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PROGRAMA DE PÓS-GRADUAÇÃO EM FISIOTERAPIA
LABORATÓRIO DE NEUROCIÊNCIAS

INFLUÊNCIA DO SISTEMA HISTAMINÉRGICO CEREBELAR NA CONSOLIDAÇÃO DA MEMÓRIA EMOCIONAL DE CAMUNDONGOS

ANNA CAROLYNA LEPESTEUR GIANLORENÇO

São Carlos
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Anna Carolyna Lepesteur Gianlorenço

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Fisioterapia da Universidade Federal de São Carlos, como parte dos requisitos para a obtenção do Título de Doutor em Fisioterapia.
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CONTEXTUALIZAÇÃO

A parte experimental do trabalho intitulado "Influência do sistema histaminérgico cerebelar na consolidação da memória emocional de camundongos" foi desenvolvida no Laboratório de Neurociências da Universidade Federal de São Carlos (UFSCar) no departamento de Fisioterapia sob orientação da Profa. Dra. Rosana Mattioli, com co-orientação da Profa. Dra. Azair Liane Matos do Canto de Souza, do Laboratório de Psicologia da Aprendizagem (LPA) no departamento de Psicologia da UFSCar. Os créditos referentes às disciplinas foram obtidos junto ao Programa de Pós- Graduação em Fisioterapia. Este trabalho contou com o apoio financeiro da Fundação de Amparo à Pesquisa do Estado de São Paulo- FAPESP, Processos 2009/15836-1 e 2009/17496-3 e do CNPq Processo 3005/432010-7. Como resultado, 5 artigos científicos foram produzidos, sendo que 4 já foram publicados em revistas de circulação internacional (qualis A2, A2, A2 e B1).

A parte experimental dos trabalhos (Apêndice G) "Developing a cognitive bias mouse model using touchscreens" e "Adult neurogenesis protects against proactive interference" foi desenvolvida no Laboratório Molecular, Cellular and System Cognition no Hospital for Sick Children, na Universidade de Toronto, no Canadá durante participação no Programa Institucional de Bolsas de Doutorado Sanduíche no Exterior (PDSE) no período de março de 2012 a fevereiro de 2013, com apoio financeiro da CAPES- Coordenação de Aperfeiçoamento de Pessoal de Nível Superior Processo 5788/11-0. Nos projetos desenvolvidos durante esse período tive participação ativa no treinamento dos animais e análise dos dados comportamentais.

No primeiro experimento, os resultados mostraram um viés cognitivo em animais submetidos a procedimentos de estresse agudo, visto que esses animais interpretaram um estimulo ambíguo como negativo. Os resultados do segundo estudo, mostraram que a neurogênese pós-treino reduz a retenção de memórias dependentes do hipocampo previamente adquiridas. Entretanto, quando os animais foram submetidos à reversão da aprendizagem, camundongos com altos níveis de neurogênese apresentaram melhor desempenho quando comparados a camundongos com níveis normais, o que demonstra que a neurogênese adulta inibe memórias antigas. Como resultado, uma nova aprendizagem pode ocorrer mais eficientemente devido a redução da interferência proativa de memórias prévias.

O uso de touchscreens na pesquisa comportamental tem crescido devido à possibilidade de utilização desse recurso em diversas tarefas em diferentes espécies, tarefas

que são baseadas em atividades realizadas em seres humanos, o que permite desenvolvimento de trabalhos translacionais. O programa permitiu a participação em seminários e congressos no departamento de Fisiologia da Universidade de Toronto, o que favorece a atualização das pesquisas desenvolvidas e contato com outros pesquisadores. Além disso, pude aprender técnicas como imunoistoquímica, perfusão transcardíaca, utilização de microscopia confocal e aperfeiçoar meu conhecimento em procedimentos de análise histológica, modelos comportamentais como o labirinto aquático, campo aberto, condicionamento de medo ao contexto e técnicas de cirurgia, procedimentos que poderão ser incorporados aos meus experimentos futuros. A minha participação foi muito produtiva para meu crescimento científico e acadêmico e como resultado, os trabalhos foram apresentados na Neuroscience Meeting e Canadian Neuroscience Meeting.

RESUMO

Esse trabalho teve como objetivo investigar a atuação do sistema neural histaminérgico na consolidação da memória emocional de camundongos. Foi realizada cirurgia esterotáxica para implantação da cânula no vérmis cerebelar de camundongos machos. No terceiro dia de recuperação, foram realizados os testes comportamentais Labirinto em Cruz Elevado (LCE) e Esquiva Inibitória (EI) em dois dias consecutivos. Imediatamente após o primeiro dia de teste, os animais receberam o tratamento farmacológico com a microinjeção de drogas histaminérgicas no vérmis cerebelar. No experimento 1, foi verificado o efeito da histamina (HA) microinjetada nas doses de 0,54 nmol, 1,36 nmol, 2,72 nmol e 4,07 nmol em camundongos submetidos ao LCE (experimento 1a) e ao teste EI (experimento 1b). No experimento 2 foi realizada microinjeção de antagonista H_1 (Clorfeniramina, CPA) nas doses de 0,016 nmol, 0,052 nmol e 0,16 nmol em camundongos submetidos ao LCE (experimento 2a) e ao teste EI (experimento 2b). No experimento 3, microinjeção de antagonista H_2 (Ranitidina, RA) nas doses de 0,57 nmol, 2,85 nmol e 5,7 nmol em camundongos submetidos ao LCE (experimento 3a) e ao teste EI (experimento 3b). No experimento 4, foi realizada microinjeção combinada de antagonista H_1 e HA em camundongos submetidos ao LCE (experimento 4a) e ao teste EI (experimento 4b); e no experimento 5, foi realizada microinjeção combinada de antagonista H_2 e HA no LCE (experimento 5a) e EI (experimento 5b). O índice de memória dos animais no LCE foi definido pela redução da exploração dos braços abertos na reexposição (T1/T2). Para o Teste de EI, o aumento ou a redução das latências foi considerado indicativo de facilitação ou prejuízo na retenção da memória. Os resultados do *experimento 1a* mostraram que no LCE, os animais do grupo controle (SAL) e dos grupos HA nas doses de 0,54 nmol e 1,36 nmol apresentaram redução da exploração dos braços abertos (BA) na reexposição, enquanto os animais que receberam HA nas doses de 2,72 e 4,07 não apresentaram diferença significativa em relação a T2. Esses resultados indicam uma inibição da memória emocional quando a histamina foi injetada no vérmis cerebelar em animais submetidos ao LCE. No *experimento 1b*, a análise estatística mostrou um aumento significativo na latência no teste de EI para os animais que receberam histamina na dose de 1,36 nmol em relação ao grupo controle. Além disso, houve diferença significativa entre os grupos microinjetados com 1,36 nmol e com 2,72 nmol e 4,07 nmol. Estes resultados indicam que a histamina microinjetada na dose de 1,36 nmol facilitou a consolidação da memória de EI em camundongos, sugerindo um papel diferente da histamina em um modelo que usa punição. No *experimento 2a*, os resultados mostraram que os grupos microinjetados com salina e CPA nas doses de 0,016 nmol, 0,052 nmol e 0,16 nmol reduziram a exploração dos BA na reexposição, o que sugere a CPA não apresentou efeitos sobre a consolidação da memória emocional. No *experimento 2b*, a CPA também não apresentou efeito nas doses utilizadas no teste de EI. No *experimento 3a*, a análise estatística indicou que os animais que receberam RA na dose de 5,7 não apresentaram redução na exploração dos BA, o que sugere prejuízo na consolidação da memória. Os resultados do *experimento 3b* mostraram que a RA na dose de 5,7 nmol prejudicou a consolidação da memória emocional no teste de EI. Os *resultados do estudo 4a* com injeção combinada de antagonista H_1 e HA em camundongos reexpostos ao LCE mostraram que os animais microinjetados com SAL+SAL, CPA+SAL e CPA+HA reduziram a porcentagem de entradas e de tempo nos braços abertos, enquanto que os animais do grupo SAL+HA não apresentaram diferença significativa na exploração dos BA na reexposição, confirmando os resultados do experimento 1a em que animais injetados com HA (4,07 nmol) apresentam prejuízo na consolidação da memória emocional. Além disso, os resultados mostraram que a CPA não alterou os parâmetros comportamentais quando aplicada por si só, e que foi capaz de reverter o déficit produzido pela histamina na consolidação da memória emocional em camundongos no LCE. No *experimento 4b* no teste de EI, os

resultados mostraram que os grupos que receberam injeção combinada de antagonista H_1 e histamina (CPA+HA) e a injeção de SAL+HA apresentaram diferença significativa no tempo de latência no segundo dia de teste em relação ao grupo controle SAL+SAL, enquanto o grupo tratado com CPA+SAL não apresentou diferença com o grupo SAL+SAL. Esses resultados mostram que a microinjeção de histamina no vérmis cerebelar aumentou o tempo de latência, e que o pré-tratamento com CPA não reverteu esse efeito. Para o *experimento 5a*, com microinjeção combinada de antagonista H_2 e HA em camundongos submetidos ao LCE, os resultados mostraram que animais microinjetados com SAL+SAL e RA+SAL reduziram a porcentagem de entrada e de tempo nos braços abertos, enquanto os grupos tratados com RA+HA e SAL+RA não apresentaram diferença entre os dias de teste nesse parâmetros. Estes resultados mostraram que a RA não alterou a consolidação da memória e não foi capaz de reverter o efeito da histamina. Já no *experimento 5b* com a microinjeção combinada de RA e HA no teste de EI, houve diferença significativa entre os grupos SAL+SAL e SAL+HA, mostrando novamente o efeito facilitador da histamina na consolidação da memória de EI. Os grupos que receberam injeção combinada de antagonista H_2 e histamina (RA+HA) e o grupo de recebeu RA+SAL não apresentaram diferença significativa em relação ao controle no tempo de latência no segundo dia de teste, o que mostra que a RA não apresentou efeito por si só, mas quando aplicada pré histamina foi capaz de reverter seu efeito. Os resultados dos diversos experimentos realizados parecem indicar efeitos da histamina em circuitos neurais diferentes em tarefas envolvendo medo ou ansiedade.

Palavras-chave: sistema histaminérgico; memória emocional; consolidação; cerebelo.

ABSTRACT

This study investigated the function of cerebellar histaminergic system on emotional memory consolidation. The cerebellar vermis of male mice were implanted with guide cannulae, and after three days of recovery, the animals were submitted to the elevated plus maze (EPM) or the inhibitory avoidance test (IA) on two consecutive days. Immediately after the first day, animals received a microinjection of histaminergic drugs into the cerebellar vermis: experiment 1, animals received microinjections of saline (SAL) or histamine (HA) (0.54, 1.36, 2.72, and 4.07 nmol/0.1 microliter); experiment 2, animals received a microinjection of SAL or the H_1 antagonist chlorpheniramine (CPA, 0.016, 0.052 or 0.16 nmol/0.1 µl); experiment 3, SAL or the H_2 antagonist ranitidine (RA, 0.57, 2.85 or 5.7 nmol/0.1 µl); experiment 4, SAL or HA 5 minutes after a pretreatment with 0.16 nmol CPA or SAL; and experiment 5, SAL or HA 5 minutes after a pretreatment with 2.85 nmol ranitidine (RA) or SAL. In the EPM, the decrease of open arm exploration (% entries and % time spent in the open arms) in Trial 2 relative to Trial 1 was used as a measure of learning and memory; while in the IA, latency to cross to the dark compartment was used to evaluate memory retention. Data were analyzed using ANOVA and Duncan's test. The results of experiment 1 showed that animals microinjected SAL and 0.54 and 1.36 nmol HA reduced percentage of open arm entries and time, while mice microinjected with HA 2.72 and 4.07 nmol did not decrease open arm exploration on trial 2; which indicates that histamine induced a dose-dependent inhibitory effect on memory consolidation. In the IA task, results showed that 1.36 nmol histamine facilitated memory consolidation, suggesting a different action of HA in a memory model that uses punishment. In the experiment 2, microinjections with CPA did not present behavioral effects in the EPM or in the IA at the doses used (0.016, 0.052 and 0.16 nmol). The results of experiment 3 showed that 5.7 nmol RA impaired memory consolidation on both protocols. The experiment 4 demonstrated that animals treated with HA did not reduce the avoidance to the open arms on retesting, and indicated that CPA did not altered behavioral parameters by itself, but the pretreatment with CPA reverted histamine-induced impairment on memory consolidation, which suggests that histamine effect on the EPM was mediated by H1 receptors. In the IA test, the results showed that the groups that received CPA+HA and SAL+HA showed a significant difference in latency on the second day of testing in relation to group SAL+SAL , while the group treated with CPA + SAL showed no difference with the control group. These results show that microinjection of histamine in the cerebellar vermis increased latency time and that pretreatment with CPA did not reverse this effect. For the fifth experiment, the results showed that animals microinjected with SAL+SAL and RA+SAL reduced the percentage of entries and time spent in open arms in the EPM while the groups treated with RA+HA and SAL+HA showed no difference between test days. These results show that RA did not alter memory consolidation and was unable to reverse the effect of histamine. In the IA, there was significant difference between SAL+SAL and SAL+HA groups, showing the facilitatory effect of histamine on memory consolidation of IA. The groups that received combined injection of RA+SAL and RA+HA showed no significant difference compared to control, which shows that the RA had no effect by itself, but when

applied before histamine was able to reverse its effect. Our results suggest different histamine effects in tasks involving anxiety or fear.

Key-words: histaminergic system; emotional memory; consolidation; cerebellum.

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LISTA DE ABREVIATURAS

LTP- Potenciação de longa duração
LTD- Depressão de longa duração
SNC- Sistema nervoso central
TM- Núcleo tuberomamilar
DAG- Diacilglicerol
PKC- Proteína cinase C
PKA- Proteína cinase A
IP3- inositol trifosfato
GABA- Ácido gama-aminobutírico
LCE - Labirinto em Cruz Elevado
I.c.v.- Intracerebroventricular
HA- Histamina
CPA - Clorfeniramina
RA- Ranitidina
SAL- Salina
AP- Antero-posterior
L- Lateral
V- Ventral
BA- Braços abertos
BF- Braços fechados
T1 - Teste 1 (Exposição)
T2 - Teste 2 (Reexposição)
i.p. - Intraperitoneal, intraperitonealmente
EBA - Entradas nos braços abertos
EBF - Entradas nos braços fechados
TBA - Tempo nos braços abertos
TBF - Tempo nos braços fechados
TC - Tempo no centro do labirinto
%EBA - Porcentagem de entradas nos braços abertos
%TBA - Porcentagem de tempo nos braços abertos
%TBF - Porcentagem de tempo nos braços fechados
EI- Esquiva inibitória
D1- dia 1
D2- dia 2
IUSDEC- curva dose efeito em U-invertido

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I - INTRODUÇÃO

1.1 Cerebelo: estrutura e função

O cerebelo, órgão do sistema nervoso supra-segmentar, deriva da parte dorsal do metencéfalo e fica situado dorsalmente ao bulbo e a ponte, contribuindo para a formação do teto do IV ventrículo. Anatomicamente, distingue-se no cerebelo uma porção ímpar e mediana, o vérmis, ligado a duas massas laterais, os hemisférios cerebelares (MACHADO, 2005).

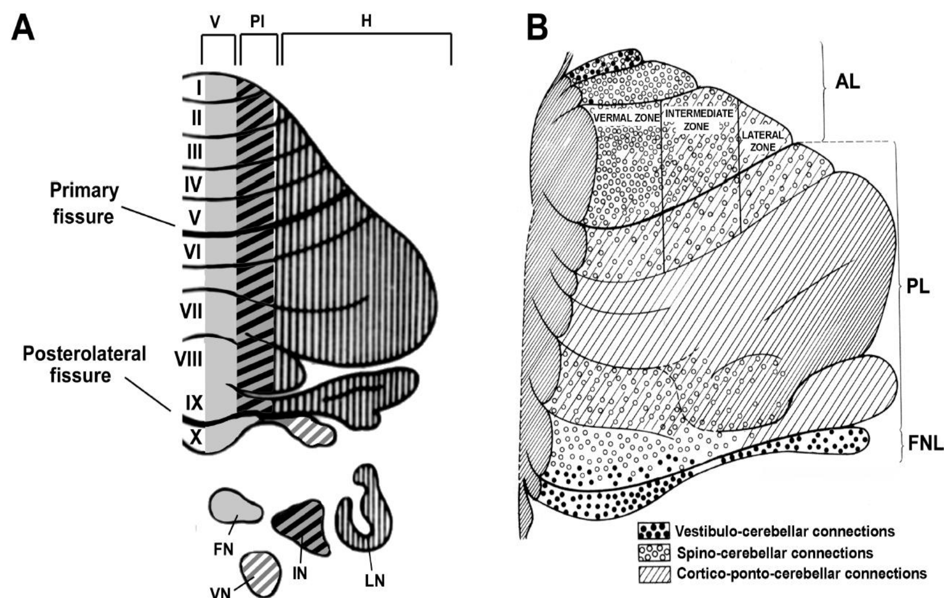

Figura 1: Subdivisão anatômica do cerebelo. **A**) subdivisão longitudinal do córtex e suas conexões com os núcleos intracerebelares e vestibular. V= vermis; PI= porção intermediária; H= hemisférios; FN= núcleo fastigial; VN= núcleo vestibular; IN= núcleo interposto; LN= núcleo lateral. Os lobos estão subdivididos em numerais romanos. **B**) representação esquemática das regiões corticais recebendo aferências vestibulares, da medula espinhal e do córtex cerebral através do núcleo pontino. AL= lobo anterior; PL= lobo posterior; FNL= lobo floculonodular (adaptado de Dow, 1942 apud Sacchetti et al., 2005).

O cerebelo conecta-se com várias áreas da medula espinhal, tronco encefálico e córtex cerebral, e é constituido de um córtex de substância cinzenta com três camadas em torno de uma região central de substância branca, com uma faixa longitudinal de córtex menos denso, o vérmis (MACHADO, 2005; ADEL e BERGMAN, 2005). As fissuras póstero- anterior dividem o cerebelo em três lóbulos anatomicamente distintos:

lobo anterior, lobo posterior e lobo flóculo-nodular. Os três lóbulos podem ser subdivididos em 10 lóbulos, com lóbulos I a V no lobo anterior, lóbulos VI a IX no lobo posterior, e lóbulo X no lobo flóculo-nodular (ADEL e BERGMAN, 2005).

A citoarquitetura do córtex cerebelar, ao contrário do córtex cerebral, é basicamente a mesma em todas a folhas e lóbulos. Da superfície para o interior, distingue-se a camada molecular, camada de células de Purkinje e camada granular. A camada média é formada por uma fileira de células de Purkinje, os elementos mais importantes do cerebelo. As células de Purkinje, piriformes e grandes, são dotadas de dentritos que se ramificam na camada molecular e de axônios que saem em direção oposta, terminando nos núcleos do cerebelo, onde exercem ação inibitória. Esses axônios constituem as únicas fibras eferentes do córtex do cerebelo. A camada molecular é formada principalmente por fibras de direção paralela e contém dois tipos de neurônios, as células estreladas e as células em cesto (apresentam sinapses axossomáticas dispostas em torno do corpo das células de Purkinje à maneira de um cesto). A camada granular é constituída principalmente pelas células granulares ou grânulos do cerebelo, células muito pequenas, numerosas que têm vários dendritos e um axônio que atravessa a camada de células de Purkinje e, ao atingir a camada molecular bifurcam-se em T - os ramos resultantes dessa bifurcação constituem as fibras paralelas. Nessa camada, existe ainda as células de Golgi, menos numerosas que as células granulares e com ramificações amplas (MACHADO, 2005).

O córtex cerebelar recebe dois sistemas de fibras aferentes: as fibras musgosas e as fibras trepadeiras. As fibras musgosas originam-se de diferentes segmentos da medula espinhal e do córtex cerebral, entre outros e representam a terminação dos feixes de fibras que penetram no cerebelo. As fibras trepadeiras são axônios de neurônios situados no complexo olivar inferior, e têm esse nome porque terminam enrolando-se em torno dos dendritos das células de Purkinje (MACHADO, 2005; ALTMANN e BAYER, 1997).

O suprimento sanguíneo do cerebelo acontece a partir de três artérias, decorrentes do sistema vertebrobasilar: a artéria cerebelar superior (supre os aspectos superiores do hemisférios cerebelares e vérmis, assim como a maioria do núcleo denteado), a artéria cerebelar antero-inferior (abastece o aspecto anterior dos hemisférios cerebelares e o lobo flóculo-nodular), e a artéria cerebelar póstero-inferior (fornece suprimento para o vermis inferior e os aspectos posteriores dos hemisférios

cerebelares, bem como uma porção variável do núcleo denteado inferomedial) (O'HALLORAN, 2012).

Com relação à sua função, os primeiros estudos sobre o cerebelo sugeriram que essa estrutura poderia servir como o local de alterações plásticas necessárias para uma ampla gama de fenômenos adaptativos e condicionados, classificados como aprendizagem motora, e ilustraram que a ablação de regiões críticas cerebelares resultou uma incapacidade de adquirir este tipo de comportamento adaptativo (BLOEDEL et al., 1979).

Grande apoio para o argumento de que o cerebelo é estrutura fundamental para os processos plásticos necessários para a aprendizagem motora foi ainda proporcionado por uma série de estudos indicando que lesões em regiões específicas do córtex cerebelar e dos núcleos do cerebelo foram capazes de eliminar seletivamente as respostas condicionadas em animais que já tinham adquirido o comportamento motor e, além disso, preveniram a aquisição em animais ingênuos (BLOEDEL et al., 1979).

Segundo revisão de Manto e colaboradores (2012), pesquisas sobre as funções do cerebelo ampliaram tremendamente durante estas últimas décadas. Os autores afirmam que o cerebelo contribui para regulação e planejamento do movimento, organização temporal e aquisição sensorial e é envolvido na previsão das consequências sensoriais da ação. O que explica os déficits clínicos apresentado por pacientes com comprometimentos cerebelares caracterizados por distúrbios na precisão e coordenação: distúrbios dos movimentos oculares, distúrbios da fala, distúrbios de movimentos dos membros, e deficiências de postura / marcha. Em resumo, a revisão destaca que as estruturas do cerebelo que controlam os movimentos dos olhos incluem o chamado vermis oculomotor (lóbulos VI e VII) e núcleo fastigial, lóbulos I-II, flóculo e paraflóculo, úvula, e nodulus; a fala é controlada pela região paravermal superior, o córtex cerebelar intermediário, e o núcleo denteado; os movimentos dos membros estão sob a supervisão do núcleo denteado, o núcleo interpósito, e córtex cerebelar intermediário e lateral; e a marcha é controlada pelo cerebelo medial e intermédio (MANTO et al., 2012).

A maioria dos pesquisadores em neurologia e saúde mental acreditava que o cerebelo estava envolvido apenas com o controle do movimento. Porém, nos últimos anos, avanços em neurociências, em tecnologias de imagem e modelos computacionais, têm permitido repetidas demonstrações de que o cerebelo participa de funções motoras e

não motoras (KOZIOL et al., 2013). Recentemente, sua participação em funções cognitivas e processos emocionais tem recebido considerável atenção. Déficits neuropsicológicos em pacientes com lesões cerebelares, ativação do cerebelo em indivíduos normais durante a realização de tarefas cognitivas e conexões anatômicas mostrando as ligações do cerebelo com outras estruturas e com o córtex cerebral suportam essa visão.

Diversas pesquisas na área da neuropsicologia realizadas em pacientes com lesões cerebelares permitiram identificar distúrbios significativos em relação à cognição e a emoção nestes pacientes (TAVANO e BORGATI, 2010; SCHMAHMANN et al., 2007; SCHMAHMANN e SHERMAN, 1998). Segundo Schmahmann e Sherman (1998), várias alterações emocionais e comportamentais têm sido descritas em pacientes com lesões cerebelares adquiridas ou congênitas, como déficits em tarefas de atenção, memória de trabalho, linguagem, processamento visuo-espacial, planejamento e raciocínio abstrato. Os autores então sugeriram que os déficits encontrados em pacientes cerebelares poderiam ser contextualizados como uma síndrome, a sindrome cerebelar afetiva cognitiva, consistindo de déficits na função executiva, processamento visual, habilidades linguísticas e processos afetivos.

Trabalhos mostraram a ativação do cerebelo (vérmis e região posterior dos hemisférios cerebelares) na realização de tarefas cognitivas, não relacionadas a atos motores (BUGALHO et al., 2006). Estudos de neuroimagem têm confirmado que o cerebelo é ativado em vários processos cognitivos, como a organização espacial, memória visual e de longo prazo, e a perturbação destas funções após danos cerebelares pode ser devido a uma ruptura das projeções cérebro-cerebelares (GORDON, 2007). Além disso, uma revisão de 275 estudos utilizando tomografia por emissao de pósitrons e ressonância magnética funcional revelou que a ativação cerebelar foi observada durante uma ampla gama de funções, incluindo a atenção orientada, olfato, fala, linguagem escrita, memória de trabalho, solução de problemas, memória espacial, memória episódica, aprendizagem de competências e aprendizagem associativa (CABEZA e NYBERG, 2000). Stoodley e Schmahmann (2009) sugerem que as regiões anteriores do cerebelo estariam envolvidas nos domínios motor e sensorial, regiões posteriores laterais mediariam as capacidades cognitivas, e o aspecto medial do cerebelo seria crítico em processos emocionais. Segundo os autores, esse modelo pode fornecer

uma estrutura útil para testar hipóteses e conceituar distúrbios neuropsiquiátricos e neuropsicológicos que podem estar presentes seguindo lesões cerebelares localizadas.

Estudos anatômicos demonstraram que o cerebelo está ligado a áreas associativas dos hemisférios cerebrais (regiões pré-frontal, occipito-parietal, temporal e límbica) (BUGALHO et al., 2006). Além disso, outros estudos têm mostrado a ligação bidirecional do cerebelo com áreas envolvidas na regulação emocional (TURNER et al., 2007; MIDDLETON e STRICK, 2001). Segundo Turner e colaboradores (2007), o cerebelo é fortemente conectado com o sistema reticular, áreas corticais associativas e áreas límbicas, como amígdala, hipocampo e núcleo septal.

1.2 Cerebelo e Memória emocional

As emoções têm papel dominante no controle comportamental, sendo extremamente importantes para a adaptação da conduta às situações de relevância e a sobrevivência do indivíduo (SACCHETTI et al., 2009). A memória emocional se refere aos processos de aquisição, consolidação e evocação de memórias associadas à experiência emocional do indivíduo. Ela pode ser avaliada com aproximações experimentais específicas que representem os "estados emocionais", analisando as respostas comportamentais e fisiológicas do indivíduo (CONDE et al., 2001).

Apesar de progressos na área, os processos de armazenamento da memória emocional e do medo não estão claramente definidos. Segundo Cahill (1995), as memórias emocionais são estabelecidas através da amígdala, porém esta estrutura parece estar relacionada a uma extensa rede (SACCHETTI et al., 2007). A influência neuromodulatória dentro da amígdala regula a consolidação da memória por meio de projeções enviadas para muitas regiões cerebrais envolvidas no armazenamento da informação (MCGAUGH, 2004).

Evidências experimentais indicam que o papel cerebelar na aprendizagem aversiva pode ser mais complexo que a simples regulação de respostas motoras. A capacidade de aprender e reter respostas de condicionamento do medo foi investigada em camundongos mutantes, caracterizados por uma deficiência primária nas sinapses feitas pelas fibras paralelas com as células de Purkinje. Nesses animais, a disfunção cerebelar prejudicou a aprendizagem do medo, sugerindo que essas sinapses estão envolvidas na consolidação da memória aversiva (SACCHETTI et al., 2004). Zhu et al.

(2007) demonstraram que a potenciação de longa duração (LTP) é o mecanismo envolvido nas memórias associativas aversivas no cerebelo, e que a transdução levando a LTP e a memória de medo são independentes da envolvida na depressão de longa duração (LTD) que é correlacionada a aprendizagem motora.

Estudos relacionam o vérmis cerebelar e a formação de memórias emocionais (SACCHETTI et al., 2002; SACCHETTI et al., 2007). Sacchetti e colaboradores (2002) demonstraram que a inativação do vérmis cerebelar após o pareamento de estímulos (choque e som) causou prejuízo na consolidação da memória. Segundos os autores, o vérmis participa da memória emocional independente de seu papel em processos motores ou sensoriais. Segundo Sacchetti e colaboradores (2009), o vérmis poderia representar uma interface entre o estímulo sensorial, o estado emocional do sujeito e as respostas motoras.

Em nosso laboratório recentemente foi realizado um estudo com o procedimento de esquiva inibitória em peixes que sofreram ablação cerebelar. Os resultados demonstraram que o cerebelo está envolvido na consolidação da memória de esquiva inibitória e/ou interpretação do valor aversivo da tarefa (GARÇÃO, 2009). Em outro estudo recente, Kellett e colaboradores (2010), investigaram se a consolidação da memória no cerebelo de coelhos era essencialmente cortical ou nuclear (núcleo anteposto anterior). Os resultados desse estudo mostraram que a consolidação da memória foi dependente do armazenamento da informação no córtex. Assim, o crescente número de estudos demonstra o interesse na investigação da participação do cerebelo na memória.

Portanto, o cerebelo apresenta importantes funções no sistema motor, assim como em processos cognitivos e emocionais. Entretanto, como o processamento de informações nos circuitos cerebelares possibilita que este exerça as funções as quais se associa não está bem fundamentado (GUEDES, 2012).

1.3 Sistema Neural Histaminérgico e sua atuação nos Processos de Aprendizagem e Memória Emocional

O sistema histaminérgico no Sistema Nervoso Central (SNC) consiste de neurônios responsáveis pela produção da histamina, uma monoamina biogênica, que tem um importante papel modulador (PRELL e GREEN, 1986). Essa amina não

atravessa a barreira hematoencefálica, sendo formada no cérebro pela descarboxilação do aminoácido L-histidina, pela enzima histidina descarboxilase. A degradação de histamina ocorre por meio da ação da enzima N-metiltransferase, e sua síntese pode ser inibida pela enzima α-fluormetilhistidina (WADA et al., 1991).

O sistema histaminérgico apresenta distribuição similar em vários vertebrados estudados (PANULA et al., 1990). Nos cérebros de mamíferos, os corpos celulares histaminérgicos são encontrados no núcleo tuberomamilar (TM) no hipotálamo posterior e possuem projeções para a maioria das regiões do sistema nervoso central. O sistema neural histaminérgico dos roedores é similar ao sistema encontrado no cérebro humano (PANULA et al., 1984).

A atuação da histamina no SNC se dá pela estimulação de quatro classes de receptores, H_1, H_2, H_3 e H_4 que diferem farmacologicamente, na localização e nas respostas intracelulares mediadas (STRAKHOVA et al., 2009; LEURS et al., 1995). Esse último tipo de receptor histaminérgico, cuja ação parecia estar restrita ao sistema imunológico (LIM et al., 2005) foi recentemente observado no SNC de ratos e humanos (STRAKHOVA et al., 2009).

Os receptores H_1 e H_2 são pós-sinapticos (BROWN et al., 2001; HILL et al, 1997), e os receptores H_3 são pré-sinapticos podendo atuar como autoreceptor ou heteroreceptor (HILL et al., 1997). Os receptores H_1 estão largamente distribuídos, com alta densidade no hipotálamo, na área septal, no núcleo medial da amígdala, cerebelo e em áreas hipocampais (BROWN et al., 2001; HAAS et al., 2008). A ativação do receptor H_1 estimula a atividade da fosfolipase C, que hidroliza o composto fosfatidil-4,5-bifosfato gerando dois segundos-mensageiros, o inositol trifosfato (IP3) e diacilglicerol (DAG). A formação do DAG estimula a atividade da proteína cinase C (PKC) que facilita a ativação dos receptores glutamatérgicos NMDA. O IP3 age em receptores do retículo endoplasmático, estimulando a liberação de Ca^{+2} no citosol. O aumento de Ca^{+2} intracelular, aumenta a atividade do trocador de Na^{+}/Ca^{+2} e também induz a produção de óxido nítrico. Ainda, a proteína G ativada pelo receptor H_1 é capaz de bloquear a abertura dos canais de K^{+} de dois poros, responsáveis pela hiperpolarização, mantendo o neurônio em estado ativado por um período prolongado. O efeito final após a estimulação deste receptor é a intensa despolarização celular, ou seja, a estimulação neuronal (BROWN et al., 2001).

Os receptores H_2 são predominantemente expressos nos gânglios da base, amídala, hipocampo e córtex. No córtex cerebelar, receptores H_2 são expressos nas células granulares e de Purkinje (BROWN et al, 2001). O receptor H_2 se acopla à proteína G para estimular a adenilato ciclase e aumentar as concentrações de AMPc intracelular que, por sua vez, ativa a proteína cinase A (PKA). As subunidades da PKA são translocadas para o núcleo, e estimulam a atividade do fator CREB, culminando em transcrição de proteínas. Este fator é importante regulador da neurofisiologia e da plasticidade neuronal. Além disto, as sub-unidades ativas da PKA, bloqueiam a condutância dos canais de K^+ ativados pelo influxo de Ca^{+2}, que são os responsáveis pela repolarização e pelo prolongamento da hiperpolarização pós-potencial de ação nestes neurônios. Finalmente, o AMPc produzido também é capaz de modificar a sensibilidade da voltagem dos canais ativados por hiperpolarização e dependente de nucleotídeo cíclico permitindo sua ativação em voltagens mais positivas, o que facilita a despolarização neuronal subsequente. Dessa forma, a estimulação dos receptores H_2 também leva à excitação neuronal, porém por mecanismos diferentes daqueles gerados pela estimulação dos receptores H_1 (BROWN et al., 2001).

Os receptores H_3 estão presentes não só nos terminais sinápticos dos neurônios do TM, como também em diversas regiões cerebrais, tais como córtex, hipocampo, giro denteado, gânglios da base, cerebelo e substância negra. Atuando como autorreceptor em neurônios histaminérgicos, os receptores H_3 têm função na regulação da síntese e liberação da histamina. Diferentes dos subtipos já citados, o receptor H_3 é acoplado a proteína G inibitória e sua estimulação leva a inibição da formação do AMPc. A ativação da proteína G inibitória também reduz a atividade dos canais de Ca^{+2} de alta voltagem, e consequentemente, reduz a fusão das membranas vesiculares e plasmática, prejudicando, dessa forma, a neurotransmissão (BROWN et al., 2001). Os receptores H_3 também podem atuar como heteroceptores, inibindo a liberação de outros neurotransmissores como glutamato, ácido gama-aminobutírico (GABA), noradrenalina, dopamina, acetilcolina, serotonina e vários peptídeos, aparentemente pela inibição dos canais de cálcio pré-sinápticos (KÖHLER et al, 2011).

Os receptores H_4 são expressos principalmente em células de origem hematopoiética, como eosinófilos e mastócitos, tendo função associada a processos inflamatórios e alérgicos (HAAS et al., 2008). Estes receptores foram identificados recentemente no SNC através da detecção do seu RNAm na amígdala, no cerebelo, no

corpo caloso, no córtex pré-frontal e tálamo, tanto em humanos, quanto em ratos. Em ambas as espécies, este receptor encontra-se em alta densidade na medula espinhal e nos gânglios da raiz dorsal, sugerindo uma possível função na mediação da nocicepção (HAAS et al., 2008).

O sistema histaminérgico está envolvido na regulação de várias funções neurológicas, como ritmo circadiano e sono, motivação e alterações comportamentais (ONODERA et al., 1994), assim como em processos de recuperação funcional (PIRATELLO e MATTIOLI, 2004). Estudos têm mostrado que o sistema histaminérgico também está relacionado com ansiedade (FAGANELLO e MATTIOLI, 2007; PRIVOU et al., 1998; IMAIZUME e ONODERA, 1993) e aprendizagem e memória (MEDALHA et al., 2000; MATTIOLI et al., 1998; DE ALMEIDA e IZQUIERDO, 1986)

O papel do sistema histaminérgico durante aprendizagem e memória tem sido estudado, porém com resultados contraditórios, que podem estar relacionados à ansiedade e ao componente emocional que cada tarefa representa. De Almeida e Izquierdo (1986) demonstraram efeito facilitador da histamina sobre a memória emocional, após injeção intracerebroventricular (icv) em ratos em um teste de esquiva ativa. Adicionalmente, os resultados obtidos por Prast e colaboradores (1996) indicaram que a histamina e a L-histidina têm um efeito facilitador na aquisição da memória de curta duração em um teste de memória social em ratos. Entretanto, os resultados do estudo de Alvarez e colaboradores (2001) indicaram ação inibitória da histamina microinjetada no hipocampo na evocação da memória de ratos. Em estudo de Serafim e colaboradores (2010), a injeção sistêmica de L-Histidina, provocou déficit de evocação da memória estado-dependente em camundongos submetidos e reexpostos ao Labirinto em Cruz Elevado (LCE).

1.4 O papel da Histamina no Cerebelo

Segundo Wada e colaboradores (1991), existem projeções de fibras contendo histamina do núcleo tuberomamilar no hipotálamo para o córtex cerebelar e núcleos profundos cerebelares, com alta densidade de terminações histaminérgicas no vérmis cerebelar e flocculus. A rede de fibras histaminérgicas é encontrada em camadas moleculares e granulares no cerebelo em várias espécies, incluindo humanos. Essas

fibras correm paralelas as camadas de fibras de Purkinje, depois atravessam perpendicularmente. As células de Purkinje no córtex cerebelar assim como os neurônios do núcleo interposto exibem receptores H_2 (SHEN et al., 2002). Células granulares são excitadas através da ativação de receptores H_1 e H_2 (TIAN et al., 2000; LI et al., 1999).

Estudos apontam importante relação entre o sistema histaminérgico e o cerebelo. Shen e colaboradores (2002) observaram efeitos excitatórios da histamina nas células do núcleo interposto de ratos via receptores H_2. Os autores sugerem que as fibras histaminérgicas podem modular a atividade neuronal no córtex cerebelar e núcleos profundos. Song e colaboradores (2006), utilizando cilíndro de equilíbrio (*rota-rod)*, observaram que o bloqueio dos receptores H_1 e H_2 do núcleo interposto cerebelar em ratos promoveu um decréscimo no desempenho motor, para habilidades de coordenação e equilíbrio. Um estudo utilizando cerebelo *in vitro* de ratos, demonstrou que a histamina promove resposta excitatória nas células de Purkinje através dos receptores H_2, uma vez que este efeito foi bloqueado pela Ranitidina, revelando a importância das fibras histaminérgicas nas atividades funcionais do cerebelo (TIAN et al., 2000). Recentemente, Quin e colaboradores (2011) demonstraram que a histamina excita neurônios do núcleo denteado do cerebelo via receptores H_2, sugerindo o envolvimento de projeções histaminérgicas para inicio e planejamento dos movimentos.

Apesar da ação da histamina e de componentes relacionados sobre os estados emocionais, de ansiedade e mecanismos de aprendizagem e memória já terem sido analisados em algumas regiões cerebrais, como o hipocampo (ALVAREZ e BANZAN, 2008; ROSTAMI et al., 2006) e a amígdala (ZARRINDAST et al., 2005; PASSANI et al., 2001), não há evidências sobre a atuação do sistema histaminérgico cerebelar na memória emocional de camundongos.

II - OBJETIVO

Objetivo geral:

▪Investigar a atuação do sistema neural histaminérgico na consolidação da memória emocional de camundongos via vérmis cerebelar;

Para isso foram realizados os seguintes experimentos:

2.2 Objetivos específicos

Experimento 1:

-1a: Verificar os efeitos dose-dependentes de histamina, microinjetada no vérmis cerebelar, sobre a consolidação da memória emocional de camundongos submetidos ao Labirinto em Cruz Elevado;

▪1b: Verificar os efeitos dose-dependentes de histamina, microinjetada no vérmis cerebelar, sobre a consolidação da memória emocional de camundongos submetidos ao Teste de Esquiva Inibitória;

Experimento 2:

▪2a: Investigar os efeitos da microinjeção do antagonista histaminérgico dos receptores H_1, Clorfeniramina, no vérmis cerebelar, sobre a consolidação da memória emocional de camundongos submetidos ao Labirinto em Cruz Elevado, e identificar dose sub-efetiva para a fase seguinte;

-2b: Investigar os efeitos da microinjeção do antagonista histaminérgico dos receptores H_1, Clorfeniramina, no vérmis cerebelar sobre a consolidação da memória emocional de camundongos submetidos ao Teste de Esquiva Inibitória, e identificar dose sub-efetiva para a fase seguinte;

Experimento 3:

▪**3a:** Investigar os efeitos da microinjeção do antagonista histaminérgico dos receptores H_2, Ranitidina, no vérmis cerebelar, sobre a consolidação da memória emocional de camundongos submetidos ao Labirinto em Cruz Elevado, e identificar dose sub-efetiva para a fase seguinte;

-**3b:** Investigar os efeitos da microinjeção do antagonista histaminérgico dos receptores H_2, Ranitidina, no vérmis cerebelar sobre a consolidação da memória emocional de camundongos submetidos ao Teste de Esquiva Inibitória, e identificar dose sub-efetiva para a fase seguinte;

Experimento 4:

▪**4a:** Verificar se os efeitos de histamina sobre a consolidação da memória emocional em camundongos submetidos ao Labirinto em Cruz Elevado podem ser revertidos com antagonista do receptor H_1 (Clorfeniramina);

▪**4b:** Verificar se os efeitos de histamina sobre a consolidação da memória emocional em camundongos submetidos ao Teste de Esquiva Inibitória podem ser revertidos com antagonista do receptor H_1 (Clorfeniramina);

Experimento 5:

▪**5a:** Verificar se os efeitos de histamina sobre a consolidação da memória emocional em camundongos submetidos ao Labirinto em Cruz Elevado podem ser revertidos com antagonista do receptor H_2 (Ranitidina);

▪**5b:** Verificar se os efeitos de histamina sobre a consolidação da memória emocional em camundongos submetidos ao Teste de Esquiva Inibitória podem ser revertidos com antagonista do receptor H_2 (Ranitidina);

III - MATERIAL E MÉTODO

3.1 Aspectos Éticos e Local da Pesquisa

O projeto de pesquisa foi aprovado pelo Comitê de Ética na Experimentação Animal da Universidade Federal de São Carlos (CEEA 049/2009). A pesquisa foi desenvolvida no Laboratório de Neurociências do Departamento de Fisioterapia com a colaboração do Laboratório de Psicologia da Aprendizagem, na Universidade Federal de São Carlos – UFSCar.

3.2 Amostra

Foram utilizados 453 camundongos ingênuos (Suíço Albino) pesando entre 25 e 35 g provenientes do Biotério Central da Universidade Federal de São Carlos. Os animais foram agrupados em 5 animais por caixa (31×20×13 cm) e permaneceram em ambiente com ciclo de luz de 12 horas (7:00 -19:00), temperatura (21 ±1°C) e umidade controladas. Alimento e água estavam disponíveis, exceto durante as breves sessões do teste. Os experimentos foram realizados na fase clara, no período entre 8 e 15 horas.

3.3 Tratamento Farmacológico

Histamina (HA) nas doses de 0,54 nmol, 1,36 nmol, 2,72 nmol e 4,07 nmol (ROSTAMI et al., 2006), Clorfeniramina (CPA) nas doses de 0,016 nmol, 0,052 nmol, 0,16 nmol (SERAFIM et al., 2012; PRIVOU et al., 1998) e Ranitidina (RA) nas doses de 0,57 nmol, 2,85 nmol e 5,7 nmol (ROSTAMI et al., 2006) (Sigma-Aldrich) foram microinjetadas em volume de 0,1µl. Os animais dos grupos controles receberam solução salina (SAL). As doses foram baseadas em estudos prévios realizados em ratos e camundongos com injeções em outras estruturas como hipocampo, amígdala e *nucleus basalis magnocellulares*.

Todas as drogas foram dissolvidas em solução salina estéril (0,9%). As soluções foram mantidas em refrigeração até o momento da sua utilização, em tubos codificados, de modo que o experimentador não teve conhecimento do conteúdo dos mesmos no momento do experimento e durante a análise das imagens gravadas.

3.4 Cirurgia e microinjeção

Os animais receberam implantação intracraniana de cânula-guia (25 gauge) de 7 mm de comprimento no vérmis cerebelar, após anestesia com solução de Cloridrato de Cetamina (50 mg/kg) e Xilasina (5 mg/kg, i.p) no volume de 10ml/kg. A cânula foi fixada no crânio do animal com cimento acrílico (JET resina e líquido polimerizante) com base nas coordenadas estereotáxicas (-6,5 AP; ±0 L; -2 V) definidas pelo Atlas de Paxinos e Franklin (2001). O mandril foi inserido no interior da cânula-guia para evitar problemas de obstrução e para reduzir contaminação. Logo após a cirurgia, os animais foram tratados com paracetamol (200 ml/mg), adicionado a água por um período de 3 dias para recuperação (concentração 0,16 mg/ml) (NUNES-DE-SOUZA et al., 2008; MESSIER et al., 1999).

O procedimento de microinjeção ocorreu no terceiro dia de recuperação dos animais e consistiu na remoção do mandril, inserção da agulha de injeção (cujo comprimento ultrapassa 2,0 mm a ponta da cânula-guia) dentro da cânula guia e a infusão das soluções no vérmis cerebelar. A agulha de injeção estava conectada por meio de tubo de polietileno (PE-10) à microsseringa Hamilton de 5 µl, e esta permanecia acoplada a uma bomba de infusão (Insight BI 2000 – Equipamentos Científicos Ltda, Brasil), programada para injetar 0,1 µl de solução durante 60 segundos. Após a infusão, a agulha de injeção permanecia por 60 segundos. O movimento de uma pequena bolha de ar no tubo de polietileno antes, durante e depois da injeção confirmava o fluxo da solução (CANTO-DE-SOUZA et al., 2002).

3.5 Equipamentos e Procedimentos

3.5.1 Labirinto em Cruz Elevado

Foi utilizado o Labirinto em Cruz Elevado, com dois braços abertos (BA) (30×5×0,25 cm) e dois braços fechados (BF) com proteções laterais de acrílico (30×5×15 cm), unidos por uma região central (5×5 cm) e elevado do solo a uma altura de 38,5 cm. O ambiente de teste foi isolado do restante da sala por uma cortina preta. Uma câmera posicionada a 90° acima do labirinto e acoplada ao computador permitiu o registro das imagens.

O experimento foi realizado em dois dias consecutivos, o teste 1 (T1) e o teste 2 (T2). Em T1 os animais foram transportados para uma sala adjacente e deixados neste ambiente por pelo menos 1 hora antes do teste. Os animais foram transportados em uma caixa individual de paredes opacas (33×15×13 cm) até o labirinto e então posicionados individualmente na plataforma central com a face voltada para o braço aberto. O teste teve duração de cinco minutos. Depois de cada teste, o labirinto foi limpo com álcool 5%. Imediatamente após a exploração ao LCE, os animais foram injetados de acordo com o tratamento farmacológico proposto. Após 24 h da realização de T1, os animais foram reexpostos ao LCE (T2).

Figura 2: Labirinto em Cruz Elevado

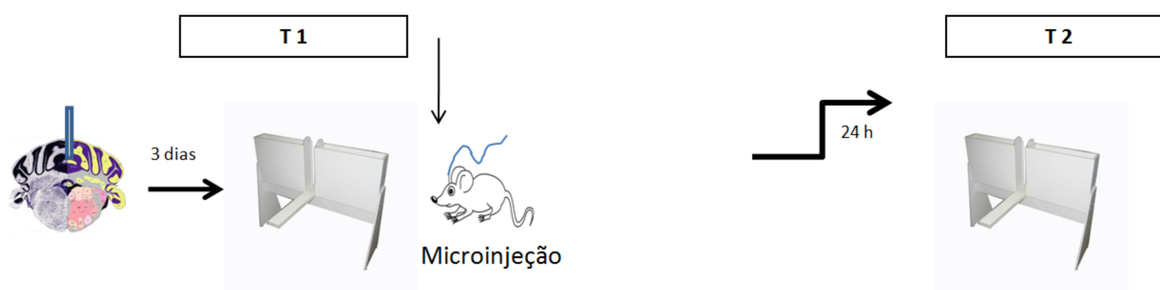

Figura 3: Protocolo experimental LCE

Análise Comportamental

Os comportamentos dos animais foram registrados, pela análise das imagens gravadas, usando um programa de computador (X-PLO-RAT, 2005) (BECERRA-

GARCIA et al., 2005) desenvolvido pelo Laboratório de Comportamento Exploratório da USP/Ribeirão Preto (http//:scotty.ffclrp.usp.br).

Cruz-Morales et al. (2002), Rodgers e Johnson (1995) e Cruz et al. (1994) em seus estudos utilizaram a análise espaço-temporal associada à análise etológica para avaliar as respostas comportamentais dos animais expostos ao LCE. Segundo Martinez (2006), o índice de memória dos animais está relacionado à exploração dos braços abertos na reexposição. Assim se o comportamento for afetado em T2 (diminuição de entradas e de tempo relativo de permanência nos braços abertos), pode-se inferir que o armazenamento e a consolidação da informação aversiva para os braços abertos, obtida na primeira exposição, foi recordada adequadamente. A exploração dos braços fechados é considerada um índice de atividade locomotora (RODGERS et al., 1997).

Na análise espaço-temporal foram quantificadas as seguintes variáveis: número de entradas nos braços abertos (EBA) e número de entradas nos braços fechados (EBF), tempo total gasto nos braços abertos (TBA), nos braços fechados (TBF) e no centro (TC) do labirinto. A entrada no braço do labirinto é definida como a presença das quatro patas fora da região central do labirinto. A partir dessas medidas foi calculada a porcentagem de entradas nos braços abertos (%EBA) = [(Frequência de entradas no braço/Total de entradas) x 100] e a porcentagem de tempo gasto nos braços abertos (%TBA) e fechados (%TBF) = [(Tempo no compartimento/300) x 100].

A análise etológica foi realizada por meio da identificação dos seguintes comportamentos dos animais durante a exploração do labirinto: Levantar (definido como a realização de um levantamento total ou parcial dos membros dianteiros), Mergulhar (definido como o movimento exploratório de cabeça e ombros para fora e debaixo do labirinto), Esticar (considerado um índice de avaliação de risco, observado quando o animal adquire uma postura exploratória, esticando todo o seu comprimento e retornando a posição anterior sem qualquer movimento das patas traseiras), Auto-limpeza (sequência típica iniciada no nariz, progredindo até as orelhas e finalizando com a limpeza de todo o corpo, incluindo o coçar-se) e Imobilidade (que ocorre quando o animal fica parado por tempo superior a 10 segundos em qualquer braço do labirinto) (CRUZ-MORALES et al. 2002; RODGERS e JOHNSON, 1995) (Figura 4).

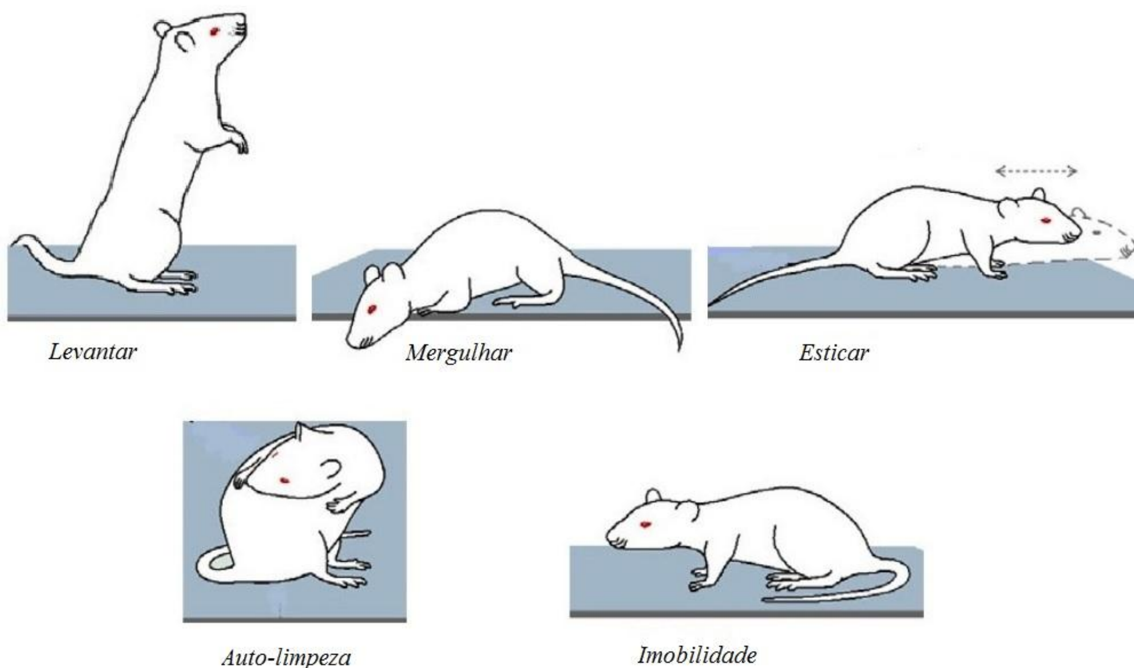

Figura 4: Representação dos comportamentos avaliados no LCE (Adaptado de Casarrubea et al., 2013)

3.5.2 Teste de Esquiva Inibitória (EI)

Para esse procedimento foi utilizada a Caixa de esquiva confeccionada em acrílico (48×24,5×25 cm) composta por dois compartimentos de mesma dimensão: um claro, sob iluminação de 450 lx, e outro escuro, com acrílico preto dotado de tampa (1 lx) Figura 5. Uma abertura na forma de porta guilhotina de 10×9 cm (base × altura) localiza-se ao nível do piso e no centro da divisão dos dois ambientes; seu acionamento se dá através do programa instalado em computador em resposta aos sensores de infravermelho. O piso é constituído por barras de aço inoxidável (2,5 mm de diâmetro) distanciadas 1 cm entre si, que liberam choques elétricos com a intensidade de 0,5 mA por 3 segundos.

O experimento foi realizado em dois dias consecutivos, o Dia 1 (D1) e o Dia 2 (D2). Em D1, os animais foram transportados para uma sala adjacente e deixados neste ambiente por pelo menos 1 hora antes do início do procedimento. No primeiro dia (D1), na primeira habituação cada animal foi transportado individualmente até a caixa e posicionado no centro da parte iluminada por 5 segundos. Então, a porta guilhotina foi aberta e o tempo de latência para o animal atravessar para o compartimento escuro foi

medido. Os animais que não atravessassem para o compartimento escuro em até 100s eram excluídos do estudo (n=4). Depois que o animal atravessasse com as quatro patas para o compartimento escuro, a porta guilhotina se fechava e o animal era retirado do aparato. A segunda habituação foi repetida 30 minutos depois, e após esse mesmo intervalo foi realizada a sessão de aquisição. Nessa sessão, imediatamente após o animal entrar no compartimento escuro a porta guilhotina foi fechada e foram liberados choques nas patas (0,5 mA por 3 segundos). Após 20 s, o animal foi removido do aparato e colocado na sua caixa moradia. Dois minutos depois, o animal foi testado seguindo o mesmo procedimento. Se ele não atravessasse para o compartimento escuro em até 120s, era considerado que a aquisição da resposta de esquiva foi obtida. Do contrário, se o animal entrasse no compartimento escuro, a porta era fechada e o mesmo choque era liberado (ZARRINDAST et al., 2002). Após esses procedimentos, o animal era retirado do aparato e a microinjeção era realizada conforme o tratamento farmacológico.

Após 24 horas (D2), um teste de retenção foi realizado para avaliar a consolidação da memória. Cada animal foi posicionado no compartimento claro por 5 s, a porta guilhotina foi aberta e a latência mensurada. Essa sessão terminava quando o animal entrava no compartimento escuro ou após 300s. Nessas sessões não foi aplicado choque. O aumento ou a diminuição das latências foi considerado indicativo de aumento ou redução na retenção da memória, respectivamente.

Os experimentos foram gravados por meio de uma câmera de vídeo digital. Posteriormente, as latências foram quantificadas pelo pesquisador através de um monitor de computador.

Figura 5: Caixa de Esquiva

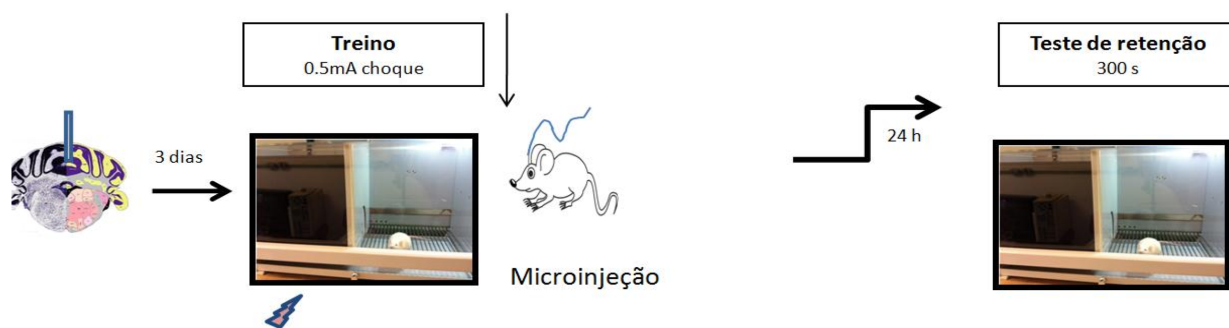

Figura 6: Protocolo experimental Esquiva Inibitória

3.6 Grupos experimentais

Os experimentos 1-5 foram realizados seguindo os mesmos procedimentos experimentais, porém com diferentes tratamentos farmacológicos.

Experimento 1

Os animais receberam injeção intra vérmis cerebelar de salina (0,9%) ou histamina (0,54; 1,36; 2,72 e 4,07 nmol/0,1µl) em T1 imediatamente após serem retirados do Labirinto em Cruz Elevado (Experimento **1a**) e do Teste de Esquiva Inibitória (Experimento **1b**).

A partir destes resultados foi definida a dose de Histamina para os experimentos 3 e 4.

Tabela 1: Grupos experimento **1a**

Grupo 1a	Teste	Tratamento	Dose	N
SAL	LCE	Salina	-------	11
HA	LCE	Histamina	0,54 nmol	15
HA	LCE	Histamina	1,36 nmol	15
HA	LCE	Histamina	2,72 nmol	16
HA	LCE	Histamina	4,07 nmol	13

Tabela 2: Grupos experimento **1b**

Grupo 1b	Teste	Tratamento	Dose	N
SAL	Esquiva	Salina	-------	11
HA	Esquiva	Histamina	0,54 nmol	11
HA	Esquiva	Histamina	1,36 nmol	9
HA	Esquiva	Histamina	2,72 nmol	10
HA	Esquiva	Histamina	4,07 nmol	10

Experimento 2

Os animais receberam injeção intra vérmis cerebelar de salina (0,9%) ou Clorfeniramina (0,016; 0,052 e 0,16 nmol/0,1µl) imediatamente após serem retirados em T1 do Labirinto em Cruz Elevado (Experimento **2a**) e do Teste de Esquiva Inibitória (Experimento **2b**).

Tabela 3: Grupos experimento 2a

Grupos 2a	Teste	Tratamento	Dose	N
SAL	LCE	Salina	-----------------	9
CPA	LCE	Clorfeniramina	0,016 nmol	9
CPA	LCE	Clorfeniramina	0,052 nmol	8
CPA	LCE	Clorfeniramina	0,16 nmol	9

Tabela 4: Grupos experimento **2b**

Grupos 2b	Teste	Tratamento	Dose	N
SAL	Esquiva	Salina	-----------------	6
CPA	Esquiva	Clorfeniramina	0,016 nmol	7
CPA	Esquiva	Clorfeniramina	0,052 nmol	7
CPA	Esquiva	Clorfeniramina	0,16 nmol	6

Experimento 3

Os animais receberam injeção intra vérmis cerebelar de salina (0,9%) ou Ranitidina (1,42; 2,85 e 5,7 nmol/0,1µl) em T1 imediatamente após serem retirados do

Labirinto em Cruz Elevado (Experimento **3a**) e do Teste de Esquiva Inibitória (Experimento **3b**).

Tabela 5: Grupos experimento **3a**

Grupos 3a	Teste	Tratamento	Dose	N
SAL	LCE	Salina	-----------------	11
RA	LCE	Ranitidina	1,42 nmol	12
RA	LCE	Ranitidina	2,85 nmol	11
RA	LCE	Ranitidina	5,7 nmol	12

Tabela 6: Grupos experimento **3b**.

Grupos 3b	Teste	Tratamento	Dose	N
SAL	Esquiva	Salina	-----------------	9
RA	Esquiva	Ranitidina	1,42 nmol	9
RA	Esquiva	Ranitidina	2,85 nmol	9
RA	Esquiva	Ranitidina	5,7 nmol	9

Para os experimentos 4 e 5 utilizamos as doses de CPA (0,16 nmol) e RA (2,85 nmol) que não apresentaram efeito sobre a consolidação da memória nos testes utilizados (LCE e EI). Assim, os antagonistas foram usados como pré-tratamento em doses sub-efetivas para verificar se o efeito da histamina poderia ser revertido.

Experimento 4

Experimento 4a: Os animais receberam injeção intra vérmis cerebelar de salina (0,9%) ou Clorfeniramina (0,16 nmol/0,1µl) imediatamente após serem retirados do Labirinto em Cruz Elevado em T1, e após 5 minutos injeção de salina ou histamina (4,07 nmol/0,1µl) na mesma estrutura.

Tabela 7: Grupos experimento **4a**

Grupo 4a	Teste	Tratamento	n
SAL+SAL	LCE	Salina+Salina	11
CPA+SAL	LCE	CPA+Salina	10
CPA+HA	LCE	CPA+HA	10
SAL+HA	LCE	Salina+HA	9

Experimento 4b: Os animais receberam injeção intra vérmis cerebelar de salina (0,9%) ou Clorfeniramina (0,16 nmol/0,1µl) em T1 imediatamente após serem retirados do Teste de Esquiva, e após 5 minutos injeção de salina ou histamina (1,36 nmol/0,1µl) na mesma estrutura.

Tabela 8: Grupos experimento **4b**

Grupo 4b	Teste	Tratamento	n
SAL+SAL	Esquiva	Salina+Salina	13
CPA+SAL	Esquiva	CPA+Salina	10
CPA+HA	Esquiva	CPA+HA	10
SAL+HA	Esquiva	Salina+HA	10

Experimento 5

Experimento 5a: Os animais receberam injeção intra vérmis cerebelar de salina (0,9%) ou Ranitidina (2,85 nmol/0,1µl) em T1 imediatamente após serem retirados do Labirinto em Cruz Elevado, e após 5 minutos injeção de salina ou histamina (4,07 nmol/0,1µl) na mesma estrutura.

Tabela 9: Grupos experimento **5a**

Grupo 5a	Teste	Tratamento	n
SAL+SAL	LCE	Salina+Salina	10
RA+SAL	LCE	RA+Salina	13
RA+HA	LCE	RA+HA	12
SAL+HA	LCE	Salina+HA	11

Experimento 5b: Os animais receberam injeção intra vérmis cerebelar de salina (0,9%) ou Ranitidina (2,85 nmol/0,1µl) em T1 imediatamente após serem retirados do Teste de Esquiva Ativa, e após 5 minutos injeção de salina ou histamina (1,36 nmol/0,1µl) na mesma estrutura.

Tabela 10: Grupos experimento **5b**

Grupo 5b	Teste	Tratamento	n
SAL+SAL	Esquiva	Salina+Salina	8
RA+SAL	Esquiva	RA+Salina	9
RA+HA	Esquiva	RA+HA	7
SAL+HA	Esquiva	Salina+HA	7

3.7 Histologia

Após o término do experimento, os animais receberam microinjeção de 0,1 µl de uma solução de 1% de azul de metileno, de acordo com o procedimento descrito para a injeção das drogas. Após receberem uma dose profunda de anestesia com solução de Cloridrato de Ketamina e Xilasina, os animais sofreram deslocamento cervical, foram decapitados e seus encéfalos removidos e acomodados em recipientes contendo solução de formalina (10%), por três dias no mínimo. Após esse período, os encéfalos foram conservados em solução de sacarose-20% 48 horas antes da análise, em solução de sacarose-30% 24 horas antes da histologia, e mantidos sob refrigeração.

Posteriormente, os encéfalos foram seccionados coronalmente na espessura de 80 micrômetros (µm) ao longo do trajeto da cânula, utilizando um micrótomo criostato (criostato ANCAP 300). As secções foram inspecionadas através de um microscópio (Olympus B202) e a visualização da dispersão do azul de metileno indicou o local da injeção. Quando a injeção não atingiu o vérmis cerebelar, o animal foi excluído do estudo.

IV - ANÁLISE ESTATÍSTICA

Todos os resultados foram inicialmente submetidos ao teste de Levene para verificar a homogeneidade. Os dados comportamentais foram analisados através da análise de variância (ANOVA) de uma via, e quando apropriado foi utilizado o teste *post hoc* de Duncan. O nível de significância adotado foi $p < 0,05$.

V - RESULTADOS

ANÁLISE HISTOLÓGICA

Ao final dos experimentos, para a análise dos dados foram utilizados 455 animais, pois porcentagem para canulação positiva foi de 88%.

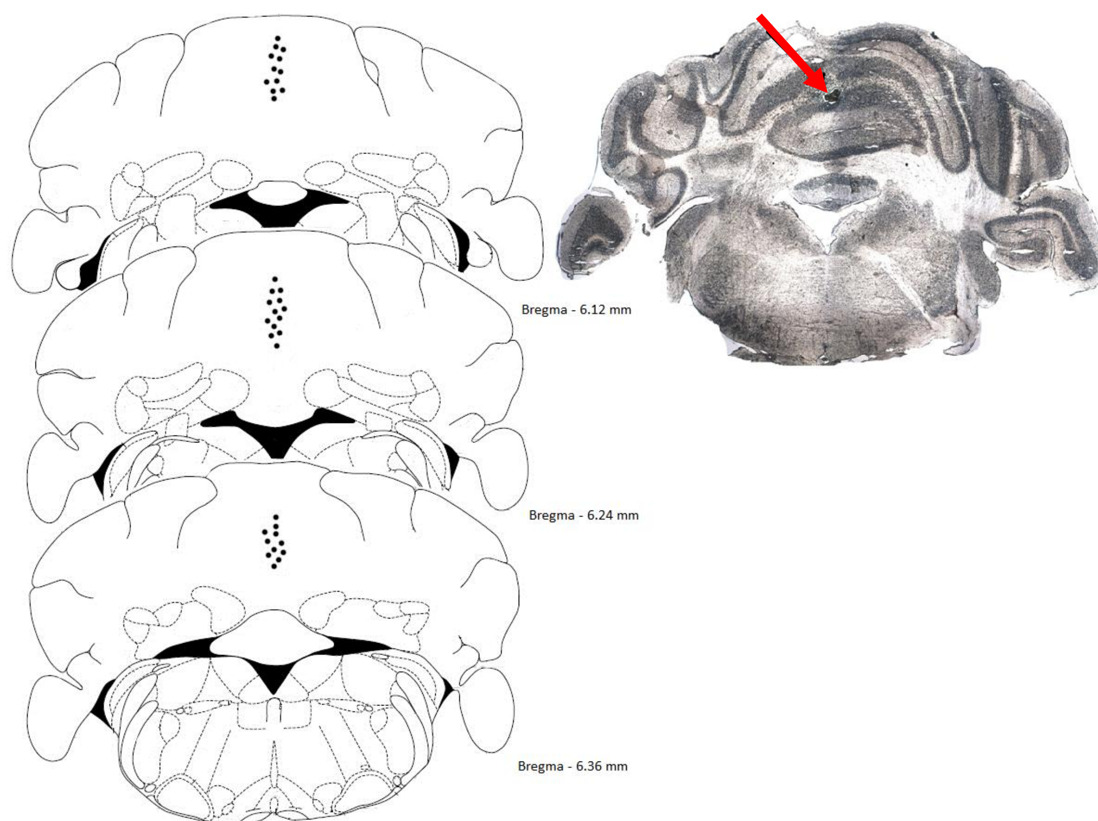

Figura 7: Figuras adaptadas do atlas de Paxinos e Franklin (2001) e fotomicrografia do cerebelo de camundongo mostrando sitio de injeção no vérmis cerebelar.

5.1 EXPERIMENTO 1a

Os resultados mostram o efeito da microinjeção de histamina no vérmis cerebelar de camundongos reexpostos ao Labirinto em Cruz Elevado (LCE).

A ANOVA de uma via não indicou diferença significativa entre os animais expostos ao LCE sem tratamento para todas as medidas avaliadas (Tabela 11), o que permitiu utilizar os resultados de T1 como *Pool* para as análises seguintes.

Para %EBA e %TBA, a ANOVA revelou diferenças entre a exposição e a reexposição ($F_{5,134}$ = 4,72, p=0,0005 e $F_{5,134}$= 6,17, p=0,000036, respectivamente). Para %EBA, o teste *post hoc* de Duncan indicou que os animais que entraram menos nos braços abertos na reexposição foram microinjetados com salina (p=0,001) e histamina na dose de 0,54 nmol (p=0,02). Além disso, a análise mostrou uma diferença significativa entre o grupo que recebeu salina e o que recebeu histamina na dose de 4,07 nmol (p=0,007). Para %TBA, houve diferença significativa nos grupos salina (p<0,001), histamina na dose de 0,54 nmol (p=0,005) e histamina na dose de 1,36 nmol (p=0,01). Nos grupos microinjetados com histamina na dose de 2,72 nmol (%EBA p=0,16; %TBA p=0,09) e na dose de 4,07 nmol (%EBA p=0,52; %TBA p=0,10) não houve diferença significativa entre os dias de teste para ambas as medidas (Figura 8 e 9).

A tabela 12 mostra os resultados para os outros comportamentos analisados. A ANOVA de uma via não indicou diferença significativa para EBF ($F_{5,134}$ = 0,61, p=0,11). Para TBA, a ANOVA indicou diferença significativa entre os dias ($F_{5,134}$ = 8,2, p=0,000001), e a análise *post hoc* revelou que esta diferença estava presente nos grupos microinjetados com salina (p<0,001), histamina na dose de 0,54 nmol (p=0,002) e histamina na dose de 1,36 nmol (p<0,001). Também foram observadas diferenças entre os dias de teste para as variáveis TBF ($F_{5,134}$ = 9,41, p < 0,00001), %TBF ($F_{5,134}$ = 9,41, p<0,00001), TC ($F_{5,134}$ = 5,85, p = 0,000063), e total de mergulho ($F_{5,134}$ = 10,39, p<0,00001). A ANOVA não detectou diferenças para EBA ($F_{5,134}$ = 1,86, p = 0,11), tempo de imobilidade ($F_{5,134}$ = 1,56, p = 0,18) e esticar total ($F_{5,134}$ = 1,13, p = 0,34).

Tabela 11: Valores da ANOVA de uma via, para as variáveis do experimento 1a analisadas na exposição sem tratamento farmacológico.

Medidas	**F**	**Valor de p**
EBA	0,72	0,55
%EBA	0,34	0,80
TBA	0,99	0,41
%TBA	0,99	0,41
EBF	0,75	0,53
TBF	0,02	0,99
%TBF	0,02	0,99
TC	2,19	0,11
Esticar total	1,79	0,14
Mergulhar total	0,71	0,55
Tempo imobilidade	0,64	0,60

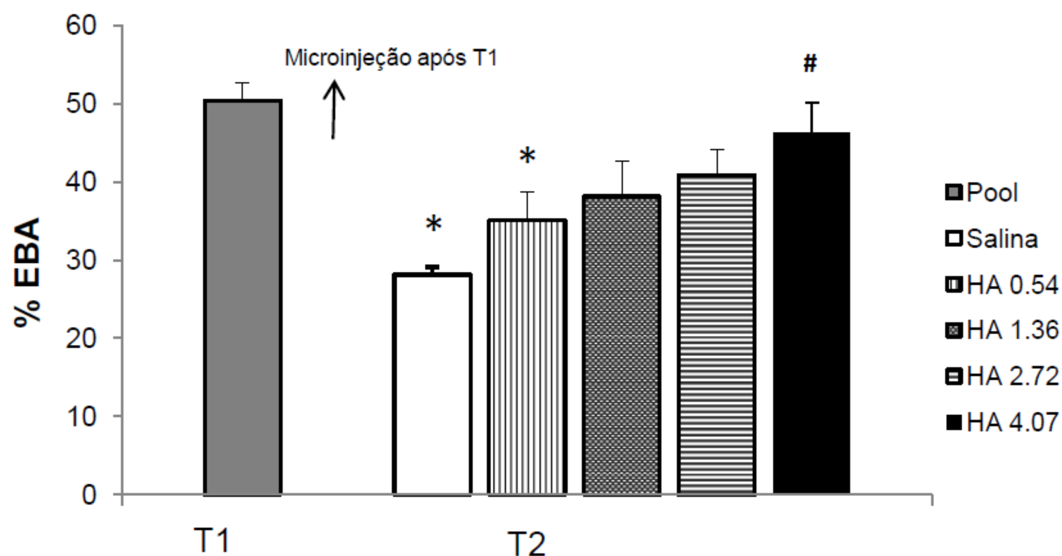

Figura 8: Médias e erro padrão da média para a porcentagem de entradas nos braços abertos de camundongos reexpostos ao LCE. Pool (todos os animais expostos ao LCE sem tratamento); Salina (microinjetada imediatamente após a exposição); HA (Histamina microinjetada imediatamente após a exposição ao LCE nas diferentes doses: 0,54 nmol; 1,36 nmol; 2,72 nmol e 4,07 nmol/0,1µl). *p<0,05 em relação a T1; #p<0,05 em relação ao grupo salina, teste de Duncan.

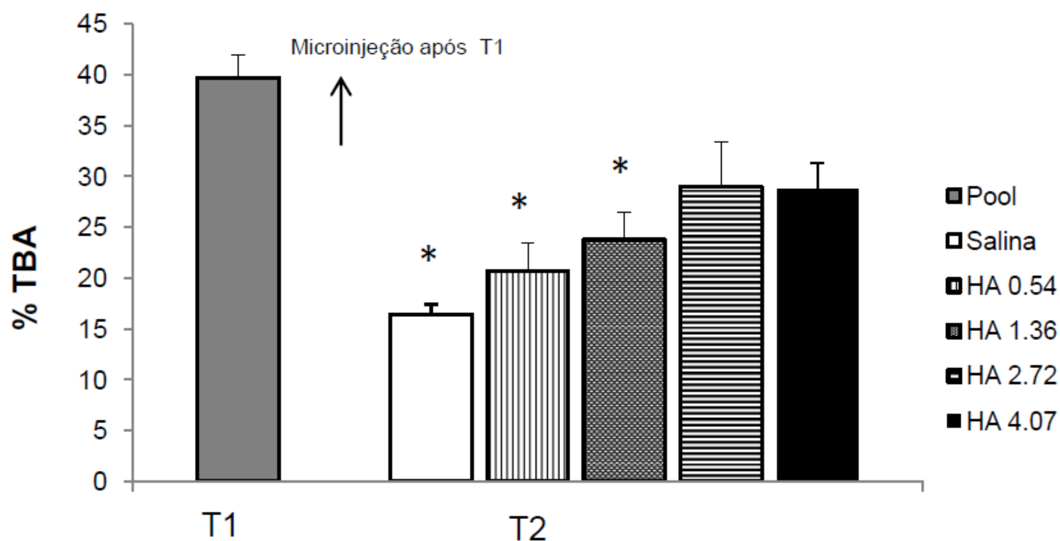

Figura 9: Médias e erro padrão da média para a porcentagem de tempo nos braços abertos de camundongos reexpostos ao LCE. Pool (todos os animais expostos ao LCE sem tratamento); Salina (microinjetada imediatamente após a exposição); HA (Histamina microinjetada imediatamente após a exposição ao LCE nas diferentes doses: 0,54 nmol; 1,36 nmol; 2,72 nmol e 4,07 nmol/0,1µl). *p<0,05 em relação a T1, teste de Duncan.

Tabela 12: Efeitos da microinjeção de histamina no vérmis cerebelar sobre o comportamento de camundongos expostos e reexpostos ao Labirinto em Cruz Elevado.

	Pool	**Salina**	**HA 0,54**	**HA 1,36**	**HA 2,72**	**HA 4,07**
EBA	8,2 ± 0,4	6,6 ± 1,4	6,3 ± 0,9	6,0 ± 1,2	7,7 ± 0,8	9,6 ± 1,2
TBA	119,0 ± 6,9	49,3 ± 9,9*	62,0 ± 9,6*	53,6 ± 12,9*	86,9 ± 13,2	86,2 ± 7,5
EBF	8,5 ± 0,5	13,9 ± 1,35	11,1 ± 1,1	10,1 ± 1,1	11,1 ± 1,5	10,7 ± 1,1
TBF	89,9 ± 5,3	178,3 ± 53,7*	166,2 ± 14,1*	163,9 ± 21,4*	134,3 ± 14,5*	125,3 ± 12,8*
%TBF	29,6 ± 1,8	59,4 ± 7,9*	53,4 ± 5,7*	54,7 ± 7,1*	44,8 ± 4,8*#	41,8 ± 4,3*#
TC	91,6 ± 0,5	72,4 ± 21,8	71,8 ± 7,5*	82,5 ± 17,5*	78,8 ± 10,5*	88,4 ± 9,6*
Esticar	9,3 ± 0,0	6,9 ± 1,1	7,9 ± 1,1	7,0 ± 0,9	9,1 ± 1,4	8,5 ± 1,6
Mergulhar	5,9 ± 0,9	0,5 ± 0,1*	1,7 ± 0,6*	2,0 ± 1,1*	1,7 ± 0,5*	1,8 ± 0,9*
Imobilidade	0,0 ± 0,1	0,0 ± 0,0	0,1 ± 0,1	0,5 ± 0,5	0,0 ± 0,0	0,0 ± 0,0

Pool (todos os animais expostos ao LCE sem tratamento); Salina (microinjetada imediatamente após a exposição); HA (Histamina microinjetada imediatamente após a exposição ao LCE nas diferentes doses: 0,54 nmol; 1,36 nmol; 2,72 nmol e 4,07 nmol/0,1µl). EBA (número de entradas nos braços abertos); TBA (tempo gasto nos braços abertos); EBF (número de entradas nos braços fechados); TBF (tempo gasto nos braços fechados); %TBF (porcentagem de tempo gasto nos braços fechados); TC (tempo gasto no centro); Esticar (total); Mergulhar (total); Tempo de imobilidade. *p<0,05 em relação a T1; #p<0,05 em relação ao grupo controle, teste de Duncan.

5.2 EXPERIMENTO 1b

Os resultados mostram os efeitos da microinjeção de Histamina no vérmis cerebelar de camundongos submetidos ao Teste de Esquiva Inibitória. A ANOVA de uma via indicou diferença significativa no tempo de latência no segundo dia entre os grupos ($F_{4, 46}$= 3,96, p=0,009). A análise *post hoc* mostrou um aumento significativo na latência para os animais que receberam histamina na dose de 1,36 nmol em relação aos animais que receberam salina (p<0,05). Além disso, houve diferença significativa entre os grupos microinjetados com histamina 1,36 nmol e os grupos que receberam histamina nas doses de 2,72 (p<0,01) e 4,07 nmol (p=0,04). Essas duas doses mais elevadas apresentaram menor tempo de latência quando comprados com a dose de 1,36 nmol (Figura 10).

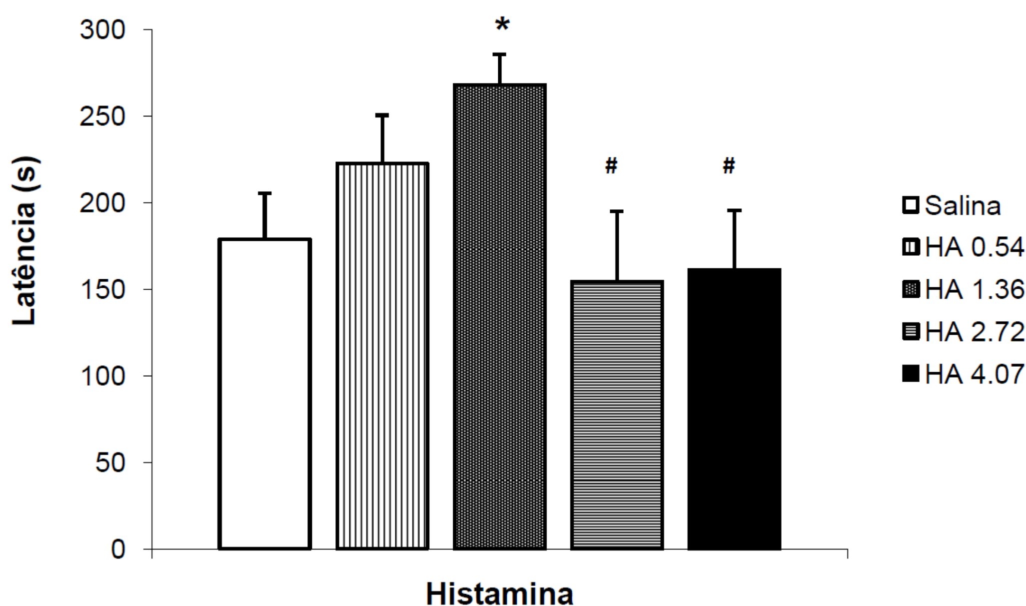

Figura 10: Efeitos da histamina (0,54; 1,36; 2,72; e 4,07 nmol/0,1µl) microinjetada no vérmis cerebelar na consolidação da memória de Esquiva Inibitória. Médias e erro padrão da média para o tempo de latência (segundos). N=9-11. *p<0,05 em relação ao grupo controle; # p<0,05 em relação à histamina 1,36 nmol, teste de Duncan.

5.3 EXPERIMENTO 2a

Os resultados mostram o efeito da microinjeção de Clorfeniramina no vérmis cerebelar de camundongos reexpostos ao Labirinto em Cruz Elevado (LCE).

A ANOVA de uma via não indicou diferença significativa entre os animais expostos ao LCE sem tratamento para todas as medidas avaliadas (Tabela 13), o que permitiu utilizar os resultados de T1 como *Pool* para as análises seguintes.

Para %EBA e %TBA, a ANOVA revelou diferenças entre a exposição (T1) e reexposição (T2) ($F_{4,65}$= 6,12, p=0,0003 e $F_{4,65}$= 6,94, p=0,0001, respectivamente). O teste *post hoc* de Duncan revelou diferenças entre a exposição e a reexposição para os grupos controle (SAL) e Clorfeniramina (CPA) nas doses de 0,016 nmol, 0,052 nmol e 0,16 nmol (Figura 11 e 12). O que indica que a microinjeção de CPA não alterou a os níveis de exploração dos braços abertos do LCE.

A ANOVA revelou diferenças entre T1 e T2 para TBA ($F_{4,65}$= 6,94, p=0,0001), TBF ($F_{4,65}$= 10,2, p<0,0001), e %TBF ($F_{4,65}$= 10,2, p<0,0001). O teste *post hoc* de Duncan revelou que essas diferenças foram observadas nos grupos salina e CPA nas doses de 0,016 nmol, 0,052 nmol e 0,16 nmol (p<0,05). Para EBA, a diferença entre a exposição e a reexposição ($F_{4,65}$= 3,96, p=0,006), foi indicada pelo teste *post hoc* apenas para o grupo salina (p=0,007) (Tabela 14).

Não houve diferenças significativas nas EBF ($F_{4,65}$= 1,08, p=0,37), TC ($F_{4,65}$= 0,33, p=0,85), esticar total ($F_{4,65}$= 1,13, p=0,34), levantar total ($F_{4,65}$= 0,58, p=0,68), e tempo total de imobilidade ($F_{4,65}$= 0,45, p=0,77). Para as variáveis tempo total de auto-limpeza e mergulhar total, a ANOVA indicou diferença entre os dias de teste ($F_{4,65}$= 2,75, p=0,04; $F_{4,65}$= 6,33, p=0,0002). O teste *post hoc* de Duncan indicou diferenças no tempo total de auto-limpeza apenas para o grupo microinjetado com salina. Na medida de mergulhar total, o teste Duncan mostrou diferença entre exposição e reexposição para todos os grupos (Tabela 14).

Tabela 13: Valores da ANOVA de uma via, para as variáveis do experimento 2a (CPA) analisadas na exposição sem tratamento farmacológico.

Medidas	**F**	**Valor de p**
EBA	0,93	0,45
%EBA	1,55	0,20
TBA	0,94	0,44
%TBA	1,25	0,30
EBF	0,81	0,52
TBF	0,33	0,85
%TBF	0,33	0,85
TC	0,57	0,68
Esticar total	1,19	0,32
Levantar total	1,07	0,38
Mergulhar total	1,37	0,25
Tempo imobilidade	0,64	0,60
Tempo auto-limpeza	1,58	0,21

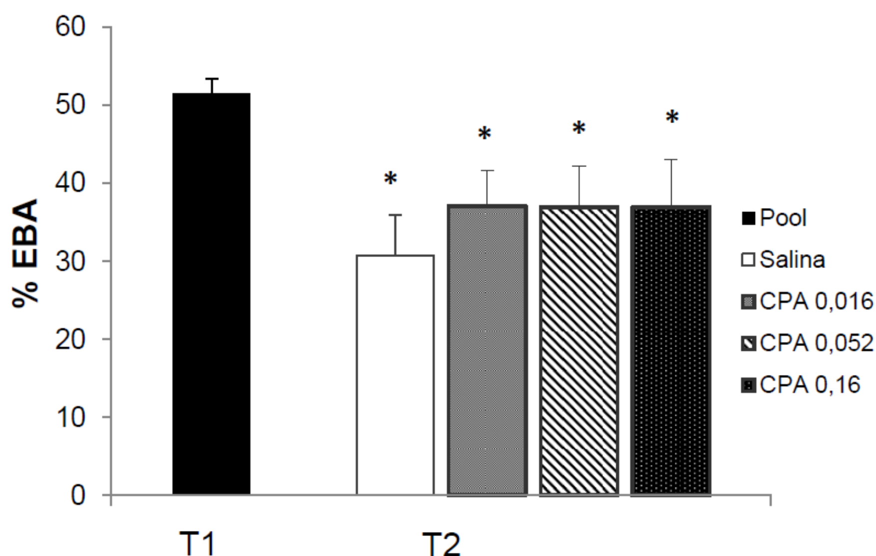

Figura 11: Médias e erro padrão da média para a porcentagem de entradas nos braços abertos de camundongos reexpostos ao LCE. Pool (todos os animais expostos ao LCE sem tratamento); Salina (microinjetada imediatamente após a exposição); CPA (Clorfeniramina microinjetada imediatamente após a exposição ao LCE nas diferentes doses: 0,016 nmol; 0,052 nmol e 0,16 nmol/0,1µl). *p<0,05 em relação a T1, teste de Duncan.

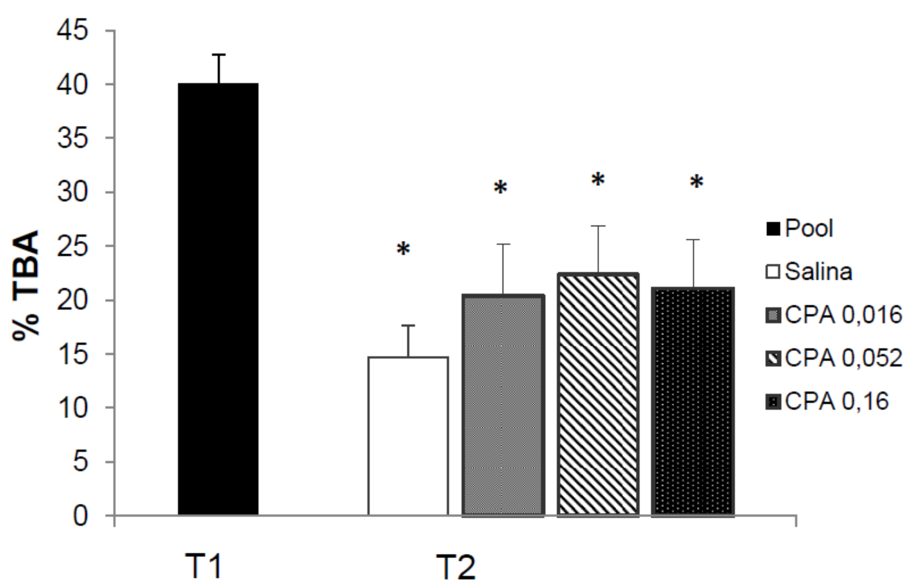

Figura 12: Médias e erro padrão da média para a porcentagem de tempo nos braços abertos de camundongos reexpostos ao LCE. Pool (todos os animais expostos ao LCE sem tratamento); Salina (microinjetada imediatamente após a exposição); CPA (Clorfeniramina microinjetada imediatamente após a exposição ao LCE nas diferentes doses: 0,016 nmol; 0,052 nmol e 0,16 nmol/0,1µl). *p<0,05 em relação a T1, teste de Duncan.

Tabela 14: Efeitos da microinjeção de Clorfeniramina no vérmis cerebelar sobre o comportamento de camundongos reexpostos ao Labirinto em Cruz Elevado.

	Pool	Salina	CPA 0,016	CPA 0,052	CPA 0,16
EBA	8,9 ± 0,6	4,0 ± 0,6*	6,6 ± 1,4	6,3 ± 1,2	6,3 ± 1,4
TBA	112,6 ± 8,3	44,2 ± 8,7*	61,1 ± 14,5*	67,2 ± 13,9*	63,3 ± 13,6*
EBF	8,3 ± 0,5	9,7 ± 1,3	10,1 ± 1,3	9,9 ± 0,8	10,2 ± 1,2
TBF	94,4 ± 5,7	173,4 ± 12,0*	150,5 ± 17,5*	152,9 ± 16,6*	150,0 ± 19,5*
%TBF	31,5 ± 1,9	57,8 ± 4,0*	50,2 ± 5,8*	51,0 ± 5,5*	50,0 ± 6,5*
TC	93,0 ± 6,4	82,4 ± 10,5	88,4 ± 12,0	80,0 ± 7,3	86,7 ± 11,7
Esticar	9,1 ± 0,8	8,2 ± 1,3	4,9 ± 1,1	6,8 ± 1,3	8,6 ± 1,5
Mergulhar	10,0 ± 1,3	2,2 ± 1,0*	2,4 ± 0,8*	2,1 ± 1,6*	4,4 ± 1,3*
Levantar	10,0 ± 1,2	12,3 ± 2,8	7,9 ± 1,8	8,1 ± 2,3	9,8 ± 2,2
Auto-limpeza	4,5 ± 1,1	15,9 ± 7,3*	6,5 ± 4,6	4,7 ± 2,1	10,6 ± 3,4
Imobilidade	0,3 ± 0,2	0,0 ± 0,0	0,4 ± 0,4	0,0 ± 0,0	0,0 ± 0,0

Pool (todos os animais expostos ao LCE sem tratamento); Salina (microinjetada imediatamente após a exposição); CPA (Clorfeniramina microinjetada imediatamente após a exposição ao LCE nas diferentes doses: 0,016 nmol; 0,052 nmol; e 0,16 nmol/0,1µl). EBA (número de entradas nos braços abertos); TBA (tempo gasto nos braços abertos); EBF (número de entradas nos braços fechados); TBF (tempo gasto nos braços fechados); %TBF (porcentagem de tempo gasto nos braços fechados); TC (tempo gasto no centro); Esticar (total); Mergulhar (total); Levantar (total); Tempo de auto-limpeza; Tempo de imobilidade. *p<0,05 em relação a T1, teste de Duncan.

5.4 EXPERIMENTO 2b

Os resultados mostram os efeitos da microinjeção de clorfeniramina no vérmis cerebelar de camundongos submetidos ao Teste de Esquiva Inibitória. A ANOVA de uma via não indicou diferença significativa no tempo de latência no segundo dia entre os grupos ($F_{3, 32}$= 0,11, p=0,95), o que sugere que não houve efeitos da CPA na consolidação da memória no Teste de Esquiva Inibitória nas doses utilizadas (Figura 13).

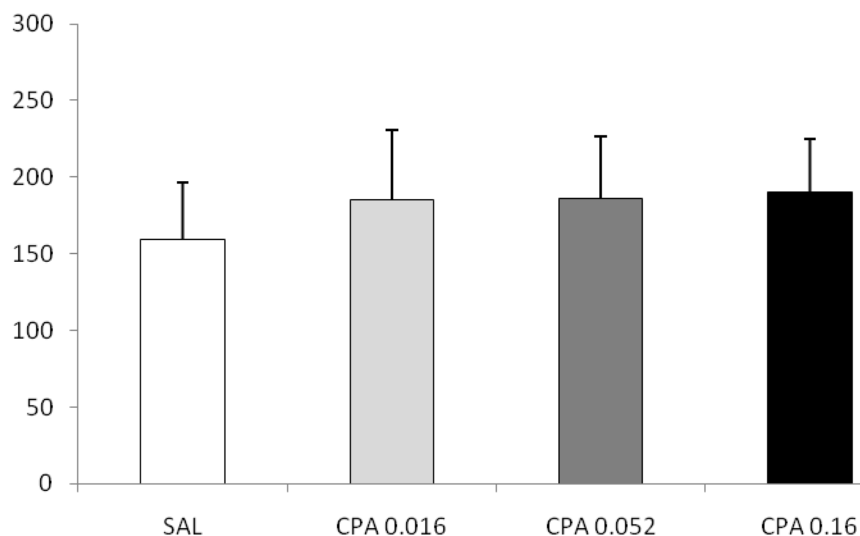

Figura 13: Efeitos da clorfeniramina (0,016; 0,052 e 0,16 nmol/0,1µl) microinjetada no vérmis cerebelar na consolidação da memória de Esquiva Inibitória. Médias e erro padrão da média para o tempo de latência (segundos). N=6-7.

5.5 EXPERIMENTO 3a

Os resultados mostram o efeito da microinjeção de Ranitidina (RA) no vérmis cerebelar de camundongos reexpostos ao Labirinto em Cruz Elevado (LCE).

A ANOVA de uma via não indicou diferença significativa entre os animais expostos ao LCE sem tratamento para todas as medidas avaliadas (Tabela 15), o que permitiu utilizar os resultados de T1 como *Pool* para as análises seguintes.

Para %EBA e %TBA, a ANOVA de uma via revelou diferenças ($F_{4,87}$ = 3,76, p=0,007 e $F_{4,87}$= 3,38, p=0,013 respectivamente). Para %EBA, o teste *post hoc* de Duncan mostrou diferenças significativas nos grupos salina (p=0,03) e nos grupos microinjetados com RA 0,57 e 2,85 nmol (p<0,05) em relação à exposição (T1). O mesmo se repetiu na análise *post hoc* para %TBA com diferença significativa entre os dias no grupo salina (p=0,027) e no grupo RA 2,85 nmol (p=0,009). Nos grupos microinjetados com RA na dose de 0,57 nmol (%EBA p=0,32; %TBA p=0,33) e na dose de 5,7 nmol (%EBA p=0,30; %TBA p=0,49) não houve diferença significativa entre os dias de teste para ambas as medidas (Figura 14 e 15).

Em relação à EBA, a ANOVA de uma via indicou diferenças ($F_{4,87}$= 2,99, p=0,02). O teste *post hoc* de Duncan revelou essa diferença em relação à exposição apenas no grupo microinjetado RA 2,85 (p=0,01).

Na variável TBA, a ANOVA evidenciou diferenças ($F_{4,87}$= 3,38, p=0,01). O teste *post hoc* de Duncan indicou diferenças em relação à exposição no grupo salina (p=0,027) e no grupo RA 2,85 nmol (p=0,025). Em relação à TBF e %TBF, a ANOVA também mostrou diferenças entre os dias ($F_{4,87}$ = 7,59, p=0,000027; $F_{4,87}$ = 7,59, p=0,000027). O teste de Duncan mostrou diferenças nos grupos salina, no microinjetado com RA nas doses 0,57 nmol e 2,85 nmol.

Para TC, a ANOVA revelou diferenças significativas entre a exposição e reexposição ($F_{4,87}$= 4,60, p=0,002) e a análise *post hoc* mostrou essa diferenças para os grupos microinjetados com RA 0,57 nmol (p=0,01) e RA 2,85 nmol (p=0,02). Além disso, houve diferença significativa entre os grupos microinjetados com RA 0,57 nmol e RA 2,85 nmol com o grupo salina. Em relação EBF, a ANOVA mostrou diferença significativa entre os dias ($F_{4,87}$= 3,22, p=0,01). O teste *post hoc* de Duncan indicou diferenças para o grupo salina (p=0,04).

No comportamento mergulhar, a ANOVA indicou diferença significativa entre os dias de teste ($F_{4,87}$= 5,14, p=0,0009). O teste de Duncan mostrou diferenças para os

grupos salina (p=0,01), microinjetado com ranitidina na dose 2,85 nmol (p= 0,01) e na dose 5,7 nmol (p=0,02). Para as variáveis Tempo de imobilidade ($F_{4,87}$= 1,30, p = 0,28) e esticar ($F_{4,87}$= 1,41, p=0,23) a ANOVA não indicou diferenças significativas (Tabela 16).

Tabela 15: Valores da ANOVA de uma via, para as variáveis analisadas na exposição sem tratamento farmacológico.

Medidas	F	Valor de p
EBA	1,39	0,26
EBF	1,20	0,32
%EBA	0,58	0,63
TBA	1,22	0,31
%TBA	1,22	0,31
TBF	0,73	0,54
TC	1,03	0,38
IMOBILIDADE	2,06	0,12
ESTICAR	0,87	0,46
MERGULHAR	0,58	0,63

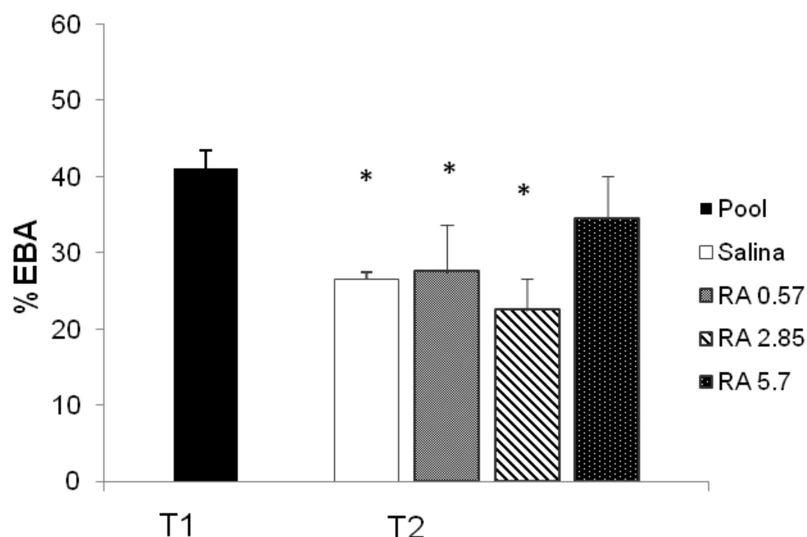

Figura 14: Médias e erro padrão da média para a porcentagem de entradas nos braços abertos de camundongos reexpostos ao LCE. T1 (Pool, todos os animais expostos ao LCE sem tratamento); Salina (microinjetada imediatamente após a exposição); RA (Ranitidina microinjetada imediatamente após a exposição ao LCE nas diferentes doses: 0,57 nmol; 2,85 nmol; e 5,7 nmol/0,1µl). *p<0,05 em relação a T1, teste de Duncan.

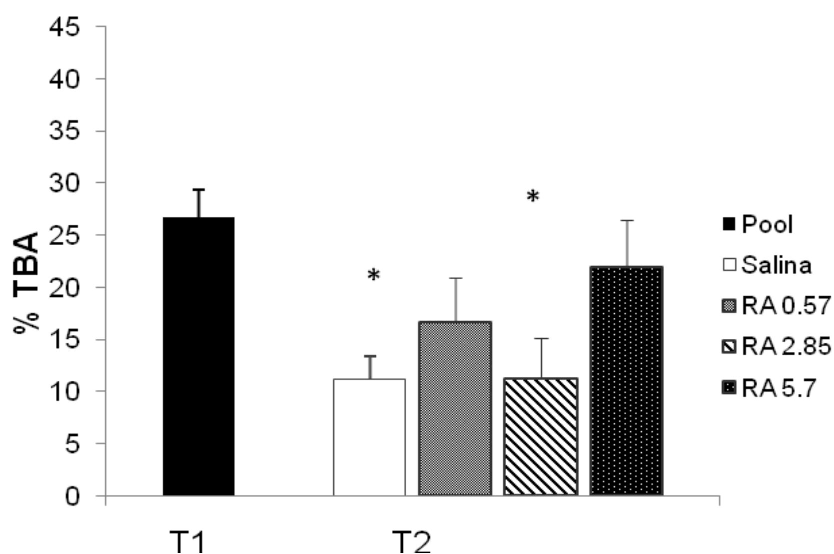

Figura 15: Médias e erro padrão da média para a porcentagem de tempo nos braços abertos de camundongos reexpostos ao LCE. T1 (Pool, todos os animais expostos ao LCE sem tratamento); Salina (microinjetada imediatamente após a exposição); RA (Ranitidina microinjetada imediatamente após a exposição ao LCE nas diferentes doses: 0,57 nmol; 2,85 nmol; e 5,7 nmol/0,1µl). *p<0,05 em relação a T1, teste de Duncan.

Tabela 16: Efeitos da microinjeção de Ranitidina (0,57 nmol; 2,85 nmol e 5,7 nmol/0,1 µl) no comportamento de camundongos expostos e reexpostos ao Labirinto em Cruz Elevado.

	Pool	SAL	RA 0,57	RA 2,85	RA 5,7
EBA	6,7 ± 0,3	4,5 ± 0,5	4,1 ± 0,6	2,6 ± 0,4*	6,6 ± 0,6
TBA	78,4 ± 1,1	33,7 ± 1,4*	50,1 ± 2,2	33,9 ± ,.9*	66,0 ± 2,0
EBF	9,0 ± 0,3	11,5 ± 0,5*	7,7 ± 0,6	8,4 ± 0,5	10,9 ± 0,5
TBF	118,6 ± 1,0	161,1± 1,7*	192,4 ± 2,8*	193,0 ± 2,0*	149,5 ± 2,3
%TBF	39,5 ± 0,6	53,7± 1,0*	64,1 ± 1,6*	64,3 ± 1,1*	49,8 ± 1,3
TC	103,1 ± 0,8	105,2± 1,6	57,5 ± 1,9*#	73,2 ± 1,7*#	84,5 ± 1,6
Esticar	7,9 ± 0,3	5,4 ± 0,5	6,3 ± 0,6	6,0 ± 0,5	9,0 ± 0,7
Mergulhar	7,3 ± 0,4	1,6 ± 0,4*	2,1 ± 0,5*	2,0 ± 0,5*	2,6 ± 0,6*
Imobilidade	0,1 ± 0,1	0,1 ± 0,2	0,4 ± 0,3	0,0 ± 0,0	0,7 ± 0,4

Pool (todos os animais expostos ao LCE em T1 sem tratamento); Salina (microinjetada imediatamente após a exposição); RA (Ranitidina microinjetada imediatamente após a exposição ao LCE nas diferentes doses: 0,57 nmol; 2,85 e 5,7 nmol/0,1µl). EBA (número de entradas nos braços abertos); TBA (tempo nos braços abertos); EBF (número de entradas nos braços fechados); TBF (tempos nos braços fechados); TC (tempo gasto no centro); ESTICAR (total de freqüência de esticadas); MERGULHAR (freqüência total de mergulhadas); IMOBILIDADE (tempo total de imobilidade). *$p<0,05$ em relação a pool (T1); #$p<0,05$ em relação ao grupo salina, teste de Duncan.

5.6 EXPERIMENTO 3b

Os resultados mostram os efeitos da microinjeção de ranitidina no vérmis cerebelar de camundongos submetidos ao Teste de Esquiva Inibitória. A ANOVA de uma via indicou diferença significativa no tempo de latência no segundo dia entre os grupos ($F_{3,32}$ = 5,48, p < 0,005). O teste de Duncan mostrou que não há diferença significativa entre os grupos salina e ranitidina 0,57 e 2,85 nmol. Entretanto, o teste indicou diferença significativa na latência para os animais que receberam ranitidina 5,7 nmol em relação aos animais controle (p=0,004). Além disso, o teste post hoc mostrou diferença entre o grupo ranitidina 5,7 nmol e 0,57 nmol e 2,85 (Figura 16).

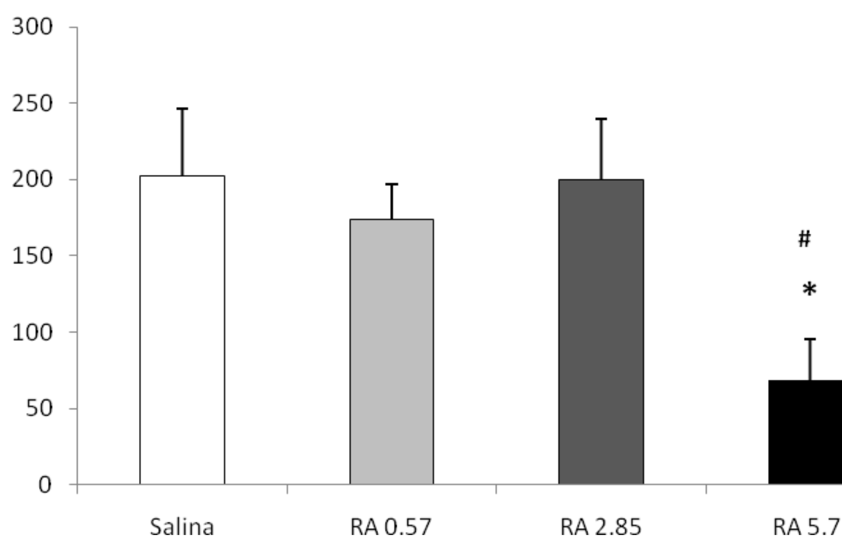

Figura 16: Efeitos da Ranitidina (0,57; 2,85 e 5,7 nmol/0,1µl) microinjetada no vérmis cerebelar na consolidação da memória de Esquiva Inibitória. Médias e erro padrão da média para o tempo de latência (segundos). N=9-11. *p<0,05 em relação ao grupo controle; # p<0,05 em relação à ranitidina 0.57 e 2,85 nmol, teste de Duncan.

5.7 EXPERIMENTO 4a

Os resultados mostram o efeito da injeção combinada de antagonista do receptor H_1 Clorfeniramina (0,16 nmol/0,1µl) e de Histamina (4,07 nmol/0,1µl) no vérmis cerebelar de camundongos reexpostos ao Labirinto em Cruz Elevado (LCE).

A ANOVA de uma via não indicou diferença significativa entre os animais expostos ao LCE sem tratamento para todas as medidas avaliadas (Tabela 17), o que permitiu utilizar os resultados de T1 como *Pool* para as análises seguintes.

A ANOVA indicou diferença na %EBA ($F_{4, 75}$= 6,83, p=0,00009). O teste *post hoc* de Duncan revelou que os animais microinjetados com SAL+SAL (p=0,02), CPA+SAL (p=0,05) e CPA+HA (p<0,001) entraram menos nos braços abertos em T2 comparados a T1, enquanto que os animais que receberam SAL+HA não reduziram a porcentagem de entradas nos braços abertos (p=0,51) e mostraram uma diferença significativa com o grupo CPA+HA (p=0,003) (Figura 17).

A Figura 18 mostra os dados de %TBA para a primeira e segunda exposição ao LCE. A ANOVA detectou diferenças entre as sessões ($F_{4, 75}$= 10,44, p=0,000001) para %TBA. A análise *post hoc* revelou que os animais que exploraram os braços abertos por menor período de tempo na reexposição foram microinjetados com SAL+SAL (p=0,007), CPA+SAL (p=0,02) e CPA+HA (p<0,0001). O grupo que recebeu SAL+HA não reduziu significativamente a porcentagem de tempo nos braços abertos (p=0,51) e apresentou diferença significativa com o grupo CPA+HA (p=0,009). Esses resultados mostram que os animais que receberam HA não reduziram a esquiva aos braços abertos na reexposição, e que a CPA não altera os parâmetros comportamentais por si só, mas reverte o prejuízo induzido pela histamina na consolidação da memória em camundongos submetidos ao LCE.

A tabela 18 mostra os resultados para as outras variáveis analisadas. A ANOVA não indicou diferença significativa para EBF ($F_{4, 75}$= 0,66, p=0,62), índex geral de atividade locomotora no LCE. A ANOVA revelou diferença entre os dias para TBA ($F_{4, 75}$ = 9,93, p=0,000002) e EBA ($F_{4, 75}$ = 7,58, p=0,00004). O teste de comparações múltiplas indicou estas diferenças para os grupos microinjetados com SAL+SAL, CPA+SAL, e CPA+HA. Além disso, foram observadas diferenças entre os grupos SAL+HA, SAL+SAL (p=0,03) e CPA+HA (p=0,0001). ANOVA mostrou diferença significativa para TBF ($F_{4, 75}$ = 15,77, p<0,00001), %TBF ($F_{4, 75}$= 15,77, p<0,00001),

TC ($F_{4,65}$= 6,15, p=0,0002), e freqüência de mergulho ($F_{4, 75}$= 9,35, p<0,00001). A ANOVA não detectou diferença entre os dias para tempo de imobilidade ($F_{4, 65}$= 1,56, p=0,19), e esticar ($F_{4 ,65}$= 2,34, p=0,06).

Tabela 17: Valores da ANOVA de uma via, para as variáveis do experimento 4a analisadas na exposição sem tratamento farmacológico.

Medidas	F	P
EBA	0,62	0,60
%EBA	0,34	0,80
TBA	0,45	0,72
%TBA	0,45	0,72
EBF	1,48	0,24
TBF	0,48	0,70
%TBF	0,48	0,70
TC	1,97	0,14
Esticar	0,49	0,69
Mergulho	0,71	0,55
Tempo de imobilidade	0,77	0,52

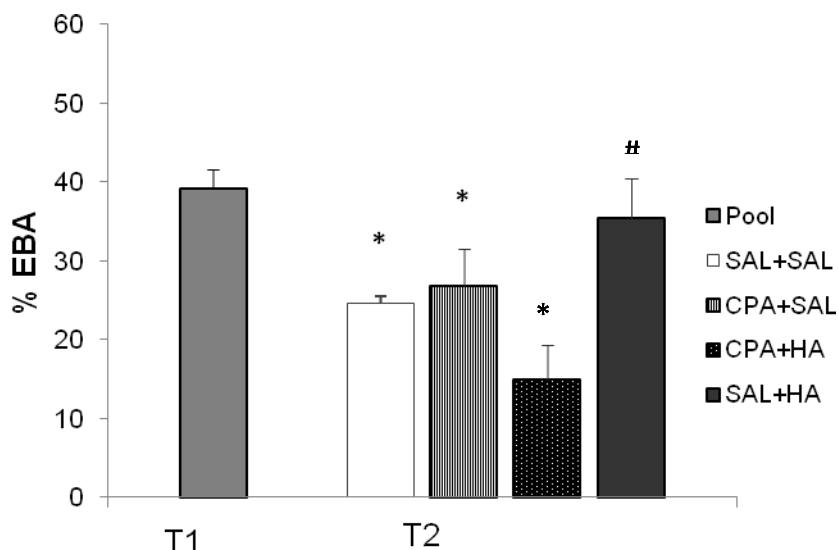

Figura 17: Médias e erro padrão da média para a porcentagem de entradas nos braços abertos de camundongos reexpostos ao LCE. Pool (todos os animais expostos ao LCE sem tratamento); Imediatamente após a exposição ao LCE, os animais receberam microinjeção: SAL+SAL; CPA+SAL (CPA 0,16 nmol/0,1µl e salina); CPA+HA (CPA 0,16 nmol/0,1µl e HA 4,07 nmol/0,1µl); SAL+HA (Salina e HA 4,07 nmol/0,1µl). *p<0,05 em relação a T1, #p<0,05 em relação ao grupo CPA+HA, teste de Duncan.

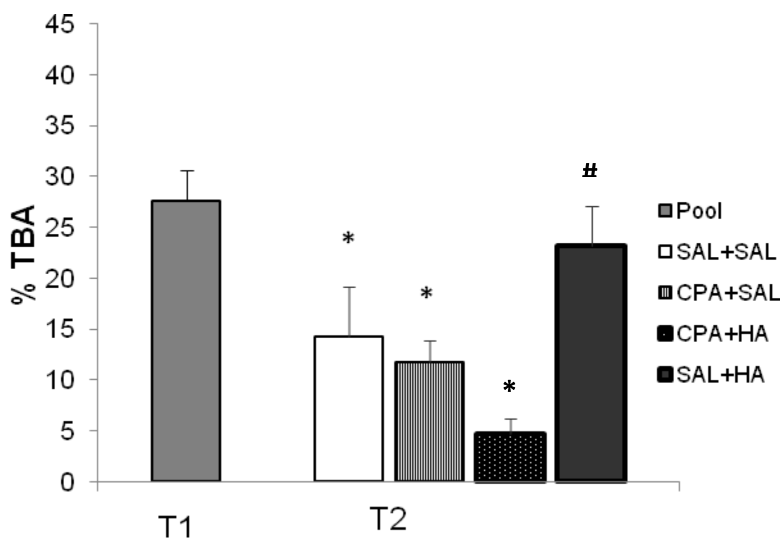

Figura 18: Médias e erro padrão da média para a porcentagem de tempo nos braços abertos de camundongos reexpostos ao LCE. Pool (todos os animais expostos ao LCE sem tratamento); Imediatamente após a exposição ao LCE, os animais receberam microinjeção: SAL+SAL; CPA+SAL (CPA 0,16 nmol/0,1µl e salina); CPA+HA (CPA 0,16 nmol/0,1µl e HA 4,07 nmol/0,1µl); SAL+HA (Salina e HA 4,07 nmol/0,1µl). *p<0,05 em relação a T1, #p<0,05 em relação ao grupo CPA+HA, teste de Duncan.

Tabela 18: Efeitos das injeções combinadas de antagonista do receptor H_1 CPA (0,16 nmol) e histamina (4,07 nmol) no comportamento de camundongos em T1 e T2 no LCE.

	Pool	SAL+SAL	CPA+SAL	CPA+HÁ	SAL+HA
EBA	6,4 ± 0,5	2,7 ± 0,6*	3,9 ± 0,8*	1,7 ± 0,5*	5,9 ± 0,9+#
TBA	84,2 ± 8,9	42,6 ± 14,8*	35,3 ± 6,3*	14,1 ± 4,4*	69,7 ± 11,7+#
EBF	9,8 ± 0,6	9,3 ± 1,9	11,7 ± 1,8	8,9 ± 0,9	11,7 ± 2,9
TBF	117,5 ± 6,8	193,6 ± 17,4*	165,1± 12,9*	217,3 ± 9,2*	181,4 ± 12,9*
%TBF	29,6 ± 1,8	64,5± 5,8*	55,0 ± 4,3*	72,4 ± 3,1*	60,5 ± 4,3*
TC	98,3 ± 5,9	63,8 ± 10,6*	99,6 ± 11,6+#	68,6 ± 8,7*	48,9 ± 7,4*
Esticar	7,5 ± 0,6	5,7 ± 1,8	4,3 ± 0,8	4,2 ± 0,8	6,7 ± 1,0
Mergulhar	4,7 ± 0,5	1,6 ± 0,6*	0,9 ± 0,4*	0,6 ± 0,4*	1,6 ± 0,7*
Tempo de imobilidade	0,1 ± 0,0	0,1 ± 0,1	0,0 ± 0,0	0,0 ± 0,0	0,9 ± 0,9

Pool (todos os animais expostos ao LCE sem tratamento); Imediatamente após a exposição ao LCE, os animais receberam microinjeção: SAL+SAL; CPA+SAL (CPA 0,16 nmol/0,1µl e salina); CPA+HA (CPA 0,16 nmol/0,1µl e HA 4,07 nmol/0,1µl); SAL+HA (Salina e HA 4,07 nmol/0,1µl). EBA (número de entradas nos braços abertos); TBA (tempo gasto nos braços abertos); EBF (número de entradas nos braços fechados); TBF (tempo gasto nos braços fechados); %TBF (porcentagem de tempo gasto nos braços fechados); TC (tempo gasto no centro); Esticar (total); Mergulhar (total); Tempo de imobilidade. *$p<0,05$ em relação a T1, +$p<0,05$ em relação ao grupo controle (SAL+SAL) em T2, #$p<0.05$ em relação ao grupo CPA+HA em T2, teste de Duncan.

5.8 EXPERIMENTO 4b

Os resultados mostram os efeitos da microinjeção combinada de antagonista do receptor H_1 Clorfeniramina (0,16 nmol/0,1µl) e de Histamina (1,36 nmol/0,1µl) no vérmis cerebelar de camundongos submetidos ao Teste de Esquiva Inibitória. A ANOVA de uma via indicou diferença significativa no tempo de latência no segundo dia entre os grupos ($F_{3, 39}$= 3,17, p=0,03). A análise *post hoc* mostrou um aumento significativo na latência para os animais que receberam CPA+HA (0,04) e SAL+HA (p=0,02) em relação ao grupo controle SAL+SAL. O grupo microinjetado com CPA+SAL não apresentou diferença significativa com o grupo SAL+SAL (p=0,45) (Figura 19). Esses resultados mostram que a CPA não alterou a consolidação da memória de Esquiva Inibitória quando aplicada com salina e não foi capaz de reverter o efeito facilitatório da histamina.

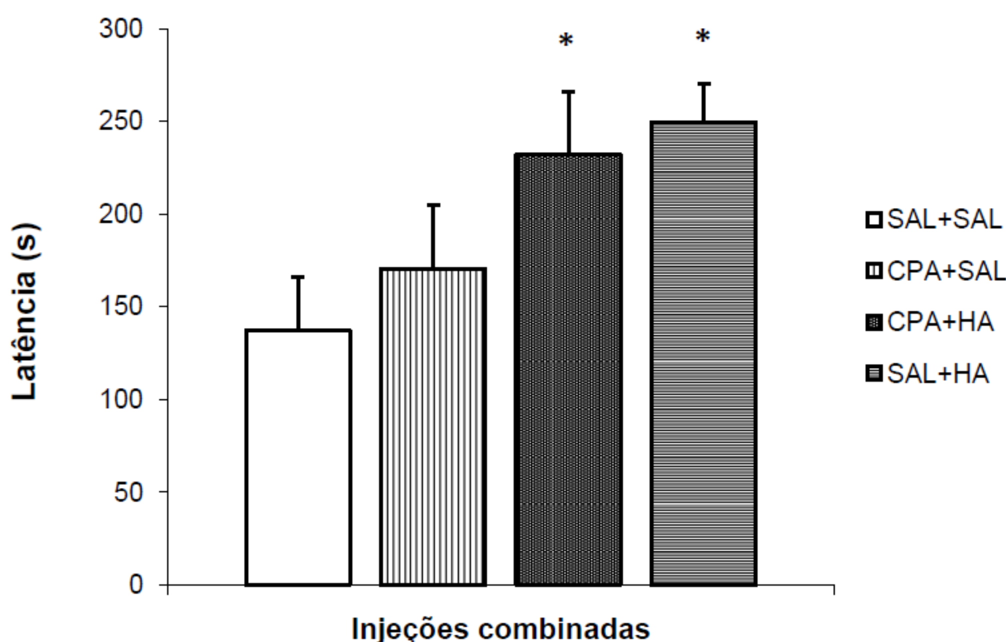

Figura 19: Efeitos da microinjeção combinada de antagonista H1 Clorfeniramina (0,16 nmol/0,1µl) e histamina (1,36 nmol/0,1µl) no vérmis cerebelar na consolidação da memória de esquiva inibitória. Médias e erro padrão da média para o tempo de latência (segundos). N=10-13. *p<0,05 em relação ao grupo controle, teste de Duncan.

5.9 EXPERIMENTO 5a

Os resultados mostraram o efeito da injeção combinada de antagonista do receptor H_2 Ranitidina (2,85 nmol/0,1µl) e de Histamina (4,07 nmol/0,1µl) no vérmis cerebelar de camundongos reexpostos ao Labirinto em Cruz Elevado (LCE).

A ANOVA de uma via não indicou diferença significativa entre os animais expostos ao LCE sem tratamento para todas as medidas avaliadas (Tabela 19), o que permitiu utilizar os resultados de T1 como *Pool* para as análises seguintes.

A ANOVA indicou diferença significativa para %EBA ($F_{4, 87}$= 3,91, p=0,006). A análise *post hoc* indicou essa diferença para os grupos microinjetados com SAL+SAL (p=0,01) e RA+SAL (p=0,03). Enquanto que os animais que receberam pré-tratamento com RA (RA+HA) (p=0,31) e os animais microinjetados com SAL+HA (p=0,13) não reduziram a %EBA (Figura 20).

A Figura 21 mostra os dados para %TBA em T1 e T2. A ANOVA detectou diferença significativa entre os dias de teste ($F_{4, 87}$= 4,49, p=0,002) na %TBA. O teste de Duncan mostrou que apenas os grupos microinjetados com SAL+SAL (p=0,007) e RA+SAL (p=0,02) mostraram uma redução na %TBA na reexposição em relação à exposição. Esses resultados mostram que o antagonista do receptor H_2 RA na dose usada nesse estudo não provocou alterações na consolidação da memória por si só, e não reverteu o prejuízo causado pela histamina no teste do Labirinto em Cruz Elevado.

A ANOVA revelou diferenças significativas entre os dias para TBA ($F_{4, 87}$ = 4,49, p=0,002). O teste de comparações múltiplas indicou que essa diferença para os grupos microinjetados com SAL+SAL (p=0,007), RA+SAL (p=0,02), e RA+HA (p=0,05). Também foram observadas diferenças entre os dias para EBA ($F_{4, 87}$= 1,93, p=0,02), TBF ($F_{4, 87}$ = 8,99, p=0,000004), %TBF ($F_{4, 87}$= 8,99, p=0,000004) e mergulhar ($F_{4, 87}$= 5,50, p=0,0005). A ANOVA não detectou diferenças entre os dias para TC ($F_{4, 87}$= 1,09, p=0,37), EBF ($F_{4, 87}$= 0,87, p=0,48), tempo de imobilidade ($F_{4, 87}$= 2,12, p=0,10), e esticar ($F_{4, 87}$= 1,65, p=0,17) (Tabela 20).

Tabela 19: Valores da ANOVA de uma via, para as variáveis do experimento 5a analisadas na exposição sem tratamento farmacológico.

Medidas	**F**	**P**
EBA	0,73	0,54
%EBA	0,19	0,90
TBA	0,64	0,59
%TBA	0,64	0,60
EBF	1,04	0,38
TBF	0,25	0,86
%TBF	0,25	0,86
TC	0,90	0,45
Esticar	1,34	0,27
Mergulhar	1,33	0,29
Tempo de imobilidade	0,64	0,60

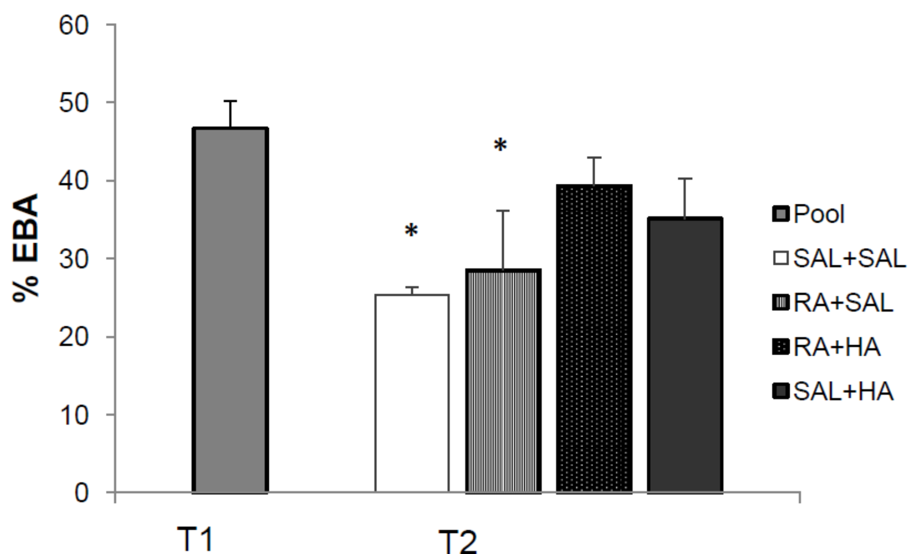

Figura 20: Médias e erro padrão da média para a porcentagem de entradas nos braços abertos de camundongos reexpostos ao LCE. Pool (todos os animais expostos ao LCE sem tratamento); Imediatamente após a exposição ao LCE, os animais receberam microinjeção: SAL+SAL; RA+SAL (RA 2,85 nmol/0,1µl e salina); RA+HA (RA 2,85 nmol/0,1µl e HA 4,07 nmol/0,1µl); SAL+HA (Salina e HA 4,07 nmol/0,1µl). *p<0,05 em relação a T1, teste de Duncan.

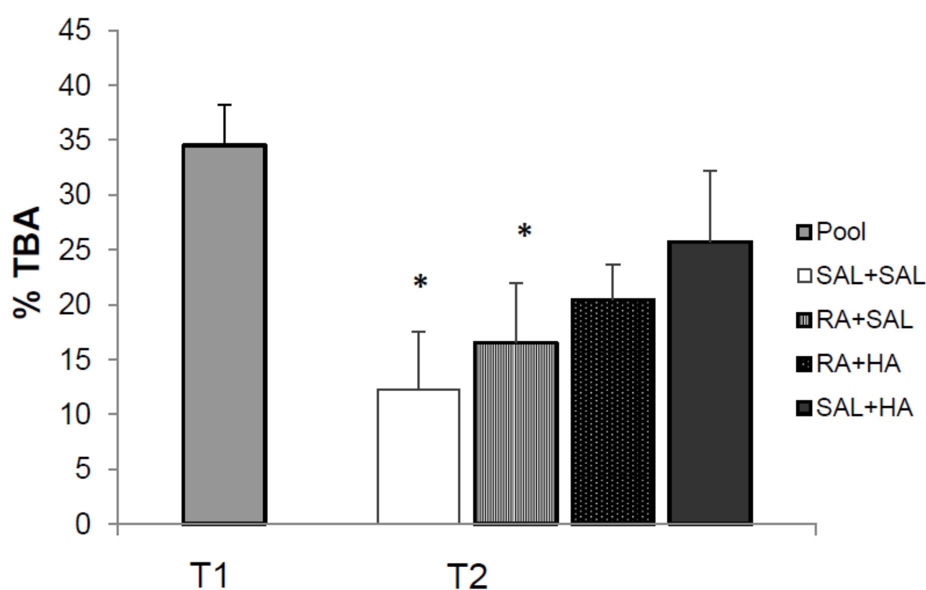

Figura 21: Médias e erro padrão da média para a porcentagem de tempo nos braços abertos de camundongos reexpostos ao LCE. Pool (todos os animais expostos ao LCE sem tratamento); Imediatamente após a exposição ao LCE, os animais receberam microinjeção: SAL+SAL; RA+SAL (RA 2,85 nmol/0,1µl e salina); RA+HA (RA 2,85 nmol/0,1µl e HA 4,07 nmol/0,1µl); SAL+HA (Salina e HA 4,07 nmol/0,1µl). *p<0,05 em relação a T1, teste de Duncan.

Tabela 20: Efeitos das microinjeções combinadas de antagonista do receptor H_2 Ranitidina (2,85 nmol) e Histamina (4,07 nmol) no comportamento de camundongos expostos e reexpostos ao Labirinto em Cruz Elevado.

	Pool	SAL+SAL	RA+SAL	RA+HÁ	SAL+HA
EBA	7,3 ± 0,6	3,4 ± 1,1*	4,7 ± 1,4	5,7 ± 0,9	4,3 ± 0,8
TBA	106,5 ± 9,6	36,9 ± 15,8*	45,9 ± 16,4*	61,3 ± 9,6*	77,2 ± 19,3
EBF	8,6 ± 0,6	7,4 ± 1,3	10,5 ± 1,7	8,3 ± 1,0	9,5 ± 1,4
TBF	92,6 ± 7,2	191,1 ± 23,8*	176,2 ± 21,8*	144,3 ± 20,4*	141,4 ± 16,4+
%TBF	30,9 ± 2,4	63,7 ± 7,9*	58,7 ± 7,3*	48,1 ± 6,8*	47,1 ± 5,5+
TC	100,9 ± 6,7	72,2 ± 17,5	75,9 ± 16,9	94,4 ± 15,5	81,4 ± 13,4
Esticar	7,0 ± 0,5	4,2 ± 0,9	6,3 ± 1,8	6,6 ± 1,1	7,3 ± 1,0
Mergulhar	6,4 ± 1,0	1,4 ± 0,6*	2,5 ± 1,3*	2,0 ± 0,6*	3,5 ± 1,1*
Tempo de imobilidade	0,0 ± 0,0	0,3 ± 0,3	0,4 ± 0,4	0,5 ± 0,3	0,0 ± 0,0

Pool (todos os animais expostos ao LCE sem tratamento); Imediatamente após a exposição ao LCE, os animais receberam microinjeção: SAL+SAL; RA+SAL (RA 2,85 nmol/0,1µl e salina); RA+HA (RA 2,85 nmol/0,1µl e HA 4,07 nmol/0,1µl); SAL+HA (Salina e HA 4,07 nmol/0,1µl). EBA (número de entradas nos braços abertos); TBA (tempo gasto nos braços abertos); EBF (número de entradas nos braços fechados); TBF (tempo gasto nos braços fechados); %TBF (porcentagem de tempo gasto nos braços fechados); TC (tempo gasto no centro); Esticar (total); Mergulhar (total); Tempo de imobilidade. $*p<0,05$ em relação a T1, $+p<0,05$ em relação ao grupo controle (SAL+SAL) em T2, teste de Duncan.

5.10 EXPERIMENTO 5b

Os resultados mostram os efeitos da microinjeção combinada de antagonista do receptor H_2 Ranitidina (2,85 nmol/0,1µl) e de Histamina (1,36 nmol/0,1µl) no vérmis cerebelar de camundongos submetidos ao Teste de Esquiva Inibitória. A ANOVA de uma via indicou diferença significativa no tempo de latência no segundo dia entre os grupos ($F_{3,\ 27}$= 3,21, p=0,03). A análise *post hoc* mostrou um aumento significativo na latência para os animais que receberam SAL+HA (p=0,007) em relação ao grupo controle SAL+SAL. Os grupos microinjetados com RA+SAL e RA+HA não apresentaram diferença significativa com o grupo SAL+SAL (p=0,26 e p=0,19) (Figura 22). Esses resultados mostram que a RA não alterou a consolidação da memória de Esquiva Inibitória por si só e foi capaz de reverter o efeito facilitatório da histamina já que quando a histamina foi microinjetada em animais pré-tratados com RA eles não apresentaram aumento da latência em relação ao grupo controle.

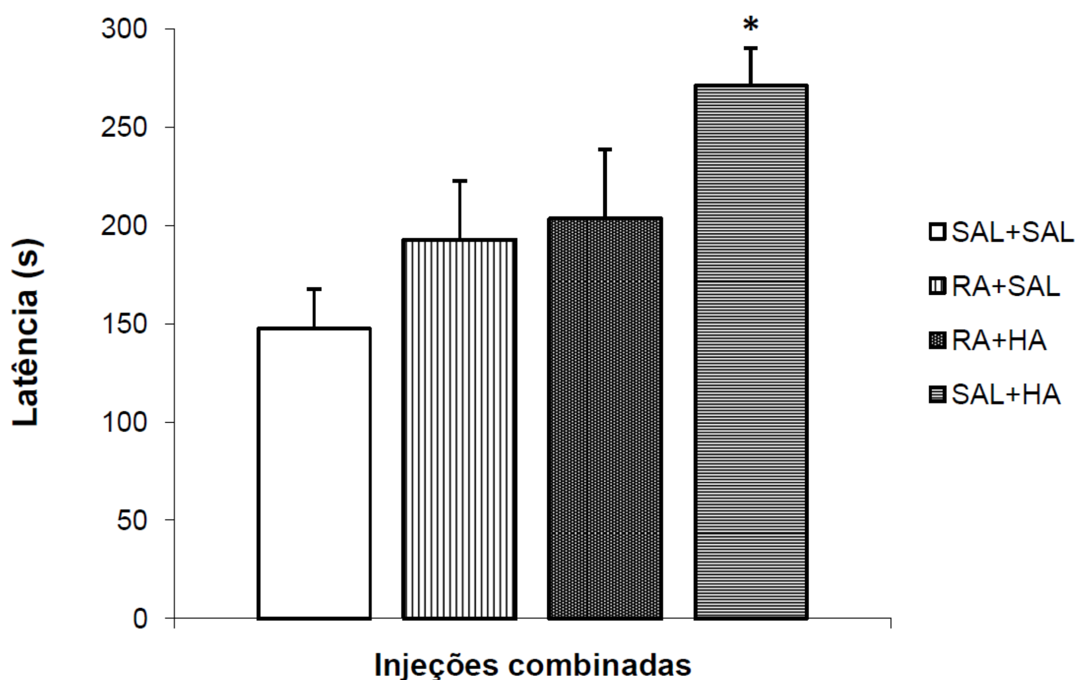

Figura 22: Efeitos da microinjeção combinada de antagonista H_2 Ranitidina (2,85 nmol/0,1µl) e histamina (1,36 nmol/0,1µl) microinjetada no vérmis cerebelar na consolidação da memória de esquiva inibitória. Médias e erro padrão da média para o tempo de latência (segundos) N=7-9. *p<0,05 em relação ao grupo controle, teste de Duncan.

Tabela 21: Síntese dos resultados apresentados

Consolidação da Memória

Experimento1	*LCE*	*EI*
HA 0,54; 1,36; 2,72; 4,07	Prejuízo na consolidação da memória dose-dependente	Efeito facilitatório na dose de 1,36 nmol
Experimento 2		
CPA 0,016; 0,052; 0,16	CPA não apresentou efeito nas doses utilizadas	CPA não apresentou efeito nas doses utilizadas
Experimento 3		
RA 0,57; 2,85; 5,7	Prejuízo na consolidação da memória na dose mais alta	Prejuízo na consolidação da memória na dose mais alta
COMBINADAS Experimento 4		
CPA+HA	CPA reverteu prejuízo causado pela HA	CPA não reverteu efeito facilitatório da HA
Experimento 5		
RA+HA	RA não reverteu prejuízo causado pela HA	RA Reverteu efeito facilitatório da HA

VI - DISCUSSÃO

Os resultados dos diversos experimentos realizados permitiram traçar uma possível ação da histamina via receptores H_1 e H_2 em dois modelos de aprendizagem que parecem indicar circuitos neurais relacionados a diferentes funções.

Em síntese, os resultados do experimento 1a mostraram que a microinjeção de histamina no vérmis cerebelar prejudicou, de forma dose-dependente, a consolidação da memória emocional de camundongos reexpostos ao Labirinto em Cruz Elevado, visto que nas doses mais elevadas, os animais não reduziram a exploração dos braços abertos do labirinto, sugerindo que esses animais não se recordaram da informação aversiva no segundo dia de exposição. No experimento 1b, os resultados mostraram que a microinjeção de histamina na dose de 1,36 nmol aumentou a latência no teste de Esquiva Inibitória, indicando que a histamina nesta dose facilitou a consolidação da memória de Esquiva Inibitória em camundongos, sugerindo um papel diferente da histamina em um modelo que usa punição.

Os resultados do experimento 2 demonstraram que a Clorfeniramina microinjetada no vérmis cerebelar não teve efeito sobre a consolidação da memória emocional dos camundongos reexpostos ao Labirinto em Cruz Elevado. Os animais que receberam microinjeção de salina e os animais que receberam CPA em três diferentes doses apresentaram comportamento semelhante, reduzindo significativamente a exploração dos braços abertos durante a reexposição ao labirinto. No Teste de Esquiva Inibitória também não foram encontradas diferenças significativas entre a latência dos animais controle e os animais tratados com CPA em nenhuma das doses utilizadas.

No experimento 3, com a microinjeção de Ranitidina, os resultados demonstraram que esse antagonista causou prejuízo na consolidação da memória emocional nos animais microinjetados com a maior dose, visto que estes não reduziram a exploração dos braços abertos do LCE na reexposição. Com relação ao Teste de Esquiva Inibitória, os animais tratados com a maior dose de Ranitidina apresentaram redução significativa na latência, inferindo prejuízo na consolidação da memória.

Os resultados do experimento 4a confirmaram os resultados do experimento 1a em que animais injetados com HA (4,07 nmol) apresentam prejuízo na consolidação da memória emocional. Além disso, os resultados mostraram que a CPA não alterou os parâmetros comportamentais quando aplicada por si só, e foi capaz de reverter o déficit

produzido pela histamina na consolidação da memória emocional em camundongos no LCE. No experimento 4b, a microinjeção de histamina no vérmis cerebelar aumentou o tempo de latência no teste de Esquiva Inibitória, e o pré-tratamento com CPA não reverteu esse efeito.

No experimento 5a, a Ranitidina não alterou a consolidação da memória e não foi capaz de reverter o efeito da histamina na memória emocional de camundongos submetidos ao LCE. No experimento 5b, os resultados mostraram que a Ranitidina não apresentou efeito por si só, mas o pré-tratamento com esse antagonista foi capaz de reverter o efeito facilitatório da histamina no teste de Esquiva Inibitória.

O sistema histaminérgico é alvo de estudos, em diversos grupos de pesquisa, que investigam seu papel nos processos de aprendizagem e memória (SERAFIM et al., 2010; ZLOMUZICA et al., 2009; PURRÓN-SIERRA et al., 2010; YANAI et al., 2008). Porém, as diferentes abordagens experimentais, espécies utilizadas e estruturas avaliadas apontam para resultados discrepantes. Além disso, segundo Passani e colaboradores (2000), a razão para as discrepâncias pode estar relacionada aos processos modulatórios que o sistema histaminérgico pode exercer pela ativação de diferentes subtipos de receptores nos diferentes sistemas envolvidos nos processos de aprendizagem e memória. Estudos realizados nosso laboratório, sugerem uma possível atuação modulatória do sistema neural histaminérgico sobre a aprendizagem emocional em peixes (FAGANELLO e MATTIOLI, 2007) e em camundongos (SERAFIM et al., 2010).

O Labirinto em Cruz Elevado é um teste que permite avaliar o comportamento do animal associado a um componente emocional, visto que o comportamento expresso durante o teste é resultante de um conflito entre a motivação para explorar o labirinto e a tendência natural em evitar os braços abertos (BERTOGLIO e CAROBREZ, 2000; LISTER, 1987). Segundo File e colaboradores (1990), depois da exploração inicial ao labirinto o animal adquire, consolida e evoca algum tipo de memória relacionada com áreas potencialmente aversivas ao labirinto.

O aumento da esquiva dos braços abertos em uma segunda exposição é observado em vários estudos sugerindo que a reexposição está associada às mudanças comportamentais indicativas de uma aprendizagem aversiva. Essa possibilidade foi reforçada quando testes separados por intervalos de 1 ou 2 semanas mostraram redução marcante da exploração aos braços abertos. Além disso, com a administração no

primeiro dia de teste de uma dose de benzodiazepínico considerada amnésica (Clordiazepóxido 75mg/kg), observou-se que esse comportamento não se manifestou (FILE, 1993; FILE et al., 1990).

Estudos realizados no nosso laboratório, avaliando a aprendizagem e memória emocional em camundongos utilizando o Labirinto em Cruz Elevado, têm demonstrado que neste modelo, a histamina parece exercer um papel inibitório na memória. Em estudo de Serafim e colaboradores (2010), o tratamento com L-histidina em T1 e T2, em camundongos reexpostos ao Labirinto em Cruz Elevado, prejudicou a memória durante a segunda exposição, indicando um déficit na evocação da memória estado dependente. De acordo com nosso estudo prévio, esse déficit pode ser mediado via receptor H_1, já que a aplicação periférica de Clorfeniramina foi capaz de reverter o efeito da L-histidina, utilizando o mesmo protocolo (GIANLORENÇO et al., 2011).

No presente estudo utilizando o LCE, os resultados do experimento 1a mostraram que a microinjeção de histamina no vérmis cerebelar prejudicou, de forma dose-dependente, a consolidação da memória emocional de camundongos reexpostos ao LCE, corroborando com possível papel inibitório da histamina na memória emocional com um componente de ansiedade.

Nossos resultados demonstram que o sistema histaminérgico cerebelar está envolvido no processo de consolidação da memória emocional. Diversas linhas de evidência apoiam o envolvimento de vérmis cerebelar na aprendizagem emocional. Além disso, estudos têm avaliado o papel do cerebelo na consolidação da memória emocional, fornecendo informações importantes sobre o papel do cerebelo na memória e explicando como os circuitos neurais estão relacionadas às memórias aversivas (STRATA et al., 2011; SACCHETTI et al., 2009, ZHU et al., 2007). Sacchetti et al. (2007) demonstraram que a inativação do vérmis cerebelar causou prejuízo na consolidação da memória de medo e a inativação combinada do cerebelo e da amígdala inibiu a consolidação quando um protocolo de intensidades maiores de choque elétrico foi aplicado. Assim, de acordo com os autores, o cerebelo influencia memórias emocionais de longo prazo, e adquire uma representação de medo duradouro, em situações importante para sobrevivência, suficiente para apoiar os processos de memória, mesmo na ausência de locais cruciais da emoção como a amígdala.

Foi anteriormente proposto que a amígdala e o cerebelo são funcionalmente interligados durante aprendizagem aversiva. De acordo com Sacchetti et ai. (2009),

vérmis e amígdala podem interagir, e a estimulação elétrica vermal modula a atividade da amígdala. Estes efeitos são mediados tanto por conexões anatômicas diretas ou indiretas entre o cerebelo e as áreas límbicas e também através das projeções paleocerebelares ascendentes para neurônios catecolaminérgicos do locus coeruleus, área tegmental ventral e substância cinzenta periaquedutal. Portanto, este resultado pode ser devido ao efeito da histamina sobre uma destas projeções do cerebelo.

Com as microinjeções com os antagonistas dos receptores H_1 e H_2, pudemos observar que as doses utilizadas de CPA não foram capazes de produzir qualquer efeito nos animais testados no LCE. Entretanto, com a ranitidina, os resultados mostraram que a dose mais alta provocou prejuizo na consolidação. Com as injeções combinadas, verificamos que os efeitos inibitórios da histamina na consolidação da memória emocional em camundongos reexpostos ao LCE foram mediados via receptor H_1, visto que com o pré-tratamento com Clorfeniramina esse efeito da histamina foi revertido, enquanto que o pré-tratamento com antagonista H_2 não promoveu efeito.

Com relação aos experimentos no LCE, analisando todos os resultados obtidos podemos concluir que a histamina promoveu um efeito inibitório dose-dependente na consolidação da memória emocional em camundongos; esse efeito foi mediado via receptor H_1; e a microinjeção de ranitidina na dose mais elevada provocou prejuízo na consolidação, possivelmente pela ampla ligação do antagonista aos receptores H_2, o que permitiu mais histamina endógena disponível na fenda para se ligar aos receptores H_1, produzindo efeito similar ao encontrado após a microinjeção de histamina exógena.

No nosso estudo, além do LCE, utilizamos um modelo amplamente utilizado para avaliar a aprendizagem e a memória: o Teste de Esquiva Inibitória, que é baseado na aversão dos roedores a áreas iluminadas, e preferência por compartimentos escuros, que representam uma área segura. Na tarefa, o animal é primeiramente exposto a caixa de esquiva. Após cruzar para o compartimento escuro, o animal aprende a associar o lado escuro da caixa (resposta exploratória inata) a um choque aplicado nas patas (estímulo incondicionado). Com isso, em uma próxima exposição ao ambiente, ele evitará expressar sua resposta inata e apresentará um aumento no tempo de latência.

No experimento 1b, os resultados mostraram que a histamina microinjetada no vérmis cerebelar facilitou a consolidação da memória na tarefa de Esquiva Inibitória e apresentou um padrão conhecido como “curva dose efeito em U-invertido” (IUSDEC), que é frequentemente observada em estudos que avaliam a ação de fármacos em

funções cognitivas como a memória. Nesse padrão os efeitos de doses crescentes de um dado composto parecem aumentar até um máximo e, em seguida, os efeitos diminuem. Esse padrão tem sido relatado em vários estudos com administração de drogas após o treino, e assim os efeitos dose-resposta nestas condições parecem estar mais relacionados às ações especificas no processo mnemônico. No nosso estudo a possibilidade de alteração em funções motoras ou sensoriais é improvável, visto que a meia-vida da histamina em condições basais é de aproximadamente 30 minutos (SCHWARTZ et al., 1991), sugerindo que os efeitos da microinjeção pós-treino são relacionados ao processo de memória.

A curva em U-invertido tem sido demonstrada com compostos que exibem efeitos contraditórios na aprendizagem e memória. Diversos estudos apresentam resultados contraditórios do sistema histaminérgico nos processos de aprendizagem e memória. Alguns trabalhos mostraram um efeito facilitatório da histamina na aprendizagem e memória de ratos e camundongos (DA SILVA et al., 2006; PRAST et al., 1996; DE ALMEIDA e IZQUIERDO,1986). Por outro lado, alguns estudos mostraram efeito inibitório da histamina nos processos de memória (ALVAREZ e BANZAN, 2008; NISHIGA et al., 2002; KAMEI e TASAKA, 1993).

Estudos relacionam o efeito em U-invertido com as alterações no nível de alerta (BALDI e BUCHERELLI, 2005). De acordo com McGaugh (1989), o alerta é um modulador endógeno nos processos de memória. O sistema histaminérgico está intimamente relacionado com a regulação do ciclo vigília-sono e o estado de alerta, e os neurônios histaminérgicos localizados no núcleo tuberomamilar e regiões próximas disparam durante a vigília (STEININGER et al., 1999). A depleção de histamina neuronal induz um aumento da quantidade de sono de onda lenta e uma diminuição no estado de vigília, e a facilitação da liberação de histamina neuronal pelo antagonista do receptor H_3 tioperamida aumenta a vigília (WADA et al., 1991; HASS et al., 2003). No nosso estudo, o aumento dos níveis de histamina poderia estimular a retenção da memória, visto que a histamina neuronal está envolvida no estado de alerta. Outro mecanismo possível para ser explorado é que a microinjeção de histamina aumentou os seus níveis na fenda sináptica e aumentou a disponibilidade de histamina para se ligar aos receptores H_3. A ativação do receptor H_3 seria responsável por inibir a síntese e liberação de histamina endógena, que alterou a sua eficácia nas dosagens mais elevadas.

Esses resultados parecem estar relacionados aos métodos utilizados, e as diferentes tarefas, que são dependentes da ativação de diferentes circuitos cerebrais. De acordo com McNaughton e Corr (2004), existe uma distinção funcional entre o medo mais relacionado com ameaças imediatas e a ansiedade mais relacionada a condições de ameaça distante ou antecipatória. Essas duas funções são executadas por dois sistemas neurais paralelos, um controlando o medo e outro a ansiedade: em níveis mais inferiores, o medo tem maior representação neural (matéria cinzenta periaquedutal, hipotálamo medial e amígdala) e em níveis mais superiores, a ansiedade tem maior representação neural (amígdala, sistema septo-hipocampal, cíngulo posterior e córtex pré-frontal) (MCNAUGHTON e CORR, 2004).

Nós sugerimos que o cerebelo pode integrar estes dois sistemas neurais, com interconexões entre vérmis cerebelar, amígdala e hipocampo com papel na memória emocional relacionada à ansiedade, enquanto que conexões entre cerebelo, matéria cinzenta periaquedutal, hipotálamo e amígdala na memória relacionada com medo.

Assim, propomos que em uma tarefa que envolve medo, a microinjeção de histamina no vérmis cerebelar pode estimular a substância cinzenta periaquedutal, os níveis da amígdala e aumentar o alerta, o que facilita a retenção da memória.

Nossa hipótese é que altos níveis de histamina no vérmis cerebelar produzem diferentes resultados nas mesmas doses, porém em modelos que avaliam funções diferentes, e que o sistema cerebelar histaminérgico está envolvido no processo de consolidação da memória diferentemente quando a memória tem um componente de ansiedade (LCE) e quando tem um componente de medo (Esquiva Inibitória).

Nos testes com Esquiva Inibitória os resultados foram diferentes, e mostraram que o pré-tratamento com CPA não reverteu o efeito facilitatório da histamina. Já o pré-tratamento com Ranitidina foi capaz de reverter esse efeito, indicando que na tarefa com componente de medo, o efeito da histamina ocorre via receptor H_2. Nos experimentos com a microinjeção dos antagonistas H_1 e H_2, Clorfeniramina e Ranitidina, não foram encontradas diferenças significativas entre a latência dos animais em nenhuma das doses de Clorfeniramina utilizadas, entretanto os animais tratados com a maior dose de Ranitidina apresentaram prejuízo na consolidação da memória, o que corrobora os resultados obtidos com a microinjeção de histamina e confirmam a participação dos receptores H_2 na consolidação da memória da tarefa de esquiva inibitória.

Nossos dados estão de acordo com os estudos que mostram que a histamina facilita a consolidação da memória de medo por meip de um mecanismo que envolve a ativação de receptores H_2. De acordo com Da Silva et al. (2006), quando infundida na região CA1 do hipocampo dorsal, a histamina melhorou a retenção da memória de esquiva inibitória de uma forma dose-dependente, e o efeito foi bloqueado por ranitidina e reproduzido por dimaprit (H_2 agonista). Giovaninni et al. (2003) demonstraram que a microinfusão do agonista de H_2 e H_3, na região CA3 do hipocampo, melhora a retenção da memória associada a um processo de condicionamento de medo contextual. No estudo de Flood et al. (1998), a infusão de dimaprit intra- septo facilitou a retenção a longo prazo.

Uma vez que as respostas cerebrais mediadas pelo receptor H_2 ocorrem através da estimulação da adenilil-ciclase, o aumento dos níveis de cAMP intracelular e activação de PKA, um mecanismo provável para os resultados comportamentais encontrados seria a ativação da via AMPc / PKA. No entanto, novos experimentos são necessários para entender as conseqüências bioquímicas da ativação dos receptores H_2 cerebelares durante a consolidação da memória.

Estes resultados indicam que a microinjeção de histamina no vermis cerebelar facilita a retenção da memória de esquiva inibitória em camundongos, não linearmente por ativação do receptor H_2, o que sugere um papel diferente para histamina em um modelo de memória que utiliza a punição.

VII - CONCLUSÃO

Em conclusão, nossos resultados demonstram que a ação da histamina nos dois modelos utilizados ocorre via receptores diferentes, H_1 para a consolidação da memória emocional testada na reexposição ao LCE e H_2 para a consolidação da memória de esquiva inibitória.

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APÊNDICE A

Estudo piloto: Efeitos da implantação da cânula no vérmis cerebelar sobre o comportamento locomotor de camundongos expostos ao Labirinto em Cruz Elevado.

Grupo	**EBF**
Cirurgia fictícia	10,66±1,33
Controle	9,97±1,61

Médias e erro padrão da média para EBF (número de entradas nos braços fechados). Cirurgia fictícia (todo procedimento cirúrgico sem implantação da cânula guia; n=6); Controle (animais submetidos à canulação expostos ao LCE sem tratamento; n=6). p= 0,66, Test- *t*.

APÊNDICE B

Média e ± erro padrão da média da medida de latência (em segundos) durante as sessões de treino na tarefa de Esquiva Inibitória nos experimentos, 1b, 2b, 4b e 5b.

	HAB1	HAB2	AQUI	TESTE
HISTAMINA	23,59	35,40	50,72	91,58
	2,39	4,87	7,28	5,10
COMBINADA H1	25,65	23,33	39,00	73,49
	2,78	1,92	6,05	6,45
COMBINADA H2	23,63	28,45	40,59	96,45
	1,98	4,59	6,52	4,57
ANTAGONISTA H1	19,80	19,80	35,60	68,93
	2,31	2,00	7,45	7,95

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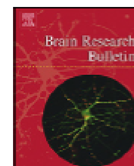

Research report

Microinjection of histamine into the cerebellar vermis impairs emotional memory consolidation in mice

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A B S T R A C T

The biogenic amine histamine is an important neurotransmitter in the central nervous system that has been implicated in learning and memory processes. Experimental evidence indicates that the role of the cerebellum may be more complex than the simple regulation of motor responses, and recent studies have demonstrated significant involvement of the cerebellum in emotional memory consolidation. This study investigated the effect of histamine microinjected into the cerebellar vermis on emotional memory consolidation in mice in the elevated plus-maze (EPM). The cerebellar vermis of male mice (Swiss Albino) were implanted with guide cannulae. The mice weighed between 25 and 30 g. After three days of recovery, behavioral tests in the EPM were performed on two consecutive days; the testing periods were called, Trial 1 and Trial 2. Immediately after Trial 1, the animals received microinjections of histamine in the cerebellar vermis (0.54, 1.36, 2.72, and 4.07 nmol/0.1 μl). On both days, the test sessions were recorded to enable analysis of behavioral measures. The decrease in open arm exploration (% entries and % time spent in the open arms) in Trial 2 relative to Trial 1 was used as a measure of learning and memory. The data were analyzed using One-way Analysis of Variance (ANOVA) and Duncan's tests. The percentage of open arm entries (%OAE) and the percentage of time spent in the open arms (%OAT) were reduced in Trial 2 relative to Trial 1 for the control group; the same was true for the group that was microinjected with histamine at doses of 0.54 (%OAE and %OAT) and 1.36 nmol (%OAT). However, when the animals received histamine at doses of 2.72 and 4.07 nmol, their open arm exploration did not decrease. No significant changes were observed in the number of enclosed arm entries (EAE), an EPM index of general exploratory activity. These results suggest that there is a dose-dependent inhibitory effect of histamine microinjected into the cerebellar vermis on emotional memory consolidation.

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1. Introduction

Histamine is a biogenic amine and an important neurotransmitter–neuromodulator in the central nervous system (CNS) [13,22]. Recent evidence has clearly established that histamine and its receptors are involved in learning and memory. Numerous experiments using different learning models and species suggest that histamine may be important for several stages of memory formation and memory retrieval during different tasks, and they emphasize the role of histamine in the physiological mechanisms of memory [1,2,23,24,39]. However, these studies have used many different behavioral tasks and produced contradictory results; for instance, both facilitatory and inhibitory effects of neuronal histamine on learning and memory have been described in animal behavior studies [1,6,15,36,46]. The mechanisms underlying these differences seem to be very complex, and the differences may be due in part to the methods used and the approaches selected in the experiments [19].

The central histaminergic nervous system originates from the tuberomammillary nucleus of the hypothalamus, and in many species, it widely innervates almost the whole brain including the cerebellum and other subcortical motor structures [38]. Previous studies have shown that the histamine-containing fibers project from the tuberomammillary nucleus to the cerebellar cortex and

Abbreviations: CNS, central nervous system; EPM, elevated plus-maze; OAE, open arm entries; OAT, open arm time; %OAE, percentage of open arm entries; %OAT, percentage of open arm time; EAE, enclosed arm entries; EAT, enclosed arm time; %EAT, percentage of enclosed arm time; CT, central area time; SAP, stretched-attend postures.

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the deep cerebellar nucleus and that the highest density of histaminergic terminations is in the vermis and flocculus [21,45]. A moderately dense network of histamine fibers has been seen in the molecular and granular layers of the cerebellum in several species including humans [14]. These fibers run parallel to the Purkinje cell layer after traversing it perpendicularly. Both Purkinje cells and neurons in the nucleus interpositus have H2 receptors [37], and granule cells are excited through both H1 and H2 receptor activation [43].

The cerebellum has traditionally been considered an important subcortical motor structure, but several lines of evidence support the view that the role of the cerebellum is more complex than previously thought and includes more than just the regulation of motor responses [34]. An increasing number of studies have demonstrated its involvement in cognitive and emotional function [35]. Functional neuroimaging studies and studies of patients with cerebellar lesions have been conducted to elucidate the role of the cerebellum in the processing of emotion [35,42,44]. According to Sacchetti et al. [32], the fact that there is a functional interconnection between the cerebellar vermis and the hypothalamus, amygdala, and hippocampus suggests that the cerebellum may play a role in an integrated network regulating emotional behavior. Moreover, Ruediger et al. [29] demonstrated that fear conditioning learning is specifically correlated with the growth of feedforward inhibition connectivity in hippocampal and cerebellar circuits.

Experimental evidence indicates that the cerebellum plays a role in emotional learning. The capacity to learn and retain fear-conditioned responses was investigated in *hotfoot* mutant mice. These animals are characterized by a primary deficiency in the synapses made by the parallel fibers onto the Purkinje cells. In these mutant mice, the cerebellar dysfunction impairs learning, which suggests that these synapses are involved in fear memory consolidation [31]. Studies have related the cerebellar vermis to emotional memory consolidation. In one study, vermis inactivation caused amnesic effects after a fear conditioning task [30]. Thus, the participation of the vermis in emotional memory is independent of its role in sensory or motor processes, and the vermis may represent an interface between sensory stimuli, emotional state, and motor responses [30,34].

Studies have demonstrated the relationship between the histaminergic system and the cerebellum. Shen et al. [37] demonstrated that histamine excites the cerebellar interpositus nucleus cells via the histamine H2 receptor mechanism. They suggested that the hypothalamocerebellar histaminergic fibers may modulate neuronal activity in the cerebellum. The results of a study by Tian et al. [43], revealed that histamine excites cerebellar Purkinje cells via H2 receptors and that the histaminergic fibers may play an important role in functional aspects of the cerebellum. In spite of these investigations, there have been no reports on the cerebellar histaminergic system and learning and memory processes.

The elevated plus-maze (EPM) is an animal model test of anxiety based on rodents' natural aversion to open spaces [18]. A general aspect of EPM exploration is that animals enter and spend less time exploring the open arms [7]. The inclusion of a retest session has been made in recent years, which is consistent with the assumption that there is a learned component underlying the exploratory behavior during EPM re-exposure [8]. According to File et al. [8], after the initial exploration of the apparatus, rodents acquire, consolidate and retrieve some kind of memory related to exploration of potentially dangerous areas of the maze. Bertoglio and Carobrez [4] use Trial 1/2 protocol to show that after a single prior non-drugged experience in the maze, mice exhibit significantly reduced open arm activity in a second trial. An increase in open arm avoidance with repeated maze exposure has been observed in several studies [7,10,16] and has been used as a measure of learning and memory [12,16,36].

The present study was designed in view of these findings to investigate the action of histamine microinjected into the cerebellar vermis on emotional memory consolidation in mice using Trial 1/2 protocol in the EPM.

2. Material and methods

2.1. Animals

Male Swiss mice (Federal University of São Carlos, UFSCar, SP, Brazil) weighing 25–35 g at the beginning of the experiments were housed in polypropylene cages (31 × 20 × 13 cm) in groups of five and maintained under a 12 h light cycle (lights on at 7:00 a.m.), in a controlled environment at temperature 23 ± 1 °C and humidity 50 ± 5%. Food and drinking water were provided *ad libitum*, except during the brief test periods. All mice were experimentally naive, and the experimental sessions were conducted during the light period of the cycle (9:00–13:00 h).

2.2. Drugs

Histamine dihydrochloride (Sigma Chemical Co., USA) was prepared in a vehicle of physiological saline. Saline solution was used as an experimental control. The doses of histamine were based on previous research [28] and on pilot work in our own laboratory. The substances were coded, and the codes were unknown to the experimenter during the tests and behavioral analysis.

2.3. Surgery and microinjection

Each mouse was implanted with a single 7 mm stainless steel guide cannula (25 gauge) under ketamine chloridrate and xylazine solution anesthesia (100 mg/kg and 10 mg/kg, respectively, delivered via i.p. injection). The stereotaxic coordinates for the cerebellar vermis were 6.5 mm posterior to bregma, 0 mm lateral to the midline, and 2.0 mm ventral to skull surface [9]. The guide cannula was fixed to the skull using dental acrylic and Jeweler's screws. A dummy cannula (33 gauge stainless steel wire) was inserted into the guide cannula at the time of surgery and served to reduce the incidence of occlusion. Postoperative analgesia was provided for 3 days by adding acetaminophen (200 mg/ml) to the drinking water in a ratio of 0.2 ml acetaminophen to 250 ml water (i.e., the final concentration was 0.16 mg/ml). Saline and drug solutions were infused into the cerebellar vermis using a microinjection unit (33 gauge cannula; Cooper's Needleworks, Birmingham, UK), which extended 2.0 mm beyond the tip of the guide cannula. The microinjection unit was attached to a 5 μl Hamilton microsyringe via polyethylene tubing (PE-10), and the administration was controlled by an infusion pump (Insight Equipamentos Científicos Ltda, Brazil) programmed to deliver a volume of 0.1 μl over a period of 60 s. The microinjection procedure consisted of gently restraining the animal, inserting the injection unit, infusing the solution, and keeping the injection needle *in situ* for a further 60 s to avoid reflux. Confirmation of successful infusion was obtained by monitoring the movement of a small air bubble inside the PE-10 tubing.

2.4. Apparatus

The EPM used was similar to that originally described by Lister et al. [18]. The EPM consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm) connected to a common central platform (5 × 5 cm). The apparatus was made of crystal acrylic and was raised to a height of 38.5 cm above floor level. All tests were conducted under moderate illumination (77 lx) as measured on the central platform of the EPM and in an environment isolated from the rest of the room by a black protective curtain.

2.5. Experimental procedure

Three days after surgery, the animals were transported to the experimental room and left undisturbed for at least 1 h before testing to facilitate adaptation. The test was performed on two consecutive days, and the trials in the EPM were denoted: Trial 1 and Trial 2. Mice were individually placed on the central platform of the maze facing the open arm and were able to explore the maze for 5 min. In Trial 1, immediately after the exposure to the EPM, animals received microinjection of saline or histamine in the cerebellar vermis (0.54, 1.36, 2.72 and 4.07 nmol/0.1 μl). Twenty-four hours later (Trial 2), mice were re-exposed to the EPM under the same experimental conditions, but they did not receive any injection. Between subjects, the maze was thoroughly cleaned with 5% ethanol and a dry cloth.

2.6. Behavioral analysis

All sessions were video recorded by a digital camera linked to a computer in an adjacent room. Images were analyzed by a highly trained observer using X-PLO-RAT, an ethological analysis pack developed at the Laboratory of Exploratory Behavior USP/Ribeirao Preto [11]. Behavioral parameters were defined in a way that was consistent with previous studies [18,26] and included the following: the frequency of open- and enclosed-arm entries (OAE and EAE) (an entry was defined as the entry of all four of an animal's paws into an arm) and total time

Table 1
One-way ANOVA statistical results for the behavior of mice with no pharmacological treatment in Trial 1.

Behavioral measures	*F*	*p*
OAE	0.72	0.55
%OAE	0.34	0.80
OAT	0.99	0.41
%OAT	0.99	0.41
EAE	0.75	0.53
EAT	0.02	0.99
%EAT	0.02	0.99
CT	2.19	0.11
SAP	1.79	0.14
Head dipping	0.71	0.55
Immobility time	0.64	0.60

spent in the open arms (OAT), enclosed arms (EAT), and central area (CT). These data were used to calculate the percentage of open arm entries (%OAE = (open entries/open + enclosed entries) × 100); the percentage of time spent in the open arms (%OAT = (open time/300) × 100); and the percentage of time spent in the enclosed arms (%EAT = (enclosed time/300) × 100). The number of stretched-attend postures (SAP; exploratory posture in which the body stretches forward and then retracts to its original position without any forward locomotion), immobility time (stillness but some movement of the chest), and the frequency of head dipping (exploratory movement of head/shoulders over the sides of the maze) were also scored. Total SAP was considered a primary index of risk assessment and head dipping was considered an index of exploratory behavior [27].

2.7. Histology

At the end of testing, all animals received a 0.1 µl infusion of 1% methylene blue according to the microinjection procedure described above. The animals received an anesthetic overdose, their brains were removed and injection sites were verified histologically according to the atlas of Franklin and Paxinos [9]. Data from animals with injection sites outside the cerebellar vermis were excluded from the study. The final sample size of each cohort ranged between 11 and 16. Histology confirmed that a total of 70 mice had accurate cannula placements in the cerebellar dorsal vermis (Fig. 1) and that the sample sizes of the different dosage cohorts were as follows: saline (n = 11), 0.54 nmol histamine (n = 15), 1.36 nmol histamine (n = 15), 2.72 nmol histamine (n = 16) and 4.07 nmol histamine (n = 13).

2.8. Statistical analysis

All results were initially submitted to Levene's test for homogeneity of variance. The data were analyzed using a One-way Analysis of Variance (ANOVA) test. When differences were indicated by significant F values, they were identified by Duncan's multiple range tests. A p value of <0.05 was required for significance.

2.9. Ethics

The experiments carried out as part of this study were approved by the Animal Ethics Commission of the Federal University of Sao Carlos (CEEA 049/09), and they are in compliance with the norms of the Brazilian Neuroscience and Behavior Society (SBNeC), which are based on the US National Institutes of Health Guide for Care and use of Laboratory Animals.

3. Results

One-way ANOVA tests showed no significant differences between groups in Trial 1 for all the measures analyzed (Table 1). Therefore, the data were *pooled* because the animals had received no pharmacological treatment at that point. Fig. 2 illustrates the effects of microinjection of histamine on %OAE in the first and second sessions. The ANOVA test showed differences between trials ($F_{5,134} = 4.72$, $p = 0.0005$). The post hoc Duncan's test indicated that the mice that entered less often into the open arms in Trial 2 in comparison with Trial 1 were microinjected with saline ($p = 0.001$) and histamine at a dose of 0.54 nmol ($p = 0.02$). Moreover, the analysis showed a significant difference between the groups that had received saline and histamine at the dose of 4.07 nmol ($p = 0.007$).

Fig. 3 shows the %OAT for the first and second sessions. ANOVA analysis detected differences between sessions ($F_{5,134} = 6.17$, $p = 0.000036$) in %OAT. Post hoc analysis revealed that animals explored the open arms for a shorter time in the second trial

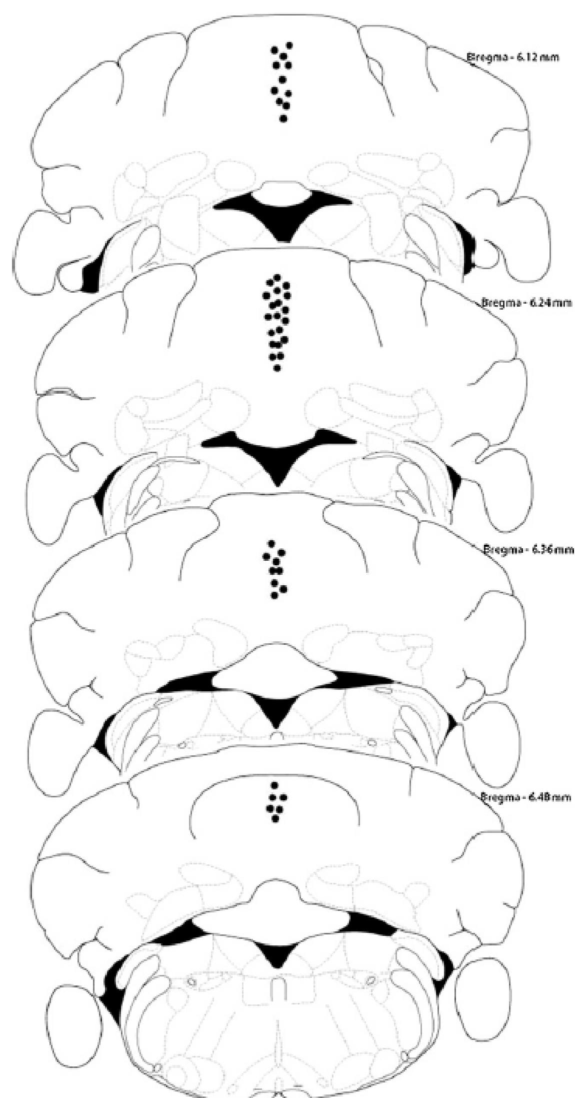

Fig. 1. Schematic representation (adapted from Franklin and Paxinos [9]) of sites of microinfusion into the cerebellums of mice. The number of points is smaller than the total number of mice because of overlaps.

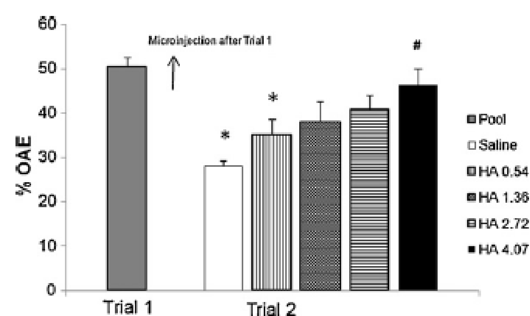

Fig. 2. Effects of microinjection of histamine (0.54, 1.36, 2.72 and 4.07 nmol) on the percentage of open arm entries (%OAE) in Trials 1 and 2 in the EPM. Data are presented as mean ± SEM. n = 11–16. * $p < 0.05$ Trial 2 versus Trial 1; # $p < 0.05$ versus control (saline) in Trial 2.

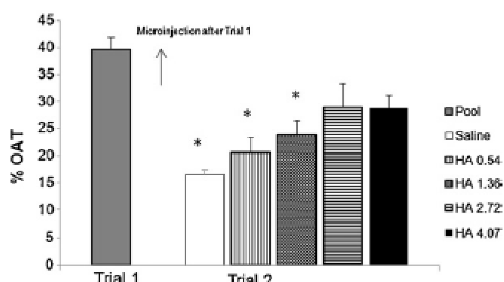

Fig. 3. Effects of microinjection of histamine (0.54, 1.36, 2.72 and 4.07 nmol) on the percentage of open arm time (%OAT) in Trials 1 and 2 in the EPM. Data are presented as mean ± SEM. n = 11–16. *$p < 0.05$ Trial 2 versus Trial 1.

when they had been microinjected with saline ($p < 0.001$), histamine at a dose of 0.54 nmol ($p = 0.005$), and histamine at a dose of 1.36 nmol ($p = 0.01$). For the groups that received histamine at doses of 2.72 nmol (%OAE: $p = 0.16$; %OAT: $p = 0.09$) and 4.07 nmol (%OAE: $p = 0.52$; %OAT $p = 0.10$), there were no differences between trials in both measures.

Table 2 shows the results for all other behaviors. ANOVA analysis did not indicate differences in EAE ($F_{5,134} = 0.61$, $p = 0.11$). The treatment did not change the locomotor activity. ANOVA analysis revealed significant differences between trials in OAT ($F_{5,134} = 8.2$, $p = 0.000001$). Post hoc comparisons indicated that this difference was present for the groups microinjected with saline ($p < 0.001$), histamine at a dose of 0.54 nmol ($p = 0.002$), and histamine at a dose of 1.36 nmol ($p < 0.001$). There were also differences between sessions in the EAT ($F_{5,134} = 9.41$, $p < 0.00001$), %EAT ($F_{5,134} = 9.41$, $p < 0.00001$), CT ($F_{5,134} = 5.85$, $p = 0.000063$), and frequency of head dipping ($F_{5,134} = 10.39$, $p < 0.00001$). ANOVA analysis did not detect any significant differences between trials in OAE ($F_{5,134} = 1.86$, $p = 0.11$), immobility time ($F_{5,134} = 1.56$, $p = 0.18$), or total SAP ($F_{5,134} = 1.13$, $p = 0.34$).

4. Discussion

The main experimental finding of this study is that the microinjection of histamine into the cerebellar vermis impairs emotional memory consolidation in mice re-exposed to the EPM because open arm exploration did not decrease in the second session. In addition, the results indicate that there is a dose-dependent effect of histamine on the inhibition of memory.

The EPM is a test that assesses behavior associated with emotion because the behavior exhibited during the test arises from a conflict between the motivation to explore the maze and the natural tendency to avoid open spaces [4,18]. The EPM is one of the most widely used behavioral tests in research on anxiety [5,25]. Recently, it has become useful for understanding the biological basis of emotion related to learning and memory [3,16,41]. During the EPM test, the animal acquires information about safe and dangerous areas in the maze. Repeated testing in the EPM provides an index of memory acquisition and retention because there are experience-dependent changes in behavior. Studies have shown that exposure to a second trial causes animals to reduce their open arm exploration [7,8,12,16]. Recently, Galvis-Alonso et al. [10] observed a reduction in open arm exploration in the second trial when the interval between sessions was 9 or 33 days, which suggests that aversion to the open arms is preserved even 33 days after the first maze exposure.

Previous studies on emotional memory performed by our research group using Trial 1/2 protocol on the EPM revealed that histamine plays an inhibitory role on emotional memory [12,36]. Also, other studies concerning the histaminergic system and memory demonstrated learning and memory impairment when histamine was administered [2,15,47]. Nishiga et al. [20] showed that rats on a histidine-deficient diet exhibited reduced hippocampal histamine contents and improved eight-arm radial maze performance. The results of a study by Liu et al. [19], which used histidine decarboxylase knockout mice, indicated that a lack of histamine improves fear conditioning consolidation. Furthermore, according to Alvarez and Banzan [1], histamine treatment interferes with the consolidation of avoidance responses and impairs latency and memory efficiency.

The microinjection of histamine into the cerebellar vermis demonstrated that the cerebellar histaminergic system is involved in the process of consolidation of emotional memory. Several lines of evidence support the involvement of the cerebellar vermis in emotional learning [32]. Moreover, studies have evaluated the role of the cerebellum in emotional memory consolidation, provided important information about the role of the cerebellum in memory and explained how neural circuits are related to aversive memories. Sacchetti et al. [33] demonstrated that cerebellar vermis blockade caused amnesia when performed immediately after the recall of fear memories and that the combined inactivation of the cerebellum and amygdala elicited amnesia of strong fear behavior. Thus, according to the authors, the cerebellum influences long-term emotional memories, and due to the strong learning, the cerebellum acquires a durable fear representation, which is sufficient to support memory processes even in the absence of sites that are crucial for emotion, such as the amygdala.

It has been previously proposed that the amygdala and cerebellum are functionally interconnected during aversive learning

Table 2
Effects of microinjection of histamine (0.54, 1.36, 2.72 and 4.07 nmol) on the behavior of mice in Trials 1 and 2 in the EPM.

	Pool	Saline	HA 0.54	HA 1.36	HA 2.72	HA 4.07
OAE	8.2 ± 0.4	6.6 ± 1.4	6.3 ± 0.9	6.0 ± 1.2	7.7 ± 0.8	9.6 ± 1.2
OAT	119.0 ± 6.9	49.3 ± 9.9*	62.0 ± 9.6*	53.6 ± 12.9*	86.9 ± 13.2	86.2 ± 7.5
EAE	8.5 ± 0.5	13.9 ± 1.35	11.1 ± 1.1	10.1 ± 1.1	11.1 ± 1.5	10.7 ± 1.1
EAT	89.9 ± 5.3	178.3 ± 53.7*	166.2 ± 14.1*	163.9 ± 21.4*	134.3 ± 14.5*	125.3 ± 12.8*
%EAT	29.6 ± 1.8	59.4 ± 7.9*	53.4 ± 5.7*	54.7 ± 7.1*	44.8 ± 4.8*#	41.8 ± 4.3*#
CT	91.6 ± 0.5	72.4 ± 21.8	71.8 ± 7.5*	82.5 ± 17.5*	78.8 ± 10.5*	88.4 ± 9.6*
SAP	9.3 ± 0.0	6.9 ± 1.1	7.9 ± 1.1	7.0 ± 0.9	9.1 ± 1.4	8.5 ± 1.6
HD	5.9 ± 0.9	0.5 ± 0.1*	1.7 ± 0.6*	2.0 ± 1.1*	1.7 ± 0.5*	1.8 ± 0.9*
Immobility time	0.0 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0

Pool (animals exposed to EPM with no pharmacological treatment); Saline (microinjected immediately after Trial 1); HA (histamine microinjected immediately after Trial 1 doses of 0.54 nmol; 1.36 nmol; 2.72 nmol and 4.07 nmol/0.1 μl); OAE (number of open arm entries); OAT (time spent in the open arms); EAE (number of enclosed arm entries); EAT (time spent in the enclosed arms); %EAT (percentage of time in enclosed arms); CT (central platform time); SAP (frequency of stretched-attend postures), HD (frequency of head dipping), immobility time. Data are presented as mean values (±SEM). The second is the unit measure of time. *$p < 0.05$, Trial 2 versus Trial 1; #$p < 0.05$, versus control (saline).

[17,40]. According to Sacchetti et al. [34], the vermis and amygdala may interact, and the vermal electrical stimulation modulates amygdala activity. These effects are mediated by both direct and indirect anatomical connections between the cerebellum and limbic areas as well as through the paleocerebellar projections to ascending catecholamine neurons of the locus coeruleus, the ventral tegmental area and the periaqueductal gray [34]. Therefore, the present results may be due to the effect of histamine on one of these cerebellar projections.

In conclusion, the results indicate that there is a dose-dependent inhibition of memory when histamine is injected into the cerebellar vermis. Therefore, it can be suggested that histamine in the cerebellum impairs the consolidation of emotional memory in mice.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Research report

Emotional memory consolidation impairment induced by histamine is mediated by H_1 but not H_2 receptors

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ABSTRACT

Histaminergic fibers are present in the molecular and granular layers of the cerebellum and have high density in the vermis and flocculus. Evidence indicates that the cerebellar vermis is involved in memory consolidation. Recently, we demonstrated that when histamine is microinjected into the cerebellar vermis it results in impaired emotional memory consolidation in mice that are submitted to the elevated plus maze (EPM). This study investigated whether histamine impairment was mediated by the H_1 or H_2 receptors. The cerebellar vermis of male mice (Swiss Albino) were implanted using a guide cannula. Three days after recovery, behavioral tests were performed in the EPM on two consecutive days (Trial 1 and Trial 2). Immediately after exposure to the EPM (Trial 1), animals received a microinjection of histaminergic drugs. In Experiment 1, saline (SAL) or histamine (HA, 4.07 nmol/0.1 μl) was microinjected 5 min after pretreatment with the H_1 antagonist chlorpheniramine (CPA, 0.16 nmol/0.1 μl) or SAL. In Experiment 2, SAL or HA was microinjected into the mice 5 min after pretreatment with the H_2 antagonist ranitidine (RA, 2.85 nmol/0.1 μl) or SAL. Twenty-four hours later, the mice were re-exposed to the EPM (Trial 2) under the same experimental conditions but did not receive an injection. On both days, the test sessions were recorded to enable analysis of the behavioral measures. The decrease in open arm exploration (% entries and % time spent in the open arms) in Trial 2 relative to Trial 1 was used as a measure of learning and memory. The data were analyzed using the two-way analysis of variance (ANOVA) and Duncan's tests. In Experiment 1, the Duncan's test indicated that the mice entered the open arms less often (%OAE) and spent less time in the open arms (%OAT) in Trial 2 after being microinjected with SAL + SAL, SAL + CPA and CPA + HA. However, the animals that received SAL + HA did not enter the open arms less frequently or spend less time in them, which was significantly different from the CPA + HA group. The results of Experiment 2 demonstrated that the %OAE and %OAT in Trial 2 were different from Trial 1 for the groups that were microinjected with SAL + SAL and SAL + RA. The animals that were microinjected with RA + HA or with SAL + HA did not show a reduction in %OAE. These results demonstrate that the animals treated with HA did not avoid the open arms less on retesting and indicated that CPA did not alter the behavior parameters but did revert the histamine-induced impairment of memory consolidation. Furthermore, the H_2 antagonist RA, at the dose used in this study, did not affect memory consolidation and failed to revert histamine-induced impairment.

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1. Introduction

Histamine is an important neurotransmitter–neuromodulator in the central nervous system (CNS). Histaminergic neurons are located exclusively in the tuberomammillary nucleus. From there, they project to all regions of the brain, including the cerebellum, which contains high density of histaminergic terminations in the vermis and flocculus (Panula et al., 1989; Wada et al., 1991). Histamine-immunoreactive fibers are located in the molecular and granular layers of the cerebellum in several species, including humans. These fibers run parallel to the Purkinje cell layer after

Abbreviations: EPM, elevated plus maze; SAL, saline; HA, histamine; CPA, chlorpheniramine; RA, ranitidine; %OAE, percentage of open arms entries; %OAT, percentage of open arm time; CNS, central nervous system; OAE, open arm entries; EAE, enclosed arm entries; OAT, time spent in the open arms; EAT, time spent in the enclosed arms; CT, time spent in the central area; %EAT, percentage of time spent in the enclosed arms; SAP, the number of stretched-attend postures; HD, head dipping.

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traversing perpendicular to it (Hass and Panula, 2003; Panula et al., 1993).

Autoradiographic mapping and in situ hybridization experiments demonstrated the presence of H_1 and H_2 receptors in the rat cerebellar cortex and deep in the cerebellar nuclei (Arrang et al., 1995; Pollard et al., 1993). These studies suggest that histamine may play an important role in modulating the excitability of cerebellar neurons. The Purkinje cells of the cerebellar cortex and the neurons in the nucleus interpositus all exhibit H_2 receptor-mediated excitatory responses when exposed to a histamine bath perfusion (Shen et al., 2002). Granule cells are excited through the activation of the H_1 and H_2 receptors (Li et al., 1999; Tian et al., 2000).

Recently, evidence has been presented indicating that the cerebellum, in addition to being a motor coordination center, is also involved in modulation of emotional behavior (Schmahmann et al., 2007; Strata et al., 2011; Tavano and Borgatti, 2010). Additionally, the vermal part of the cerebellum has been implicated in emotional or fear-related behaviors (Sacchetti et al., 2009; Storozheva, 2009). According to Sacchetti et al. (2005), the fact that there is a functional interconnection between the cerebellar vermis and the hypothalamus, amygdala, and hippocampus suggests that the cerebellum may play a role in the integrated network that regulates emotional behavior.

Studies have related the cerebellar vermis to emotional memory consolidation (Sacchetti et al., 2002; Storozheva, 2009). In one study, inactivation of the vermis caused amnesic effects after a fear-conditioning task (Sacchetti et al., 2002). Other findings indicate that after training the cerebellar vermis is selectively involved in long-term memory formation for certain types of defense behaviors (Storozheva, 2009). Thus, the participation of the vermis in emotional memory may be independent of its role in sensory or motor processes, and the vermis may represent an interface between sensory stimuli, the emotional state, and motor responses (Sacchetti et al., 2005, 2009).

The neural histaminergic system is involved in several behavioral and neurobiological functions, such as arousal, food intake, motor activity, and nociception (Brown et al., 2001; Hass and Panula, 2003). However, the part histaminergic circuits play in mnemonic effects is complex. Histamine seems to have different effects in distinct brain regions and may have modulatory effects that differ according to the memory type. The exact role of this neurotransmitter in learning processes and memory consolidation, the action of the receptor subtypes and how they affect key circuits related to a specific memory system are not well understood (Köhler et al., 2011).

Histaminergic modulation of learning and memory was studied using lesions and pharmacological interventions in the tuberomammillary nucleus and other decisive brain regions. However, the role of the cerebellar histaminergic system on memory has not been investigated. In our first study, microinjection of histamine into the cerebellar vermis demonstrated that the cerebellar histaminergic system is involved in the process of consolidation of emotional memory. These results indicated that there was a dose-dependent inhibition of memory consolidation when histamine was injected into the cerebellar vermis in mice re-exposed to the elevated plus maze (Gianlorenço et al., 2011a). Therefore, in the present study we investigated whether histamine impairment was mediated by the H_1 and/or H_2 receptors.

2. Materials and methods

2.1. Subjects

Male Swiss mice (Federal University of São Carlos, UFSCar, SP, Brazil) weighing 25–35 g at the beginning of the experiments were housed in polypropylene cages (31 cm × 20 cm × 13 cm) in groups of five and were maintained under a 12 h light cycle (lights on at 7:00 a.m.) in a controlled environment at a temperature of 23 ± 1 °C and a humidity level of $50 \pm 5\%$. Food and drinking water were provided ad libitum, except during the brief test periods. All mice were experimentally naive, and the experimental sessions were conducted during the light period of the cycle (9:00–13:00 h).

2.2. Drugs

Histamine dihydrochloride, the H_1 receptor antagonist, chlorpheniramine maleate salt and the H_2 receptor antagonist ranitidine hydrochloride (Sigma Chemical Co., USA) were prepared in a vehicle of physiological saline. Saline solution was used as an experimental control. The doses were based on previous research (Gianlorenço et al., 2011a) and on pilot work in our laboratory. The substances were coded, and the experimenter was blinded to the codes when the tests and behavioral analysis were performed.

2.3. EPM apparatus

The EPM used was similar to the one originally described by Lister (1987). The EPM consisted of two open arms (30 cm × 5 cm × 0.25 cm) and two enclosed arms (30 cm × 5 cm × 15 cm) that were connected to a common central platform (5 cm × 5 cm). The apparatus was made of crystal acrylic and was raised 38.5 cm above floor level.

2.4. Stereotaxic surgery and drug infusion

Mice were intraperitoneally anesthetized using ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg) solution in association with local anesthesia (3% lidocaine with norepinephrine 1:50,000) and were placed in a Stoelting stereotaxic instrument. A single, 7 mm, stainless steel guide cannula (25 gauge) was implanted in the cerebellar vermis according to the following coordinates from the mouse brain atlas (Franklin and Paxinos, 2001): 6.5 mm posterior to the bregma, 0 mm lateral to the midline, and 2.0 mm ventral to the skull surface. The guide cannula was fixed to the skull using dental acrylic and jeweler's screws. A dummy cannula (33 gauge stainless steel wire) was inserted into the guide cannula at the time of surgery to reduce the incidence of occlusion. Postoperative analgesia was provided for 3 days by adding acetaminophen (200 mg/ml) to the drinking water in a ratio of 0.2 ml acetaminophen to 250 ml water (i.e., the final concentration was 0.16 mg/ml).

Saline and drug solutions were infused into the cerebellar vermis using a microinjection unit (33 gauge cannula; Insight Equipamentos Científicos Ltda, Brazil), which extended 2.0 mm beyond the tip of the guide cannula. The microinjection unit was attached to a 5-μl Hamilton microsyringe via polyethylene tubing (PE-10), and the administration was controlled by an infusion pump (Insight Equipamentos Científicos Ltda, Brazil) that was programmed to deliver a volume of 0.1 μl over a period of 60 s. The microinjection procedure consisted of gently restraining the animal, inserting the injection unit, infusing the solution, and keeping the injection needle in situ for a further 60 s to avoid reflux. Confirmation of successful infusion was obtained by monitoring the movement of a small air bubble inside the PE-10 tubing.

2.5. General conditions and data collection

Three days after surgery, the animals were transported to the experimental room and left undisturbed for at least 1 h before testing to facilitate adaptation. The test was performed on two consecutive days, and the trials in the EPM were denoted Trial 1 and Trial 2. Mice were individually placed on the central platform of the maze facing the open arm and were able to explore the maze for 5 min.

In Trial 1, immediately after exposure to the EPM, the animals received a microinjection of the drugs. In Experiment 1, saline (SAL) or 4.07 nmol histamine (HA) was microinjected 5 min after pretreatment with 0.16 nmol chlorpheniramine (CPA). In Experiment 2, SAL or 4.07 nmol HA was microinjected 5 min after pretreatment with 2.85 nmol ranitidine (RA). Twenty-four hours later (Trial 2), the mice were re-exposed to the EPM under the same experimental conditions as in Trial 1 with the exception that they did not receive an injection. Between subjects, the maze was thoroughly cleaned with 5% ethanol and a dry cloth. All tests were conducted under moderate illumination (77 lx) as measured on the central platform of the EPM and in an environment isolated from the rest of the room by a black protective curtain.

All sessions were video recorded with a digital camera that was linked to a computer in an adjacent room. Images were analyzed by a highly trained observer using X-PLO-RAT, which is an ethological analysis software package developed at the Laboratory of Exploratory Behavior USP/Ribeirao Preto (Garcia et al., 2005). Behavioral parameters were defined in a way that was consistent with previous studies (Lister, 1987; Rodgers and Johnson, 1995) and included the following observations: the frequency of open- and enclosed-arm entries (OAE and EAE) (an entry was defined as the entry of all four of an animal's paws into an arm) and the total amount of time spent in the open arms (OAT), enclosed arms (EAT), and central area (CT). These data were used to calculate the percentage of open arm entries (%OAE = (open entries/open + enclosed entries) × 100), the percentage of time spent in the open arms (%OAT = (open time/300) × 100), and the percentage of time spent

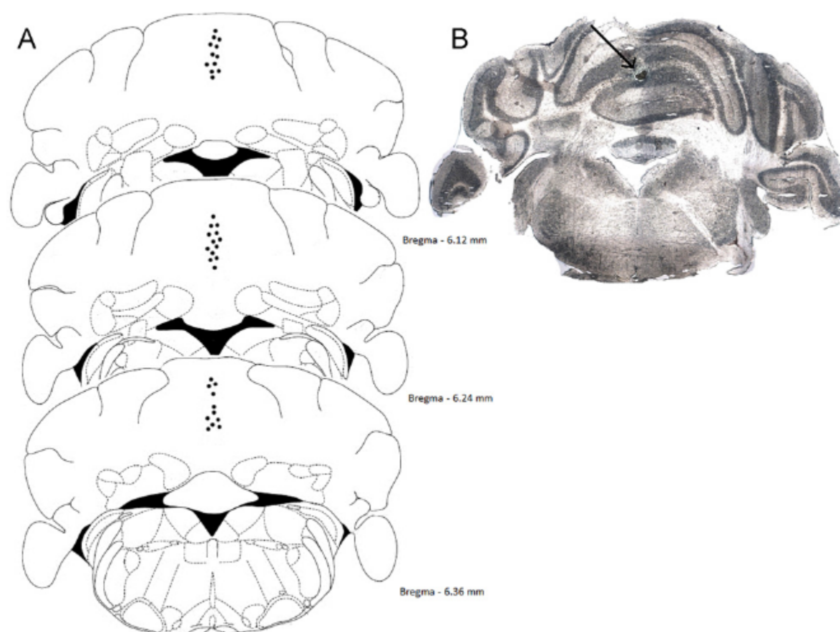

Fig. 1. (A) Schematic representation (adapted from Franklin and Paxinos, 2001) of sites of microinfusion (filled circles) into the cerebella of mice. The number of points is smaller than the total number of mice because of overlap. (B) Photomicrograph showing a typical injection site (indicated by an arrow) in the cerebellar vermis.

in the enclosed arms (%EAT = (enclosed time/300) × 100). The number of stretched-attend postures (SAP; exploratory posture in which the body stretches forward and then retracts to its original position without any forward locomotion), immobility time (stillness but some movement of the chest), and the frequency of head dipping (HD; exploratory movement of head/shoulders over the sides of the maze) were also scored. Total SAP was considered a primary index of risk assessment, and head dipping was considered an index of exploratory behavior (Rodgers et al., 1997).

2.6. Histology

At the end of testing, all animals received a 0.1 μl infusion of 1% methylene blue according to the microinjection procedure described above. The animals received an anesthetic overdose, their brains were removed and the injection sites were verified histologically according to the atlas of Franklin and Paxinos (2001). Data from animals with injection sites outside the cerebellar vermis were excluded from the study. The final sample size of each cohort ranged between 9 and 13. Histology confirmed that a total of 87 mice had accurate cannula placement mainly in the anterior and central vermis in the lobules V and VI (Fig. 1).

2.7. Statistical analysis

All results were initially analyzed using the Levene's test for homogeneity of variance. The data were analyzed using the two-way repeated measures ANOVA (factor 1: treatment; factor 2: trial). When differences were indicated by significant *F* values, they were further analyzed using Duncan's multiple range tests. A *p* value of less than 0.05 was considered significant.

2.8. Ethics

The experiments performed as part of in this study were approved by the Animal Ethics Commission of the Federal University of Sao Carlos (CEEA 049/09) and are in compliance with the norms of the Brazilian Neuroscience and Behavior Society (SBNeC), which are based on the US National Institutes of Health Guide for Care and use of Laboratory Animals.

3. Results

3.1. Experiment 1: pretreatment with the H_1 receptor antagonist CPA prevents the HA-induced impairment of memory consolidation

As summarized in Fig. 2A and B and Table 1, the two-way ANOVA revealed no significant differences among groups in Trial 1 for all the measures analyzed. However, there were differences in the %OAE between trials ($F_{1,38} = 22.89$, $p = 0.00003$). The post hoc Duncan's test indicated that the mice entered the open arms less often in Trial 2 compared to Trial 1 when microinjected with SAL + SAL ($p = 0.007$), CPA + SAL ($p = 0.04$), and CPA + HA ($p = 0.0004$). However, no change in the percentage %OAE was observed in the animals

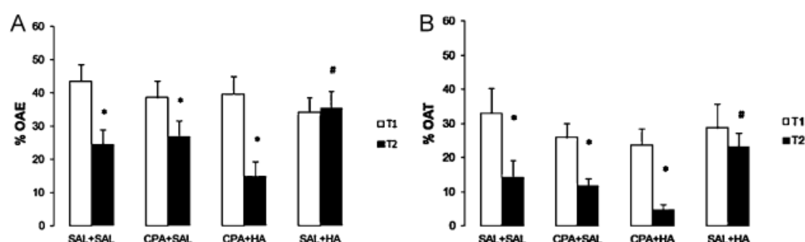

Fig. 2. Effects of the combined injection of the H_1 antagonist CPA (0.16 nmol) and histamine (4.07 nmol): (A) on the percentage of open arm entries (%OAE) and (B) on the percentage of open arm time (%OAT) in Trials 1 and 2 in the EPM. Data are presented as the mean ± SEM. $n = 9–11$. $^*p < 0.05$ Trial 2 versus Trial 1; $^\#p < 0.05$ versus CPA + HA group in Trial 2, Duncan's test.

Table 1
Effects of the combined injection of the H_1 antagonist CPA (0.16 nmol) and histamine (4.07 nmol) on the behavior of mice in Trials 1 and 2 in the EPM.

	SAL + SAL		CPA + SAL		CPA + HA		SAL + HA	
	T1	T2	T1	T2	T1	T2	T1	T2
OAE	6.5 ± 0.7	2,7 ± 0.6*	7.4 ± 1.5	3.9 ± 0.8*	5.9 ± 1.2	1.7 ± 0.5*	5.7 ± 1.0	5.9 ± 0.9*,#
OAT	99.2 ± 21.6	42.6 ± 14.8*	77.8 ± 12.4	35.3 ± 6.3*	71.3 ± 14.6	14.1 ± 4.4*	86.4 ± 21.1	69.7 ± 11.7#
EAE	9.0 ± 1.3	9.3 ± 1.9	11.3 ± 1.3	11.7 ± 1.8	8.2 ± 0.7	8.9 ± 0.9	10.9 ± 0.7	11.7 ± 2.9
EAT	11.8 ± 16.6	193.6 ± 17.4*	114.3 ± 9.1	165.1 ± 12.9*	112.1 ± 9.7	217.3 ± 9.2*	132.0 ± 17.1	181.4 ± 12.9*
%EAT	37.3 ± 5.5	64.5 ± 5.8*	38.1 ± 3.0	55.0 ± 4.3*	37.4 ± 3.2	72.4 ± 3.1*	44.0 ± 5.7	60.5 ± 4.3*
CT	89.0 ± 10.3	63.8 ± 10.6	107.9 ± 6.1	99.6 ± 11.6	116.6 ± 15.5	68.6 ± 8.7*	81.6 ± 11.8	48.9 ± 7.4
SAP	6.7 ± 1.3	5.7 ± 1.8	8.8 ± 1.3	4.3 ± 0.8*	7.4 ± 1.2	4.2 ± 0.8	7.2 ± 1.1	6.7 ± 1.0
HD	6.9 ± 0.9	1.6 ± 0.6*	4.1 ± 0.8	0.9 ± 0.4*	4.9 ± 1.1	0.6 ± 0.4*	2.6 ± 1.1	1.6 ± 0.7
Immobility	0.2 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.9

The data are presented as the mean values (±SEM). Immediately after exposure to the EPM (T1), the animals received a microinjection of the following drugs: SAL + SAL; CPA + SAL (CPA 0.16 nmol/0.1 μl and saline); CPA + HA (CPA 0.16 nmol/0.1 μl and HA 4.07 nmol/0.1 μl); SAL + HA (saline and HA 4.07 nmol/0.1 μl). OAE (number of open arm entries), OAT (time spent in the open arms), EAE (number of enclosed arm entries), EAT (time spent in the enclosed arms), %EAT (percentage of time in enclosed arms), CT (central platform time), SAP (frequency of stretched-attend postures), HD (frequency of head dipping), and immobility (total time). The second is the unit measure of time.

* $p < 0.05$ Trial 2 versus Trial 1.

\+ $p < 0.05$ versus control group (SAL + SAL) in Trial 2.

\# $p < 0.05$ versus the CPA + HA group in Trial 2.

that received SAL + HA ($p = 0.84$) and the %OAE was significantly different from the CPA + HA group ($p = 0.007$) (Fig. 2A).

Fig. 2B shows the %OAT for the first and second sessions. The ANOVA revealed differences in the %OAT between trials ($F_{1,38} = 19.27$, $p = 0.0001$). Post hoc analysis determined that animals explored the open arms for a shorter time in the second trial when they had been microinjected with SAL + SAL ($p = 0.02$), CPA + SAL ($p = 0.05$) and CPA + HA ($p = 0.01$). The group that received SAL + HA did not spend less time in the open arms ($p = 0.46$), and the %OAT was significantly different from the CPA + HA group ($p = 0.02$). These results demonstrate that the animals treated with HA did not avoid the open arms more during retesting, indicating that the CPA did not alter behavioral parameters but prevented the histamine-induced impairment of memory consolidation.

Table 1 shows the results for all other behaviors. The ANOVA showed no significant differences in EAE among groups ($F_{1,38} = 1.54$, $p = 0.22$), which is an EPM index of general exploratory activity. The ANOVA revealed significant differences between trials in OAT ($F_{1,38} = 18.53$, $p = 0.0001$) and OAE ($F_{1,38} = 25.24$, $p = 0.00001$). Post hoc comparisons indicated that these differences were present for the groups that were microinjected with SAL + SAL, CPA + SAL, and CPA + HA. Additionally, there were significant differences between the groups SAL + HA and SAL + SAL (OAE, $p = 0.03$) and between SAL + HA and CPA + HA (OAE, $p = 0.004$; OAT, $p = 0.02$). Furthermore, there were differences between sessions for EAT ($F_{1,38} = 63.42$, $p < 0.00001$), %EAT ($F_{1,38} = 63.42$, $p < 0.00001$), CT ($F_{1,38} = 14.39$, $p = 0.0005$), total SAP ($F_{1,38} = 7.41$, $p = 0.01$), and frequency of head dipping ($F_{1,38} = 36.27$, $p < 0.00001$). The ANOVA did not detect any significant differences in immobility time ($F_{1,38} = 0.84$, $p = 0.37$) between trials.

3.2. Experiment 2: the effects of pretreatment with the H_2 receptor antagonist RA on HA-impairment of memory consolidation

The two-way ANOVA test revealed that there were no significant differences between the groups in Trial 1 for any of the measures analyzed (Table 2). However, there were differences in the %OAE ($F_{1,45} = 15.31$, $p = 0.0003$) between trials. The post hoc analysis indicated that differences in the %OAE existed in groups that were microinjected with SAL + SAL ($p = 0.02$) and RA + SAL ($p = 0.05$). The animals that received pretreatment with RA (RA + HA) ($p = 0.23$) and animals microinjected with SAL + HA ($p = 0.21$) did not have a reduced %OAE (Fig. 3A).

Fig. 3B shows the %OAT for the first and second sessions. The ANOVA revealed that there were differences in the %OAT between sessions ($F_{1,45} = 20.53$, $p = 0.00005$). Duncan's test indicated that the animals that were microinjected with SAL + SAL ($p = 0.002$), RA + SAL ($p = 0.04$), and RA + HA ($p = 0.03$) exhibited a decreased %OAT in Trial 2 relative to Trial 1. These results indicated that the H_2 antagonist RA, at the dose used in this study, did not have an effect on memory consolidation and that it failed to prevent entirely histamine impairment.

Table 2
Effects of the combined injection of the H_2 antagonist RA (2.85 nmol) and histamine (4.07 nmol) on the behavior of mice in Trials 1 and 2 in the EPM.

	SAL + SAL		RA + SAL		RA + HA		SAL + HA	
	T1	T2	T1	T2	T1	T2	T1	T2
OAE	6.1 ± 0.9	3.4 ± 1.1	7.2 ± 1.4	4.7 ± 1.4	8.5 ± 1.0	5.7 ± 0.9	7.1 ± 1.4	4.3 ± 0.8
OAT	120.0 ± 23.3	36.9 ± 15.8*	94.3 ± 21.2	45.9 ± 16.4*	120.4 ± 19.4	61.3 ± 9.6*	95.5 ± 16.0	77.2 ± 19.3
EAE	6.7 ± 0.8	7.4 ± 1.3	9.1 ± 1.3	10.5 ± 1.7	8.9 ± 5.4	8.3 ± 1.0	9.1 ± 1.2	9.5 ± 1.4
EAT	89.0 ± 15.1	191.1 ± 23.8*	87.7 ± 14.5	176.2 ± 21.8*	89.1 ± 15.4	144.3 ± 20.4*	99.9 ± 15.0	141.4 ± 16.4
%EAT	29.7 ± 5.0	63.7 ± 7.9*	29.2 ± 4.8	58.7 ± 7.3*	29.7 ± 5.1	48.1 ± 6.8*	33.3 ± 5.0	47.1 ± 5.5
CT	91.0 ± 10.3	72.2 ± 17.5	118.0 ± 20.5	75.9 ± 16.9	90.5 ± 12.5	94.4 ± 15.5	104.6 ± 9.8	81.4 ± 13.4
SAP	7.5 ± 1.0	4.2 ± 0.9	7.5 ± 1.0	6.3 ± 1.8	5.4 ± 1.1	6.6 ± 1.1	7.9 ± 1.1	7.3 ± 1.0
HD	10.9 ± 3.2	1.4 ± 0.6*	7.3 ± 1.8	2.5 ± 1.3*	5.4 ± 1.2	2.0 ± 0.6	3.3 ± 1.1	3.5 ± 1.1
Immobility	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0	0.4 ± 0.4	0.1 ± 0.1	0.5 ± 0.3	0.0 ± 0.0	0.0 ± 0.0

The data are presented as the mean values (±SEM). Immediately after exposure to the EPM (T1), the animals received a microinjection of the following drugs: SAL + SAL, RA + SAL (RA 2.85 nmol/0.1 μl and saline), RA + HA (RA 2.85 nmol/0.1 μl and HA 4.07 nmol/0.1 μl), SAL + HA (saline and HA 4.07 nmol/0.1 μl). OAE (number of open arm entries), OAT (time spent in the open arms), EAE (number of enclosed arm entries), EAT (time spent in the enclosed arms), %EAT (percentage of time in enclosed arms), CT (central platform time), SAP (frequency of stretched-attend postures), HD (frequency of head dipping), and immobility time. The data are presented as mean values (±SEM). The second number is the unit measure of time.

* $p < 0.05$ for Trial 2 versus Trial 1.

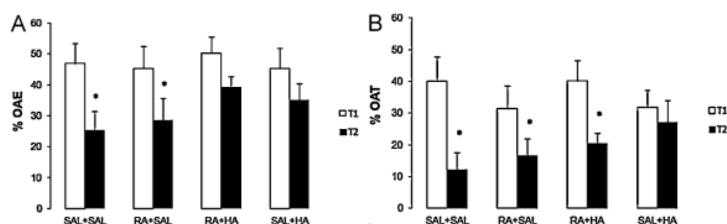

Fig. 3. Effects of the combined injection of the H_2 antagonist RA (2.85 nmol) and histamine (4.07 nmol): (A) on the percentage of open arm entries (%OAE) and (B) on the percentage of open arm time (%OAT) in Trials 1 and 2 in the EPM. Data are presented as the mean ± SEM. $n = 10–13$. $^{*}p < 0.05$ Trial 2 versus Trial 1, Duncan's test.

The ANOVA revealed significant differences in the OAT between trials ($F_{1,45} = 22.33$, $p = 0.00003$). Post hoc comparisons indicated that significant differences existed for the groups microinjected with SAL + SAL ($p = 0.001$), RA + SAL ($p = 0.05$), and RA + HA ($p = 0.02$). Additionally, differences between sessions for EAT ($F_{1,45} = 31.83$, $p = 0.000001$), %EAT ($F_{1,45} = 31.83$, $p < 0.00001$), OAE ($F_{1,45} = 11.78$, $p = 0.001$) and the frequency of head dipping ($F_{1,45} = 19.48$, $p = 0.0001$) were detected. The ANOVA did not indicate any significant differences between trials in the CT ($F_{1,45} = 3.09$, $p = 0.09$), EAE ($F_{1,45} = 24$, $p = 0.63$), immobility time ($F_{1,45} = 3.44$, $p = 0.07$), or total SAP ($F_{1,45} = 1.61$, $p = 0.21$).

4. Discussion

The primary findings of the present study are that pre-treatment with the H_1 antagonist CPA was able to completely abolish the effect on intra-cerebellar histamine impaired emotional memory consolidation in mice submitted to the EPM, whereas combined microinfusion with the H_2 antagonist RA failed to reverse the histamine effect.

In the EPM, memory acquired during the first exposure is related to an anxious emotional state. The behaviors expressed during the test are due to a conflict between motivation to explore the maze and the natural tendency to avoid open spaces (Bertoglio and Carobrez, 2000; Lister, 1987). According to File et al. (1990), after the initial exploration of the apparatus, rodents acquire, consolidate and retrieve some memory related to exploration of potentially dangerous areas of the maze. Several studies show that EPM-experienced animals exhibit a significant decrease in %OAE and %OAT during retesting (Bertoglio and Carobrez, 2000; Galvis-Alonso et al., 2010). In a recent study, Gazarini et al. (2011) demonstrated that pretest and posttest dorsal hippocampus anisomycin infusion do not interfere with the further avoidance to open arms exhibited by rats in the EPM retest, and according to the authors, the test/retest protocol in the EPM is an effective tool that can be used to investigate memory.

This study confirms our early results, which demonstrated that animals microinjected with 4.07 nmol histamine did not explore the open-arms less during retesting in the EPM, indicating that histamine has an inhibitory effect on memory (Gianlorenço et al., 2011a). However, the combined injection with saline (SAL + HA) induced a less pronounced effect on memory consolidation, since there was an absence of a significant difference between the control group (SAL + SAL) and the group treated with HA.

Histaminergic circuits play a complex role in the mnemonic system. Histamine has different effects in distinct brain regions and may produce modulatory effects that differ according to memory type (Benetti et al., 2012; Da Silva et al., 2006; Köhler et al., 2011). Our results show that the histaminergic system may have an inhibitory effect in the memory consolidation process with an anxiety component. The histaminergic projections to the cerebellar vermis, amygdala and hippocampus can be involved in the modulation of this role. It has been previously proposed that the amygdala and cerebellum are functionally interconnected during aversive learning (Turner et al., 2007). According to Sacchetti et al. (2009), the vermis and amygdala may interact, and the vermal electrical stimulation modulates amygdala activity. These effects are mediated by both direct and indirect anatomical connections between the cerebellum and the limbic areas.

Evidence demonstrates the existence of the histaminergic receptors H_1, H_2 and H_3 in the cerebellum of rodents (Arrang et al., 1995; Pollard et al., 1993). Generally, H_1 and H_2 receptors excite or potentiate excitatory impulses (Gianlorenço et al., 2011b; Pollard et al., 1993), while H_3 activation mediates autoinhibition of TMN neurons (Arrang et al., 1985; Hass and Panula, 2003). According to a recent review, the H_1 receptor is the predominant histaminergic receptor in the cerebellum (Köhler et al., 2011).

In Experiment 1, the microinfusion of CPA did not alter behavioral parameters by itself, but when CPA was administrated prior to HA it reduced the open arm exploration and showed a significant difference from the group that was microinjected with HA only. Therefore, pre-treatment with CPA abolished the inhibitory effect of HA on memory consolidation, which indicates that this receptor is possibly involved in histamine induced memory impairment.

One possible mechanism to be explored is whether the administration of the H_1 antagonist CPA enhanced histamine levels in the synaptic cleft and increased the synaptic availability of histamine and its binding to H_3 receptors. Activation of H_3 receptors would be responsible for inhibition of the synthesis and release of endogenous histamine, which reverses the memory impairment. However, since CPA is not highly selective for H_1 receptors, we cannot exclude the possibility of interactions with other neurotransmitters systems, such as the serotoninergic and cholinergic systems (Benetti et al., 2012; Hasenöhrl et al., 1999).

Evidence regarding the role of the histaminergic system in the learning and memory process is controversial. In addition, the function of the histaminergic receptors and how they affect the memory system is still unclear (Köhler et al., 2011). Some studies that investigated the role of the H_1 receptor in memory found that the intraventricular infusion of CPA improved water maze performance in aged rats (Hasenöhrl et al., 1999) and improved the one-trial step through passive avoidance retention (Zarrindast et al., 2002). However, blockade of the H_1 receptor impaired working and reference memory (Chen et al., 2001). Studies with H_1 knockout mice demonstrated that their inhibitory avoidance performance was unaffected (Yanai et al., 1998) and that their object recognition performance was impaired (Daí et al., 2007). In the EPM, systemic CPA did not have an effect on emotional memory (Gianlorenço et al., 2011b).

Studies have examined the effects of H_2 antagonists on cognitive performance. In the study by Flood et al. (1998), intra-septal infusion of dimaprit (H_2 agonist) facilitated long-term retention of an avoidance learning task. The results from Alvarez and Banzan (2008) demonstrated that pretreatment with RA blocked the inhibitory effect of histamine on memory consolidation. Recently, Benetti et al. (2012) suggested that activation of postsynaptic H_2 receptors within the nucleus basalis magnocellularis by

endogenous histamine is responsible for the potentiated expression of fear responses.

In Experiment 2, the H_2 antagonist RA did not have an effect on open arm exploration by itself and when RA was administered before histamine, RA was not able to reverse the inhibitory effect of histamine on memory. This suggests that H_2 receptors in the cerebellar vermis likely do not play a role in the inhibitory effect of histamine on the EPM.

Emotional memory plays an important role in controlling behavior and it is critical for the survival of individuals and of species. An emotional memory deficit could lead to the exposure of an individual to numerous dangerous situations while the persistence of an aversive memory is a considerable factor associated with the development of anxiety and fear disorders, including phobias and posttraumatic stress disorder (PTSD). Therefore, in view of our results, there is a potential importance of these histamine-modulated effects leading to emotional memory erasure. Further research is warranted to better understand the importance of the histaminergic system to trauma-related disorders.

5. Conclusion

In conclusion, the results of the present study demonstrate that H_1 receptors in the cerebellar vermis mediate memory consolidation impairment induced by histamine in mice re-exposed to the EPM.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Effect of histamine H1 and H2 receptor antagonists, microinjected into cerebellar vermis, on emotional memory consolidation in mice

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Abstract

This study investigated the effects of histamine H1 or H2 receptor antagonists on emotional memory consolidation in mice submitted to the elevated plus maze (EPM). The cerebellar vermis of male mice (Swiss albino) was implanted using a cannula guide. Three days after recovery, behavioral tests were performed in the EPM on 2 consecutive days (T1 and T2). Immediately after exposure to the EPM (T1), animals received a microinjection of saline (SAL) or the H1 antagonist chlorpheniramine (CPA; 0.016, 0.052, or 0.16 nmol/0.1 µL) in Experiment 1, and SAL or the H2 antagonist ranitidine (RA; 0.57, 2.85, or 5.7 nmol/0.1 µL) in Experiment 2. Twenty-four hours later, mice were reexposed to the EPM (T2) under the same experimental conditions but they did not receive any injection. Data were analyzed using one-way ANOVA and the Duncan test. In Experiment 1, mice microinjected with SAL and with CPA entered the open arms less often (%OAE) and spent less time in the open arms (%OAT) in T2, and there was no difference among groups. The results of Experiment 2 demonstrated that the values of %OAE and %OAT in T2 were lower compared to T1 for the groups that were microinjected with SAL and 2.85 nmol/0.1 µL RA. However, when animals were microinjected with 5.7 nmol/0.1 µL RA, they did not show a reduction in %OAE and %OAT. These results demonstrate that CPA did not affect behavior at the doses used in this study, while 5.7 nmol/0.1 µL RA induced impairment of memory consolidation in the EPM.

Key words: Chlorpheniramine; Ranitidine; Cerebellar vermis; Emotional memory consolidation

Introduction

The central histaminergic nervous system originates from the tuberomammillary nucleus (TMN) of the hypothalamus, and, in many species, it widely innervates almost the whole brain including the cerebellum and other subcortical motor structures (1). The four histamine receptors identified as H1, H2, H3, and H4 subtypes are expressed in the brain (1,2). H1 and H2 receptors potentiate excitatory inputs while H3 receptors down regulate histamine synthesis and release as well as the releases of other neurotransmitters (3). Because the H4 receptor has been recently discovered in the mammalian central nervous system, its role in the brain remains unclear.

The neural histaminergic system is involved in several behavioral and neurobiological functions, such as arousal, food intake, motor activity, and nociception (1,4). However, the part that histaminergic circuits play in mnemonic effects is complex. Histamine seems to have different effects in distinct brain regions and may have modulatory effects that differ according to memory type. The exact role of this neurotransmitter in learning processes and memory consolidation and the action of the receptor subtypes and how they affect key circuits related to a specific memory system are not well understood (5).

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Previous studies have shown that the histamine-containing fibers project from the tuberomammillary nucleus to the cerebellar cortex and the deep cerebellar nucleus, with a high density of histaminergic terminations in the vermis and flocculus (6,7). A moderately dense network of histamine fibers has been seen in the molecular and granular layers of the cerebellum in several species including humans (1). These fibers run parallel to the Purkinje cell layer after traversing it perpendicularly.

Autoradiographic mapping and *in situ* hybridization experiments demonstrated the presence of H1 and H2 receptors in the rat cerebellar cortex and deep in the cerebellar nuclei (8). These studies suggest that histamine may play an important role in modulating the excitability of cerebellar neurons. The Purkinje cells of the cerebellar cortex and the neurons in the nucleus interpositus all exhibit H2-receptor-mediated excitatory responses when exposed to a histamine bath perfusion (9). Granule cells are excited through the activation of H1 and H2 receptors (10,11).

The cerebellum has traditionally been considered an important motor structure, but several lines of evidence support the role of the cerebellum as more complex than previously thought and includes more than just the regulation of motor responses (12). An increasing number of studies have demonstrated its involvement in cognitive and emotional functions. Functional neuroimaging studies and studies of patients with cerebellar lesions have been conducted to elucidate the role of the cerebellum in the processing of emotion (13-15). Moreover, Ruediger et al. (16) demonstrated that fear conditioning learning is specifically correlated with the growth of feedforward inhibition connectivity in hippocampal and cerebellar circuits.

Experimental evidence indicates that the cerebellum plays a role in emotional learning. The capacity to learn and retain fear-conditioned responses was investigated in *hotfoot* mutant mice. These animals are characterized by a primary deficiency in the synapses made by parallel fibers onto the Purkinje cells. In these mutant mice, the cerebellar dysfunction impairs learning, which suggests that these synapses are involved in fear memory consolidation (17). Studies have related the cerebellar vermis to emotional memory consolidation, since vermis inactivation caused amnesic effects after a fear conditioning task (18). Thus, the participation of the vermis in emotional memory is independent of its role in sensory or motor processes, and the vermis may represent an interface between sensory stimuli, emotional state, and motor responses (12,18).

Histaminergic modulation of learning and memory was studied using lesions and pharmacological interventions in the tuberomammilary nucleus and other decisive brain regions. In our first study (19), microinjection of histamine into the cerebellar vermis demonstrated that the cerebellar histaminergic system is involved in the process of consolidation of emotional memory. These results indicated that there was a dose-dependent inhibition of memory consolidation when histamine was injected into the cerebellar vermis in mice reexposed to the elevated plus maze (EPM). Therefore, in the present study, we investigated the effects of H1 and/or H2 receptor antagonists on emotional memory consolidation.

Material and Methods

Animals

Male Swiss mice (Universidade Federal de São Carlos, Brazil) weighing 25-35 g at the beginning of the experiments were housed in polypropylene cages ($31 \times 20 \times 13$ cm) in groups of five and were maintained under a 12:12-h light-dark cycle (lights on at 7:00 am) in a controlled environment at a temperature of $23 \pm 1°C$ and a humidity level of $50 \pm 5\%$. Food and drinking water were provided *ad libitum*, except during the brief test periods. All mice were experimentally naive, and the experimental sessions were conducted during the light period of the cycle (9:00 am to 1:00 pm).

Drugs

The H1 receptor antagonist chlorpheniramine (CPA) maleate salt and the H2 receptor antagonist ranitidine hydrochloride (RA; Sigma Chemical Co., USA) were prepared using saline as vehicle. Saline (SAL) was used as an experimental control. The doses were based on previous research (20) and on pilot work in our laboratory. The substances were coded, and the experimenter was blinded to the codes when the tests and behavioral analysis were performed.

EPM apparatus

The EPM used was similar to the one originally described by Lister (21). The EPM consisted of two open arms ($30 \times 5 \times 0.25$ cm) and two enclosed arms ($30 \times 5 \times 15$ cm) that were connected to a common central platform (5×5 cm). The apparatus was made of crystal acrylic and was raised 38.5 cm above floor level.

Stereotaxic surgery and drug infusion

Mice were intraperitoneally anesthetized using 100 mg/kg ketamine hydrochloride and 10 mg/kg xylazine solution in association with local anesthesia (3% lidocaine with norepinephrine 1:50,000) and were placed in a Stoelting stereotaxic instrument. A single, 7-mm stainless steel guide cannula (25 gauge) was implanted in the cerebellar vermis according to the following coordinates from the mouse brain atlas (22): 6.5 mm posterior to the bregma, 0 mm lateral to the midline, and 2.0 mm ventral to the skull surface. The guide cannula was fixed to the skull using dental acrylic and jeweler's screws. A dummy cannula (33-gauge stainless steel wire) was inserted into the guide cannula at the time of surgery to reduce the

incidence of occlusion. Postoperative analgesia was provided for 3 days by adding acetaminophen (200 mg/mL) to the drinking water in a ratio of 0.2 mL acetaminophen to 250 mL water (i.e., the final concentration was 0.16 mg/mL).

Saline and drug solutions were infused into the cerebellar vermis using a microinjection unit (33-gauge cannula; Insight Equipamentos Científicos Ltda., Brazil), which extended 2.0 mm beyond the tip of the guide cannula. The microinjection unit was attached to a 5-μL Hamilton microsyringe via polyethylene tubing (PE-10), and the administration was controlled by an infusion pump (Insight Equipamentos Científicos Ltda.) that was programmed to deliver a volume of 0.1 μL over a period of 60 s. The microinjection procedure consisted of gently restraining the animal, inserting the injection unit, infusing the solution, and keeping the injection needle *in situ* for a further 60 s to avoid reflux. Confirmation of successful infusion was obtained by monitoring the movement of a small air bubble inside the PE-10 tubing.

General conditions and data collection

Three days after surgery, the animals were transported to the behavioral space and left undisturbed for at least 1 h before testing, to facilitate adaptation. The test was performed on 2 consecutive days, and the trials in the EPM were denoted Trial 1 and Trial 2. Mice were individually placed on the central platform of the maze facing the open arm and were able to explore the maze for 5 min.

In Trial 1, immediately after exposure to the EPM, the animals received a microinjection of the drugs as follows: in Experiment 1, SAL or 0.016, 0.052, or 0.16 nmol/0.1 μL CPA, and in Experiment 2, SAL or 0.57, 2.85, or 5.7 nmol/0.1 μL RA. Twenty-four hours later (Trial 2), the mice were reexposed to the EPM under the same experimental conditions as in Trial 1, with the exception that they did not receive an injection. Between subjects, the maze was thoroughly cleaned with 5% ethanol and a dry cloth. All tests were conducted under moderate illumination (140 lux) as measured on the central platform of the EPM and in an environment isolated from the rest of the room by a black protective curtain.

All sessions were video recorded with a digital camera that was linked to a computer in an adjacent room. Images were analyzed by a highly trained observer using X-PLO-RAT, which is an ethological analysis software package developed at the Laboratory of Exploratory Behavior, USP, Ribeirão Preto (23). Behavioral parameters were defined in a way that was consistent with previous studies (21,24) and included the following observations: the frequency of open- and enclosed-arm entries (OAE and EAE; where an entry was defined as the entry of all four of an animal's paws into an arm) and the total amount of time spent in the open arms (OAT), enclosed arms (EAT), and central area (CT). These data were used to calculate the percentage of OAE [%OAE = (open entries/open + enclosed entries) × 100], the percentage of OAT [%OAT = (open time/300) × 100], and the percentage of EAT [%EAT = (enclosed time/300) × 100]. The number of stretched-attend postures (SAP; an exploratory posture in which the body stretches forward and then retracts to its original position without any forward locomotion), immobility time (stillness but some movement of the chest), and the frequency of head dipping (HD; exploratory movement of head or shoulders over the sides of the maze) were also scored. Total SAP was considered a primary index of risk assessment, and HD was considered an index of exploratory behavior.

Histology

At the end of testing, all animals received a 0.1-μL infusion of 1% methylene blue according to the microinjection procedure described earlier. The animals received an anesthetic overdose, their brains were removed, and the injection sites were verified histologically according to the atlas of Franklin and Paxinos (22). Data from animals with injection sites outside the cerebellar vermis were excluded from the study. The final sample size of each cohort ranged between 9 and 12. Histology confirmed that a total of 78 mice had accurate cannula placement in the cerebellar dorsal vermis (Figure 1).

Statistical analysis

All results were initially analyzed using the Levene test for homogeneity of variance. Data were analyzed using one-way ANOVA. When differences were indicated by significant *F* values, they were further analyzed using the Duncan multiple range test. A P value of less than 0.05 was considered to be significant.

Ethics

The experiments performed as part of this study were approved by the Animal Ethics Commission of the Universidade Federal de São Carlos (CEEA #049/09) and were in compliance with the norms of the Brazilian Neuroscience and Behavior Society, which are based on the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Results

Experiment 1: effects of the H1 receptor antagonist CPA on memory consolidation

One-way ANOVA showed no significant difference between the groups in Trial 1 for all the measures analyzed (Table 1). Therefore, the data were pooled because the animals had received no pharmacological treatment at that point. Data are summarized in Figure 2A and B, and Table 2. ANOVA showed differences in %OAE between sessions ($F_{4,65}$ = 6.12, P = 0.0003). The *post hoc* Duncan test indicated that all groups entered the open arms less often in Trial 2 in comparison with Trial 1.

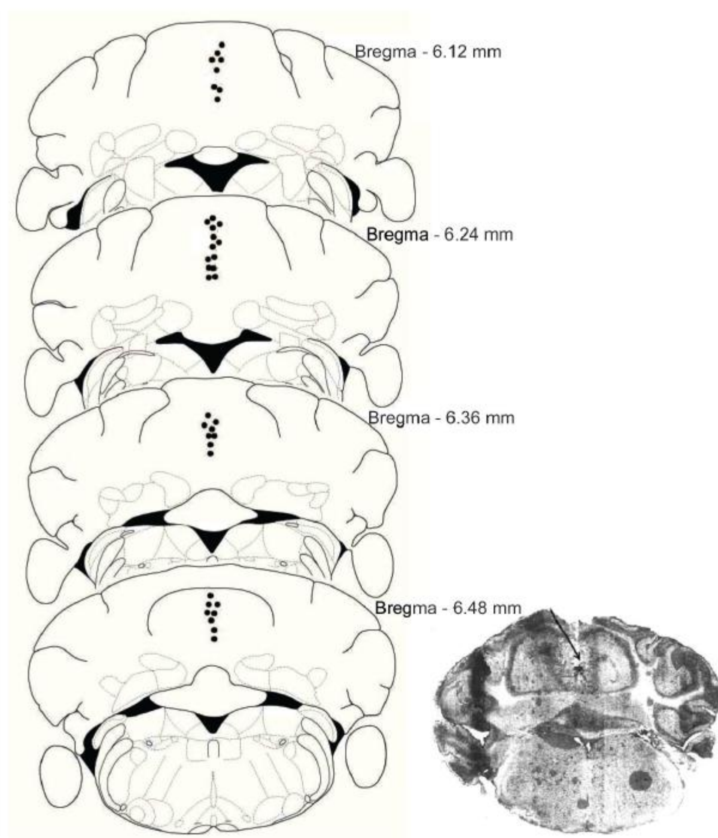

Figure 1. *A*, Schematic representation (adapted from Ref. 22) of sites of microinfusion (filled circles) into the cerebellum of mice. *B*, Photomicrograph showing a typical injection site (indicated by an arrow) in the cerebellar vermis.

Figure 2B shows %OAT for the first and second sessions. ANOVA detected differences in %OAT between sessions ($F_{4,65}$=6.94, P=0.0001), and *post hoc* analysis determined that animals explored the open arms for a shorter time in the second trial when they had been microinjected with SAL or CPA (0.016, 0.052, and

Table 1. One-way ANOVA statistical results for the behavior of mice with no pharmacological treatment in Trial 1.

Behavioral measures	Experiment 1		Experiment 2	
	F	P	*F*	P
OAE	0.93	0.45	1.39	0.26
%OAE	1.55	0.20	0.58	0.63
OAT	0.94	0.44	1.22	0.31
%OAT	1.25	0.30	1.22	0.31
EAE	0.81	0.52	1.20	0.32
EAT	0.33	0.85	0.73	0.54
%EAT	0.33	0.85	0.73	0.54
CT	0.57	0.68	1.03	0.38
SAP	1.19	0.32	0.87	0.46
Head dipping	1.37	0.25	0.58	0.63
Immobility time	0.64	0.60	2.06	0.12

OAE: number of open-arm entries; %OAE: percentage of OAE; OAT: time spent in the open arms; %OAT: percentage of OAT; EAE: number of enclosed-arm entries; EAT: time spent in the enclosed arms; %EAT: percentage of EAT; CT: central platform time; SAP: frequency of stretched-attend postures.

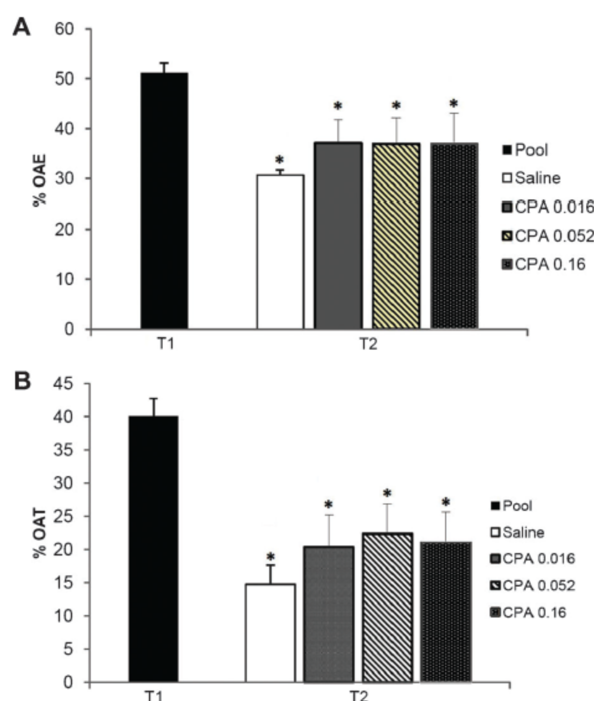

Figure 2. Effects of H1 antagonist chlorpheniramine (CPA; 0.016, 0.052, or 0.16 nmol/0.1 µL) microinjected into the cerebellar vermis on *A*, the percentage of open-arm entries (%OAE) and *B*, the percentage of time spent in the open arms (%OAT) in Trials 1 and 2 in the elevated plus maze. Pool: animals exposed to the elevated plus maze with no pharmacological treatment. Data are reported as means ± SE (n = 9). *P < 0.05 Trial 2 *vs* Trial 1 (Duncan test).

0.16 nmol/0.1 µL). These results demonstrate that CPA did not alter behavioral parameters at the doses used.

Table 2 shows the results for all other behaviors. ANOVA did not indicate differences in EAE ($F_{4,65}$ = 1.08, P = 0.37), which is an EPM index of general exploratory activity. ANOVA revealed significant differences between trials in OAT ($F_{4,65}$ = 6.94, P = 0.0001) and OAE ($F_{4,65}$ = 3.96, P = 0.006). Furthermore, there were differences between sessions for EAT ($F_{4,65}$ = 10.20, P < 0.0001), %EAT ($F_{4,65}$ = 10.20, P < 0.0001), and frequency of HD ($F_{4,65}$ = 6.33, P = 0.0002). ANOVA did not detect any significant differences in immobility time ($F_{4,65}$ = 0.45, P = 0.77), CT ($F_{4,65}$ = 0.33, P = 0.85), and total SAP ($F_{4,65}$ = 1.13, P = 0.34) between trials.

Experiment 2: effects of the H2 receptor antagonist RA on memory consolidation

One-way ANOVA did not determine that there were significant differences between the groups in Trial 1 for any of the measures analyzed (Table 1). ANOVA indicated that there were differences in %OAE ($F_{4,87}$ = 3.76, P = 0.007) between trials. The *post hoc* analysis indicated that differences in %OAE existed in groups that were microinjected with SAL (P = 0.03), and 0.57 and 2.85 nmol/0.1 µL RA (P < 0.05). The animals that received treatment with 5.7 nmol/0.1 µL RA did not have a reduced %OAE (Figure 3A).

Figure 3B shows %OAT for the first and second sessions. ANOVA determined that there were differences in %OAT between sessions ($F_{4,87}$ = 3.38, P = 0.013). The Duncan test indicated that the animals that were microinjected with SAL (P = 0.03) and 2.85 nmol/0.1 µL RA (P = 0.009) exhibited a decreased %OAT in Trial 2 relative to Trial 1, while the groups microinjected with 0.57 and

Table 2. Effects of H1 antagonist chlorpheniramine (CPA) microinjected into the cerebellar vermis on the behavior of mice in Trials 1 and 2 in the elevated plus maze (EPM).

	Trial 1	Trial 2			
	Pool	SAL	CPA 0.016	CPA 0.052	CPA 0.16
OAE	8.9 ± 0.6	4.0 ± 0.6*	6.6 ± 1.4	6.3 ± 1.2	6.3 ± 1.4
OAT	112.6 ± 8.3	44.2 ± 8.7*	61.1 ± 14.5*	67.2 ± 13.9*	63.3 ± 13.6*
EAE	8.3 ± 0.5	9.7 ± 1.3	10.1 ± 1.3	9.9 ± 0.8	10.2 ± 1.2
EAT	94.4 ± 5.7	173.4 ± 12.0*	150.5 ± 17.5*	152.9 ± 16.6*	150.0 ± 19.5*
%EAT	31.5 ± 1.9	57.8 ± 4.0*	50.2 ± 5.8*	51.0 ± 5.5*	50.0 ± 6.5*
CT	93.0 ± 6.4	82.4 ± 10.5	88.4 ± 12.0	80.0 ± 7.3	86.7 ± 11.7
SAP	9.1 ± 0.8	~~1.3~~	4.9 ± 1.1	6.8 ± 1.3	8.6 ± 1.5
Head dipping	10.0 ± 1.3	2.2 ± 1.0*	2.4 ± 0.8*	2.1 ± 1.6*	4.4 ± 1.3*
Immobility time	0.3 ± 0.2	0.0 ± 0.0	0.4 ± 0.4	0.0 ± 0.0	0.0 ± 0.0

Immediately after ~~the~~ exposure to the EPM, animals received microinjection of CPA (0.016, 0.052, or 0.16 nmol/0.1 µL). Pool: animals exposed to EPM with no pharmacological treatment; SAL: saline; OAE: number of open-arm entries; OAT: time spent in the open arms; EAE: number of enclosed-arm entries; EAT: time spent in the enclosed arms; %EAT: percentage of time spent in EAT; CT: central platform time; SAP: frequency of stretched-attend postures. Data are reported as means ± SE in seconds. *P < 0.05 Trial 2 *vs* Trial 1 (Duncan test).

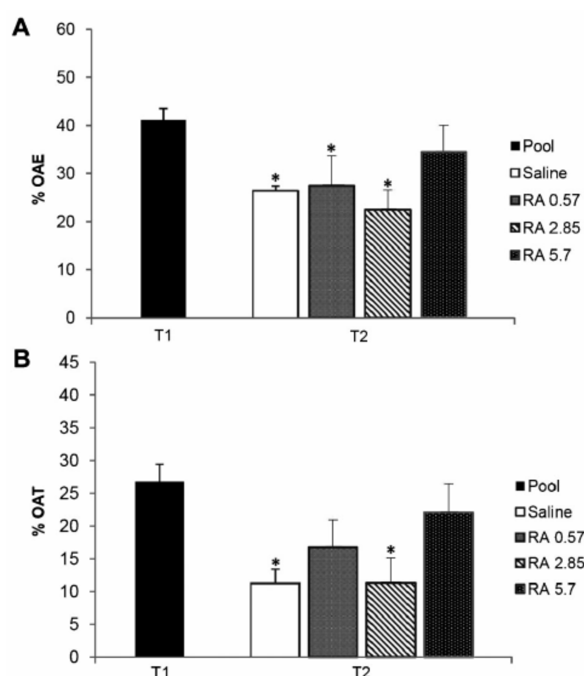

Figure 3. Effects of H2 antagonist ranitidine (RA; 0.57, 2.85, or 5.7 nmol/0.1 µL) microinjected into the cerebellar vermis on *A*, the percentage of open-arm entries (%OAE) and *B*, the percentage of time spent in the open arms (%OAT) in Trials 1 and 2 in the elevated plus maze. Pool: animals exposed to the elevated plus maze with no pharmacological treatment. Data are reported as means ± SE (n=9-12). *P<0.05 Trial 2 *vs* Trial 1 (Duncan test).

5.7 nmol/0.1 µL RA did not reduce %OAT. These results demonstrated that the H2 antagonist RA, at the dose of 5.7 nmol/0.1 µL, impairs memory consolidation in mice reexposed to the EPM.

Table 3 shows the results for all other behaviors. ANOVA revealed significant differences in the OAT between trials ($F_{4,87}=3.38$, P=0.01). *Post hoc* comparisons indicated that differences existed for the groups microinjected with SAL (P=0.03) and 2.85 nmol/0.1 µL RA (P=0.03). Additionally, differences between sessions for OAE ($F_{4,87}=2.99$, P=0.02), EAT ($F_{4,87}=7.59$, P<0.001), %EAT ($F_{4,87}=7.59$, P<0.001), CT ($F_{4,87}=4.60$, P=0.002), and EAE ($F_{4,87}=3.22$, P=0.01) and the frequency of HD ($F_{4,87}=5.14$, P=0.0009) were detected. ANOVA did not detect any significant differences between trials in immobility time ($F_{4,87}=1.30$, P=0.28) or total SAP ($F_{4,87}=1.41$, P=0.23).

Discussion

The primary findings of the present study are that infusion into the cerebellar vermis with the H1 antagonist CPA (0.016, 0.052, and 0.16 nmol/0.1 µL) did not show any behavioral effects, whereas microinjection with the H2 antagonist 5.7 nmol/0.1 µL RA impaired emotional memory consolidation in mice reexposed to the EPM.

In the EPM, the behavior expressed during the test are due to a conflict between motivation to explore the maze and the natural tendency to avoid open spaces (24,25). According to File (26), after the initial exploration of the apparatus, rodents acquire, consolidate, and retrieve some memory related to exploration of potentially dangerous areas of the maze. Several studies show that EPM-experienced animals exhibit a significant decrease

Table 3. Effects of H2 antagonist ranitidine (RA) microinjected into the cerebellar vermis on the behavior of mice in Trials 1 and 2 in the elevated plus maze (EPM).

	Trial 1	Trial 2			
	Pool	SAL	RA 0.57	RA 2.85	RA 5.7
OAE	6.7 ± 0.3	4.5 ± 0.5	4.1 ± 0.6	2.6 ± 0.4*	6.6 ± 0.6
OAT	78.4 ± 1.1	33.7 ± 1.4*	50.1 ± 2.2	33.9 ± 1.9*	66.0 ± 2.0
EAE	9.0 ± 0.3	11.5 ± 0.5*	7.7 ± 0.6	8.4 ± 0.5	10.9 ± 0.5
EAT	118.6 ± 1.0	161.1 ± 1.7*	192.4 ± 2.8*	193.0 ± 2.0*	149.5 ± 2.3
%EAT	39.5 ± 0.6	3.7 ± 1.0*	64.1 ± 1.6*	64.3 ± 1.1*	49.8 ± 1.3
CT	103.1 ± 0.8	105.2 ± 1.6	57.5 ± 1.9*#	73.2 ± 1.7*#	84.5 ± 1.6
SAP	7.9 ± 0.3	5.4 ± 0.5	6.3 ± 0.6	6.0 ± 0.5	9.0 ± 0.7
Head dipping	7.3 ± 0.4	1.6 ± 0.4*	2.1 ± 0.5*	2.0 ± 0.5*	2.6 ± 0.6*
Immobility time	0.1 ± 0.1	0.1 ± 0.2	0.4 ± 0.3	0.0 ± 0.0	0.7 ± 0.4

Immediately after the exposure to the EPM, animals received microinjection RA (0.57, 2.85 or 5.7 nmol/0.1 µL). Pool: animals exposed to EPM with no pharmacological treatment; SAL: saline; OAE: number of open-arm entries; OAT: time spent in the open arms; EAE: number of enclosed arm entries; EAT: time spent in the enclosed arms; %EAT: percentage of EAT; CT: central platform time; SAP: frequency of stretched-attend postures. Data are reported as means ± SE in seconds. *P<0.05 Trial 2 *vs* Trial 1; #P<0.05 *vs* control (SAL; Duncan test).

in %OAE and %OAT during retesting (19,20,25). Therefore, the test/retest protocol in the EPM has demonstrated usefulness to investigate both short- and long-term memory components (27).

Histaminergic projections to the cerebellar vermis, amygdala, and hippocampus can be involved in the modulation of emotional memory consolidation. It has been previously proposed that the amygdala and cerebellum are functionally interconnected during aversive learning (12,15). According to Sacchetti et al. (28), the vermis and amygdala may interact, and the vermal electrical stimulation modulates amygdala activity. These effects are mediated by both direct and indirect anatomical connections between the cerebellum and the limbic areas.

Evidence demonstrates the existence of the histaminergic receptors H1, H2, and H3 in the cerebellum of rodents (8). Generally, H1 and H2 receptors excite or potentiate excitatory impulses, while H3 activation mediates autoinhibition of TMN neurons (6,8).

Evidence regarding the role of the histaminergic system in the learning and memory process is controversial, and the function of the histaminergic receptors and how they affect the memory system is still unclear (5). H1 receptors mediate actions on brain activity, and classic antihistamines such as CPA act as H1 antagonists (1). Several studies have demonstrated the anxiolytic effects of CPA in behavioral tests (1,29). Furthermore, CPA involvement has been proposed in spatial learning and emotional memory processes; however, results in this area are indefinite. For instance, the pharmacological blockade of the H1 receptor with CPA improved spatial learning in the Morris water maze (30), but it conversely impaired spatial learning in the eight-arm radial maze (31). In a recent study, Serafim et al. (20) showed that microinjection of CPA in the amygdala impaired emotional memory performances at a dose of 0.16 nmol/0.1 µL, and suggested that the H1 receptors in the amygdala are not implicated in anxiety-like behaviors but are involved in emotional states induced by the T1/T2 EPM protocol in mice. However, in the present study, CPA microinjected into the cerebellar vermis did not affect behavioral measures in mice reexposed to the EPM.

Some studies have examined the role of H2 receptors on cognitive performance. In the study by Benetti et al. (32), the H2 receptor agonist ampthamine improved fear memory expression, and the authors suggested that activation of postsynaptic H2 receptors within the nucleus basalis magnocellularis by endogenous histamine is involved in the expression of fear responses. Another H2 receptor agonist, dimaprit, improved extinction of aversive memory, while the H2 receptor antagonist, RA, impaired this memory (33,34). Da Silva et al. (35) showed that knockout mice lacking the H1 and H2 receptor subtypes enhanced learning and memory in auditory and contextual fear conditioning tests, but impaired learning of the Barnes maze and short-term memory of an object recognition test. In addition, electrophysiological examination indicated that both knockout mice H1 and H2 showed impaired long-term potentiation in CA1 areas of the hippocampus (35). Recently, Da Silveira et al. (36) showed that the H1 receptor antagonist, pyrilamine, the H2 receptor antagonist, RA, and the H3 receptor agonist, imetit, injected in the CA1 region immediately, 30, 120, or 360 min posttraining, blocked long-term memory retention in a time-dependent manner (30-120 min) without affecting general exploratory behavior, anxiety state, or hippocampal function.

In the present study, animals that received 5.7 nmol/ 0.1 µL RA did not reduce open-arm exploration during retesting on the apparatus, which demonstrates that RA microinjected into the cerebellar vermis impairs emotional memory consolidation in mice reexposed to the EPM. Using a similar protocol, we recently demonstrated that animals microinjected with histamine intra cerebellar vermis did not avoid the open arms less on retesting, and pretreatment with CPA abolished the inhibitory effect of histamine on memory consolidation, while pretreatment with RA did not show any memory effect, which indicated that the H1 receptor is probably involved in histamine-induced emotional memory impairment (37). The present results did not reveal memory effects for the two lower doses of RA and further indicate that H1 mediation seems to be the main mechanism involved in EPM memory impairment. Therefore, the results of the higher dose of RA may be due to a massive blockade of H2 receptors by RA, which induced endogenous histamine to bind only to the H1 receptors reproducing the same kind of effect found with microinfusion of exogenous histamine.

Because emotional memory has an important role in controlling behavior and it is critical for survival, a memory deficit can expose the animal to a needlessly dangerous situation while persistence of an aversive memory is a considerable factor associated with the development of anxiety and fear disorders, including phobias and post-traumatic stress disorder. Therefore, in view of our results, these histamine-modulated effects leading to memory erasure are potentially important and may give clues to new studies regarding the histaminergic system and trauma-related disorders (37).

In conclusion, the results of the present study demonstrate that H2 receptor antagonist ranitidine in the cerebellar vermis impaired emotional memory consolidation in mice reexposed to the EPM.

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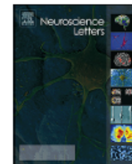

Intra-cerebellar microinjection of histamine enhances memory consolidation of inhibitory avoidance learning in mice via H2 receptors

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HIGHLIGHTS

- Histaminergic fibers are present in the molecular and granular layers of the cerebellum.
- Histamine microinjected into the cerebellar vermis enhanced consolidation of inhibitory avoidance memory.
- Histamine's effect was a non-linear response called an "inverted U-shaped dose effect curve".
- Pretreatment with H2 antagonist was able to prevent this effect.
- H2 receptors mediated the facilitation on memory consolidation induced by histamine.

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ABSTRACT

Studies have demonstrated the relationship between the histaminergic system and the cerebellum, and we intend to investigate the role of the cerebellar histaminergic system on memory consolidation. This study investigated the effect of intra-cerebellar microinjection of histamine on memory retention of inhibitory avoidance in mice, and the role of H1 and H2 receptors in it. The cerebellar vermis of male mice were implanted with guide cannulae, and after three days of recovery, the inhibitory avoidance test was performed. Immediately after a training session, animals received a microinjection of histaminergic drugs: in the experiment 1, saline (SAL) or histamine (HA 0.54, 1.36, 2.72 or 4.07 nmol); experiment 2, SAL or 1.36 nmol HA 5 min after a pretreatment with 0.16 nmol chlorpheniramine (CPA) or SAL; and experiment 3, SAL or 1.36 nmol HA 5 min after a pretreatment with 2.85 nmol ranitidine (RA) or SAL. Twenty-four hours later, a retention test was performed. The data were analyzed using one-way analysis of variance (ANOVA) and Duncan's tests. In experiment 1, animals microinjected with 1.36 nmol HA showed a higher latency to cross to the dark compartment compared to controls and to 2.72 and 4.07 nmol HA groups. In experiment 2, the combined infusions revealed difference between control (SAL + SAL) and SAL + HA and CPA + HA; while in the experiment 3 the analysis indicated differences in retention latency between mice injected with SAL + SAL and SAL + HA. The groups that received the H2 antagonist RA did not show difference compared to control. These results indicate that 1.36 nmol HA enhances memory consolidation of inhibitory avoidance learning in mice and that the pretreatment with H2 antagonist RA was able to prevent this effect.

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1. Introduction

Brain histaminergic neurons are located in the tuberomammillary nucleus (TMN) of the posterior hypothalamus, from where their efferent fibers project to almost the entire brain [24]. This feature is consistent with the histamine function in several physiological functions, including arousal, body temperature, sleep–wake cycle, and cognition [13,21,40]. There is consistent evidence that the neural histaminergic system modulates learning and memory; however, the exact role of histamine in these processes, the action

Abbreviations: AAS, ascending arousal system; CPA, chlorpheniramine; EPM, elevated plus maze; IUSDEC, inverted U-shaped dose effect curve; i.c.v., intracerebroventricular; HA, histamine; RA, ranitidine; TMN, tuberomammillary nucleus.

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of the receptors subtypes and how they affect key circuits related to a specific memory system are not well understood [14,17].

The cerebellum is considered as an important motor structure, but several lines of evidence support the assertion that the role of the cerebellum includes more than the regulation of motor responses [30,37,39]. In humans, cerebellar areas around the vermis are activated during learning and mental recall of emotional personal episodes [5,28,34]. Lesions of the cerebellar vermis may affect retention of a fear memory without altering baseline motor/autonomic responses to the frightening stimuli in both human and animal models [8,30]. Other findings indicate that cerebellar vermis is selectively involved in long-term memory formation in certain types of defense behavior after training [35].

Studies have demonstrated the relationship between the histaminergic system and the cerebellum [11,23,38]. Histamine-immunoreactive fibers are seen in the molecular and granular layers of the cerebellum in several species including humans [14,23,24]. Autoradiographic mapping and in situ hybridization experiments have demonstrated the presence of H1, H2 and H3 receptors in the rat cerebellar cortex and deep cerebellar nuclei [1,27], and suggest that histamine may play an important role in modulating the excitability of cerebellar neurons. We recently showed that histamine microinjected into the cerebellar vermis impaired emotional memory consolidation in the elevated plus-maze (EPM), and that this effect was mediated by H1 receptor [10,11]. Therefore, in the present study, we investigated the effect of histamine microinjected into the cerebellar vermis on memory retention of inhibitory avoidance in mice, and the role of H1 and H2 receptors in it.

2. Material and methods

2.1. Animals

Male Swiss mice (Federal University of Sao Carlos, UFSCar, SP, Brazil) weighing 25–35 g were maintained under a 12 h light cycle (lights on at 7:00 a.m.), in a controlled environment at a temperature of $23 \pm 1\,^{\circ}$C and humidity of $50 \pm 5\%$. All mice were experimentally naive, and the experimental sessions were conducted during the light period of the cycle (9:00–15:00 h).

2.2. Drugs

Histamine dihydrochloride, the H1 receptor antagonist chlorpheniramine maleate salt and the H2 receptor antagonist ranitidine hydrochloride (Sigma Chemical Co., USA) were prepared in a vehicle of physiological saline [10]. Saline solution was used as an experimental control.

2.3. Surgery and microinjection

Mice were intraperitoneally anesthetized using ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg) solution, associated with local anesthesia (3% lidocaine with norepinephrine 1:50,000), and placed in a Stoelting stereotaxic instrument. A single 7 mm stainless steel guide cannula (25 gauge) was implanted in the cerebellar vermis following the coordinates from the mouse brain atlas [26]: 6.5 mm posterior to bregma, 0 mm lateral to the midline, and 2.0 mm ventral to skull surface. The guide cannula was fixed to the skull using dental acrylic and jeweler's screws. A dummy cannula (33 gauge stainless steel wire) was inserted into the guide cannula to reduce the incidence of occlusion. Postoperative analgesia was provided for 3 days by adding acetaminophen (200 mg/ml) to the drinking water in a ratio of 0.2 ml acetaminophen to 250 ml water (i.e., the final concentration was 0.16 mg/ml).

Saline and drug solutions were infused into the cerebellar vermis using a microinjection unit (33 gauge cannula), which extended 2.0 mm beyond the tip of the guide cannula. The microinjection unit was attached to a 5 μl Hamilton microsyringe via polyethylene tubing, and the administration was controlled by an infusion pump programmed to deliver a volume of 0.1 μl over 60 s [11].

2.4. Inhibitory avoidance task

Rodents have an aversion to brightly illuminated areas, and preference for the dark environments, which represent secure areas [15]. In the inhibitory avoidance task, during the acquisition trial, an instinctive response is punished by a foot shock in the dark compartment, and in the retention trial, animal is returned to the area and avoidance of the punished context is observed.

The apparatus consisted of an acrylic box ($48 \times 24.5 \times 25$ cm) with two compartments of the same size, one light (under illumination 400 lx) and one dark (with black acrylic), separated by a guillotine door (9×10 cm). The floor was made of stainless-steel rods (2.5 mm in diameter) spaced 1 cm apart that delivered electric shocks at an intensity of 0.5 mA for 3 s. The box was connected to a computer containing software (Insight Equipamentos Científicos Ltda, Brazil) that triggered the functions of the apparatus, and a camera recorded the experiment.

Animals were placed in the apparatus after 1 h of habituation in the experimental room. Each animal was gently placed in the light compartment for 5 s, after which the guillotine door was lifted, and the latency of the animal crossing to the dark (shock) compartment was timed. Animals that waited more than 100 s to cross to the other side were excluded ($n = 2$). Once the animal crossed to the next compartment with all four paws, the door was closed and the mouse was taken to its home cage. The habituation trial was repeated after 30 min and was followed after the same interval by the acquisition trial during which the guillotine door was closed and a foot shock (0.5 mA, 3 s) was delivered immediately after the animal had entered the dark compartment. After 20 s, the mouse was removed from the apparatus and placed in the home cage. Two min later, the animal was retested in the same way as before; if the mouse did not enter the dark compartment of the period of 120 s, a successful acquisition of inhibitory avoidance response was recorded. Otherwise, when the mouse entered the dark compartment a second time, the door was closed and it received the same shock as above. After retesting, if the animal acquired inhibitory avoidance successfully, it was removed from the apparatus and injected via the guide cannula, as the following: experiment 1, saline (SAL) or histamine (HA 0.54, 1.36, 2.72 or 4.07 nmol); experiment 2, saline (SAL) or 1.36 nmol histamine (HA) 5 min after a pretreatment with 0.16 nmol chlorpheniramine (CPA) or SAL; and experiment 3, SAL or 1.36 nmol HA 5 min after a pretreatment with 2.85 nmol ranitidine (RA) or SAL. Four animals that did not acquire the inhibitory avoidance successfully were excluded.

Twenty-four hours after training, a retention test was performed to determine memory consolidation. Each animal was placed in the light compartment for 5 s, the door was opened, and the latency was measured for entering into the dark compartment. The test session ended when the animal entered the dark compartment or remained in the light compartment for 300 s.

2.5. Histology

At the end of testing, all animals received a 0.1 μl infusion of 1% methylene blue according to the microinjection procedure described above. The animals received an anesthetic overdose, their brains were removed and injection sites were verified histologically [26]. Data from animals with injection sites outside the cerebellar

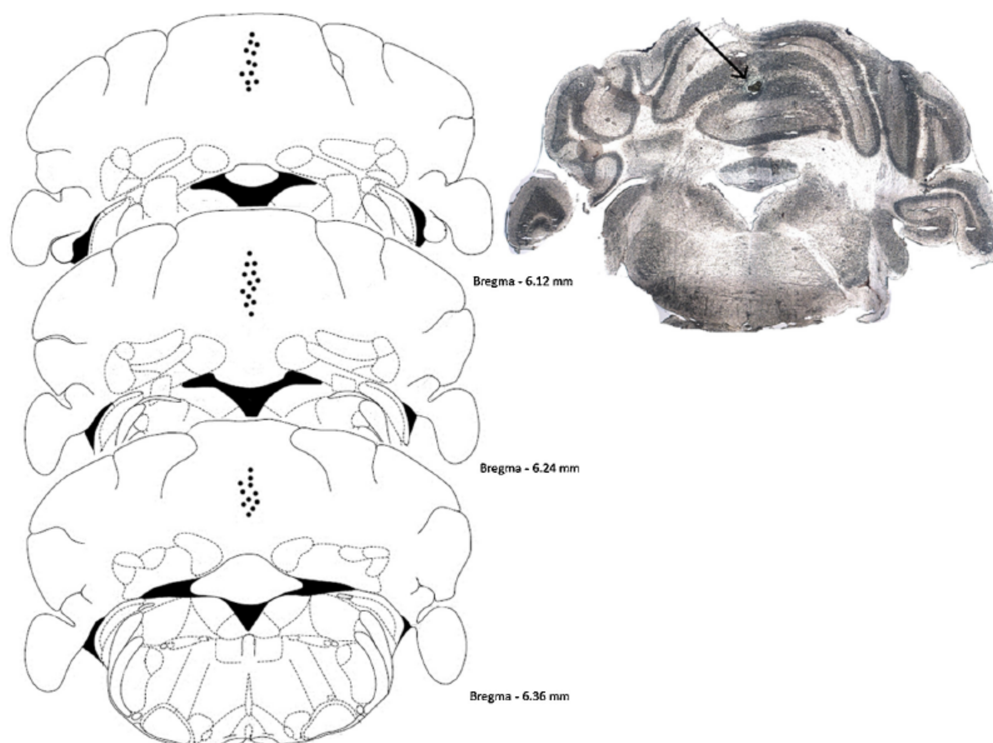

Fig. 1. (A) Schematic representation (adapted from Paxinos and Franklin [25]) of sites of microinfusion (filled circles) into the cerebella of mice. (B) Photomicrograph showing a typical injection site (indicated by an arrow) in the cerebellar vermis.

vermis were excluded. The final sample size of each cohort ranged between 7 and 13. Histology confirmed that a total of 125 mice had accurate cannula placements mainly in the anterior and central vermis in the lobules V and VI (Fig. 1).

2.6. Statistical analysis

All results were initially submitted to Levene's test for homogeneity of variance. The data were analyzed using a one-way analysis of variance (ANOVA) test. When differences were indicated by significant *F* values, they were identified by Duncan's multiple range tests. A *p* value of <0.05 was required for significance.

3. Results

3.1. Experiment 1: effects of histamine on memory retention of inhibitory avoidance task

One-way ANOVA indicated a significant difference among results obtained with histamine (0.54, 1.36, 2.72 and 4.07 nmol/0.1 μl) and with saline (control group) ($F_{4,46} = 3.96$, $p = 0.009$). Post hoc analysis showed a significant increase of retention latency for animals that received 1.36 nmol histamine in relation to the control group ($p < 0.05$). In addition, there were differences between groups microinjected with 1.36 nmol histamine and with 2.72 nmol ($p < 0.01$) and 4.07 nmol ($p = 0.04$) histamine. These two higher doses showed a decrease of latency time in relation to 1.36 nmol histamine (Fig. 2).

3.2. Experiment 2: effects of pretreatment with the H_1 receptor antagonist CPA on HA-facilitation on memory retention

ANOVA indicated difference among groups ($F_{3,39} = 3.17$, $p = 0.03$), and Duncan's test revealed a significant increase of retention latency for animals that received CPA + HA ($p = 0.04$) and SAL + HA ($p = 0.02$) in relation to the control group SAL + SAL. Also, there was no difference between control and CPA + SAL ($p = 0.45$) (Fig. 3). These results show that CPA did not affect memory consolidation and it was not able to abolish the histamine effect.

3.3. Experiment 3: pretreatment with the H_2 receptor antagonist RA prevents the HA-facilitation on memory retention

The analysis showed significant difference among groups ($F_{3,27} = 3.21$, $p = 0.03$), and between groups injected with SAL + HA and with SAL + SAL ($p < 0.01$). There was no difference between SAL + SAL and RA + SAL and RA + HA ($p = 0.26$; $p = 0.19$) (Fig. 4). These results show that RA did not affect memory consolidation and it did prevent the effect of histamine on memory retention.

4. Discussion

The main findings of the present study are that intra-cerebellar injection of 1.36 nmol histamine facilitates memory consolidation of inhibitory avoidance task (confirmed in the three experiments performed), that suggests a different role of histamine in a memory model that uses punishment; the pretreatment with H2 antagonist RA was able to abolish this effect, while the combined microinfusion with H1 antagonist CPA fails to prevent the histamine effect.

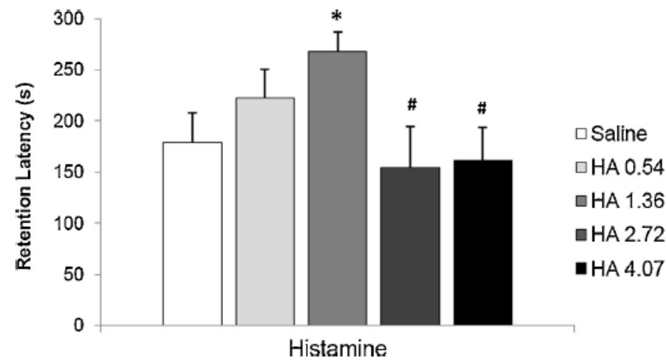

Fig. 2. Effects of histamine (0.54, 1.36, 2.72 and 4.07 nmol/0.1 μl) microinjected into the cerebellar vermis on memory retention of an inhibitory avoidance task. n = 9–11. Data are presented as mean ± SEM. ANOVA, Duncan's test. *Significant difference from saline control group, $p < 0.05$; #Significant difference from 1.36 nmol histamine, $p < 0.05$.

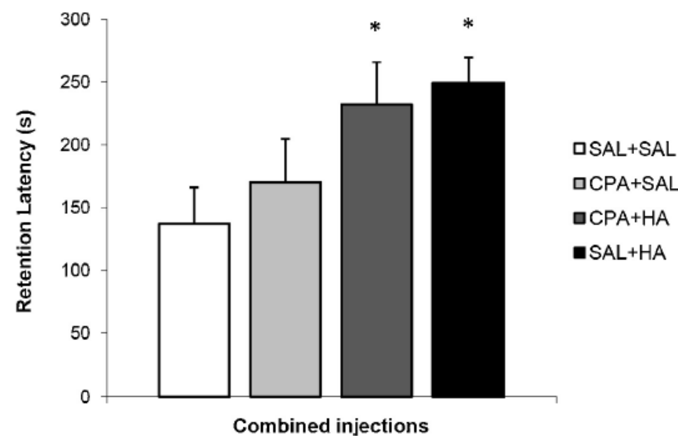

Fig. 3. Effects of combined injection of H1 antagonist CPA (0.16 nmol) and histamine (1.36 nmol) on memory retention of an inhibitory avoidance task. n = 10–13. Data are presented as mean ± SEM. ANOVA, Duncan's test. *Significant difference from saline control group, $p < 0.05$.

Our results of histamine microinjection showed a non-linear response called an "inverted U-shaped dose effect curve" (IUSDEC), which is frequently reported when studying the actions of pharmacological treatments on cognitive functions such as memory. The effects of increasing dosages of a given compound appear to increase up to a maximum and then the effects decrease [2]. Most interpretations of the IUSDEC rely on the effects of emotional arousal modifications [2]. According to McGaugh [20],

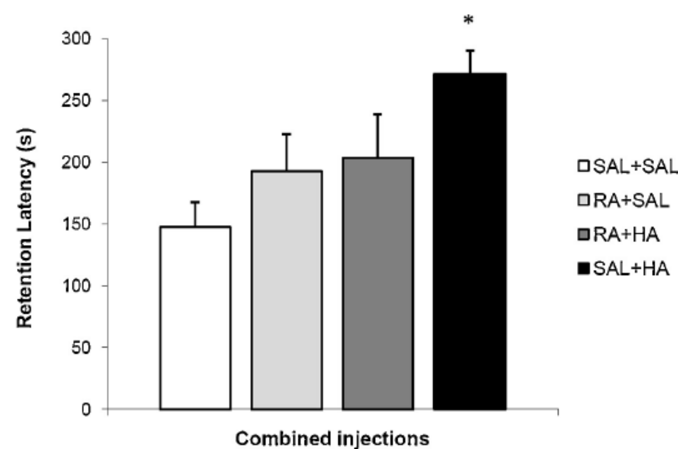

Fig. 4. Effects of combined injection of H2 antagonist RA (2.85 nmol) and histamine (1.36 nmol) on memory retention of an inhibitory avoidance task. n = 7–9. Data are presented as mean ± SEM. ANOVA, Duncan's test. *Significant difference from saline control group, $p < 0.05$.

arousal has been invoked as an essential endogenous modulator of memory processes. The "arousal hypothesis" of IUSDEC is based on the finding that the arousal state can interact with exogenously supplied mnemoactive or presumptive mnemoactive agents to alter their effectiveness at any given dosage.

IUSDEC has been reported many times following post-training administration, and the dose-response effects measured in these experimental conditions can be closely related to specific actions on mnemonic processing. In this study, the possibility of alteration in motor or sensory functions is unlikely; since histamine half-life is about 30 min in basal conditions [32], and in our previous study histamine did not affect locomotor activity in the EPM [10,11], we suggest that the effects of post-training microinjection of histamine are related to mnemonic processing.

According to Steininger et al. [33], histamine neurons located in the TMN and surrounding regions fire tonically during waking. The histaminergic system is closely related to the regulation of the sleep–wake cycle and the control of arousal [16,18,25,36]. The depletion of neuronal histamine induces an increase in the amount of slow-wave sleep and a decrease in wakefulness [16], and the facilitation of neuronal histamine release by the H3 receptor antagonist thioperamide enhances wakefulness [18]. In our study the increase of histamine levels may stimulate memory retention in view of the fact that neuronal histamine is involved in arousal control.

Another possible mechanism to be explored is that the microinjection of histamine enhanced its levels in the synaptic cleft and increased synaptic availability of histamine and its binding to H3 receptors. The activation of H3 receptor would be responsible to inhibit the synthesis and release of endogenous histamine, which altered its effectiveness at the higher dosages.

According to Baldi and Bucherelli [2], IUSDEC has been reported for compounds that exhibit opposite effects on learning and memory. Several studies demonstrated contradictory effects of the histaminergic system on learning and memory processes. Some reports demonstrate a facilitatory effect of histamine on learning and memory in rats and mice. Prast et al. [29] reported that an intracerebroventricular (i.c.v.) injection of histamine facilitated short-term memory in a social memory test. In a study by DeAlmeida and Izquierdo [7], post-training i.c.v. administration of histamine facilitated the retention test performance of step-down inhibitory avoidance behavior in rats. In a recent study by Bonini et al. [4], histamine administered in the hippocampus after non-reinforced retrieval facilitated memory extinction in a dose-dependent manner.

In contrast, some studies showed an inhibitory effect of histamine on mnemonic processes. Nishiga et al. [22] showed that rats on a histidine-deficient diet exhibited a reduced hippocampal histamine content and an improved eight-arm radial maze performance. Serafim et al. [31] demonstrated that systemic injections of L-histidine induced a state-dependent memory retrieval deficit in mice re-exposed to the EPM, and we recently found that intra-cerebellar histamine impaired emotional memory consolidation in a dose-dependent manner in the EPM, by H1 receptor activation [10].

These discrepancies seem to be related to the methods used, the approaches selected in the experiments, and, primarily, the different tasks, which are dependent on the functioning of different brain areas. For instance, according to McNaughton and Corr [19], there is a functional distinction that makes fear more likely to be engaged with more immediate threats and anxiety more likely to be engaged under conditions of distant or anticipated threat. These two functions are executed by two parallel neural systems, one controlling fear and one anxiety: at the lower levels, fear has a greater neural representation (periaqueductal gray, medial hypothalamus and amygdala) and at the higher levels, anxiety has a greater neural representation (amygdala, septo-hippocampal system, posterior cingulate and prefrontal cortex) [19].

We suggest that cerebellum may integrate these two neural systems, with interconnection between the cerebellar vermis, the amygdala and hippocampus playing a role in emotional memory related to anxiety, whilst the connections between cerebellum, periaqueductal gray, hypothalamus and amygdala related to fear. Therefore, the high levels of histamine in the cerebellar vermis produced different results at the same dose, but in models that assess different functions, and the cerebellar histaminergic system is involved in the process of memory consolidation differently when the emotional memory has an anxiety component (EPM) [11] or when it has a fear component (inhibitory avoidance) [41]. We propose that in a learning task that involves fear, the microinjection of histamine into the cerebellar vermis can stimulate the periaqueductal gray, the amygdala level and increase arousal, which improves memory retention.

Our data agree with reports showing the histamine enhances fear memory consolidation through a mechanism that involves activation of H2 receptors. According to Da Silva et al. [6] when infused into the CA1 region of the dorsal hippocampus, histamine improved memory retention of inhibitory avoidance in a dose-dependent manner, and the effect was blocked by ranitidine and mimicked by dimaprit (H2 agonist). Giovaninni et al. [12] showed that the stereotaxically localized micro-infusion of H2 and H3 agonist in the CA3 region of the hippocampus improves retention of the memory associated with a contextual fear conditioning procedure. In study of Flood et al. [9], infusion of dimaprit intra-septum facilitated long term retention in avoidance learning task. Since the brain H2 receptor-mediated responses occur through stimulation of adenylyl cyclase, increase in cAMP intracellular levels and PKA activation [3,6], a likely candidate is the cAMP/PKA pathway. However, further experiments are necessary to understand the biochemical consequences of cerebellar H2 receptors activation during memory consolidation.

In conclusion, these results indicate that histamine microinjected into the cerebellar vermis facilitates the memory retention of inhibitory avoidance in mice, non-linearly by H2 receptor activation, which suggests a different role for histamine in a memory model that uses punishment.

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Developing a cognitive bias mouse model using touchscreens

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Introduction

Cognitive Bias

Emotions arise in important situations to the organism, in the sense that they may influence survival and reproductive success. The primary function of emotions in these contexts is widely hypothesized to be to guide the animal's behaviour in order to achieve survival goals - the attainment of valuable resources/rewards, and the avoidance of harm/punishment - perhaps by providing a 'common currency' that the animal uses to determine which behaviour or sequence of behaviours is most likely to enhance survival (Medl et al., 2009, Cardinal et al., 2002).

Emotion can influence cognitive processes, particularly in situations requiring decision making under uncertainty which reveal the close interplay between cognitive and emotional processes (Enkel et al., 2010). According to Mendi et al. (2009), alongside subjective, behavioural and physiological components, it has been recognized in human psychology that cognitive processes influence and are influenced by emotional state, and the use of the term 'cognitive' broadly refers to information processing including attention, learning, memory and decision-making (Shettleworth, 1998). Studies of human psychology provide the main source of information about the effects of emotional states on cognitive function (Mendi et al., 2009). Recent research is increasingly seeking parallels between humans and animals in studies of emotion and cognition (Henry et al., 2007, Desiré et al., 2002).

Human studies have found that the valence of an individual's emotional state appears to influence a number of cognitive processes including attention, memory, and judgment (Mendi et al., 2009). Moreover, information processing by humans can be

biased by their emotions. There is evidence that affective state influences memory retrieval, with happier people being more likely to recall positive memories and unhappy or depressed people more likely to recall negative ones (Denny and Hunt, 1992; Mineka et al., 1998). Furthermore, people in a negative state are more likely to make negative judgments about future events or ambiguous stimuli ('pessimism') than people in positive states who show more optimistic judgments and interpretations (MacLeod and Byrne, 1996; Nygren et al., 1996).

Negative feelings are reliably correlated with pessimistic cognitive biases, defined as the increased expectation of bad outcomes (Bateson et al., 2011). This bias, reflecting selective imposition of negative meanings on ambiguity, has proven characteristic of clinical and subclinical anxiety dysfunction (MacLeod and Mathews, 2012). It is also particularly evident in depression, in which a common characteristic is a negatively distorted interpretation of ambiguous information (Lawson et al, 2002; Chan et al, 2008; Dearing and Gotlib, 2009). The cognitive theory of depression emphasizes this negative cognitive bias toward pessimistic judgments as a key factor leading to the pathological condition (Beck, 2008). Understanding of the neurobiological mechanisms of this bias could thus provide a new approach for therapeutic intervention.

Neurophysiological work in monkeys and functional neuroimaging in humans implicate the amygdala, cingulate cortex, insula, and several prefrontal regions in decision making under ambiguity and risk (Ernst and Paulus, 2005; Bach et al, 2009; Sugrue et al, 2005; Opris and Bruce, 2005; Chaudhry et al, 2009); however, the neurobiological mechanisms underlying the negative cognitive bias are poorly understood. Recent findings suggest an amygdala activation bias to negative stimuli, using noradrenergic-glucocorticoid challenges to pharmacologically mimic

neurobiological stress conditions (Kukolja et al, 2008), which underscores the potential of stress to negatively bias neural function.

The use of cognitive bias as a measure of negative emotional states across species suggests that animals could be regarded as exhibiting emotions (Bateson et al., 2011). Animal studies have demonstrated that negative states can induce a negative interpretation of ambiguous information (Brilot et al., 2010) that can be defined as the tendency to interpret ambiguous stimuli as though they were threats. Measuring corresponding patterns of behavior with similar methods could bridge the gap between the psychological phenomenon in humans and its underlying biological basis in animal models.

According to Brilot et al. (2010), a subject is regarded as showing a pessimistic judgment bias if it demonstrates a higher probability of exhibiting the response appropriate to the CS- stimulus than the same subject in a more positive affective state or when compared to a control subject in a more positive state. Cognitive biases are observed in clinical populations where anxious individuals adopt a more pessimistic interpretation of ambiguous stimuli and depressed individuals adopt both a more pessimistic interpretation of ambiguous aversive stimuli and a less optimistic interpretation of ambiguous appetitive stimuli. In the study of Hymel and Sufka (2012), the authors showed that the cognitive biases of more pessimism and less optimism can be reversed in the depression- like phase by imipramine using chicks.

The touchscreen cognitive testing method

It is well understood that the use of animal models is an essential component in the quest to understand the brain mechanisms of cognition. In addition, such models are necessary to test potential therapeutic agents in a direct and expedient manner that is not possible in human subjects (Bussey et al. 2008). Creating more translatable behavioral tasks for testing rodents might provide results that are more interpretable, yielding more useful information regarding which treatments might aid human patients (Bartko et al. 2010).

Bussey et al. (1994, 1997) developed the touchscreen method for rats, and more recently for mice (Bussey et al. 2001), creating a cognitive testing method for rodents resembling as close as possible those used in humans. This computer-automated behavioral testing method allows computer-graphic stimuli to be presented to a rodent so that it may respond to the computer screen via a nose-poke directly to the stimulus. The method has been used in several behavioral, lesion and pharmacological studies (Bussey et al. 2008).

In view of these findings, the aim of this study is to develop a more complex behavioural assay of depression and anxiety than those typically employed with a method to measure and potentially modulate cognitive bias in mice using touchscreens.

Methods

All procedures were approved by the Animal Care Committee at the Hospital of Sick Children. Hybrid C57 B/6 x 129 SVEV mice were bred in the colony at The Hospital for Sick Children and maintained on a 12 hr light/dark cycle (lights on at 07:00 hrs) and housed 3-5 animals per cage. Adult male mice aged 8 weeks at the start of the experiment were used for all experimental groups. During an initial acclimation period, all mice were provided food and water *ad libitum*. One week prior to the start of the experiment, all mice were handled 2min/day by the experimenter.

Apparatus

Testing was conducted in a touchscreen-based automated operant system for mice (Bussey-Saksida Touch Systems, Lafayette Instrument, Lafayette Indiana). The apparatus consisted of a standard modular testing chamber housed within a sound- and light-attenuating box (40×34×42 cm; Med Associates Inc., St. Albans, Vermont). The inner operant chamber consisted of a metal frame, clear Perspex walls, and a stainless steel grid floor. The box was fitted with a fan (for ventilation and masking of extraneous noise) and a pellet receptacle (magazine), which was illuminated by a 3-W light bulb and fitted with a photocell head entry detector attached to a peristaltic pump-based liquid-reward dispenser (situated outside of the box). A 3-W houselight and tone generator (Med Associates) were fitted to the back wall of the chamber. Behavioral testing programs were controlled with a computer software program (Bussey-Saksida Touch Systems, Lafayette Instrument, Lafayette Indiana).

At the end of the box opposite the magazine was a flat-screen monitor equipped with an infrared "touchscreen" (16 cm high and 21.20 cm wide; Craft Data Limited, Chesham, UK) mediated by ELO touchscreen software (ELO Touchsystems Inc.). Since the "touchscreen" uses infrared photocells, the mouse was not required to exert any pressure on the monitor screen in order for a nose-poke to be detected. Furthermore, black Perspex "masks" with response windows were placed over the screen, through which the mouse could make a nose-poke toward the screen. The mask (h 11.80 cm × w 22.8 cm) had three response windows (h 5.80 cm × w 5.0 cm) which were positioned 0.8 cm apart from one another and located 3.0 cm from the sides of the mask.

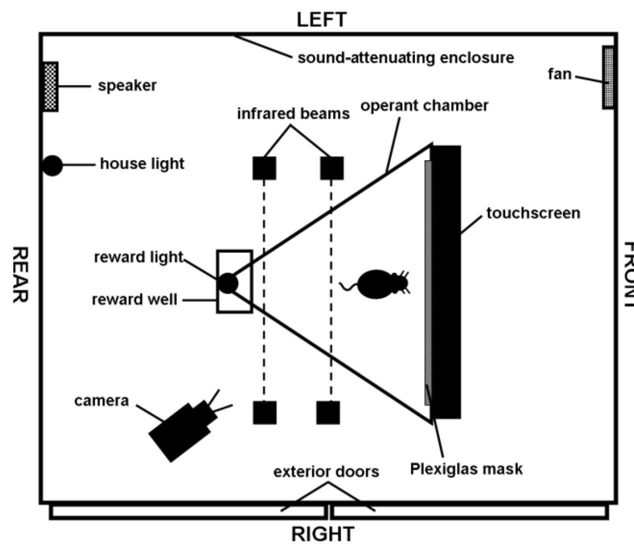

Figure 1: Schematic representation of the aparattus

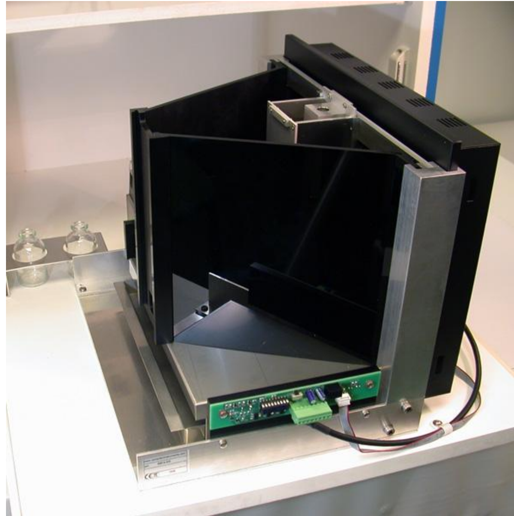

Figure 2: Touchscreen Box

Pretraining Procedure

Starting five days before the beginning of pretraining, mice were put on a mild food-restriction protocol during which each mouse was fed 3-4g of mouse chow per day. Under this protocol, all mice remained above 90% of their free-feeding weight. .

Mice received two 15 minute sessions, one per day, of chamber habituation during which the screen was blank and the three hole mask was in place. After that, they received three-five 15 minute sessions, one per day, of liquid reward training during which a delivery of 7 microliters of strawberry milkshake (Neilson diary) occurred according to a 10 sec fixed-interval schedule of reinforcement with an initial delivery of 150 microliters of strawberry milkshake. During liquid reward training, the screen was blank and the three hole mask was in place, and presentation of a 3Khz tone of 1 s duration coincided with illumination of the reward light.

Mice then received touchscreen training during which achromatic visual stimuli were presented one at time in one of the three locations of the three hole mask. Mice received 30-trial sessions during which the location of the presented stimulus was pseudorandomized and remained on screen until a touch was made. Responses to a blank location had no effect. The parameters of these sessions included a 3KHz tone of 1 s duration which coincided with illumination of the reward light upon touching a location containing a stimulus, no prime, and 7 microliter strawberry milkshake delivery for each correct response. The stimuli used were from a bank of 40 stimuli created by the Bussey-Saksida Touch Systems. To aide learning, a small amount of confectioner's sugar moistened with water was applied to the touchscreens (in the center of the three response windows) starting on session 1 and lasting until consistent responding occurred. These pretraining sessions continued until all mice reached a criterion of 30 trials within 30 minutes two times.

Size Visuomotor Conditional Learning (VMCL) Training

During VMCL size training, mice received 40-trial sessions with a maximum of 30 min (when correction trials were in place) with the three hole mask in place. An initial delivery of 150 microliters of strawberry milkshake occurred at the start of the session which was followed by the presentation of a sample stimulus in the centre location which remained on the screen until a touch was made to it. Following a touch to the sample stimulus, two flanking dummy choices stimuli (black letter S) appeared, one to the right and the other to the left of the centre sample stimulus. On S+ trials, the sample stimulus was a large white square (5.4 cm x 5.4 cm), while on S- trials, the sample stimulus was a small white square (1.62 x 1.62 cm). On S+ trials, mice had to touch the choice stimulus located to the right of the sample stimulus to receive a large

reward of milkshake (10 microliters). On S- trials, mice had to touch the choice stimulus located to the left of the sample stimulus to receive a small reward of milkshake (1.5 microliters). As soon as the correct choice stimulus was touched, all three stimuli (sample and 2 choice stimuli) disappeared and milkshake reward delivery occurred along with a 3Khz tone of 1 sec duration. If an incorrect response was made, all three stimuli (sample and 2 choice stimuli) disappeared and the houselight was illuminated for the duration of a 5 sec timeout period. During all sessions, an intertrial interval of 5 sec was employed and half of the trials were S+ trials and the remaining half S- trials pseudorandomized such that no more than three trials of the same type occurred consecutively. Behavioural measures of performance consisted of S+ and S- trial accuracy, correct touch latency (time to touch a choice stimulus), and reward latency (time to collect strawberry milkshake reward). Criterion performance consisted of 85% accuracy on both S+ and S- trials two times. Once mice achieved criterion performance with the aid of correction trials, they graduated to sessions without correction trials until criterion was met another two times. When in place, a correction trial occurred when an incorrect response was made consisted of presentation of the exact same trial until a correct response occurred. Performance on correction trials did not count towards accuracy, correct touch latency, or reward latency.

Shape Visuomotor Conditional Learning (VMCL) Training

During VMCL Shape training, mice received 40-trial sessions with a maximum of 30 min (when correction trials were in place) with the three hole mask in place. An initial delivery of 150 microliters of strawberry milkshake occurred at the start of the session which was followed by the presentation of a sample stimulus in the centre location which remained on the screen until a touch was made to it. Following a touch

to the sample stimulus, two flanking dummy choices stimuli (black letter S) appeared, one to the right and the other to the left of the centre sample stimulus. On S+ trials, the sample stimulus was a white triangle (3 cm X 3 cm;), while on S- trials, the sample stimulus was a white rectangle (3 cm X 3 cm). On S+ trials, mice had to touch the choice stimulus located to the right of the sample stimulus to receive a large reward of milkshake (10 microliters). On S- trials, mice had to touch the choice stimulus located to the left of the sample stimulus to receive a small reward of milkshake (1.5 microliters). As soon as the correct choice stimulus was touched, all three stimuli (sample and 2 choice stimuli) disappeared and milkshake reward delivery occurred along with a 3Khz tone of 1 sec duration. If an incorrect response was made, all three stimuli (sample and 2 choice stimuli) disappeared and the houselight was illuminated for the duration of a 5 sec timeout period. During all sessions, an intertrial interval of 5 sec was employed and half of the trials were S+ trials and the remaining half S- trials pseudorandomized such that no more than three trials of the same type occurred consecutively.

Behavioural measures for VMCL task performance consisted of S+ and S- Trial percentage accuracy, correct choice touch latency (latency to touch the correct flanking Choice Stimulus from the time of presentation on the touchscreen), and reward latency (latency of reward well head entries following illumination of the reward well light). VMCL training continued until all mice within a cage reached a performance criterion of 85% accuracy on both S+ and S- trials on two non-consecutive training sessions with the aid of correction trials. When in place, a correction trial occurred when an incorrect Choice Stimulus response was made and consisted of presentation of the exact same trial until a correct response occurred. Performance on correction trials did not count towards behavioral performance measures. Once all mice within a cage achieved the

above mentioned performance criterion, they graduated to VMCL training sessions without the aid of correction trials and training continued until all mice within a cage reached a performance criterion of 85% accuracy on both S+ and S- trials on two non-consecutive training sessions.

Probe test

Restraint stress

At probe, mice were separated into two groups, control and stress, equating for the number of times criterion was achieved during VMCL training, to prevent heterogeneity between groups. The stress group was submitted to 30 min of restraint and then immediately received probe testing, while the control group received probe testing without any manipulation (Probe 1). The Size probe test consisted of 11 pseudorandomized trials during which 5 un-rewarded presentations of an intermediate sized square (45, 55, 65, 75, or 85% of the size of the S+ stimulus) occurred as the sample stimulus along with 3 regular rewarded S+ trials and 3 regular rewarded S- trials. Thus, all five intermediate sized squares were presented once during probe sessions while a regular S+ or S- trial occurred prior to the first un-rewarded trial as well as in between each un-rewarded trial in a pseudorandomized order. The same procedure was repeated 24 hours later (Probe 2).The Shape probe test also consisted of 11 pseudorandomized trials during which 5 un-rewarded presentations of an ambiguous white arrow stimulus (3 cm X 3 cm that was constructed by merging the S+ triangle stimulus with the S- rectangle stimulus) occurred as the sample stimulus along with 3 regular rewarded S+ trials and 3 regular rewarded S- trials. A regular S+ or S- trial occurred prior to the first un-rewarded trial as well as in between each un-rewarded trial

in a pseudorandomized order. The same procedure was repeated 24 hours later (Probe 2). Behavioural measures for the six rewarded VMCL trials of the ambiguous probe test session consisted of S+ and S- Trial Accuracy, Correct Choice Touch Latency (latency to touch the correct flanking Choice Stimulus from the time of presentation on the touchscreen), and Reward Latency (latency of reward well head entries following illumination of the reward well light). For the five un-rewarded ambiguous trials, behavioural measures included Sample Touch Latency (latency to touch the ambiguous Sample Stimulus from the time of presentation on the touchscreen), choice of right or left flanking Choice Stimulus, and Choice Touch Latency (latency to touch a flanking Choice Stimulus from the time of presentation on the touchscreen).

Foot shock

Following Probes 1 and 2 of restraint stress, all mice were retrained on their respective VMCL task, size or shape, until they achieved 85% accuracy on S+ and S- trials two times without the aid of correction trials. The probe tests were then repeated but the stress group was submitted to 3 foot shocks of 0.5 mA for 2 s (Probe 3). The same procedure was repeated 24 hours later (Probe 4). The same general probe test procedures as discussed above were employed.

Statistical analysis

The data were analyzed using SPSS. Analysis of variance (ANOVA) was used to analyze the % accuracy and latency data during learning using Session and Trial-type (S+ or S-) as within-subjects factors. Chi-square tests were used to compare the right/left choices of the stressed and control animals at probe. Independent t-tests were

used to analyze the sample touch latency and the open field data. $P<0.05$ was considered to be significant.

Results

Pretraining

The liquid reward training took 3 days and animals performed the touchscreen training for 9-12 days.

Training

Animals in the VMCL size task took 34.5 ± 3 days (mean ± SEM) to reach criteria twice without the aid of correction trials, and in the VMCL shape, the subjects took 45.7 ± 3.7 days (mean ± SEM). The performance across sessions during training showed that animals were able to learn both tasks, and they reached performance criterion faster in the size compared to the shape version (Figure 3a and b). The S+ trials were acquired significantly faster than the S- trials (Figure 4a and b) as evidenced by significant within-subjects effects of Trial-type in both the size [$F(1,30)= 179.0$, $p<0.001$)] and the shape conditions [$F(1,21)=68.7$, $p<0.001$)]. Mice were also quicker during the S+ trials to touch the location leading to reward [Size: $F(1,19)= 36.1$, $p<0.001$; Shape: $F(1,11)= 15.0$, $p<0.01$] and to approach the reward well [Size: $F(1,19)= 33.8$, $p<0.001$; Shape: $F(1,9)= 19.7$, $p<0.01$] (Figure 5a and b) as evidenced by significant within-subjects effects of Trial-type in both the size and shape conditions. This suggests that mice detected and preferred the greater reward provided by the S+ trials.

A) B)

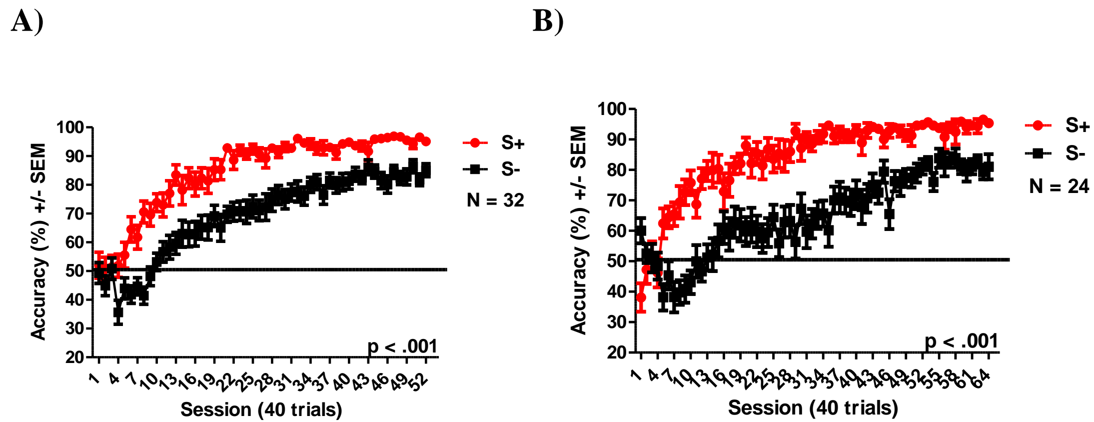

Figure 3: Percentage accuracy of animals across sessions during training in the A) VMCL Size and B) VMCL Shape task. Data are presented as mean±SEM.

A) B)

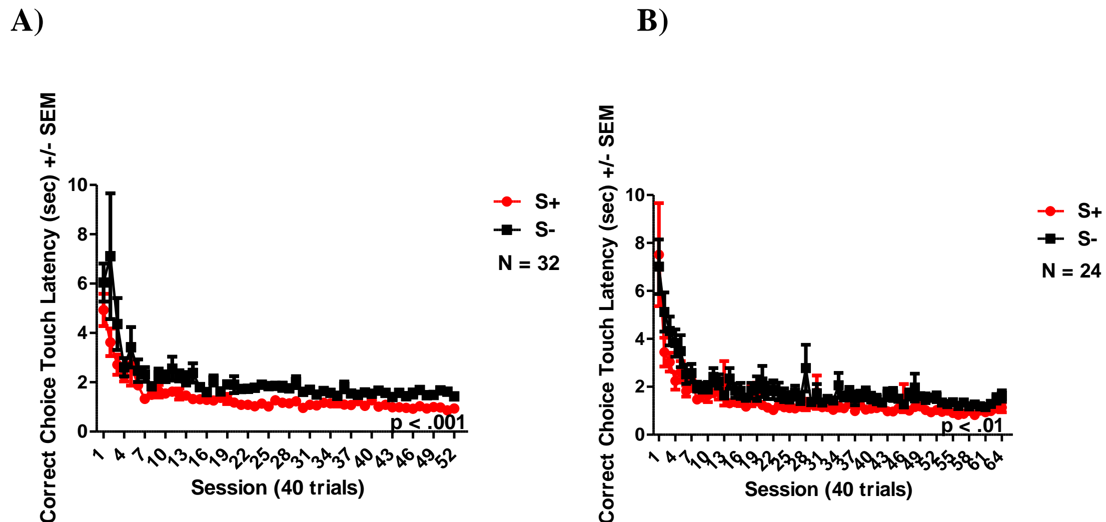

Figure 4: Correct choice touch latency (seconds) of the animals across sessions during training in the A) VMCL Size and B) VMCL Shape task. Data are presented as mean±SEM.

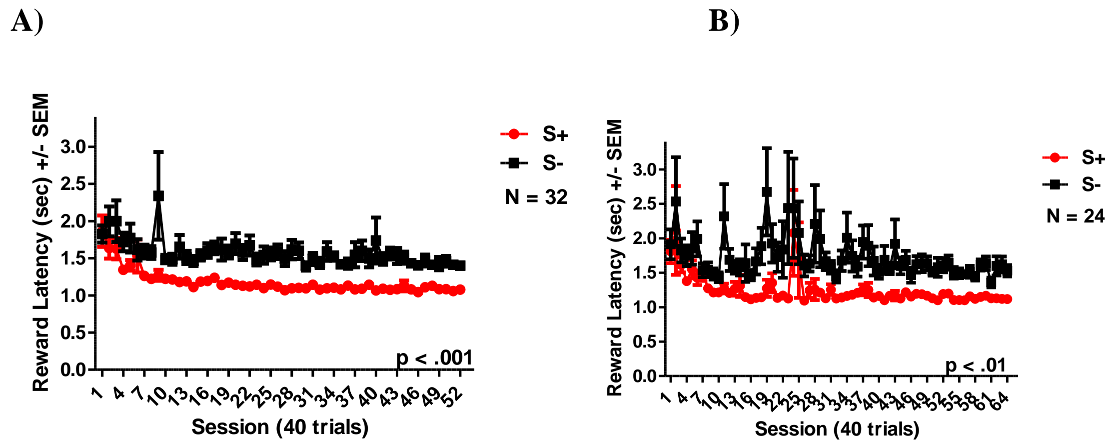

Figure 5: Reward latency (seconds) of animals across sessions during training in the A) VMCL Size and B) VMCL Shape task. Data are presented as mean±SEM.

Probe test

Figure 6 represents the percentage choices to the right during the VMCL size probe sessions, and it reveals that after restraint in day 2 [X^2 (1, N=31)= 4.57, p=0.03)] and after shock (day 1 [X^2 (1, N=31)= 7.39, p=0.007)] and shock day 2 [X^2 (1, N=31)= 7.39, p=0.007)]), the animals made significantly fewer choices to the right compared to control animals when the 45% ambiguous stimulus was presented. This suggests that the negative affective state induced by restraint and foot shock resulted in a 'pessimistic' bias, i.e. a decreased tendency to associate the 45% ambiguous probe stimulus with the greater reward.

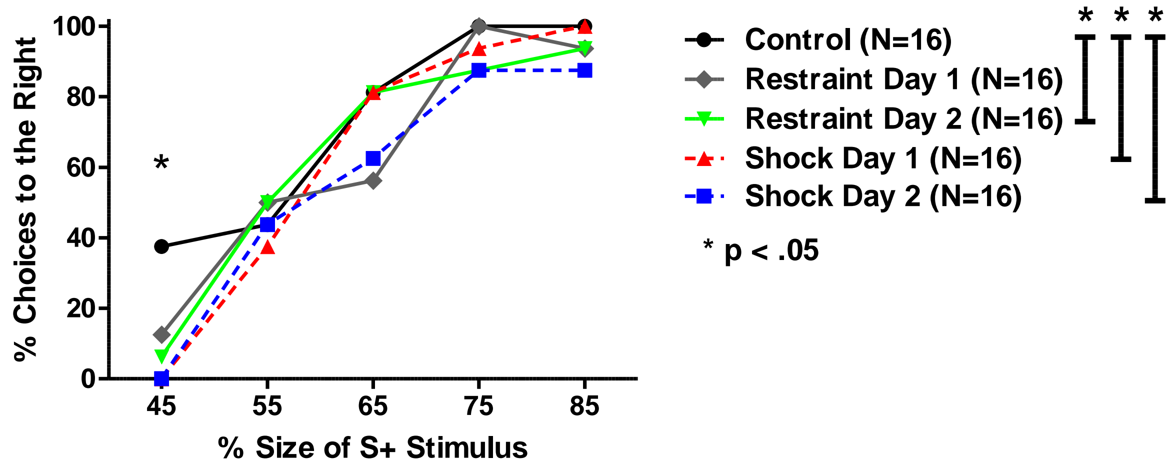

Figure 6: Percentage of choices to the right in the VMCL Size probe test. *p<0.05.

Similarly, the negative affective state induced a decreased latency to approach the sample probe stimulus leading to the lesser reward (i.e. left choice stimulus in the shocked condition) [Shock day 1: t(30)= - 2.2, p=0.03; Shock day 2: t(30)= -2.0, p=0.05)] (Figure 7).

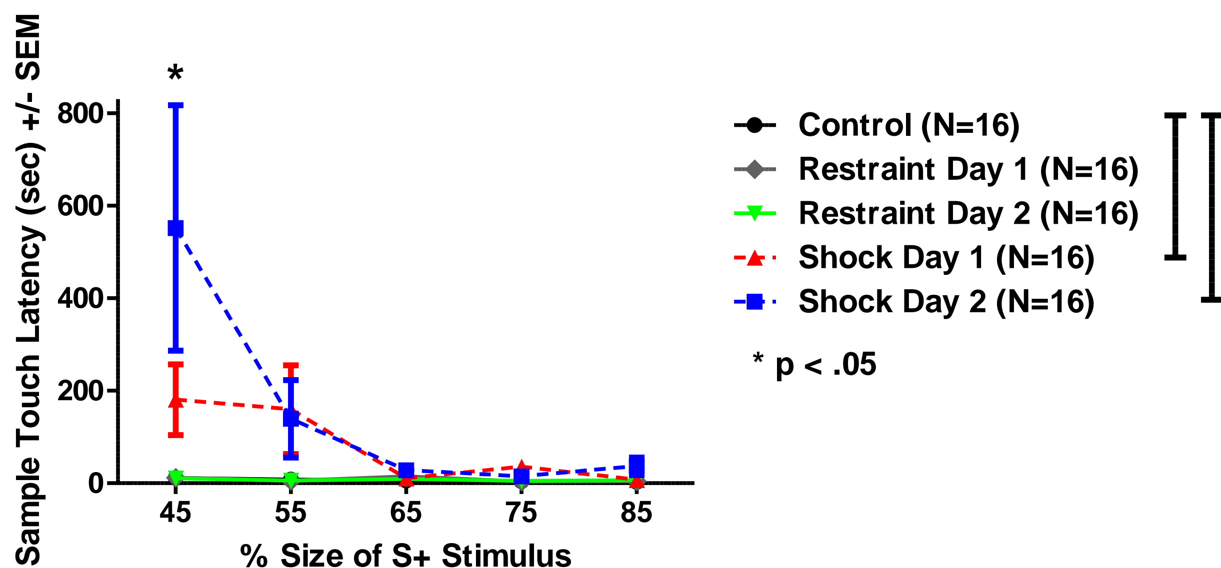

Figure 7: Sample touch latency (seconds) in the VMCL Size probe test. *p<0.05.

Performance during the regular VMCL size trials during probe showed no difference among groups in % accuracy, except for a difference between control animals and those that had received shock on day 2 [t(30)= 2.1, p= 0.05) (Figure 8).

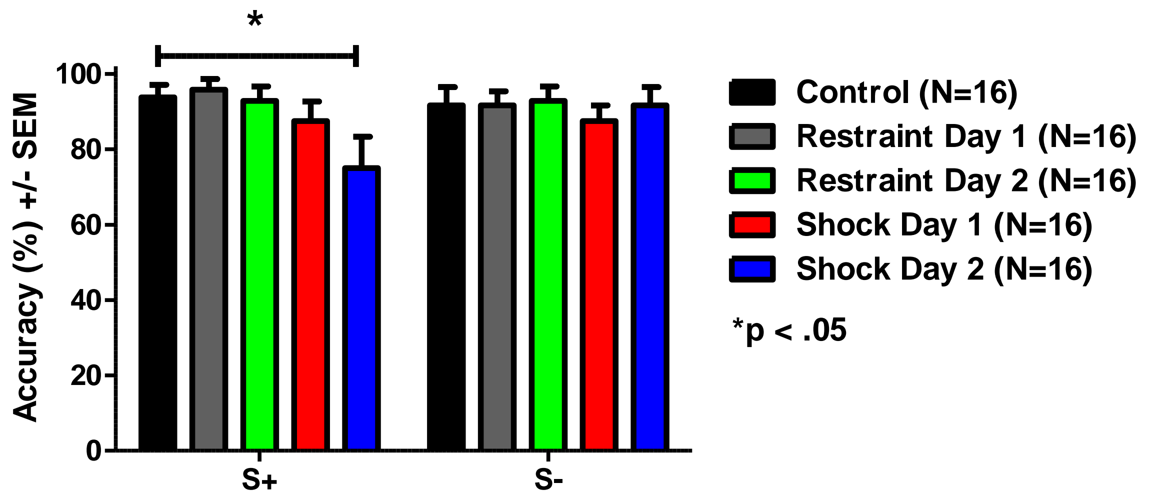

Figure 8: Percentage of accuracy in the VMCL Size probe test. *p<0.05.

Figure 9 shows the % choices to the right during the VMCL shape probe. Statistical analysis indicated that there were no significant differences between the groups for the first presentation of the ambiguous stimulus. Also, there were no differences between groups in the latency data or the % accuracy in the regular VMCL trials, except for a difference between control animals and those that had received shock on day 1 [t(22)= -2.1, p=0.05) (Figure 10).

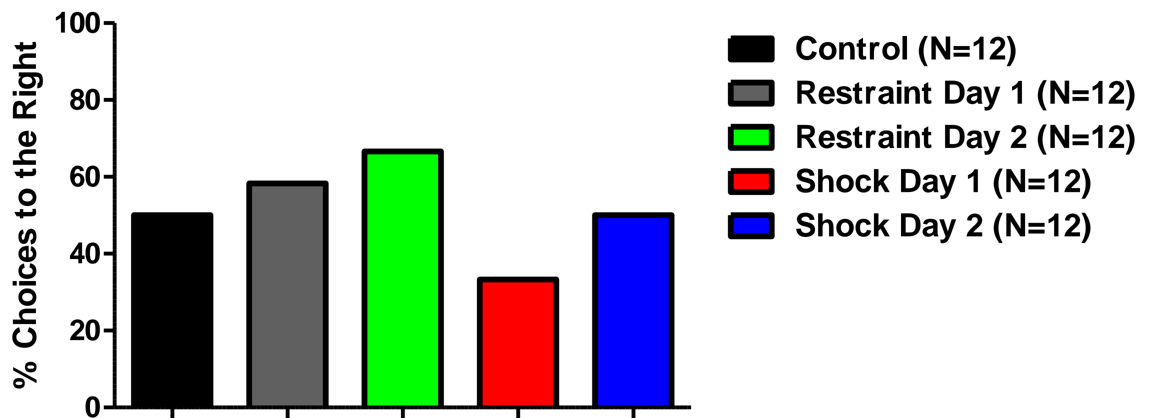

Figure 9: Percentage of choices to the right in the VMCL Shape probe test**.**

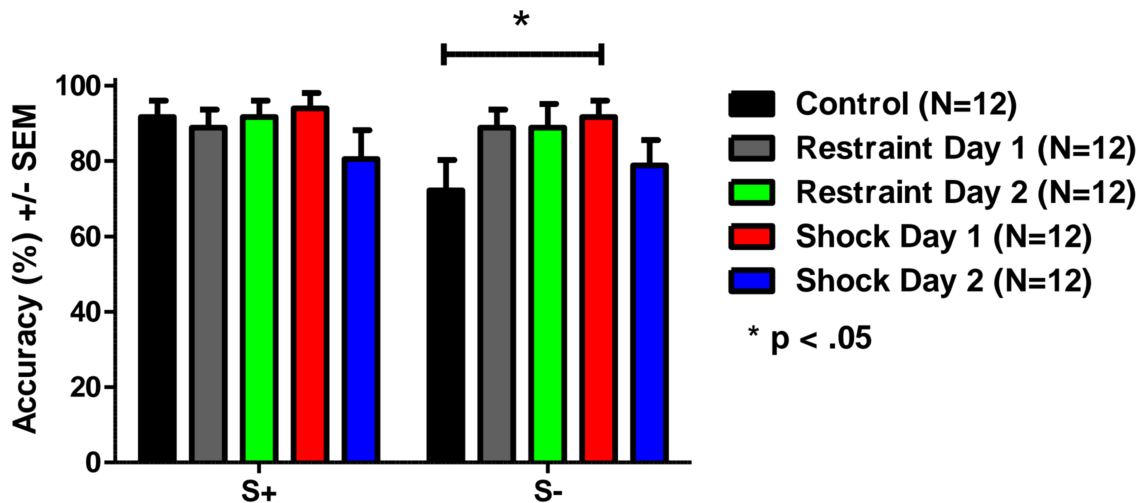

Figure 10: Percentage of accuracy in the VMCL Shape probe test. $p<0.05$.

Open Field

Shocked, but not restrained animals, showed a significant difference in the time spent exploring the zones of the open field compared to control animals {[Zone 1 - Shock day 1: $t(16)= -2.2$, $p= 0.05$; Shock day 2: $t(16)= -2.5$, $p= 0.02$; Zone 2 - Shock day 1: $t(16)= 2.3$, $p= 0.04$; Shock day 2: $t(16)= 2.4$, $p= 0.03$; Zone 3 - Shock day 2: $t(16)= 2.9$, $p= 0.01$]} (Figure 12). Shocked animals also traveled a lesser distance in ten

minutes than unshocked controls [Shock day 1: t(16)= 4.0, p= 0.001; Shock day 2: t(16)= 2.8, p= 0.01] (Figure 13).

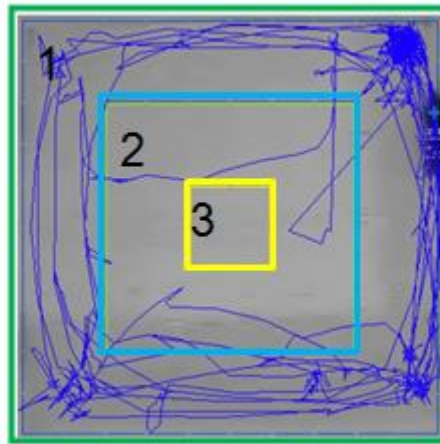

Figure 11: Representative behavior of shocked animals in the different zones of the open field.

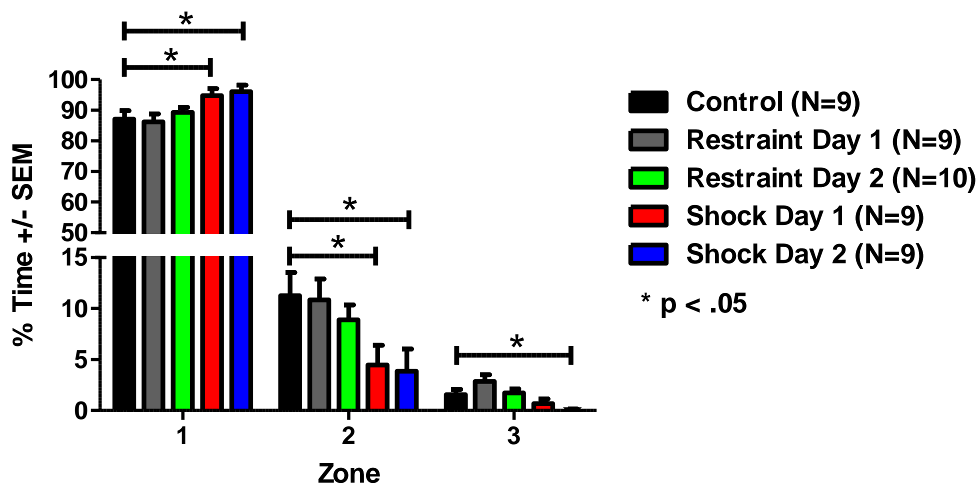

Figure 12: Percentage of time spent in the different zones of the open field. P<0.05.

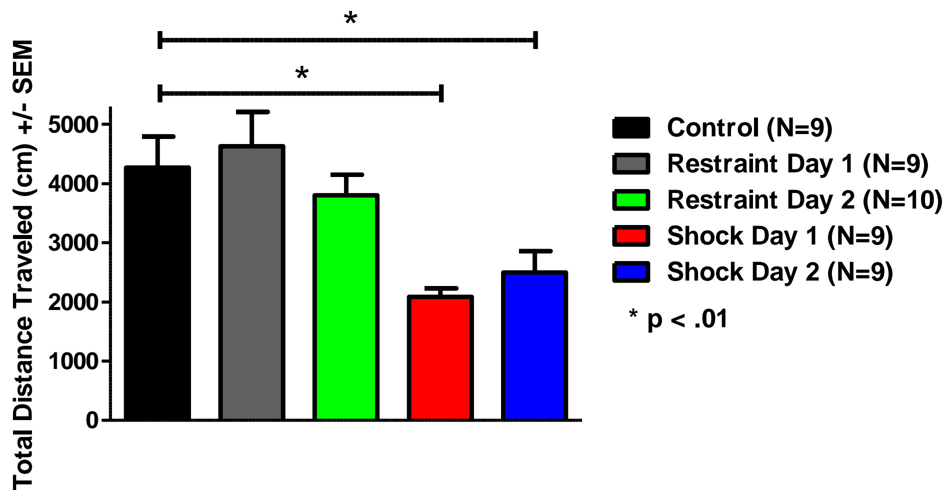

Figure 13: Total distance travelled of animals in the open field. $p<0.01$.

Discussion

Our results showed a significant pessimistic bias in mice subjected to a negative state induction using the size stimuli, and this effect was evident in the choice and latency data, and it was selective to the smallest (45%) ambiguous probe stimulus. However, in the shape task there was no difference among groups when the ambiguous stimulus was presented during the test.

Studies of human psychology provide the main source of information about the effects of emotional states on cognitive function. A consistent finding from human studies is that the valence of an individual's emotional state appears to influence a number of cognitive processes including attention, memory and judgment. Many brain areas, such as amygdala, prefrontal and orbitofrontal cortex, anterior cingulate, insula, nucleus accumbens, ventral tegmental area, and periaqueductal gray, and brain systems including dopamine, noradrenaline, opioid and serotoninsystems, appear to be involved

in the processing of affective information, including the encoding of reward value and decision-making (Paul et al., 2005, Rangel et al., 2008).

Numerous studies have investigated judgment bias in several animal species, with some studies aimed at welfare assessment, and others interested in cognitive bias in animal models of human affective disorders. Judgment bias in this context refers to the propensity of a subject to show behavior indicating anticipation of either relatively positive or relatively negative outcomes in response to affectively ambiguous stimuli (Mendl et al., 2009). Such propensities can be operationally defined as 'optimism' or 'pessimism', respectively (Matheson et al., 2008), with the caveat that this should not be taken to imply a conscious equivalent of these states as experienced by humans.

Harding et al. (2004) was the first study published on cognitive bias in animals, and introduced the judgment bias experimental paradigm that has formed the basis for most subsequent studies. In this paradigm, animals learn that one cue predicts a positive event and another cue predicts a less positive or negative event, and these animals are then tested with the presentation of ambiguous cues . This study showed a 'pessimistic' response bias in rats that were housed in unpredictable conditions, as judged by their lever-press response to ambiguous tones.

In a study using starlings, the birds were trained in a discrimination task, in which a background shade determined the amount of food reward (small or large). The animals were tested with intermediates background shades, and a reduction in environmental conditions had no effect on 'pessimism'. However, the authors suggested that individual differences in performance of stereotypies did predict 'pessimism' (Brilot et al., 2010).

Recently, Bateson et al. (2011) demonstrated that agitated honeybees are more likely to classify ambiguous odor stimuli as predicting punishment, and also have lower levels of hemolymph dopamine, octopamine, and serotonin. Salmeto et al (2011), suggest that it is possible to evaluate cognitive bias in chicks and associate such biases with anxiety- and depression-like states. In their model, they correlate increased latencies to respond to aversive ambiguous cues with 'pessimism' and anxiety, and increased latencies to respond to both aversive and appetitive ambiguous cues with decreased 'optimism' and a depression-like state.

Enkel et al. (2010) using congenitally helpless rats, a genetic animal model of depression, showed a negative response bias as evidenced by decreased positive and increased negative responding. Moreover, the treatment with a combined noradrenergic-glucocorticoid challenge, mimicking stress-related changes in endogenous neuromodulation, biased rats away from positive responding. This response shift was accompanied by neuronal activation in dentate gyrus and amygdala.

In our study, mice subjected to a negative state induction interpreted the smallest (45%) ambiguous size probe stimulus as more like the S-, expecting a small reward, compared to the control group, and we suggest that the negative state induction produced a pessimistic-like state.

According to Mendl et al. (2009), the absolute affective value of the reinforcers may influence the type of bias that is observed. For example, if the less positive/negative reinforcer is mild, there may be a general tendency for all subjects to respond to intermediate ambiguous probes and those close in appearance to the positive training cue, as if they predict the positive reinforcer because the cost of getting it wrong and receiving the negative reinforcer is relatively small. Affective-induced

differences in response may only appear at the probes closest to the negative training cue where the perceived likelihood of the negative outcome occurring is highest, as we observed in our probe test.

On the other hand, if the negative reinforcer is more severe (e.g. noise, shock), all subjects may be more likely to respond cautiously to intermediate probes and those closest in appearance to the negative cue to minimize the chance of receiving the negative reinforcer, and hence affect-induced biases will be more likely to appear at probes nearest to the positive training cue.

Our results show that the touchscreen-based VMCL size task offers much promise as a novel measure of the effect an animal's emotion has on judgment and decision-making. The task also allows for generalization across species, measures emotional valence rather than arousal, and can potentially assess 'optimism' and positive emotions, important behavioural measures that are not easily assessed with other tasks.

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Adult neurogenesis protects against proactive interference

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Introduction

Adult neurogenesis, new neurons continually born throughout adulthood in mammals, has emerged as an integral and crucial process within the hippocampus. The dentate gyrus in the hippocampus is one of two brain regions with lifelong neurogenesis demonstrated in rodents, primates and humans (Aimone, Wiles and Gage, 2006). Despite an increasing amount of information about the characteristics of the newborn granule cells, the specific contribution of their robust generation to memory formation by the hippocampus remains unclear.

Computational studies predict a role for new neurons in pattern separation and interference reduction on the basis of the unique properties of these cells (Luu e al., 2012, Becker 2005). One such property is the ability of new neurons to undergo a complete turnover while they grow and become transformed from one developmental stage to another during the course of several days to weeks (Deng et al. 2010).

Recent work in rodents has shown that hippocampal neurogenesis facilitates pattern separation while impairing pattern completion (Creer et al., 2010; Nakashiba et al., 2012). It has been proposed that the high activity level of newborn neurons facilitates their ability to encode and parse similar episodic events at the cost of recalling previously-learned information (Luu et al., 2012).

Emerging evidence suggests that neurogenesis is important for pattern separation and for mitigating interference when similar items must be learned at different times. Lu et al. (2012) showed that irradiated rats exhibited proactive interference from previously-learned odor pairs, demonstrated by significantly impaired ability to overcome interference during learning of a second list of odors in a different context. According to the authors, neurogenesis is critical for resolving interference between overlapping associations learned at different times.

The hippocampal circuitry is in a constant state of flux, and this plasticity has potential implications for the clearance of outdated memories. The fidelity of a hippocampus-dependent memory may be decreased by adding new neurons (Epp et al., 2013).

Therefore, we sought to determine whether increasing neurogenesis after learning a hippocampal-dependent paired-associates task would decrease the stability of the memory for the task, and if so, whether that would lead to an improvement in reversal learning.

Participants

The participants were male hybrid C57 B/6 x 129 SVEV mice that were bred in the colony at The Hospital for Sick Children. All mice were 8 weeks of age at the start of the experiment. Mice were housed 3-5 animals per cage with water available *ad libitum* in a temperature and humidity controlled vivarium. Mice were maintained on a 12 hr light/dark cycle (lights on at 07:00 hrs) and tested 5-6 days per week between the hours and 9am and 4pm. Four days prior to the start of the experiment, all mice were handled daily for 2 min by an experimenter and food restriction commenced. Under food restriction, each mouse received approximately 3g of rodent chow per day following their training sessions, and weights were monitored daily to ensure that no mouse dropped below 90% of *ad libitum* weight. Food restriction was in effect for the duration of the experiment. All experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) and approved by the Animal Care Committee at the Hospital for Sick Children.

Apparatus

Four touchscreen-equipped automated operant chambers for mice (Bussey-Saksida Touch Systems, Lafayette Instrument, Lafayette, Indiana) were used (Figure 1). Each apparatus consisted of a triangular modular operant chamber housed within a sound- and light-attenuating box (40×34×42 cm; Med Associates Inc., St. Albans, Vermont). The modular operant chamber consisted of a metal frame, black Plexiglas walls, and a stainless steel grid floor. The box was fitted with a fan (for ventilation and masking of extraneous noise) and a peristaltic pump-based liquid reward well, which was illuminated by a 3-W light bulb and fitted with a photocell head-entry detector. A 3-W houselight and tone generator (Med Associates Inc., St. Albans, Vermont) were fitted to the back wall of the chamber. Behavioral testing programs were controlled with a computer software program (Bussey-Saksida Touch Systems, Lafayette Instrument, Lafayette, Indiana).

At the front of the box opposite the liquid reward well was a flat-screen monitor equipped with an infrared touchscreen (16 cm high and 21.20 cm wide; Craft Data Limited, Chesham, UK) running ELO touchscreen software (ELO Touchsystems Inc.). Given that the touchscreen used infrared photocells, the mouse was not required to exert significant pressure on the screen in order for a response to be detected. A black Plexiglas slide-in mask with response windows was placed over the screen through which the mouse could make a nose-poke response. The mask (h 11.80 cm × w 22.8 cm) had three response windows (h 5.8 cm × w 5.8 cm) which were positioned 0.8 cm apart from one another and located 3.0 cm from the sides of the mask.

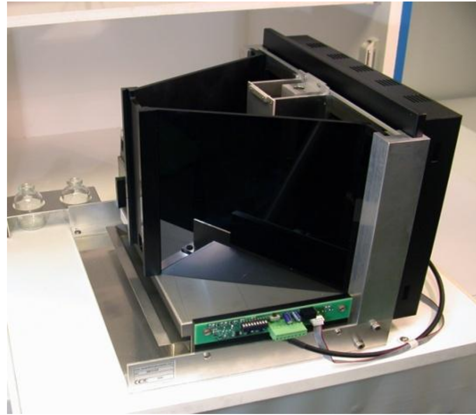

Figure 1: Bussey Touch Screen Mouse Chamber used for testing

Pretraining Procedures

Habituation

Mice were exposed to the inside of the operant chamber for 15 min per day for 2 days during which the touchscreen was blank and the three-hole response mask was in place.

Light-Reward training

Seven days following the start of food restriction, mice began light-reward training. Each session commenced with the delivery of 25µl of strawberry milkshake (Neilson Dairy, Toronto, Ontario) into the reward well concomitant with the illumination of the reward well light and the presentation of a 3Khz tone of 1 sec duration. After a head entry into the reward well was detected, the light was extinguished and a fixed-interval (10 sec) schedule of reinforcement was initiated during which illumination of the reward well and sounding of the tone indicated the availability of 7µl of strawberry milkshake. Mice received one 15 min session per day

for 3 days during which the touchscreen was blank and the three-hole response mask was in place.

A decrease in Reward Latency (latency of reward well head entries following illumination of the reward well light) and an increase in the number of rewards obtained within a session reflected learning of the light-reward association.

Touch-Light-Reward training

Mice were trained to touch an achromatic visual stimulus presented on the touchscreen one at a time in one of the three locations of the three-hole response mask. During each trial, the location of the presented stimulus was pseudorandomized and remained on the screen until a touch to the stimulus was made. A touch response to the stimulus resulted in delivery of 7μl of strawberry milkshake in the reward well, illumination of the reward well light, and presentation of the concomitant 3Khz tone of 1 sec duration. There was a 5 sec inter-trial interval (ITI) which commenced as soon as a head entry into the reward well was detected. Responses to a blank location had no effect. The stimuli used were from a bank of 40 achromatic computer-generated visual stimuli (5 cm x 5 cm) created by the Bussey-Saksida Touch Systems (Lafayette Instrument, Lafayette, Indiana). To facilitate learning, a small amount of confectioner's sugar moistened with water was applied to the touchscreens (in the center of the three response windows). Each daily session consisted of 30 trials or a maximum of 30 min, whichever came first. Training continued until all mice within a cage reached a performance criterion of 30 trials within a 30 min session on non-consecutive days. A further decrease in Reward Latency, a decrease in Touch Latency (latency to touch a stimulus once it appeared on the touchscreen), and an increase in the number of trials completed within a session reflected learning of the touch-light-reward association.

Paired-Associate Learning (PAL) task training

The PAL task required mice to learn three stimulus-place associations. The three-hole response mask was employed such that there were three possible locations, each one associated with one of three visual stimuli (5 cm x 5 cm). Each PAL task training session commenced with the delivery of 7μl of strawberry milkshake (Neilson Dairy, Toronto, Ontario) into the reward well concomitant with the illumination of the reward well light and the presentation of a 3Khz tone of 1 sec duration. After a head entry into the reward well was detected, the light was extinguished and following a 5 sec ITI, the first PAL trial began with presentation of two of the three visual stimuli, one in the correct location and the other in an incorrect location. The third location remained blank and responses to it had no effect. If the correct stimulus was touched, both stimuli disappeared and a delivery of milkshake (7μl) in the reward well occurred concomitant with illumination of the reward well light and presentation of the 3Khz tone of 1 sec duration. There was a 5 sec ITI which commenced as soon as a head entry into the reward well was detected. If the incorrect stimulus was touched, both stimuli disappeared and the houselight was illuminated for the duration of a 5 sec timeout period, which was then followed by a 5 sec ITI. Each session consisted of 36 trials with a maximum of 30 min.

There were six different trial types (Figure 2a) with each one occurring six times per session in a pseudorandomized order such no more than three trials of the same type occurred consecutively. A correction trial occurred when an incorrect response was made and consisted of presentation of the exact same trial until a correct response occurred. Performance on correction trials did not count towards behavioral performance measures. Behavioural measures for PAL task performance consisted of

percentage accuracy, number of correction trials, correct touch latency (latency to touch correct stimulus from the time of presentation on the touchscreen), and reward latency (latency of reward well head entries following illumination of the reward well light). PAL training continued until all mice within a cage reached a performance criterion of 80% accuracy with all 36 trials completed per session.

Neurogenesis Manipulation

Wheel running is a well-validated method to increase hippocampal neurogenesis in rodents (Van Praag et al., 1999). After PAL training, animals were separated into two groups, sedentary and runner. The groups were counterbalanced to ensure that mice in both groups performed equally well during the final four sessions of PAL task acquisition. The sedentary group was left in their standard home cages for 30 days while being maintained on mild food restriction. The runner group had free access to a running wheel inside their cage for 30 days while being maintained on mild food restriction. Each running wheel was linked to a computer to track distance traveled. After 30 days, animals received retraining on the original PAL task for 7 days and then received training on the Reversal PAL task.

Paired-Associate Learning (PAL) task reversal training

The same general task parameters as those used for the original PAL task were employed except that the correct locations were reversed for the six different trial types (Figure 2b).

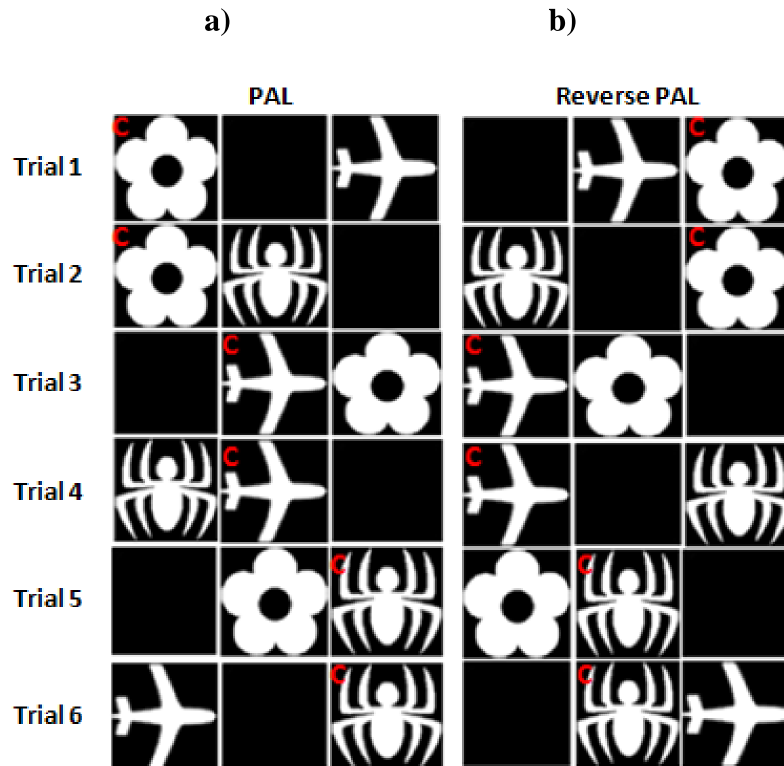

Figure 2: An Illustration of the six possible pseudorandomized trials for a) PAL and b) reverse PAL, with the correct response for each trial identified with a C in the top left corner.

Statistical analysis

The data were analyzed using SPSS. Analysis of variance (ANOVA) and dependent and independent t-tests were used to analyze the results. $P<0.05$ was considered to be significant.

Results

PAL Task Training

The mice were able to acquire the PAL task to criterion, with accuracy increasing to 80% over 49 sessions (Figure 3). Correction trials were in place for

incorrect responses in order to facilitate learning. The number of correction trials decreased over time, with very few correction trials required by the 49th session (Figure 4).

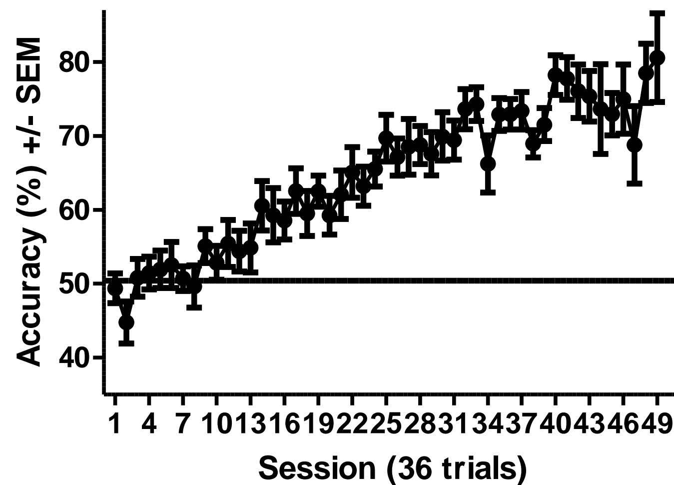

Figure 3: PAL learning curve illustrating average accuracy for each session during PAL training. Chance performance is 50%. Data consists of mean±SEM for 49 sessions, with 36 trials per session.

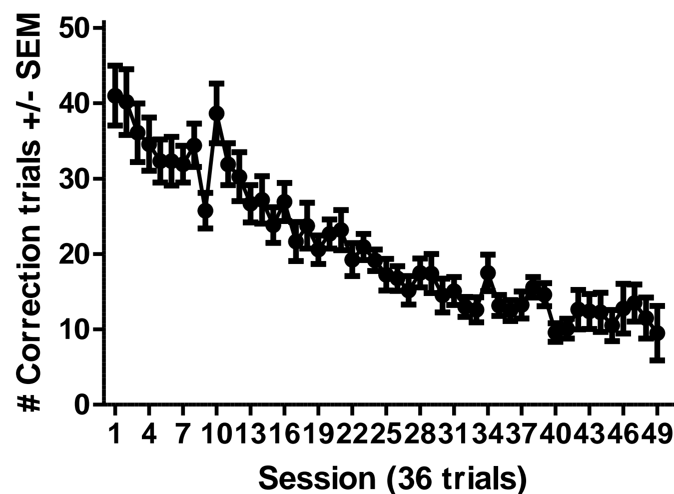

Figure 4: Curve representing the average number of correction trials required during PAL training. Data consists of mean ±SEM over 49 sessions, consisting of 36 trials for each session.

PAL and Reverse PAL (post-running)

Mice in the sedentary and runner groups performed comparably on the PAL task during the last 4 sessions of pre-running task acquisition [t(29)= 0.10, p=0.91] (Figure 5). During the first session of post-running PAL retraining, the accuracy of mice in both groups significantly decreased relative to their performance 30 days earlier [Runners: t(15)= 8.10, p<0.001; Sedentary: t(14)= 6.30, p<0.001]. However, the sedentary mice performed significantly better than the runners on the first session of PAL retraining [t(29)= -2.54, p=0.02] (Figure 5). Additionally, while the runner mice performed no differently than chance (50%) [t(15)= 0.71, p=0.49], the sedentary mice performed at accuracy levels significantly greater than chance [t(14)= 4.54, p<0.001] on the first session of PAL retraining.

An ANOVA was run using Session as a within-subject factor and Group (runner or sedentary) as a between-subjects factor. Over the course of the 7 PAL retraining sessions, accuracy significantly improved for mice in both groups, as evidenced by a significant within- subjects effect of Session [F(6,174)= 11.21, p<0.001] withno interaction with Group.The sedentary mice continued to significantly outperform the runner mice during the remaining PAL retraining sessions as indicated by a significant between-subjects effect of Group [F(1,29]= 24.20, p<0.001] (Figure 5).

Mice in the sedentary and runner groups required comparable numbers of correction trials during the last 4 sessions of pre-running PAL task acquisition [t(29)= -0.38, p=0.71] (Figure 6). During the first session of post-running PAL retraining, the number of correction trials required for mice in both groups significantly increased relative to the number required 30 days earlier [Runners: t(15)= -8.24, p<0.001; Sedentary: t(14)= -5,78, p<0.001]. However, mice in the sedentary group required

significantly fewer correction trials than those in the runner group on the first session of PAL retraining [t(29)= 3.06, p=0.005] (Figure 6). Over the course of the 7 PAL retraining sessions, the number of correction trials required significantly decreased for mice in both groups, as evidenced by a significant within-subjects effect of Session [F(6,174)= 15.70, p<0.001] with no interaction with Group. The sedentary mice continued to require significantly fewer correction trials than the runner mice during the remaining PAL retraining sessions, as revealed by a significant between-subjects effect of Group [F(1,29]= 22.89, p<0.001] (Figure 6).

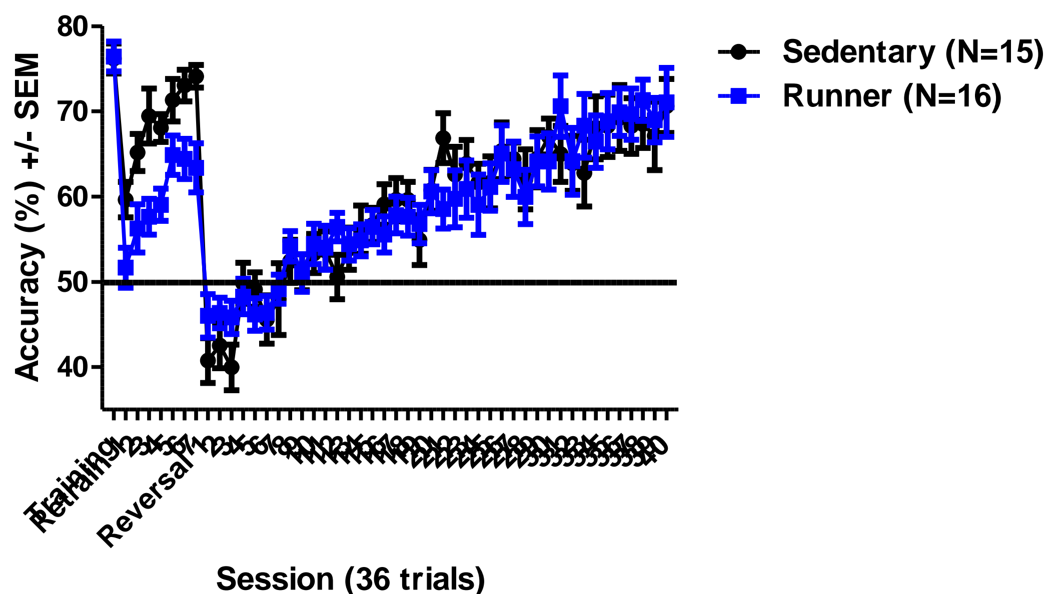

Figure 5: Learning curve for PAL and reversal PAL for sedentary and runner mice indicating average accuracy for each session during pre-running training, and post-running retraining and reversal of the PAL task. Chance performance is 50%. Data consists of mean±SEM for the last 4 PAL pre-running training sessions, 7 PAL post-running retraining sessions and 39 post-running reversal PAL training sessions, with 36 trials per session.

During the first session of PAL reversal training, the accuracy of mice in both groups significantly decreased relative to their performance on the last day of PAL retraining [Runners: t(15)= 3.80, p=0.002; Sedentary: t(14)= 11.90, p<0.001]. However, during the first three sessions of PAL reversal training, mice in the runner group performed significantly better than those in the sedentary group, as evidenced by a significant between-subjects effect of Group [F(1,29)= 4.09, p=0.04]. Additionally, while the runner mice performed no differently than chance (50%) [t(15)= -1.56, p=0.14], the sedentary mice performed at accuracy levels significantly lower than chance [t(14)= -3.53, p=0.003] on the first session of PAL reversal training.

Thereafter the runners and sedentary mice displayed no difference in accuracy with mice in both groups reaching approximately 75% accuracy by the final session of PAL reversal training, as indicated by a significant within-subjects effect of Session [F(36, 792)= 15.69, p<0.001] with no interaction with Group (Figure 5).

During the first session of PAL reversal training, the number of correction trials required for mice in both groups significantly increased relative to the number required during the final session of retraining of the original PAL task [Runners: t(15)= -4.24, p=0.001; Sedentary: t(13)= -5.27, p<0.001]. However, during the first three sessions of PAL reversal training, mice in the runner group required significantly fewer correction trials than those in the sedentary group, as evidenced by a significant between-subjects effect of Group [F(1,28)= 10.16, p= 0.004]. Thereafter, the number of correction trials required for mice in both groups decreased at a comparable rate, as indicated by a significant within-subjects effect of Session [F(36, 792)= 20.31, p<0.001] with no between-subjects effect of group and no interaction (Figure 6).

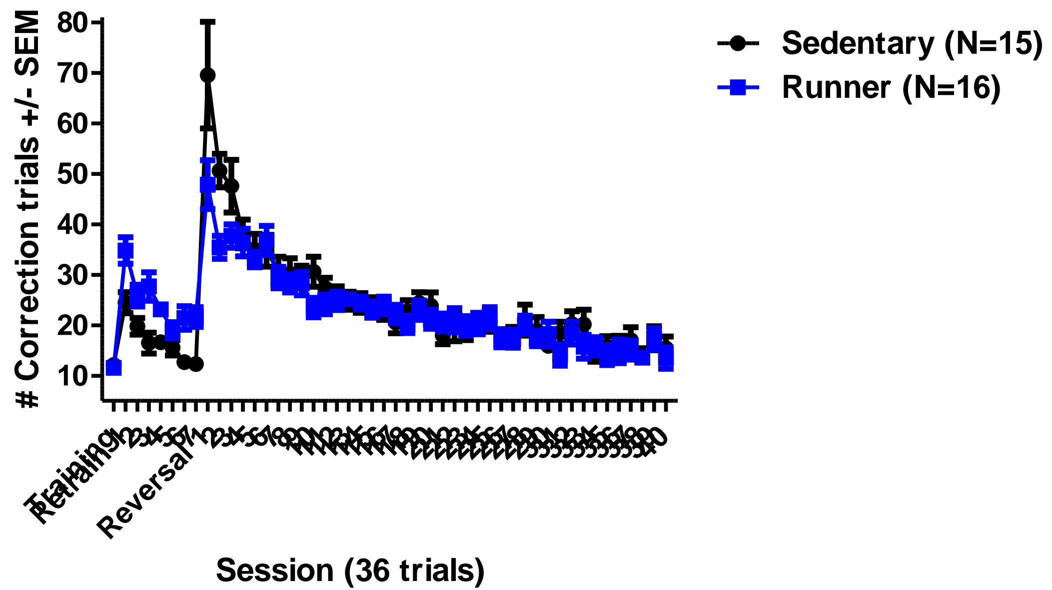

Figure 6: Post-running curve for the average number of correction trials required during pre-running PAL task training, and post-running PAL retraining and reversal sessions. Data consists of mean ±SEM for the last 4 PAL pre-running training sessions, 7 PAL post-running retraining sessions and 39 post-running reversal PAL training sessions, with 36 trials per session.

Discussion

Our results show that a post-training increase of neurogenesis causes a decrease in retention of previously acquired hippocampus-dependent memories. However, when subjected to reversal learning, mice with elevated levels of neurogenesis outperformed mice with normal levels of neurogenesis. The results described here show that adult neurogenesis aids in the clearance or inhibition of outdated memories. As a result, new

learning can occur more efficiently due to decreased proactive interference from the previous memories.

The hippocampus and specifically the dentate gyrus are thought to play a role in the ability to distinguish between various object-location representations that are similar in nature (Lee and Solivan, 2010). However, the role that hippocampal neurogenesis may play in pattern separation, pattern completion, and in the ability to perform an object-location paired-associate task has not been widely investigated.

The Paired Associate Learning (PAL) task is a hippocampal-dependent memory task involving the presentation of pairs of stimuli during the training phase. During the test phase, single stimuli are presented and the ability of subjects to recall the associated stimulus pair is observed. Object-place PAL tasks involve the ability to learn the association between an object and location, which have been utilized in touchscreen-based mouse models (Bartko et al, 2010). Previous research has shown that individuals with early onset Alzheimer's disease may be impaired on PAL tasks (Talpus et al, 2009), and some studies have shown that the PAL neuropsychological test is able to identify and predict early-onset Alzheimer's disease (Swainson et al, 2001; Talpus et al, 2009).

Recent studies have employed a touchscreen-based mouse PAL task that resembles the object-location paired-associate tasks used in humans. This provides a translational model that could be important for future research related to the field (Bartko et al, 2010 and Talpus et al, 2009). In the mouse model of the PAL task, the mice are required to learn the association between a visual stimulus and a particular spatial location on the touchscreen. Previously, PAL tasks have been used to identify the role of the hippocampus in identifying object-location paired associates (Talpus et al, 2009). This was investigated by deactivating the the hippocampus. Hippocampal

impairment resulted in impaired performance on PAL tasks in touchscreen-based mouse models (Talpus et al, 2009).

The hippocampus is involved in the formation of flexible, contextually-complex memories and there is evidence that discrete neuronal representations within the dentate gyrus form the basis of these memories (Leutgeb et al., 2007). An important feature of hippocampus-based memories is that by virtue of their distinctiveness and contextual richness, they are readily distinguished from other memories. When the hippocampus is impaired, memories become more schematic, susceptible to interfering influences, and, as a result, subject to inaccuracy (Winocur et al., 2007).

According to Winocur et al. (2012), the new neurons in the hippocampus are part of the mechanism that controls interference in memory. The authors suggest that rats engaged in running activity exhibited increased neuronal growth and protection from memory impairment, and that hippocampal cells generated in adulthood play a role in differentiating between conflicting, context-dependent memories, providing further evidence of the importance of neurogenesis in hippocampus-sensitive memory tasks.

We showed that increasing neurogenesis levels with running after learning a hippocampal-dependent paired-associates task decreased the stability of the memory for the task, which lead to an improvement in reversal learning. These findings support the notion that newly generated cells play a role in modulating interfering influences and minimizing the effect of competing memories during selective recall. This view is supported by recently developed computational models which, while differing from one another in several respects, suggest that adult-born hippocampal cells are essential to the process of reducing the adverse effects of interference during learning and memory.

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ANEXO A

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Parecer da Comissão de Ética em Experimentação Animal nº 041/2009

Protocolo nº 049/2009

A Comissão de Ética em Experimentação Animal da Universidade Federal de São Carlos – CEEA/UFSCar – na sua 36ª. Reunião, ocorrida em 20/10/2009, **APROVOU** o trabalho intitulado **"Influência do Sistema Neural Histaminérgico Cerebelar na Memória Emocional de camundongos"**, apresentado por Anna Carolyna Lepesteur Gianlorenço, Rosana Mattioli e Azair Liane Matos do Canto de Souza.

São Carlos, 26 de outubro de 2009.

Profª. Drª. Keico Okino Nonaka

Presidente da Comissão de Ética em Experimentação Animal