou vírus, associada a fatores genéticos, está relacionada a alta incidência de TEA em crianças [14, 15]. Estima-se que o TEA afete 1% da população mundial e seus principais sintomas e morbidades associadas representam custos elevados à saúde pública [8, 20]. Apesar de uma variedade de anormalidades ter sido descrita em pacientes com TEA, incluindo alterações neuroanatômicas, neuroquímicas e moleculares, não existem biomarcadores disponíveis para diagnosticar esse transtorno. A exposição pré-natal ao ácido valpróico (VPA) tem sido utilizada como um modelo de autismo e têm contribuído para um maior entendimento sobre a fisiopatologia desse transtorno [2].

Alterações em diferentes sistemas de neurotransmissores, como o serotoninérgico, dopaminérgico, GABAérgico e glutamatérgico foram estudadas e relatadas no TEA. O papel desses sistemas no sistema nervoso central (SNC), além de interações sinápticas, inclui o controle de mecanismos relacionados à migração de células neurais e posicionamento do encéfalo imaturo [9]. Surpreendentemente, o sistema histaminérgico recebeu pouca atenção e poucos estudos sobre TEA e histamina estão disponíveis na literatura [6].

Diferentes desordens neurológicas são caracterizadas por alterações no sistema histaminérgico [23]. Pacientes com doença de Alzheimer apresentam número reduzido de neurônios histaminérgicos e redução nos níveis de histamina, níveis elevados de histamina são encontrados em diferentes estruturas encefálicas de amostras *postmortem* de pacientes com doença de Parkinson e distribuição alterada dos receptores

histaminérgicos foi detectada em pacientes com esquizofrenia [16, 28, 32]. Além disso, após estudos experimentais, o uso de ligantes dos receptores histaminérgicos (H1R, H2R, H3R e H4R) têm sido propostos como nova opção terapêutica para estas desordens [24].

Recentemente, foi relatado que o uso do ciproxifan, um antagonista do receptor H3R, foi capaz de atenuar alterações comportamentais causadas pela exposição pré-natal ao VPA em camundongos [5]. Contudo, não existem relatos disponíveis acerca da concentração de histamina ou expressão de receptores histaminérgicos nos encéfalos do modelo animal ou amostras *postmortem* de pacientes. Avaliar um possível envolvimento do sistema histaminérgico na fisiopatologia do TEA poderia contribuir para uma maior sobre esse transtorno e indicar como antagonistas dos receptores histaminérgicos podem melhorar características do tipo autista.

Uma das hipóteses que visa explicar os mecanismos de desenvolvimento do TEA é o envolvimento de componentes do sistema imunológico [13]. Microglia ativada em amostras encefálicas *postmortem* [34], histórico familiar de doenças autoimunes [33] e níveis anormais de citocinas inflamatórias [27] são algumas das evidências encontradas entre pacientes com TEA. Apesar da expressão do H4R no encéfalo ainda ser discutida, a ligação entre esse receptor e diversas funções imunológicas, como a quimiotaxia e mobilização celular [11], aumento da expressão de moléculas de adesão [4] e modulação da liberação de citocinas e quimiocinas [22], justifica seu estudo no modelo VPA de autismo um transtorno altamente associado com alterações no sistema imunológico.

Nosso objetivo foi mensurar a concentração de histamina e do RNAm dos receptores H3R e H4R em diferentes estruturas encefálicas do modelo VPA de autismo. Amostras de córtex, estriado e hipocampo foram usadas para essas análises. Além disso, nós usamos camundongos H3R KO nos testes com aparato de três câmaras e *marble*

burying para avaliar o papel desse receptor na sociabilidade e comportamento estereotipado.

Métodos

Animais experimentais

- Modelo VPA

Camundongos *Swiss* foram obtidos da Universidade Federal de Pelotas e acasalados, sendo que a gestação foi confirmada pela presença de tampão vaginal no dia embrionário 0 (E0). No E11, fêmeas prenhes receberam uma injeção intraperitoneal de 500 mg/kg de VPA (Sigma-ALdrich, St Louis, MO, USA) dissolvido e salina. Fêmeas controles receberam um volume equivalente de salina. Para evitar o efeito da ninhada, cada animal utilizado foi de uma ninhada diferente. Os animais foram mantidos em um ciclo claro/escuro de 12 horas com temperatura controlada (22°C) e livre acesso à comida e água. Amostras de córtex, estriado e hipocampo foram removidas, congeladas em nitrogênio líquido e armazenadas a -80°C para análises de qPCR e HPLC. Esse estudo foi aprovado pelo comitê de ética do Hospital de Clínicas de Porto Alegre, sob o número de protocolo 12-0433.

- Animais H3R KO

Camundongos adultos H3R KO foram adquiridos através da *Johnson and Johnson Pharmaceutical Research and Development* (La Jolla, CA), acasalados e mantidos na unidade de experimentação animal da Universidade de Helsinque. Os H3R KO derivam das linhagens de camundongo 129/Ola e C57BL/6J.

Extração de RNA e síntese de cDNA

O RNA total das estruturas encefálicas foi extraído de acordo com as orientações do fabricante do RNeasy mini kit (Qiagen, Hilden, Germany). As amostras de RNA foram armazenadas a -20ºC até o momento do uso. A síntese do cDNA foi realizada a partir de 1 µg das amostras encefálidas dos camundongos do modelo VPA, utilizando o kit *Superscript III Reverse Transcriptase* (Invirogen).

PCR em tempo real

A técnica de PCR em tempo real foi realizada com o sistema de PCR em Tempo Real *LightCycler 480* e *o Master kit LightCycler 480 SYBR green I* (Roche Applied Science). Os *primers* para amplificação foram desenhados através do *Primer-BLAST* (NCBI) e as sequências são mostradas na Tabela (1 no artigo em inglês e 3 na tese). A peptidil prolil isomerase A (PPIA) foi usada como controle. Os ciclos utilizados foram os seguintes: 95 °C por 30 s e 45 ciclos de 95 °C por 10 s e 62 °C por 45 s. A análise da curva de dissociação foi realizada (aumento de 0,2 °C por segundo, de 60 °C a 95 °C com leituras de fluorescência contínuas) ao fim dos ciclos para assegurar que apenas um amplicon foi obtigo. Todas as reações foram realizadas em duplicata. Os dados foram avaliados com o software *LightCycler 480*, versão 1.5.

HPLC

O conteúdo de histamina das amostras de córtex cerebral, estriado e hipocampo foi analisado por HPLC seguindo protocolo descrito anteriormente [29, 35]. As amostras foram homogeneizadas por sonicação com ácido perclórico 2%, centrifugadas por 30 minutos a 15,000 *g* numa temperatura de 4°C e filtradas através de um filtro PVDF de 0,45-µm (Pall Life Sciences, Ann Arbor, MI, USA) antes de serem avaliadas no sistema de HPLC. O sistema utilizado foi o Shimadzu LC20AD e o software de análise foi o LCSOLUTION 1.21 (Shimadzu, Kyoto, Japan). Para a normalização dos resultados da

análise de HPLC, foi utilizado o kit BCA (Thermo Fisher Scientific Inc., Rockford, IL, USA) para medir a concentração proteica.

Testes Comportamentais

Três câmaras

Neste teste foi utilizado um aparato com um tamanho total de 628 × 456 × 220 (comprimento, largura e altura em milímetros) dividido em três compartimentos para avaliação de sociabilidade e interesse por novidade social. As aberturas entre os compartimentos possibilitam que os animais os explorem. Primeiramente, os animais foram ambientados por 5 minutos no aparato. Após a ambientação, o teste de sociabilidade foi iniciado com uma gaiola vazia (objeto) posicionada em uma das câmaras laterais e uma gaiola com um camundongo *Swiss* (animal desconhecido 1) na câmara lateral oposta. O animal desconhecido 1 não teve nenhum tipo de contato prévio com o animal avaliado pelo teste. Durante 10 minutos foi cronometrado o tempo que o animal testado gastou explorando cada câmara, bem como interagindo com o objeto ou com o animal desconhecido 1. A avaliação do interesse por novidade social começou imediatamente após o final do teste de sociabilidade. O animal desconhecido 1 permaneceu em sua gaiola (a partir de agora é denominado como animal conhecido) e um novo animal desconhecido (animal desconhecido 2) foi colocado na gaiola da câmara oposta (que estava vazia anteriormente). O tempo que o animal testado gastou explorando cada câmara, bem como interagindo com o animal conhecido ou com o animal desconhecido 2 foi cronometrado por 10 minutos.

Marble burying

Os Camundongos foram ambientados individualmente, por dez minutos, em uma caixa com o fundo coberto por aproximadamente 4 cm de maravalha. Após a ambientação, os animais foram retirados da caixa e 20 bolinhas de gude foram

acomodadas em um arranjo 4x5 em distâncias iguais. Após os 10 minutos de duração do teste, dois observadores contatam o número de bolinhas de gude que estavam cobertas em mais de 50% pela maravalha. Após cada teste, as bolinhas eram limpas com álcool 70%.

Resultados

H3R, H4R e Histamina

Os níveis do RNAm do H3R nos camundongos no córtex cerebral e hipocampo do grupo VPA não foram diferentes dos camundongos controles ($p>0,05$). Contudo, um aumento significativo nos níveis do RNAm do H3R foi detectado no estriado dos camundongos VPA ($p<0,05$) (Figura 1, versão em inglês). Não houve diferença estatisticamente significativa entre os grupos quando o RNAm do H4R foi analisado no córtex cerebral (Controle: 0,001777 ± 0,0006385, VPA: 0,0006227 ± 0,0002518; $p>0,05$), estriado (Controle: 0,00605 ± 0,003882, VPA: 0,003244 ± 0,001463; $p>0,05$) e hipocampo (Controle: 0,002532 ± 0,001498, VPA: 0,000298 ± 0,0001325; $p>0,05$). Os níveis de histamina foram analisados em amostras homogeneizadas do córtex cerebral, estriado e hipocampo de camundongos controles e VPA. Comparados ao grupo controle, animais VPA apresentaram maior concentração de histamina no estriado ($p<0,05$). Não foi detectada diferença entre os grupos em outras estruturas cerebrais (Figura 2, versão em inglês).

Teste três câmaras

Sociabilidade

WT C57BL/6J e H3R KO permaneceram na câmara central por curtos períodos de tempo, frequentemente menos que 100 s. Assim, os animais ficaram mais envolvidos na exploração do novo ambiente e seu conteúdo. Os camundongos WT C57BL/6J passaram significativamente mais tempo na câmara com o camundongo novo 1 que na câmara com o objeto (316 ± 20s e 182 ± 12s, respectivamente; $p<0,05$). Além disso, esses animais exploraram mais o camundongo novo 1 que o objeto (192 ± 23s e 70 ± 8s, respectivamente; $p<0,05$). Camundongos H3R KO apresentaram comportamento semelhante ao WT C57BL/6J, passaram mais tempo na câmara do camundongo novo 1 que na câmara com o objeto (335 ± 16s e 161 ± 20s, respectivamente; $p<0,05$) e interagindo mais com o camundongo novo 1 que o objeto (252 ± 8s e 99 ± 12s, respectivamente; $p<0,05$). Quando o IS foi calculado, não foi detectada diferença entre os camundongos WT C57BL/6J e H3R KO (Figura 3, versão em inglês).

Novidade Social

O camundongo WT C57BL/6J preferiu a câmara com o camundongo novo 2 que a câmara com o camundongo conhecido, indicando um interesse por novidade social e/ou formação da memória social (306 ± 14s e 205 ± 15s, respectivamente; $p<0,05$). Em conformidade, esses animais passaram mais tempo explorando o camundongo novo 2 que o camundongo conhecido (185 ± 22s e 97 ± 16s, respectivamente; $p<0,05$). O camundongo H3R KO não apresentou diferença no tempo que passou nas câmaras com o camundongo novo 2 ou o camundongo conhecido (242 ± 17s e 238 ± 27s, respectivamente; $p=0,072$). Além disso, não foi detectada diferença quando os tempos explorando o camundongo novo 2 e camundongo conhecido foram comparados (143 ±

25s e 141 ± 21s, respectivamente; p=1,000). Quando o INS foi calculado, camundongos WT C57BL/6J alcançou maior escore que animais H3R KO (Figura 4, versão em inglês).

Teste *Marble burying*

Afim de avaliar o comportamento repetitivo, os animais foram submetidos ao teste *marble burying*. Não foi detectada diferença significativa entre animais H3R KO e WT C57BL/6J (p>0,05), ambos os grupos enterraram aproximadamente 50% das bolinhas (Figura 5).

Discussão

Considerando o papel da histamina na modulação de diversas funções fisiológicas, e relatos do envolvimento do sistema histaminérgico na fisiopatologia de diferentes desordens neurológicas, incluindo doença de Alzheimer, doença de Parkinson e esquizofrenia, faz sentido investigar este sistema no TEA [23]. O presente trabalho relata níveis anormais de RNAm de H3R e histamina no estriado do modelo VPA. Nós também verificamos que o H3R pode influenciar o interesse por novidade social, uma característica alterada no TEA, já que camundongos H3R KO demonstraram comportamento alterado quando testados para esse parâmetro.

A histamina é responsável por modular diferentes comportamentos, como cognição, sono e vigília e nocicepção. A liberação dessa amina biogênica, bem como de outros neurotransmissores é regulada negativamente pela ativação do H3R [23]. No presente trabalho, nós detectamos um nível elevado de histamina no estriado do modelo VPA, mas também detectamos níveis altos de RNAm do H3R nessa estrutura. Esses

foram achados surpreendentes, como o bloqueio do H3R, ou sua deficiência, levam a um aumento na liberação de histamina; é possível esperar uma redução no conteúdo de histamina em uma estrutura onde a expressão do RNAm do H3R é elevada. Contudo, essa alta expressão do RNAm poderia ser uma alteração compensatória em consequência aos efeitos do VPA a nível proteico. Além disso, é válido lembrar que nós não podemos afirmar qual seria a fonte exata de histamina detectada nas estruturas encefálicas do modelo VPA. Existem evidências que apontam para o envolvimento do sistema imunológico na fisiopatologia do TEA [13] e um papel para mastócitos não pode ser descartado. Altos níveis do peptídeo neurotensina, um fator de ativação dos mastócitos foi detectado no soro de pacientes com TEA [1].

Infusão de histamina no estriado ventral aumenta a taxa de liberação de acetilcolina nessa estrutura em ratos [26]. Além disso, a administração de agonistas inversos do H3R, que subsequentemente levam a um aumento na liberação de histamina, produz o mesmo efeito na atividade colinérgica. A histamina também pode modular a atividade GABAérgica no estriado. Na verdade, altas densidades de H3R são encontradas em neurônios GABAérgicos do estriado. A ativação desses receptores diminui a liberação de gaba por essas células. Isso vai ao encontro de diversos relatos que mostram deficiência da atividade GABAérgica no TEA [25]. Surpreendentemente, a análise por cromatografia líquida-espectrometria de massa (LC-MS) mostrou que camundongos BTBR, um modelo de TEA, apresentam níveis reduzidos de acetilcolina e não são diferentes dos animais controles em relação ao conteúdo de histamina no córtex pré-frontal [21].

Estudos farmacológicos com agonistas inversos do H3R, bem como o uso de animais H3R KO permitiram uma maior compreensão sobre o papel fisiológico desse receptor no SNC [24]. Memória e aprendizado, nocicepção e regulação do ciclo sono-vigília são alguns dos processos que envolvem o H3R [31]. No presente estudo,

camundongos VPA apresentaram maior nível de RNAm do H3R no estriado. O aumento da expressão RNAm do H3R em diferentes áreas do encéfalo já foi associado com um efeito neuroprotetor após indução de isquemia e epilepsia em ratos [18, 19]. Necrose hipóxico-isquêmica no estriado já foi descrita em associação com o TEA [7], o que poderia levar a uma regulação pós-isquêmica do H3R.

Apesar de a literatura apontar para diferentes alterações em várias estruturas encefálicas de pacientes com TEA, anormalidades no estriado são particularmente relevantes em função da sua provável conexão com um dos principais sintomas do TEA. O aumento da taxa de crescimento de áreas do estriado em indivíduos com TEA é associado à severidade dos comportamentos repetitivos apresentados por esses indivíduos [17].

Recentemente, foi relatado que uma cultura de microglia é capaz de expressar H4R, bem como os outros três receptores de histamina, e que a presença de histamina pode aumentar suas expressões de uma maneira dose-dependente [12]. A baixa especificidade apresentada pelos anticorpos de receptores histaminérgicos atualmente disponíveis comercialmente torna difícil a detecção do H4R a nível proteico [30]. Contudo, a literatura apresenta alguns relatos da presença do RNAm desse receptor em cerebelo e hipocampo humano [10], bem como no hipocampo do camundongo CD1 [36]. Nenhuma diferença entre grupos foi detectada quando o RNAm do H4R foi avaliado no córtex cerebral, hipocampo e estriado.

Concluindo, nós descrevemos níveis elevados de histamina no estriado de camundongos VPA, bem como um aumento na expressão do RNAm do H3R nessa mesma estrutura. Um possível papel para o H3R na sociabilidade pode ser hipotetizado, já que camundongos H3R KO não demonstraram interesse por novidade social durante o teste três câmaras. Este é o primeiro relato sobre anormalidades no sistema histaminérgico

de um modelo animal de autismo. Mais investigações são necessárias para corroborar esses dados iniciais.

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9. DISCUSSÃO

O presente trabalho investigou o sistema histaminérgico no modelo VPA de autismo, bem como o efeito de um agonista inverso do H3R sobre o comportamento desses animais. Os resultados obtidos são os primeiros dados disponíveis na literatura sobre este tema **(ARTIGO 1)**.

Diversas hipóteses foram levantadas para explicar o desenvolvimento do TEA e, ao longo dos anos, muitas delas foram refutadas (Roehr, 2013). O importante papel de neurotransmissores no desenvolvimento e maturação do encéfalo faz com que alterações em diferentes sistemas de neurotransmissão sejam cada vez mais investigadas e consideradas importantes para o desenvolvimento do TEA (Pardo and Eberhart, 2007).

O **ARTIGO 2** desta tese levanta a hipótese de que agonistas inversos do H3R possam vir a ser utilizados como tratamento do TEA. A ausência de avanços no desenvolvimento de terapias inovadoras para o tratamento de sintomas do TEA fortalece essa hipótese. Diversos ligantes de receptores histaminérgicos já foram testados como tratamento para uma variedade de desordens neurológicas em pesquisas clínicas e experimentais **(ARTIGO 1)** e alguns deles, como o pitolisant (Schwartz, 2011), se mostraram muito promissores. Outros estudos experimentais com diferentes modelos de autismo, e que avaliem mais parâmetros relacionados ao TEA, serão necessários para a elaboração de estudos clínicos.

O sistema histaminérgico recebeu pouca atenção, quando se trata da pesquisa sobre o TEA. Isso é um fato surpreendente, já que esse sistema modula importantes funções fisiológicas, além de ser capaz de regular outros sistemas de neurotransmissão, como o GABAérgico e glutamatérgico, reconhecidamente alterados no TEA.

Já foi demonstrado que o uso da tioperamida, agonista inverso do H3R, aumenta a liberação de GABA e histamina na área hipotalâmica anterior de ratos (Yamamoto et

al., 1997). Além disso, a histamina foi descrita como potenciador das respostas do receptor GABAa (Saras et al., 2008). O uso de outro agonista inverso do H3R, o clobenpropit, aumenta a liberação de GABA como forma de proteção à excitotoxicidade mediada por NMDA através da rota cAMP/PKA em cultura de neurônios corticais (Dai et al., 2007a). Em relação ao sistema glutamatérgico, a histamina estimula o transportador astrocítico de glutamato 1 e, após 24h de exposição, reduz significativamente o conteúdo extracelular de glutamato e protege contra a excitotoxicidade causada pela morte celular em cultura de células (Fang et al., 2014). Foi demonstrado também que a ativação do H3R pelo agonista immepip inibe a liberação do glutamato pelos sinaptossomas no estriado de ratos (Molina-Hernández et al., 2001). Estes relatos podem ser importantes para a pesquisa envolvendo TEA já que, como mencionado acima, alterações nos sistemas GABAérgico e glutamatérgico podem ter papel crucial na fisiopatologia do TEA.

Através dos resultados do **ARTIGO 3** podemos inferir que o sistema histaminérgico está afetado no estriado do modelo VPA, já que foram constatados níveis elevados de histamina e do mRNA do H3R nessa estrutura encefálica. É possível que essas alterações não possuam responsabilidade direta pelas alterações comportamentais deste modelo, mas, como citado acima, alterações no sistema histaminérgico podem refletir em anormalidades de outros sistemas de neurotransmissão. Como perspectiva para estudos futuros, fica a necessidade de avaliar o efeito do tratamento do ciproxifan sobre outros sistemas de neurotransmissores do modelo VPA.

10. CONCLUSÕES

O **ARTIGO 1** desta tese revisa o papel do sistema histaminérgico na fisiopatologia de diferentes desordens neurológicas e aponta o uso de diversos ligantes de receptores histaminérgicos como medidas terapêuticas em estudos experimentais e clínicos. Além disso, nesse artigo é realizado um levantamento sobre todos os estudos disponíveis na literatura envolvendo o sistema histaminérgico e TEA. Essa informação foi importante para demonstrar como esse sistema de neurotransmissão estava negligenciado nas pesquisas com TEA e, consequentemente, apontar a necessidade de mais estudos envolvendo as duas áreas.

Baseado em informações provenientes da revisão da literatura descrita no artigo 1, os experimentos publicados no **ARTIGO 2** foram planejados. Esse estudo mostrou que o CPX, agonista inverso do H3R, é capaz de reverter importantes prejuízos comportamentais apresentados por camundongos que sofreram exposição pré-natal ao VPA. Quando tratado com uma dose de 3 mg/kg de CPX antes de testes comportamentais, esse modelo de autismo apresentou um melhor comportamento social e redução do comportamento repetitivo. Esses dados iniciais abrem novas perspectivas para estudos subsequentes que visem avaliar ainda mais o possível uso dessa droga para o tratamento de características do tipo autista.

No **ARTIGO 3** foram utilizados animais HR3 KO e o modelo VPA. Os animais H3R KO apresentaram baixo interesse por novidade social quando foram submetidos a testes comportamentais. Os animais VPA foram reservados para análises moleculares que demonstraram as seguintes alterações no estriado: 1) aumento no conteúdo de histamina e 2) níveis elevados de mRNA do H3R. A relevância dessas alterações ainda precisa ser averiguada. Sabe-se que, além da HA, o sistema histaminérgico é capaz de regular a

liberação de outros neurotransmissores. Isso abre a possibilidade de que os prejuízos apresentados pelo modelo não sejam resultados diretos de anormalidades do sistema histaminérgico, e sim resultantes de uma desregulação causada em outros sistemas de neurotransmissão.

Estes achados contribuem para investigações futuras sobre a fisiopatologia do TEA, bem como apontam para um possível novo tratamento para características do tipo autista. Mais estudos são necessários para corroborar e expandir esses dados iniciais.

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