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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS:

FISIOLOGIA ANIMAL COMPARADA

LABORATÓRIO DE NEUROCIÊNCIAS

PARTICIPAÇÃO DOS NÚCLEOS COLINÉRGICOS DO PROSENCÉFALO BASAL NO DESEMPENHO COGNITIVO E LOCOMOTOR DE CAMUNDONGOS GENETICAMENTE MODIFICADOS

Tese de conclusão de doutorado apresentada pelo MSc Gustavo Morrone Barbat Parfitt como parte dos requisitos para obtenção do título de doutor em Ciências Fisiológicas: Fisiologia Animal Comparada desenvolvida sob orientação da Prof.ª Dr.ª Daniela Marti Barros e co-orientação da Prof.ª Dr.ª Ana Paula Horn

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"Os conceitos e princípios fundamentais da ciência são invenções livres do espírito humano."
Albert Einstein

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SÚMARIO

RESUMO GERAL

Esta tese de doutorado objetivou compreender como a transmissão tônica colinérgica influencia aspectos cognitivos e comportamentais em camundongos. No capítulo 1 desta tese, fazemos uma revisão sobre a modulação colinérgica, gabaérgica e glutamatérgica do septo medial e as implicações no aprendizado aversivo. O capítulo 2, trata sobre os aspectos cognitivos como memória espacial e de trabalho usando como modelo animais geneticamente modificados para o gene do transportador vesicular de acetilcolina (VAChT). Visando entender tais mecanismos, foram gerados animais em que a transmissão colinérgica foi abolida no prosencéfalo basal. Para tanto, foram cruzados animais VAChT $^{flox/flox}$ com animais VAChT$^{Nkx2.1\text{-}Cre}$ gerando a linhagem VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$. Camundongos VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ tem a proteína VAChT deletada no prosencéfalo basal, causando alteração na liberação de ACh. Neste estudo estes animais foram submetidos a diversas tarefas comportamentais; primeiramente avaliamos a memória espacial no teste de labirinto aquático de Morris (LAM). Nesta tarefa, os animais não apresentaram prejuízos na aquisição e retenção da memória espacial. No entanto, quando o teste reverso foi realizado, no qual a plataforma foi deslocada para o quadrante oposto, os animais apresentaram prejuízos de aquisição e retenção da memória espacial. Posteriormente, estes animais foram submetidos ao teste de pareamento do objeto no lugar, do inglês *object-in-place paired-associated learning* (PAL), que consiste em um tarefa *touchscreem* para avaliação do aprendizado espacial, apresentando déficits na capacidade de adquirir este aprendizado espacial. Além disso, foi testada nestes animais a capacidade de desenvolver potencialização de longa duração (LTP), *in vivo.* Esses animais possuem a capacidade de desenvolver LTP, mas não conseguem mantê-la, como observado pelo decaimento da fase tardia da mesma. Para avaliar qual a contribuição da transmissão colinérgica septo-hipocampal nos déficits observados, foram gerados animais VAChT (*Knockout*) KOs seletivamente para o hipocampo. Para tanto, animais VAChT$^{flox/flox}$ foram injetados com um vetor viral AAV8 contendo o transgene da Cre ou da GFP. Estes animais foram testados nos mesmos comportamentos mencionados acima. Na tarefa de LAM os animais AAV8-Cre não

apresentaram déficits, tendo comportamento semelhante ao controle. No entanto, similarmente ao animais VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$, durante o teste reverso foram observados prejuízos na aquisição e retenção no LAM. Posteriormente, nestes animais, foi realizada a tarefa de PAL. Nessa tarefa, foi observada uma correlação entre os níveis da proteína VAChT e a performance dos animais. Os níveis da proteína VAChT foram medidos no hipocampo e córtex dos animais por *Western Blotting*. Ainda, foram realizadas as tarefas de memória de trabalho na tarefa de LAM e de alternações espontâneas. Em ambas as tarefas tanto os animais VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$,quanto os animais AAV8-Cre, apresentaram prejuízos na memória de trabalho. No capítulo 3 desta tese, foram avaliados aspectos da atividade locomotora nestes animais com deleção da proteína VAChT no prosencéfalo basal ou seletivamente na via septo-hipocampal. Para esse objetivo, tanto os animais VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ quanto os animais AAV8-CRE foram submetidos ao teste de campo aberto. Neste teste, os animais VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ apresentaram um aumento na atividade locomotora em relação aos controles VAChT$^{flox/flox}$. Entretanto, nos animais AAV8-CRE não foram observadas alterações na atividade locomotora. Posteriormente a atividade locomotora durante o período de 24h foi avaliada, neste período os animais VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ apresentaram um aumento da atividade locomotora durante a noite. Em conclusão, essa tese mostrou que a transmissão colinérgica, especialmente através da via septo hipocampal, é crucial para o aprendizado reverso na tarefa de LAM e para o bom desempenho da tarefa de PAL, além de ter importância na memória de trabalho, bem como tem um papel importante no controle da atividade locomora provavelmente mediado pela transmissão cortical.

Palavras Chaves : Ach, memória espacial, sistema colinérgico, memória de trabalho

ABSTRACT

The present PhD thesis had as the main aim understands how the cholinergic tone influences the cognitive function and behavioural aspects in mice. In the Chapter 1, a review concerning the populations of cholinergic, gabaergic and glutamatergic neuron of the medial septum and, their modulation in hippocampal function and aversive learning was performed. In the Chapter 2, cognitive aspects such as, spatial memory and working memory were studied using VAChT mutant mice. To understand the cholinergic role in these aspects, VAChT $^{flox/flox}$ mice were interbreed with VAChT$^{Nkx2.1\text{-}Cre}$ mice to generate the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mouse line. VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mouse line does not express VAChT in the basal forebrain, compromising the ACh release. In this work, the animals were submitted to different behavioural tasks; initially the spatial memory was evaluated in the Morris water maze task (MWM). In this task, no alteration in the acquisition and retention in the spatial memory were observed. However, when the reversal test, in which the platform in transferred to the opposite quadrant, was performed the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ animals displayed impairment in spatial memory acquisition and retention. Hereinafter, these animals were tested in the PAL task, a touchscreen task to evaluate the spatial memory, displaying impairments in the acquisition of the task. Moreover, the capacity to develop LTP was also tested *in vivo,* although these animals have the capacity to develop the LTP they cannot sustain it, as a decay is observed in the late phase. To evaluate the participation of cholinergic septum-hippocampal transmission in the observed deficits, animals with the selective deletion of VAChT in the hippocampus were evaluated. To that purpose, VAChT$^{flox/flox}$ mice were injected with AAV8 viral vector containing the CRE or GFP transgene in the medial septum. These animals were tested in the same aforementioned behavioural tasks. In the MWM the AAV8-Cre animals did not present alterations having a similar behaviour as the controls whereas, during the reversal task, similarly to VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice, impairment in the acquisition and retention in MWM was observed. Subsequently, the PAL task was performed in these animals. In this task a positive correlation between the level of the protein VAChT and the performance in the task was observed. In addition, the VAChT levels

were measured in the hippocampus and cortex through western blotting. Further on, the animals were submitted to tasks to evaluate the working memory performance in the MWM and in the spontaneous alternations. In the tasks, both VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ and the AAV8-Cre animals showed impairments in working memory. In the Chapter 3, locomotor activity aspects were evaluated in VAChT deleted mice for the basal forebrain or selectively in the hippocampus. For this purpose, both VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ and AAV8-CRE animals were submitted to the open field task. In this task, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ showed an increase in the locomotor activity related to the VAChT$^{flox/flox}$ controls. However, in the AAV8-CRE animals no alterations were observed in the locomotor activity. In addition, the 24 h locomotor activity was evaluated. During this period the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ showed an increase in the locomotor activity through the dark period. In conclusion, this work demonstrated that the cholinergic tone, specifically in the hippocampus, is crucial for the reversal learning and for the optimum performance in the PAL task. Besides of being important for the working, also has an important role in the control of locomotor activity probably mediated by the cortical cholinergic transmission.

Keywords: Ach, working memory, spatial memory; cholinergic system

LISTA DE ABREVIATURAS

AC – Via Comissural

ACh – Acetilcolina

AMPA - Alfa-Amino-3-Hidroxi-Metil-5-4-Isoxazolpropiónico

CA1 – Corno De Amon 1

CamKII – Proteína Cinase Dependente De Cálcio E Calmodulina

CHAT - Colina Acetil Transferase

CHT1 - Transportador De Alta Afinidade De Colina

CS - Estímulo Condicionado

DB – Banda Diagonal De Brocca

DG - Região Do Giro Dentado

EC - Córtex Entorrinal

EPSP – Potencial Pós-Sinaptico Excitatório

ERK - Cinase Ativada Por Estímulos Extracelulares

IPSP - Potencial Pós-Sináptico Inibitório

LAM – Labirinto Aquático De Morris

LTD - Depressão De Longa Duração

LTP – Potencialização de Longa Duração

MF – Fibras Musgosas

mPFC - Região Medial De Córtex Pré-Frontal

MS – Septo Medial

mTOR - Proteína Alvo Da Rapamicina Em Mamíferos

NBM - Núcleo Basal Magnocelular

NGF - Fator De Crescimento Neural

NMDA - N-metil D-Aspartato

PAL – Pareamento Do objeto No Lugar (tradução de *object-in-place paired-associated learning)*

PB - Prosencéfalo Basal

PKA - Proteína Cinase Dependente De Ampc

PKC - Proteína Cinase Dependente De Cálcio

SI – Substância Inominata

SNC – Sistema Nervoso Central

SP - Plasticidade Sináptica

STP - Potencialização De Curta Duração

SRC - Proto-oncogene tyrosine-protein cinase

VAChT - Transportador Vesicular De Ach

VTA - Área Tegmental Ventral

WM - Memória De Trabalho

1. INTRODUÇÃO GERAL

1.1 Aprendizado e memória

O aprendizado é o processo pelo qual o sistema nervoso central (SNC) processa informações sensoriais do ambiente para adquirir conhecimento ou executar tarefas (MEDINA et al., 2008). O aprendizado, quando bem sucedido gera um traço mnemônico, que pode ser evocado para a utilização da informação previamente armazenada (MEDINA et al., 2008). Entretanto, entre o processo de aquisição e evocação, essa memória passa por um tempo de maturação em regiões específicas do SNC. Esse tempo de maturação é chamado de consolidação, e é o tempo necessário para que a rede neural em formação adquira a estabilidade necessária para ser utilizada (MCGAUGH, 2000). No entanto algumas memórias precisam ser utilizadas antes do período total de consolidação e por isso, durante a formação de uma memória, duas redes neuronais são formadas em paralelo. Uma dessas redes compreende a **memória de curta duração**, a qual pode ser utilizada imediatamente não deixando traços; a segunda rede, a qual é utilizada para a **memória de longa duração**, começa a ficar disponível certo período após a consolidação (IZQUIERDO et al., 1998a, 1998b). O processo de consolidação da memória é um processo identificado há mais de 100 anos, e essa identificação foi um processo crucial para a descoberta das bases neuro-anatômicas da aquisição e consolidação da memória. Consolidação é o processo pelo qual as memórias são estabilizadas, processo que pode durar até seis horas (IZQUIERDO et al., 2008; MCGAUGH, 2000). Durante a consolidação , as memórias em formação estão mais vulneráveis a interferências. Os primeiros estudos os quais postularam as estruturas cerebrais envolvidas na memória foram realizados pelo pesquisador Penfield em 1954. Seus experimentos postularam o envolvimento do córtex temporal medial na memória. Posteriormente, estudos envolvendo indivíduos com lesões cerebrais seletivas, implicaram o hipocampo, bem como outras estruturas do lobo temporal medial, como o córtex entorrinal e parahipocampal, nos processos de aquisição e consolidação da memória. Entretanto, foi observado que pacientes com lesões no hipocampo não perdiam suas memórias remotas, o que foi considerado um

indicativo que as memórias são armazenadas somente por um período temporário no hipocampo (MILNER; SQUIRE; KANDEL, 1998; SQUIRE, 2009).

O ponto de partida para o estudo das bases moleculares da consolidação da memória foi a descoberta do modelo *in vitro* da potencialização de longa duração (LTP), fenômeno eletrofisiológico que se baseia na aplicação de um breve estímulo de alta frequência em qualquer uma das vias do hipocampo induzindo um fortalecimento das sinapses que pode durar horas. Simultaneamente, com a utilização de modelos animais, de ferramentas farmacológicas e de biologia molecular foi demonstrado que os mecanismos da LTP e da formação da memória compartilham bases moleculares similares (IZQUIERDO; MEDINA, 1997). Um exemplo é o clássico mecanismo que descreve a ativação de receptores NMDA durante a LTP *in vitro*, o qual é também é observado no aprendizado na tarefa do labirinto aquático de Morris. Posteriormente, foi também observado o aparecimento da LTP durante o aprendizado *in vivo* (MORRIS et al., 1986).

Para o desenvolvimento da LTP e da formação da memória faz-se necessária a ativação de receptores alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico (AMPA). A ativação dos receptores AMPA causa uma despolarização da membrana, o que possibilita a ativação dos receptores NMDA. Ainda, é observado um consequente aumento da atividade das proteínas cinases CamKII e proteína cinase dependente de cálcio (PKC) (IZQUIERDO et al., 2006). Além disso, durante a consolidação da memória, também são observados dois picos de ativação da proteína cinase dependente de AMPc (PKA), a qual é requerida imediatamente e de três à seis horas após o aprendizado (BERNABEU et al., 1997).

1.2 Neuroquímica e anatomia do sistema colinérgico

O sistema colinérgico está envolvido com diversas funções no SNC, entre as quais estão incluídas a plasticidade neuronal e o comportamento. Ainda, o sistema autônomo também possui neurônios colinérgicos os quais realizam, por exemplo, o controle de secreções glandulares (WESS; EGLEN; GAUTAM, 2007). A principal característica do sistema colinérgico é a presença de neurônios capazes de sintetizar, armazenar e liberar o neurotransmissor acetilcolina (ACh). Ainda, são encontrados neste sistema neurônios com a capacidade de liberar outros

neurotransmissores, como o glutamato (ALLEN; ABOGADIE; BROWN, 2006; REN et al., 2011). Além disso, há casos em que células não neuronais secretam ACh como é observado em cardiomiócitos (ROY et al., 2013). A síntese do neurotransmissor ACh, dá-se a partir da captação do aminoácido colina, processo realizado pelo transportador de alta afinidade de colina (CHT1) (PRADO et al., 2013) e do grupamento acetil, proveniente do metabolismo da acetil-Coa. Essa reação é catalisada pela enzima colina acetil transferase (CHAT) ocorrendo no citoplasma e nos terminais nervosos (PRADO et al., 2013). Antes de sua liberação, a ACh é acumulada em vesículas nos terminais nervosos. A molécula mediadora da translocação das moléculas de ACh para as vesículas é o transportador vesicular de ACh (VAChT). Este transportador faz a troca de dois prótons por cada molécula de ACh transportada, e este processo é conhecido por ser uma etapa crucial para a liberação de ACh (PRADO et al., 2013) (Fig. 1).

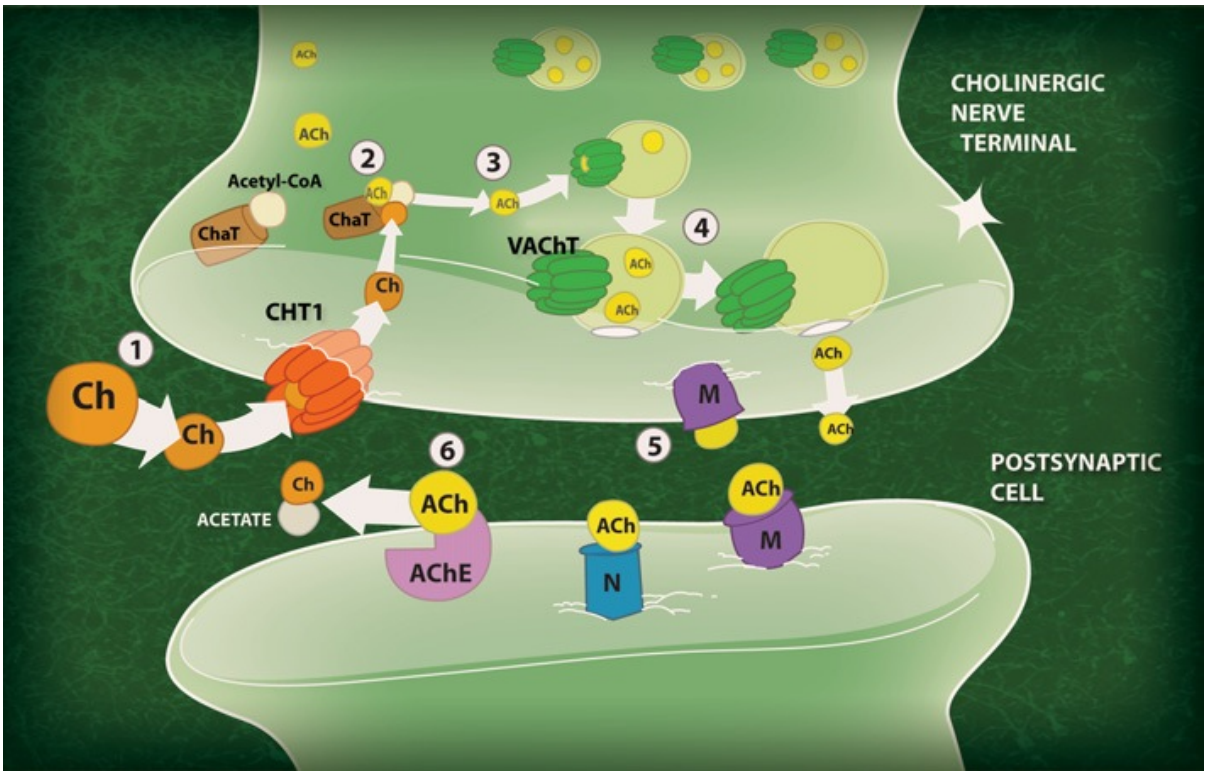

Figura 1. Processos de síntese, empacotamento, liberação e degradacao de acetilcolina. 1- Captação do aminoácido colina pelo CHT1; 2- Síntese da ACh apartir da colina captada e da Acetil-CoA pela enzima ChaT; 3- Captação intravesicular de ACh pelo transportador VAChT; 4,5- Liberação da ACh na fenda sináptica e ativação de receptores muscarínicos e/ou nicotinícos; 6- Degradação da ACh pela enzima colinesterase em colina e acetato. Prado et al., 2013.

Após sua liberação na fenda sináptica, a ACh, pode atuar tanto em receptores muscarínicos como em receptores nicotínicos. Os receptores muscarínicos são classificados em dois grupos dependendo de sua atividade. Os subtipos M1, M3 e M5 pertencem ao grupo considerado receptores excitatórios e são acoplados preferencialmente ás proteínas G da família G_q/G_{11} (WESS; EGLEN; GAUTAM, 2007). Os receptores muscarínicos do subtipo M2 e M4 acoplam preferencialmente a proteínas G da família G_i/G_0 , e apresentam um caráter predominante inibitório (WESS; EGLEN; GAUTAM, 2007). A ativação destes receptores pelo agonista ACh induz a diferentes eventos fisiológicos no sistema nervoso central e nos tecidos alvo do sistema autônomo parassimpático em diferentes situações (WESS; EGLEN; GAUTAM, 2007). Os receptores muscarínicos tem ampla distribuição no SNC. O M1, o qual é o subtipo predominantemente pós-sináptico, apresentam uma ampla distribuição em diferentes regiões do SNC com uma proeminente expressão no hipocampo e córtex cerebral, além da amígdala e nos *medium spiny neurons* do estriado (SPINDEL, 2012). Já o receptor M2, considerado preferencialmente pré-sináptico, tem uma expressão esparsa no hipocampo e no córtex cerebral. Similarmente, o receptor M3, que apresenta formas pré-sinápticas e pós-sinápticas, tem sua expressão direcionada ao hipocampo e ao córtex cerebral (SPINDEL, 2012; WESS; EGLEN; GAUTAM, 2007). O receptor M4 se expressa em áreas corticais incluindo o hipocampo e amígdala, com uma proeminente expressão nos neurônios do estriado e também possui projeções pré-sinápticas e pós-sinápticas (TICE et al., 1996). Os receptores M5 possuem menor expressão no SNC em geral, especificamente na substância *nigra* e área ventral tegmental (VTA)(TICE et al., 1996).

A anatomia do sistema colinérgico no SNC é classificada de acordo com o padrão de inervação da população de neurônios colinérgicos, sendo classificada em seis subgrupos Ch1-Ch6 (MESULAM et al., 1983) (Fig. 2).

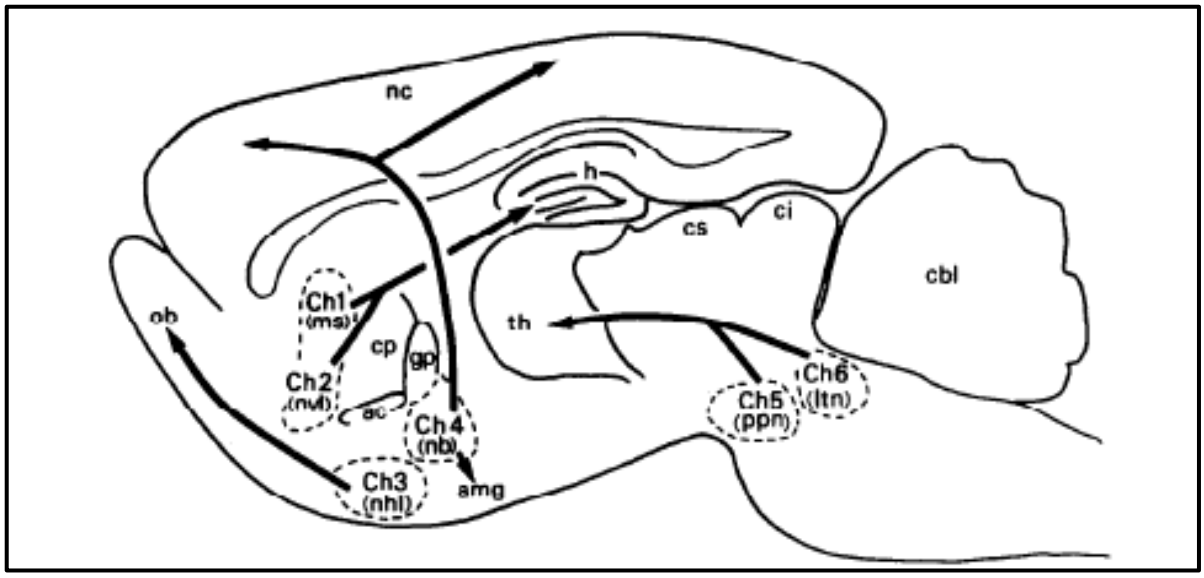

Figura 2. Neuroanatomia do sistema colinérgico no sistema nervoso central e padrão de inervação colinérgica. Ch1-Ch2 grupo de neurônios colinérgicos com origem no septo medial e banda diagonal, os quais inervam o hipocampo e uma pequena porção do córtex cingulado; Ch3 porção dos neurônios da banda diagonal os quais inervam o bulbo olfatório; Ch4 neurônios localizados no núcleo basalis magnocelularis e substância inominata os quais inervam o córtex e amídala; Ch5-Ch6 neurônios do tronco encefalico com invervação ao tálamo. Adaptado de (MESULAM et al., 1983).

O grupamento Ch1 (septo medial) e Ch2 (braço vertical da banda diagonal de Brocca) apresentam projeções para as regiões do hipocampo e do córtex cingulado (WOOLF; ECKENSTEIN; BUTCHER, 1984). Já o grupamento Ch3 (braço horizontal da banda diagonal de Brocca) apresenta um grupamento de neurônios ventral ao grupo Ch2 com projeções para o bulbo olfatório (MESULAM et al., 1983). Os neurônios do grupamento Ch4 (Núcleo basalis magnocelularis/substância *innominata*) inervam a maioria do córtex cerebral, além da região da amígdala (MESULAM et al., 1983). Ainda, os grupamentos Ch5-Ch6 são componentes do núcleo pedúnculo pontino e inervam principalmente o tálamo. Recentemente, foram observadas inervações para o estriado e neoestriado provenientes desses grupamentos (DAUTAN et al., 2014; MESULAM et al., 1983). Diferentemente dos grupamentos Ch1-Ch6, a inervação colinérgica para o estriado é principalmente local, o estriado possui um população em torno de 5% de interneurônios colinérgicos com uma vasta arborização capaz de cobrir uma grande área

(SPINDEL, 2012). Em relação à distribuição dos axônios colinérgicos nas diferentes regiões, no córtex todas as camadas recebem inervação colinérgica, sendo essa a mais proeminente na camada V. No hipocampo, a maioria dos terminais se localiza no extrato lacunoso molecular e oriens, enquanto pouca inervação se observa nas células piramidais. Na amígdala, são observadas poucas conexões colinérgicas, entretanto se observam projeções em todos os subnúcleos da amídala com maior densidade na região do núcleo basolateral (SPINDEL, 2012).

1.3 Sincronia Neuronal

1.3.1 Efeitos da transmissão colinérgica sobre a sincronia neuronal e plasticidade sináptica

A sincronia neuronal está relacionada com diferentes tipos de processamento cognitivo, como no aprendizado, memória e atenção além de participar nos movimentos corporais voluntários (HASSELMO, 2005). A atividade oscilatória de populações neuronais é um dos eventos relacionados à sincronia neuronal e é responsável pela coordenação da população neuronal para executar funções cognitivas (COLGIN, 2013). Por exemplo, durante a apresentação de um estímulo condicionado (CS) no teste de medo condicionado, a amígdala lateral e a região CA1 do hipocampo tornam-se sincronizadas em ritmo *theta*, o que parece melhorar a comunicação entre estas regiões durante a evocação desta memória aversiva (SEIDENBECHER; LAXMI; STORK, 2003).

Evidências sólidas da modulação colinérgica no córtex e hipocampo por diferentes tipos de oscilações têm sido demonstradas (FISAHN et al., 1998, 2002; GIVENS; OLTON, 1994, 1990). Projeções provenientes do septo medial para o hipocampo estão implicadas na modulação de oscilações neuronais no hipocampo. O ritmo *theta,* que é um tipo de oscilação neuronal, entre 4–12-Hz, é sugerido que seu o surgimento no hipocampo esteja corelacionado a evocação da memória. No entanto, este tipo de oscilação também está ligado a outros fenômenos como a ansiedade. Embora a função do ritmo *theta* ainda esteja em debate, alguns autores sugerem que este esteja coordenando às diferentes fontes de informação multimodal que chegam ao hipocampo e ao mesmo tempo ligando-as ao estado emocional (COLGIN, 2013) (Fig. 3).

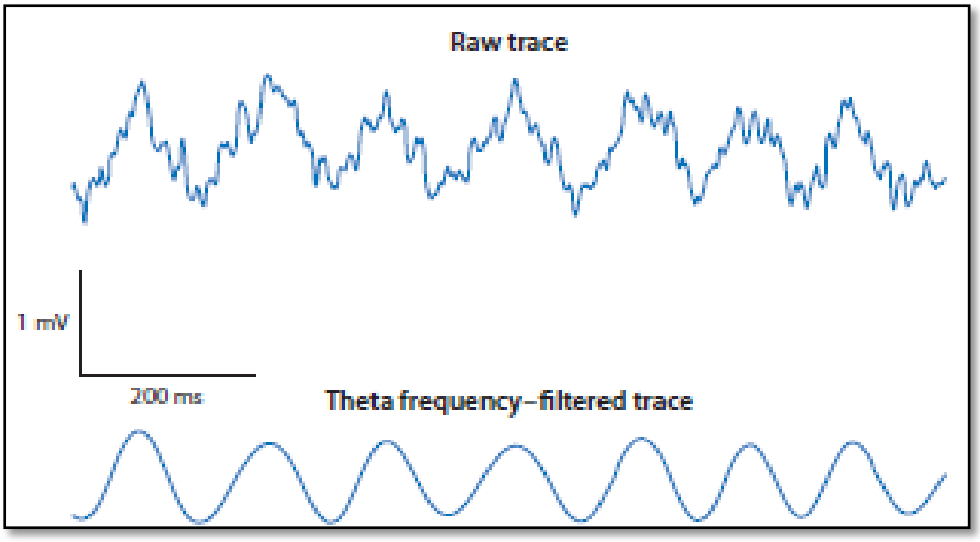

Figura 3 Exemplo de ritmo theta no hipocampo adquirido durante a exploração de um ambiente novo. Adaptado de Colgin, 2013.

O ritmo *theta* pode ser modulado pela sinalização colinérgica provavelmente pela aumento da sua amplitude. Em acordo com a essa observação, neurônios colinérgicos do septo medial não apresentam taxa de disparo em ritmo *theta* e apresentam uma baixa taxa de disparos. Assim, lesões seletivas aos neurônios colinérgicos do septo medial (MS) prejudicam o *theta power* mas deixam intacta a frequência do *theta* no hipocampo (BRAZHNIK; FOX, 1999; LEE et al., 1994; SIMON et al., 2006). Além disso, a estimulação dos neurônios colinérgicos do septo medial suprime as *sharp wave ripples* e acabam suprimindo o *poder* das ondas supra *theta* e oscilações lentas na região CA1 levando a consequente predominância das oscilações na faixa *theta* (VANDECASTEELE et al., 2014). Desta mesma maneira, a liberação fásica de ACh no hipocampo está altamente acoplada com o ritmo *theta* entretanto, essa liberação fásica não é responsável pela geração do ritmo *theta* devido ao atraso observado entre a liberação fásica de Ach e a iniciação do *theta* em mamíferos (ZHANG; LIN; NICOLELIS, 2010). Além disso, a inativação farmacológica de ambos MS e núcleo basal magnocelular (NBM) prejudica a memória e, mais especificamente, a lesão no MS diminui o *power* do ritmo *theta* no hipocampo o que foi correlacionado com o desempenho da memória (GIVENS; OLTON, 1994, 1990). A VTA também participa da geração do ritmo *theta* no hipocampo, sendo esse efeito é considerado indireto e relacionado às

conexões da VTA ao MS, pois foi observado que o efeito encontra-se abolido quando o MS é inativado (FITCH et al., 2006; ORZEŁ-GRYGLEWSKA et al., 2012). Além disso, as vias dopaminérgicas do MS e da banda diagonal de Brocca (DB) modulam neurônios que apresentam ritmo *theta* no MS, através da ativação de receptores D1/5 (FITCH et al., 2006).

A plasticidade sináptica (SP) é um dos mecanismos pelos quais o sistema nervoso é capaz de modificar a valência das sinapses e, consequentemente, modificar as redes neurais. A SP é um conjunto de mecanismos essenciais pelo qual o SNC pode formar, perder, potencializar ou enfraquecer conexões dos circuitos neuronais. Especificamente, a LTP é uma forma de plasticidade sináptica relacionada a diferentes tipos de plasticidade, que são dependentes de experiência e pode ser induzidas pelo aprendizado em diversos paradigmas (MALENKA; BEAR, 2004; WHITLOCK et al., 2006).

No hipocampo, a transmissão colinérgica possui uma importante participação na manutenção da LTP (AUERBACH; SEGAL, 1994, 1996; DREVER; RIEDEL; PLATT, 2011; LEUNG et al., 2003; MARTYN et al., 2012; OVSEPIAN; ANWYL; ROWAN, 2004). A sinalização muscarínica foi primeiramente implicada em estudos utilizando carbacol, um agonista muscarínico que tem a capacidade de simular a LTP elétrica na região CA1 (AUERBACH; SEGAL, 1994, 1996). Este tipo de LTP possui mecanismos similares a LTP elétrica, como aumento da resposta de receptores AMPA e ativação de receptores NMDA (DREVER; RIEDEL; PLATT, 2011). Em modelos geneticamente modificados, a deleção da proteína VAChT no prosencéfalo basal (PB) interrompe a liberação de ACh, prejudicando a LTP *in vitro* no hipocampo (MARTYN et al., 2012). Além disso, a ativação de receptores M1 facilita a indução da LTP no hipocampo, através da inibição de canais de cálcio de baixa condutância SK (BUCHANAN et al., 2010; GIESSEL; SABATINI, 2010). Essa ativação acontece pelo recrutamento da proteína cinase PKC, a qual inibe os canais SK tendo como efeito final um melhoramento da função dos receptores NMDA (BUCHANAN et al., 2010; GIESSEL; SABATINI, 2010).

O sistema colinérgico também contribui para outros tipos de plasticidade sináptica, como a depressão de longa duração (LTD) (CARUANA; WARBURTON; BASHIR, 2011; DICKINSON et al., 2009; HUANG; HSU, 2010; JO et al., 2010;

SCHEIDERER et al., 2008; VOLK et al., 2007). A LTD está correlacionada com o aprendizado e armazenamento de informações e se relaciona a processos que requerem flexibilidade cognitiva, como a extinção da memória e a flexibilidade comportamental (COLLINGRIDGE et al., 2010). Similarmente ao observado na LTP, o carbacol tem a capacidade de induzir LTD no hipocampo, o que se relaciona com a sinalização muscarínica (CARUANA; WARBURTON; BASHIR, 2011; JO et al., 2010). A ativação dos receptores muscarínicos induzida por carbacol na LTD, induz a endocitose de receptores NMDA (JO et al., 2010). Conjuntamente, ocorre a fosforilação da subunidade GluA2 dos receptores AMPA o que causa sua endocitose (DICKINSON et al., 2009). Estes mecanismos mostram que a ativação de receptores muscarínicos diminui a transmissão glutamatérgica no hipocampo causando a LTD. A síntese proteica também e um fator essencial para LTD, sendo que a ativação de receptores muscarínicos induz a síntese proteica durante a LTD (VOLK et al., 2007). Os mecanismos que causam a indução da síntese proteica envolvem a ativação das cinases ERK, mTOR e Src, e se sabe que as atividades destas cinases são requeridas para o desenvolvimento da LTD e LTP (SCHEIDERER et al., 2008; VOLK et al., 2007).

Os receptores muscarínicos do tipo M2 tem um papel crucial para a plasticidade sináptica no hipocampo. A falta deste receptores reduz a LTP e interrompe a potencialização de curta duração (STP) na via Schaffer-CA1, a qual leva informação da região CA3 para região CA1, através da disrupção da inibição gabaérgica nos neurônios da região CA1 (SEEGER et al., 2004). O bloqueio de receptores M2 também é capaz de prejudicar a LTP induzida por Cch (AUERBACH; SEGAL, 1996). Além disso, o receptor M2 parece modular ambos os EPSP e IPSP, com proeminente efeito sobre o IPSP (SEEGER et al., 2004). Zheng e colaboradores (2012) demonstraram que os receptores M2 podem regular diferencialmente a LTP na via AC-CA3 e MF-LTP, contribuindo para o desenvolvimento da AC-CA3 LTP e reduzindo a intensidade da MF-LTP (ZHENG; WESS; ALZHEIMER, 2012).

1.4 Efeitos da transmissão colinérgica sobre a memória de trabalho

A memória de trabalho (WM) pode ser definida em animais como um tipo de memória de curta duração, para objetos, estímulos ou locais, sendo utilizada para

resolver ou realizar uma dada tarefa. Estas memórias, se não forem necessárias para próxima seção da tarefa não são armazenadas. Pode ser distinguida da memória de referência que é necessária para a etapa subsequente da tarefa e portanto precisa ser armazenada (DUDCHENKO, 2004). Estudos demonstram fortes evidências do envolvimento do hipocampo neste tipo de memória de trabalho, a ativação de receptores NMDA, assim como na memória do longa duração, tem participação nas sub-regiões do hipocampo (KESNER et al., 1996). Outras regiões também são importantes para a memória de trabalho, assim lesões na região medial do córtex pré-frontal (mPFC) e regiões infra e prelímbicas do mPFC induzem déficits na WM (KESNER et al., 1996).

O envolvimento do prosencéfalo basal (PB) colinérgico na WM é suportado por diversos trabalhos empregando diferentes métodos, abrangendo desde lesões do PB, manipulações farmacológicas e recentemente manipulações genéticas (DURKIN; TOUMANE, 1992; DURKIN, 1994; GUZMAN et al., 2011). Embora algumas controvérsias ainda permaneçam em relação aos estudos utilizando lesões, muitos outros suportam o papel do sistema colinérgico na WM . O aumento transitório da atividade colinérgica durante e após tarefas, em que a WM é requerida, podem ser observadas tanto no córtex como no hipocampo (DURKIN; TOUMANE, 1992; DURKIN, 1994). Posteriormente, estudos utilizando microdiálise confirmaram o aumento da liberação de ACh durante e depois procedimentos em que a WM era requerida, esse aumento pode auxiliar na manutenção da memória de trabalho durante a realização de várias tarefas (CHANG; GOLD, 2004; FADDA; MELIS; STANCAMPIANO, 1996; HIRONAKA et al., 2001)

Estudos utilizando ferramentas farmacológicas para inativação ou realização de lesões inespecíficas do prosencéfalo basal (PB) demonstraram o envolvimento destas regiões na WM. A inativação transitória do MS/DB ou do NBM prejudica similarmente a memória de trabalho, e mais especificamente lesões do MS diminuem o *theta* power, o que está correlacionado ao desempenho na WM (GIVENS; OLTON, 1994, 1990). Ainda, lesões combinadas do PB (ambos MS/DB e NBM) prejudicam a WM, mas poupam a memória de referência (KNOWLTON et al., 1985). Lesões específicas ao MS também prejudicam a WM, entretanto, lesões do NBM prejudicam a WM somente de maneira *delay* independente, o que pode refletir problemas em outros domínios cognitivos como atenção (DUNNETT;

EVERITT; ROBBINS, 1991; DUNNETT; ROGERS; JONES, 1989; NUMAN; QUARANTA, 1990).

Embora estes estudos apontem para um proeminente papel do PB na WM, dada a população de células não colinérgicas nesta região, métodos de estudos mais seletivos foram requeridos para explorar a função colinérgica. O advento de toxinas seletivas possibilitou pela primeira vez endereçar essa questão e isolar a contribuição colinérgica para a WM (WENK et al., 1994). Lesões colinérgicas seletivas no MS, com a toxina colinérgica 192 IgG-saporina, a qual é captada seletivamente por neurônios colinérgicos expressando os receptores de baixa afinidade p75 (WENK et al., 1994), prejudica o desempenho da WM em diferentes tarefas, o que implica a inervação colinérgica ao hipocampo ou córtex cingulado nestas tarefas (CHANG; GOLD, 2004; SHEN et al., 1996; WALSH et al., 1996). Em contraste, alguns trabalhos não observaram efeitos na WM após a ablação dos neurônios colinérgicos do MS, mesmo quando altos níveis de deleção foram observados (CHAPPELL et al., 1998; MCMAHAN; SOBEL; BAXTER, 1997).

Lesões inespecíficas no NBM mostraram déficits independentes do *delay* na WM (DUNNETT, 1985). Entretanto, as projeções corticais colinérgicas do NBM foram também analisadas por Chudasama e colaboradores (2004) em uma tarefa combinada de atenção e memória, em que lesões no NBM induziram déficits *delay* dependentes na WM somente quando os animais foram submetidos a uma alta demanda atencional na tarefa (CHUDASAMA et al., 2004).

Entretanto, algumas questões ainda permanecem quanto a extensão das lesões em relação aos déficits na WM e o quanto estas lesões podem abolir funcionalmente a liberação de ACh. Craig e colaboradores (1999) observaram que somente com altos níveis de lesão no PB, incluindo o MS e o NBM/SI, foram observados déficits na tarefa de WM no labirinto radial (WRENN; WILEY, 1999). Wrenn e colaboradores (1999) também relataram que mesmo altos níveis de lesão seletivamente ao MS não foram suficientes para causar prejuízos na WM (WRENN; WILEY, 1999). As variações nas observações em diferentes trabalhos podem ser explicadas pelo trabalho de Chang e colaboradores (2004), no qual mesmo com lesões seletivas com altos níveis de eficiência em neurônios colinérgicos, confirmados pela imunomarcação com CHAT, os animais ainda retinham 40% de

níveis residuais de liberação de ACh no hipocampo, mesmo assim estes indivíduos tiveram prejuízo na tarefa de alternação espontânea, os quais puderam ser revertidos com o tratamento com inibidores da colinesterase (CHANG; GOLD, 2004). Este relato reabre a necessidade de métodos mais seletivos para estudar o papel da transmissão colinérgica na WM. Assim, a possibilidade de deleção condicional de moléculas participantes da síntese da ACh pode ser uma resolução para esta questão, uma vez que já foi relatado que a deleção da proteína VAChT termina com a liberação de ACh em camundongos (GUZMAN et al., 2011).

Manipulações genéticas como a superexpressão de VAChT, a qual aumenta a liberação de ACh, prejudicam a WM na tarefa de Labirinto Aquático de Morris (LAM) e na tarefa de alternações espontâneas, além de outros domínios cognitivos, sugerindo que os níveis de ACh precisam ser propriamente regulados para manter a função do SNC (KOLISNYK et al., 2013). Adicionalmente, o *knockdown* da proteína VAChT em 40% não altera o desempenho na tarefa de alternâncias espontâneas (DE CASTRO et al., 2009).

O bloqueio da ativação dos receptores muscarínicos com escopolamina demonstra que a ativação destes receptores é importante durante as tarefas de labirinto radial e alternância espacial, as quais avaliam memória de trabalho (BYMASTER et al., 1993; GRANON et al., 1995; WIRSCHING et al., 1984). Ainda, infusões de escopolamina no mPFC produzem déficits na WM, o qual não estão relacionados a prejuízos atencionais (CHUDASAMA et al., 2004).

Diversos trabalhos têm demonstrado a participação dos receptores muscarínicos M2 na memória de trabalho em diferentes tarefas (BAINBRIDGE et al., 2008; SEEGER et al., 2004). Ainda, os receptores M2 estão envolvidos no mecanismo pelo qual o estradiol melhora a performance na WM, melhorando a ligação dos receptores NMDA ao glutamato induzida pelo estradiol (DANIEL; DOHANICH, 2001; DANIEL; HULST; LEE, 2005).

Os receptores muscarínicos M1 participam da memória de trabalho em diferentes tarefas. A ativação ou bloqueio farmacológico do receptor M1 mostra que a ativação destes receptores é essencial durante as tarefas de alternação espacial e *three-panel runway,* as quais testam memória de trabalho em roedores (BYMASTER et al., 1993; OHNO; YAMAMOTO; WATANABE, 1994; OHNO et al., 1994; UKAI;

SHINKAI; KAMEYAMA, 1995). Ainda, o bloqueio do receptor M1 no córtex infra límbico induz um decaimento na performance na tarefa de alternações espontâneas enquanto, a ativação destes melhora a performance neste tarefa (WALL; FLINN; MESSIER, 2001). Estes relatos demostram que a ativação dos receptores M1 modula a performance em tarefas em que a memória de trabalho é utilizada. Entretanto, o primeiro relato utilizando animais M1 nocaute *(*KO) não reportou alterações na WM, por outro lado animais com deleção similar demostraram prejuízos na tarefa de Win-shift no labirinto radial (ANAGNOSTARAS et al., 2003; MIYAKAWA et al., 2001). A despeito da importância para plasticidade neuronal, nenhum estudo demonstrou participação dos receptores M3, M4 e M5 na WM.

1.5. Sistema colinérgico sobre a memória espacial de longa duração

A memória espacial é um tipo de memória declarativa dependente do hipocampo, estando envolvida na aquisição e evocação de informações relacionadas ao espaço (MORRIS et al., 1982). O principal fluxo de informação sensorial sobre o ambiente flui da camada II do córtex entorrinal (EC) para o hipocampo na região do giro dentado (DG), pela via perforante. A partir deste ponto, o DG se conecta com a região CA3 para depois levar a informação a região CA1 (NAKAZAWA et al., 2004). Posteriormente, a região CA1 remete fibras ao EC chegando às camadas 5/6 do EC, não obstante a informação chega simultaneamente às regiões CA3 e CA1 diretamente das camadas superficiais do EC (NAKAZAWA et al., 2004). A correlação entre o fluxo da informação sensorial ao hipocampo e a memória espacial pode ser correlacionada com a descoberta de neurônios no hipocampo e EC que aumentam sua taxa de disparos em regiões específicas de um ambiente, tornando-se menos ativas em outras posições neste mesmo ambiente chamadas, *place cells* CA1 (NAKAZAWA et al., 2004). Além disso, a descoberta do fenômeno de LTP no hipocampo e posterior correlação com a memória espacial no LAM, demonstra que o bloqueio farmacológico de receptores NMDA bloqueia a indução da LTP e prejudica a aquisição da memória nessa tarefa (MORRIS et al., 1986). Essa relação foi posteriormente comprovada em animais NMDAR1 nocautes seletivamente para região CA1 do hipocampo, nos quais a transmissão sináptica se mostrava preservada, mas foram observados prejuízos na indução da LTP e de aquisição da memória espacial (TSIEN; HUERTA; TONEGAWA, 1996). Além disso, as cascatas de sinalização posteriores a ativação

dos receptores NMDA no hipocampo, como as cinase cálcio-calmodulina dependente II (CamKII), também estão envolvidas com a plasticidade sináptica hipocampal e formação da memória espacial (SILVA; STEVENS; TONEGAWA, 1992).

O estudo da relação entre a memória e o sistema colinérgico teve um impulso inicial com a descoberta de lesões em núcleos colinérgicos do PB em pacientes com a doença de Alzheimer, esta caracterizada pelo declínio cognitivo e perda de memória (COYLE; PRICE; DELONG, 1983). De encontro a isso, o desempenho no LAM sofre um declínio com o envelhecimento dos animais e se correlaciona com o número de neurônios Chat/NGFr positivos no PB. Além disso, o tratamento de animais velhos com o fator trófico NGF, melhora a desempenho destes animais no LAM conjuntamente com melhora nos níveis de atrofia de neurônios colinérgicos do NBM (FISCHER et al., 1987, 1992). Além disso, animais com disrupção em um dos alelos do gene do NGF apresentam déficits na tarefa de LAM e perda de neurônios colinérgicos no septo medial (CHEN et al., 1997). Além disso, animais idosos KO para o receptor nicotínico β2 apresentam a performance prejudicada na tarefa de LAM (ZOLI et al., 1999).

Estudos utilizando lesões dos núcleos colinérgicos do PB também endereçaram a questão do papel do sistema colinérgico na memória espacial. Lesões não específicas no NBM prejudicam a aquisição da tarefa de LAM, o que pode ser atenuado com o transplante de células produtoras de ACh no córtex dos animais lesionados (WINKLER et al., 1995). Além disso, lesões seletivas em neurônios colinérgicos do NBM causam déficits na aquisição do LAM e labirinto radial, no entanto lesões similares realizadas no septo medial falharam em observar prejuízos (BERGER-SWEENEY et al., 1994; MURRAY; FIBIGER, 1985). Ainda, lesões inespecíficas do MS/DB prejudicam o aprendizado no LAM, o que poderia ser explicado pela lesão concomitante de células gabaérgicas e glutamatérgicas presentes no MS/DB (HAGAN et al., 1988). Em fato, as células colinérgicas do MS/DB parecem ter um papel mais importante na manutenção da memória espacial, já que lesão nesta população celular não afeta o aprendizado no LAM , mas prejudica a memória remota (LECOURTIER et al., 2011). Além disso, manipulações farmacológicas dos receptores colinérgicos, além do bloqueio sistêmico ou central de receptores nicotínicos ou muscarínicos, prejudicam a aquisição e retenção da

memória espacial (DECKER; MAJCHRZAK; ANDERSON, 1992; DECKER; MAJCHRZAK, 1992; NAKAGAWA; TAKASHIMA, 1997).

Mais recentemente, manipulações genéticas abordam a questão do sistema colinérgico e da memória espacial. Animais em que a liberação tônica de ACh foi abolida no PB demostram apenas déficits moderados na tarefa de LAM, enquanto que os mesmos animais tem um prejuízo na tarefa reversa em que a plataforma é deslocada para o quadrante oposto (MARTYN et al., 2012). Além disso, a disrupção da transmissão colinérgica tônica prejudica a LTP no hipocampo (MARTYN et al., 2012). Entretanto, animais KO para o receptores muscarínicos M1, M3, M4, além de receptores nicotínicos β2, não apresentam déficits na tarefa de LAM (ANAGNOSTARAS et al., 2003; KOSHIMIZU; LEITER; MIYAKAWA, 2012; MIYAKAWA et al., 2001; YAMADA et al., 2001; ZOLI et al., 1999).

O teste de pareamento de objeto ao lugar, do inglês *object-in-place paired-associated learning* (PAL), é uma tarefa *touchscreen* recentemente implementada para testar a memória espacial em roedores pelo seu alto grau de translacionalidade. Nesta tarefa, o animal tem que associar uma gravura específica a sua posição espacial para receber uma recompensa. Dentre as bases neuro anatômicas para a performance na tarefa de PAL, o hipocampo é essencial para a performance na tarefa. Além disso, a ativação de receptores glutamatérgicos AMPA e NMDA é requerida para a manutenção desta (TALPOS et al., 2009). Corroborando com estes achados, lesões no hipocampo não parecem afetar a aquisição da tarefa, mas prejudicam a evocação desta quando realizadas após os animais terem apreendido esta tarefa (DELOTTERIE et al., 2015). Além disso, concordando com estes dados, animais com a neurogênese bloqueada no hipocampo anteriormente ao início da tarefa de PAL, não tem prejuízos na aquisição desta (CLELLAND et al., 2009). Além disso, o estriado dorsal está envolvido na aquisição da tarefa possivelmente participando do componente motivacional desta, desde que animais com lesões excitotóxica nesta região não adquirem a tarefa e tem um aumento no tempo de latência para o recolhimento da recompensa (DELOTTERIE et al., 2015). Mutações no gene DGL2, da família do disk, causam prejuízos na aquisição da tarefa de PAL em camundongos, similarmente a humanos com mutações neste gene (NITHIANANTHARAJAH et al., 2013).

Os receptores muscarínicos estão envolvidos com a evocação da memória espacial na tarefa de PAL, mais especificamente o receptor M1 parece ser crucial para a performance no PAL (BARTKO et al., 2011a). O bloqueio de receptores M1 em animais já treinados na tarefa prejudica a performance, entretanto animais KO para o receptor M1 não possuem déficits na aquisição e performance da tarefa (BARTKO et al., 2011a, 2011b). Entretanto, o bloqueio de ambos receptores colinérgicos nicotínicos e muscarínicos no hipocampo não prejudica a performance em animais já treinados na tarefa (TALPOS et al., 2009).

1.6. Sistema Cre-LOX

O uso de animais geneticamente modificados na pesquisa biomédica tem se popularizado nos últimos anos. A disponibilidade comercial de diferentes linhagens de camundongos e o aparecimento de novas tecnologias, as quais facilitam a obtenção destes animais, tem impulsionado o uso de animais geneticamente modificados. Além disso, o aumento da complexidade e especificidade requeridas para a resolução dos novos problemas científicos ocasionaram uma demanda para o uso destes animais (TSIEN; HUERTA; TONEGAWA, 1996).

Um dos modelos de animais mais úteis no estudo da biologia de determinado gene e também sua implicação para doenças são os animais *knockout*. Estes animais possuem mutações em um gene endógeno específico as quais, interrompem a função deste gene. Estes animais foram gerados pela manipulação de células tronco, em que uma construção contendo regiões homólogas ao gene e mutações introduzidas é transfectada nestas células. Posteriormente, estas células são selecionadas e introduzidas em embriões no estágio de blastocisto, os quais são transferidos para “mães de aluguel” para geração destes embriões (DOYLE et al., 2012).

No entanto, esta estratégia gera animais *knockout* para todas as células do corpo do animal, o que pode ser uma desvantagem em alguns casos. Um exemplo a ser citado, é o caso da deleção da proteína VAChT, a qual causa letalidade pós-natal (PRADO et al., 2006). Para superar estes problemas foi criado um sistema para deleção condicional de um dado gene, o sistema Cre-Lox. Cre-Lox é um sistema presente nos bacteriófagos que permite a recombinação de genes bacterianos por este vírus. Parte deste sistema, a enzima Cre é uma recombinase

que reconhece pequenas sequências de nucleotídeos chamadas LoxP, recombinando as regiões flanqueadas por estas sequências(DOYLE et al., 2012). O uso do transgene da Cre recombinase em camundongos possibilitou juntamente com o flanqueamento de partes específicas de genes com sequências LoxP uma recombinação gênica específica, para geração de knockout condicionais. Um exemplo no caso do problema da proteína VAChT foi usar o sistema Cre-Lox para flanquear dois exóns específicos do gene, gerando uma linhagem VAChT flox-flox e fazer o cruzamento com uma linhagem transgênica Cre sobre controle do promotor do receptor dopaminérgico D2. Como resultado, causou a deleção do VAChT seletivamente no estriado, evitando a letalidade (GUZMAN et al., 2011).

Diante do exposto acima nesta introdução, esse trabalho se propôs a utilizar linhagens de camundongos knockout condicionais para a proteína VAChT em regiões específicas do SNC para avaliar o papel de circuitos colinérgicos no comportamento. Este trabalho buscou avaliar a participação do sistema colinérgico do prosencéfalo basal nos processos de memória e atividade motora utilizando métodos de ótima especificidade. Mesmo que muito estudados o papel do sístema colinérgico nesse processo ainda está em debate, sendo que, nosso trabalho procurou agregar novos conhecimentos a este tema.

2. OBJETIVOS

2.1 Objetivo Geral

- Caracterizar o papel da liberação de acetilcolina nos núcleos basais do prosencéfalo sobre o desempenho cognitivo e motor em camundongos knockouts para o transportador de acetilcolina (VAChT) no prosencéfalo e nos núcleos colinérgicos

2.2 Objetivos específicos

1) Avaliar por imunofluorescência e *imunoblotting* os níveis das proteínas VAChT e CHAT no SNC nos camundongos $VAChT^{nk\text{-}Cre\text{-}flox/flox}$.
2) Determinar a função cognitiva dos camundongos $VAChT^{nk\text{-}Cre\text{-}flox/flox}$ nos testes de labirinto aquático de Morris (LAM) e PAL.
3) Verificar a função cognitiva dos camundongos $VAChT^{flox/flox}$ injetados com AAV-Cre no septo medial através dos testes LAM e de PAL.
4) Investigar por imunofluorescência os níveis das proteínas VAChT e CHT1 no SNC dos camundongos $VAChT^{flox/flox}$ injetados com AAV-Cre no septo medial ou NBM.
5) Determinar os níveis de VAChT e CHAT no SNC dos camundongos $VACh^{Tflox/flox}$ injetados com AAV-Cre no MS/DB.
6) Verificar a atividade locomotora dos camundongos $VAChT^{nk\text{-}Cre\text{-}flox/flox}$ no teste campo aberto e em caixas metabólicas.
7) Avaliar a atividade locomotora dos camundongos $VAChT^{flox/flox}$ injetados com AAV-Cre no teste de campo aberto.

CAPÍTULO 1

REVIEW PAPER

Medial septum modulation by cholinergic, GABAergic and glutamatergic systems in the sensorial information processing mechanisms: implications for aversive learning

(Este manuscrito será submetido à revista *Frontiers in Neural Circuits*)

Running title: Medial septum in aversive learning

Authors: Gustavo M. Parfitt[1], André P. Koth[1], Carolina Silva Peixoto[1], Ana Paula Horn[1, 2], Daniela M. Barros[1, 2]

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[1] Programa de Pós-graduação em Ciências Fisiológicas, Fisiologia Animal Comparada - Laboratório de Neurociências - (FURG)

[2] Instituto de Ciências Biológicas, Universidade Federal do Rio Grande (FURG), Rio Grande, RS, 96210-900, BRAZIL.

Correspondence concerning this article should be addresssed to Daniela Martí Barros - Laboratório de Neurociências, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande (FURG) – Av Itália, Km 8 - Rio Grande, RS, 96203-900, BRAZIL

e-mail: barrosdm@yahoo.com.br

ABSTRACT

The medial septum (MS) and diagonal vertical band of Brocca (DB) are part of the basal forebrain nuclei and have a diverse population of neurons in an intricate network. Such neuronal population contain cholinergic, GABAergic and glutamatergic neurons, they project mainly to the hippocampus through the fimbria fornix, supracalosal stria and ventral pathway. In this review we discussed the mechanisms by which the medial septum modulates the neuronal activity in the hippocampus focussing in the mechanisms of neuronal plasticity and brain rhythms. We also discuss an emerging field in the cholinergic studies which is the modulation of GABAergic cells activity by cholinergic transmition. Lastly, we present behavioural evidences relating MS modulation of fear learning more specifically fear conditioning and inhibitory avoidance. In general, the medial septum and cholinergic cortical projections activation seem to be particularly significant for behaviour relevant stimuli processing. In addition, during the aversive conditioning the MS/DB is involved in the molecular mechanisms of plasticity and neuronal synchrony supporting the brain areas where the information is processed.

Keywords: synaptic plasticity; aversive learning; medial septum

1. INTRODUCTION

The medial septum (MS) and diagonal vertical band of Brocca (DS) are part of the basal forebrain nuclei and have a diverse population of neurons in an intricate network (Colom et al., 2005). Such neuronal population contain cholinergic, GABAergic and glutamatergic neurons, they project mainly to the hippocampus through the fimbria fornix, supracalosal stria and ventral pathway (Niewiadomska et al., 2009). MS/DB has been implicated in some aspects of the hippocampal function and its related behaviours. The processing of information is a particular role of the MS/DB which is important to different aversive paradigms as inhibitory avoidance, fear conditioning and others (Calandreau et al., 2006; Mitsushima et al., 2013; Lovett-Barron et al., 2014). In this review we discussed at first the mechanism by which the medial septum modulate the neuronal activity in the hippocampus focussing in the mechanisms of neuronal plasticity and brain rhythms. In this section, we describe the different neuronal population of the MS and their contributions for these mechanisms in the hippocampus. In a second part we discuss an emerging field in the cholinergic studies which is the modulation of GABAergic cells activity by cholinergic transmition. Lastly, we present behavioural evidences relating MS modulation of fear learning more specifically fear conditioning and inhibitory avoidance.

2. ACTIVATION OF THE BASAL FOREBRAIN CONSEQUENCES ON NEURONAL OSCILLATIONS AND SYNAPTIC PLASTICITY

2.1 Medial septum activation and its consequences for the hippocampus processes

2.1.1 Cholinergic projections

The neuronal synchrony is related to different types of cognitive processing and behaviours, such as learning, memory, attention and voluntary movement (Hasselmo, 2005). The oscillatory activity is one of the events related to neuronal synchrony and, is responsible by the coordination of the neuronal population to execute cognitive functions (Colgin, 2013). For example, during the CS presentation in fear conditioning, the lateral amygdala and the CA1 region of the hippocampus

become synchronized at theta rhythm which may improve the communication between these regions during memory retrieval (Seidenbecher et al., 2003).

Solid evidences have shown the cholinergic modulation in different types of oscillation in both, cortex and hippocampus (Givens and Olton, 1990, 1994; Fisahn et al., 1998, 2002). Cholinergic septal projections to the hippocampus have been implicated in the modulation of hippocampal oscillations, as an example; the theta rhythm a type of the oscillations ranging from ~ 4– 12-Hz, is modulated by cholinergic signalling probably by the enhancement of its amplitude. In agreement, cholinergic neurons from the MS do not fire at theta range and display a slow fire rate, even thought, selective lesions of cholinergic neurons in the MS can disrupt the theta power, but spare the theta frequency (Simon et al., 2006; Brazhnik and Fox, 1999; Lee et al., 1994). Moreover, stimulation of medial septal cholinergic neurons suppresses the sharp wave ripples and, reduces the power of supra theta and slow oscillations in the CA1 region leading to a predominance of theta oscillations (Vandecasteele et al., 2014). In the same manner, phasic ACh release to the hippocampus was found to be highly coupled with theta rhythm, induced by tail pinch, however, this phasic realise is not responsible by the generation of theta due to a delay in the phasic realise and the theta initiation (Zhang et al., 2010). In addition, transient pharmacological inactivation of both MS and NBM can disrupt memory, and more specifically the MS lesion can diminish the theta power in the hippocampus which was correlated with the memory performance (Givens and Olton, 1990, 1994). Ventral tegmental area (VTA) region also participates in theta generation in the hippocampus, this effect is indirect and it is related to VTA-septal connections, since the effect is abolished when the MS is inactivated (Fitch et al., 2006; Orzeł-Gryglewska et al., 2012). In addition, cathecominergic innervation, via D1/5 receptors seems to tonic regulate the theta burst basal occurrence in the MS/DB (Fitch et al., 2006).

Pharmacological evidence also has shown that the activation of cholinergic receptors is able modulate different types of oscillations in the hippocampus. Besides of theta modulation, cholinergic transmission can modulate of gamma oscillations, which are important for cortical processing. This modulation is dependent of muscarinic activation with subsequent augmentation of Icat and Ih currents, mainly through M1 receptors. Others muscarinic receptors did not seem to

be important for this phenomenon (Fisahn et al., 1998, 2002; Fellous and Sejnowski, 2000).

In the nervous system, synaptic plasticity (SP) is one of the mechanisms that are able to change the weight of the synapses, consequently, modifying them. SP is an essential conjunct of mechanisms by which the CNS form, get rid, potentiate and/or weak connections of neuronal circuits. Specifically, long term potentiation (LTP) is a form of synaptic plasticity related with different types experience dependent plasticity, and can be induced by fear conditioning and inhibitory avoidance paradigms (Malenka and Bear, 2004; Whitlock et al., 2006).

Previously, the participation of cholinergic system in synaptic plasticity was described by different experimental conditions for example, the addition of low concentrations of carbacol (Cch), a general muscarinic agonist, induces LTP in hippocampal slices *in vitro* (Auerbach and Segal, 1994, 1996). The LTP induced by Cch (Cch-LTP) is a pure postsynaptic type of plasticity, related to the increase in AMPAr response and, depends on NMDAr activation (Drever et al., 2011). The abolishment of ACh release by the deletion of its vesicular transporter VAChT, disrupts the LTP *in vitro* (Martyn et al., 2012). In addition, to these *in vitro* observations, *in vivo* LTP protocols confirmed the role of cholinergic signalling in LTP modulation. It was observed that free walking mice showed a reduced LTP in the hippocampus after MS/DB cholinergic lesions or after the block of muscarinic receptors (Leung et al., 2003). Similar observation was made, concerning the muscarinic receptors, using a weak LTP training in anesthetised mice. Furthermore, MS stimulation reduces the threshold for the LTP induction and this facilitation has been shown to be dependent of muscarinic receptors (Ovsepian et al., 2004; Leung et al., 2003).

Cholinergic transmission also has a role in other type of plasticity long term depression (LTD). LTD is correlated with learning and information storage, it has been related to processes which require cognitive flexibility such as extinction and behaviour flexibility (Collingridge et al., 2010). Carbacol also can induce LTD in the hippocampus and medial pre frontal cortex (mPFC) which is called muscarinic LTD (mLTD) (Jo et al., 2010; Caruana et al., 2011). Moreover, the endocytosis of NMDA receptors has been shown to be an important mechanism for mLTD. During mLTD

the activation of muscarinic receptors, thought the release of Ca^{2+} from intracellular stores, induces recruitment of the hippocalcin protein to the cell surface membrane causing a NMDARs endocytosis (Jo et al., 2010). This induced dissociation of hippocalcin from the PSD-95 enables NMDARs to dissociate from PSD-95, which results in the binding of B-adaptin to NMDARs to promote its endocytosis (Jo et al., 2010). The dephosphorylation and posterior endocytosis of GluA2-containing AMPA receptors is also been suggested as mechanism of mLTD in the hippocampus. The activation of muscarinic receptors induces the interaction of GluA2 subunit with the protein liprin-α, process mediated by GRIP, a PDZ containing protein that commands GluA2 membrane trafficking, this interaction cause the GluA2 dephosphorylation , possibly by LAR receptor tyrosine phosphatase (Dickinson et al., 2009). This mechanism of dephosphorylation of the GluA2 cause its subsequent internalization (Dickinson et al., 2009) (fig 1).

The cholinergic m-LTD is dependent on ERK, mTOR and Src kinase family activation, since the blocking of these kinases impairs the LTD development (Volk et al., 2007; Scheiderer et al., 2008). Protein synthesis is also involved in CCh LTD, raising the possibility that the activation of these aforementioned kinases is triggering new protein synthesis (Volk et al., 2007). Surprising, Cch LTD in the hippocampus does not require either PLC activation or PKC activity fact also observed in the muscarinic mediated GluA2 internalization (Scheiderer et al., 2008; Dickinson et al., 2009). Interestingly, the co-activation of M1 and α1 receptors, by agonists at low concentrations, induces LTD mediated by ERK activation. On the other hand, in the mPFC the mLTD has the participation of PLC, PKC and, the NOS-sCG-PKG signalling is crucial for its development (Huang and Hsu, 2010; Caruana et al., 2011).

Evidences of Gq coupled muscarinic receptors, such as M1 receptors, participation in LTP and LTD have been demonstrated in the past years. On the other hand, the hippocampal LTP, induced with theta burst stimulation, is just mildly affected by the ablation of the M1 receptors while the LTP induced by HFS do not display alterations (Anagnostaras et al., 2003). In fact, it has been known that the Carbacol can enhance the LTP and lower the threshold to induce it (Auerbach and Segal, 1994; Shinoe et al., 2005). Even though, some studies have been demonstrated that M1 or M3 KOs have an intact LTP, carbachol enhanced LTP is

not present in mice lacking M1 receptors but, it is still present in M3 lacking mice, pointing to a specific role of M1 in this enhancement (Auerbach and Segal, 1994; Shinoe et al., 2005). In addition, M1 receptor activation is able to facilitate the LTP induction in the CA1 region, and this facilitation is due to the inhibition of small-conductance Ca^{2+}-activated SK channels and K^+ channels (Buchanan et al., 2010; Giessel and Sabatini, 2010). Moreover, this inhibition of both SK channels and K^+ channels seems to enhance NMDARs function and be dependent of PKC recruitment (Buchanan et al., 2010; Giessel and Sabatini, 2010). Some authors may argue that M1 receptors are not required for LTP, but is required by other forms of plasticity, such as mglut LTD and mLTD (Volk et al., 2007; Kamsler et al., 2010; Jo et al., 2010). A possible mechanism involving M1 signalling for CCh-induced LTD is the glur1 endocytosis (Volk et al., 2007). Besides, M1 receptors are required for the Schaffer collateral mglut LTD, the M1 receptor activation maintain the basal levels of PKC activity in the CA3 region, which is essential for mglut LTD, allowing the LTD development after mglut stimulation (Kamsler et al., 2010). The M1 receptor is also involved to carbacol induced LTD in the mPFC and in its facilitation (Huang and Hsu, 2010; Caruana et al., 2011). Despite of the contribution for different physiological events, there is no strong evidence of the participation of M3/M5 for the hippocampal LTP and LTD (Luo et al., 2008).(fig 1b)

M2 receptors have an established role in synaptic plasticity in the hippocampus. The lack of M2 receptor reduces the LTP, and abolishes the short-term potentiation (STP) in the Schaffer-CA1 region, via disruption of GABAergic inhibition of CA1 neurons (Seeger et al., 2004). Blocking of M2 receptors also is able to disrupt the mLTP induced with carbacol (Auerbach and Segal, 1996). In addition, the M2 receptor modulate both EPSP and IPSP, with proeminent effect over the IPSP (Seeger et al., 2004). Zheng (2012) reported that M2 regulate differently the AC-CA3 LTP and MF-LTP, contributing to AC-CA3 LTP and reducing the MF-LTP (Zheng et al., 2012).

AChE is part of the cholinergic machinery and is able regulate the level of cholinergic tone in the CNS. In despite of its canonical function, it has been related with the modulation of synaptic plasticity and neuronal synchrony in the CNS. The AChE blocker donepezil, enhances the early LTP but does not alter late LTP in the hippocampus, furthermore donepezil prevents the LTP decay in old mice (Kroker et

al., 2012; Barnes et al., 2000). In addition, both donepezil and physiostigmine induce gamma oscillations in the CA3 region of the hippocampus, with the participation of muscarinic and GABAa receptors (Spencer et al., 2010). Moreover, physiostigmine facilitates the LTD in the mPFC (Caruana et al., 2011). Mice overexpressing the AChE-R variant have an enhancement in LTP in the CA1 region, also AChE-R variant seems to be involved with the stress enhancement of tbs-LTP in the hippocampus (Nijholt et al., 2004).

Nicotinic receptors have an established role in synaptic plasticity, its acute or chronicle activation can either enhance or facilitate the LTP *in vitro* and, enable LTP in conditions where is difficult to induce, for example in old mice (McKay et al., 2007). Nicotinic receptors can boost the STP to a LTP in pyramidal neurons of the CA1 region (Ji et al., 2001). Moreover, the nicotinic current is able drive an intracellular increase of calcium in synergism with NMDAR-mediated calcium increase, which boosts the LTP (Ji et al., 2001). Presynaptic nicotinic receptors are also capable of modulate the synaptic activity through the increase of glutamate release probability (Dani and Bertrand, 2007). Moreover, due to the high expression of nicotinic receptors in GABAergic neurons, nicotinic currents are thought to regulate the activity of these neurons (Ji et al., 2001). This regulation can either prevent the STP or reduce the LTP (Ji et al., 2001) but, in some cases cause disinhibitions in the pyramidal neurons by the reduction of GABAergic activity (Ji and Dani, 2000).

Some reports raise evidences that cholinergic modulation of synaptic plasticity is highly dependent on time, thus depending on the time of its activation nicotinic receptors can either induce LTP or LTD (Gu and Yakel, 2011; Ge and Dani, 2005). Ge and Dani (2005) used a protocol to induce STP and observed a STP boost to LTP when the ACh was delivered 2-4 seconds prior the stimulation. However, when the ACh is delivered and the stimulation is done in continuity with the ACh induced burst a LTP is observed whereas, when the stimulation was made in no continuity with the ACh burst the stimulation turned into a LTD. The nicotinic boost of STP seems to have the participation of NMDA receptors, since they are required to its occurrence (Ge and Dani, 2005). Similarly, cholinergic stimulation 120 ms prior the Schaffer collaterals (SCs) stimulation lead to LTP α7 dependent; when the stimulation was 10 ms before the SC stimulation lead to STD also dependent of α7;

when a 10 ms stimulation was given 10 ms after the SC a LTP was induced with muscarinic dependence (Gu and Yakel, 2011). Nicotinic enhancement of LTP in the hippocampus is dependent of calcium release from RyR-sensive calcium stores, α7 receptors are also necessary for this enhancement (Welsby et al., 2006). Moreover, PKA and ERK 1/2 activation are required to nicotinic enhancement of LTP (Welsby et al., 2006). Transposing this *in vitro* information to an *in vivo* view, it points that during the processing of information by the hippocampus the cholinergic stimulation paired with the information processing, improves the its processing in hippocampus. However, the disruption of this pairing causes decay in the information relevance.

2.1.2 Glutamatergic projections and GABAergic projections

The MS/DB neuronal population is composed by around of 30% of GABAergic neurons and, these neurons contain GAD and some also contain parvalbumin (PV+), that have been used as markers (Colom et al., 2005; Hangya et al., 2009). GABAergic cells from the MS project to the hippocampus and innervate only other GABAergic interneurons, these connections disinhibit pyramidal neurons by triggering IPSP in the GABAergic interneurons of the hippocampus (Toth et al., 1997; Turi et al., 2014). This disinhibition is regulated by the activation of presynaptic GABAb receptors with the subsequent diminishing of GABA realise by the terminals (Turi et al., 2014). In addition, MS/DB GABAergic neurons are involved with the theta oscillations in the hippocampus, with two different patterns of firing; one coupled to the peak and the other with the trough of theta waves (Borhegyi et al., 2004; Kaifosh et al., 2013). Also, PV^+ positive GABAergic neurons in the medial septum contain the pacemaker channel HNC and, have a temporal correlation with the hippocampal theta oscillations (Hangya et al., 2009). The presence hyperpolarization activated cations HNC channels in the MS interneurons, plus the fact that these neurons fire at theta frequencies point towards function of these neuronal population as pacemaker during theta rhythm (Simon et al., 2006). An important characteristic of the MS/DB GABA is that they are activated by behaviour relevant sensory stimulus such as, an air puff, which has been used as US in head fixed mice conditioning. In addition, Kaifosh and colleagues identified novel inputs to the MS prevenient from the hypothalamus and brain stem that may be way how these stimuli arrive the MS (Kaifosh et al., 2013). Interestingly, disruption in the septo-hipocampal GABAergic

projections did not disrupt the hippocampal LTP but increased it (Vega-flores et al., 2013).

The GABAergic activity in the MS/DB can be modulated by diverse stimuli coming from outside and inside the nucleus. Tonic local cholinergic transmission in the MS/DB activate the GABAergic population of neurons with apparently no influence in the cholinergic local population. This cholinergic driven excitation is related to the M3 activation in the GABAergic neurons (Wu et al., 2000; Alreja et al., 2000). Also MS/DB GABAergic neurons are modulated by somatostatin (SOM+). This release disrupt the rhythmic firing activity of MS GABAergic neurons and the theta power in the hippocampus (Bassant et al., 2005). Moreover, the MS GABAergic projections also receive connections from hippocampal SOM+ GABAergic neurons, that may help to synchronise the septo-hipocampal activity, although these neurons are not relevant for the MS sensory activation reported by Kaifosh, 2013 (Gulyas et al., 2003). MS/DB GABAergic cells also receive histaminergic innervation prevenient from the tuberomammillary nucleus, histamine release drives a general depolarization in MS-GABA neurons through H1/2 receptors (Xu et al., 2004). Part of these effects on MS-GABA neurons were found to be indirect effect of cholinergic MS/DB neurons activation, mediated by the activation of M3 receptors (Xu et al., 2004).

The presence of glutamatergic neurons in the MS/DB was only recently reported, the majority of these neurons express the glutamatergic marker Vglut2 however, a small portion of glutamate positive neurons also cooexpress GAD or CHAT which indicate the possibility of coorealise of these neurotransmitters (Colom et al., 2005). MS/DB Glutamatergic neurons display some connections with the hippocampus but, intraseptal connections with cells bodies of PV+ neurons are more observed (Hajszan et al., 2004; Colom et al., 2005). In addition, glutamatergic neurons from MS-DB display different patterns of firing, with a considerable population of fast firing neurons. In some cases, functional glutamatergic septo hippocampal connections were observed, with the ability of generate AMPA mediated responses in CA3 pyramidal neurons (Huh et al., 2010). Interestingly, some of glutamatergic neurons are synchronised at theta range, similarly to GAD+ neurons of the MS/DB (Huh et al., 2010; Popova et al., 2013). Similarly, glutamatergic transmission can drive the activation of pacemaker cells in MS/DB,

possible GABAergic. In addition to the fact, they are activated by cholinergic transmission prevenient from the MS (Manseau et al., 2005; Huh et al., 2010; Popova et al., 2013). These evidences of glutamatergic participation on intraseptal network and hippocampal physiology point towards a role of this population in the hippocampal theta.

2.2 Cholinergic control of GABAergic transmission in the cortex and hippocampus

Cholinergic modulation in the auditory cortex (AC) is essential for normal auditory function and plasticity in the AC (Kilgard and Merzenich, 1998; Leach et al., 2013). Electric stimulation in the nucleus basalis (NB) paired with a specific tone, alters auditory cortex responses to auditory stimuli by sustained reorganization of receptive fields (Kilgard and Merzenich, 1998; Froemke et al., 2013). That reorganization of the auditory cortex driven by NB stimulation could help in the discrimination of behaviour relevant stimuli. In addition, the AC is engaged in auditory fear conditioning (AFC), by sending projections to the lateral nucleus of amygdala helping integrate the CS stimulus (Quirk et al., 1997; Kim and Jung, 2006). Moreover, the US can modify the AC processing. During the auditory fear conditioning the unconditioned stimulus, in this case a foot shock drives a desisnhibition of the AC through L1 interneurons activation. The basal forebrain seems to play a key role in the activation of the interneurons in the L1 layer of the auditory cortex, through nicotinic receptors activation, that triggers the inhibition of PV+ positive interneurons in the L2/3 layer (Letzkus et al., 2011). This interneuron inhibition in the L2/3 layer disinhibits the L2/3 layers pyramidal neurons which may enhance the sensory processing in the AC. This mechanism has implications for the auditory fear conditioning acquisition (Letzkus et al., 2011) (Fig 2a).

The mechanisms concerning cholinergic modulation in GABAergic transmission in the cortex were also investigated using optogenetics combined with *in vitro* acute slices (Arroyo et al., 2012; Bennett et al., 2012; Brombas et al., 2014). The cholinergic modulation in the L2/3 cortical layer fast spiking (FS) interneurons has been described by different groups. Optogenetically, activation of BF fibbers terminals, in the cortex, lead to a prolonged inhibition of FS L2/3 interneurons by the activation of late spiking (LS) L1 interneurons with involvement of non-α7 nicotinic receptors activation. However an inhibition of pyramidal L2/3 neurons was also found

probably due to the inactivity of FS interneurons in acute slices (Arroyo et al., 2012; Bennett et al., 2012). In addition, this activation involves first fast α7 stimulation and subsequently slow and delayed non-α7 stimulation in L1 interneurons, this difference in the transmission kinetics is occurring probably due to the extrasinaptic localization of non- α7 receptors. Thus, volume of cholinergic transmition is responsible by the slow stimulation, whereas the synaptic α 7 receptors are responsible by the fast stimulation of LS L1 interneurons (Bennett, 2012). In the L1 layer of somatosensory cortex ACh transmition seems, at rest potential, to activate neuroglialform (NGFM) and c-ACs interneurons through nicotinic receptors. However, when under activity NGFM cells are inhibiting by ACh and, c-AC activity are enhanced (Brombas et al., 2014). This cholinergic related inhibition of NGFM cells is mediated by M1 receptor activation, with a subsequent activation of calcium dependent SK potassium channels (Brombas et al., 2014). Since the L1 NGFM interneurons inhibit L2/3 pyramidal neurons through GABA-A and B receptors, ACh can abolish NGCS inhibition of L2/3 pyramidal neurons when these cells are active. Moreover, c-ACs can inhibit NGCS but cannot inhibit the L2/3 pyramidal neurons. Thus, this dual effect of Ach in different interneurons types in the L1 can contribute to the mechanism of pyramidal disinaptic disinhibition.

In addition, Letzkus and colleagues (2011) also found a similar foot-shock response in the L1 layer of the visual cortex. In the V1 visual cortex, it was reported that BF activation during visual stimuli alters GABAergic response. Interestingly, BF activation enhances the non-FS responses in the visual cortex L1 in non-optimal visual stimulus, a similar enhancement in the non-FS L2/3 was observed, however in L2/3 pyramidal neurons a decay in the late phase of the stimulus was observed (Kimura et al., 2014). These alterations suggest that the cholinergic transmition controls the durations of visual response in the V1 cortex.

In the hippocampus cholinergic transmission plays a role in the excitation of stratum oriens O-LM interneurons with strong implications for the hippocampus processing during fear conditioning. Early studies showed pharmacological evidences of muscarinic activation of hippocampal CA1 interneurons causing IPSP in principal pyramidal cells (Pitler and Alger, 1992; Behrends and ten Bruggencate, 1993). However, a dual effect of medial septum activation is observed in these hippocampal interneurons, either depolarization or inhibition or a biphasic response,

first inhibition followed by a slow depolarization (Widmer et al., 2006). In addition, muscarinic signalling mimics this dual effect in hippocampal interneurons, the muscarinic mediated depolarization is intermediated by the inactivation of K currents but, a hyperpolarization of interneurons was also observed (McQuiston and Madison, 1999). These reports did not relate these dual effects to a particular interneuron morphology or layer in the hippocampus, with the exception of O-ML cells in the stratus oriens (McQuiston and Madison, 1999; Widmer et al., 2006). Further on, it was demonstrated that O-ML cells presented after depolarization response and increase in the firing rate in response to muscarinic stimulation. This muscarinic response was mediated by M1/M3 receptors through the inactivation of potassium conductance and activation of cationic currents (Widmer et al., 2006; Lawrence et al., 2006a, 2006b). Recently, it was reported that Som+ positive interneurons participate from CFC acquisition and recall in the CA1 region of the hippocampus, rather them PV+ cells that seem do not participate from CFC acquisition. The US during the CFC activates these SOM+ neurons, this response is driven by medial septum cholinergic neurons triggering muscarinic signalling with apparently no participation of nicotinic receptors (Lovett-Barron et al., 2014). In agreement with the aforementioned *in vitro* reports, Lovett-Barron and colleagues imply the SOM+ O-LM cells as pivotal for this mechanism, as they were activated by the US. The potential role of this inhibition in distal dendrites in the LM could be the barrage of sensory US input from the entorrinal cortex favouring the CS encoding in the hippocampus (Lovett-Barron et al., 2014). Even that PV+ cells did not participate form CFC acquisition, they seen to have a role in working memory since, the muscarinic signalling in hippocampal PV+ positive cells is important for working memory (Yi et al., 2014). In Addition, CCK+ interneurons participate from the Carbachol induced *theta* in the hippocampus (Fig. 2b) (Nagode et al., 2014).

3. BEHAVIOURAL EVIDENCE

The observation that behavioural relevant stimuli trigger ACh release to the hippocampus and cortex simultaneously, and the fact that this phasic ACh release is abolished with habituation, indicate that novel stimuli mobilise the cholinergic population in the BF (Inglis and Fibiger, 1995; Acquas, 1996). This mobilization of the cholinergic basal forebrain by novel behaviour relevant stimuli leads us to reflect its role in different types of learning. It has been observed that fear conditioned

animals showed increased ACh levels after conditioned stimuli presentation and, also a positive correlation between the Ach levels and FC learning is observed during the development (Acquas, 1996; Takase et al., 2014). During learning in the IA a long lasting increase in the Ach levels in the dorsal hippocampal is observed (Mitsushima et al., 2013). In addition, systemic blocking of muscarinic receptors disrupt fear conditioning and inhibitory avoidance learning (Soares et al., 2006).

3.1 Cholinergic modulation in the amygdala

The amygdala is involved in different types of aversive learning, such as contextual fear conditioning, auditory fear conditioning and inhibitory avoidance. In addition, lesions in the NBM which provides cholinergic input to the amygdala disrupt avoidance learning (Vazdarjanova and Mcgaugh, 1999; Wilensky et al., 1999; Power and McGaugh, 2002). The amygdala has been reported to process both the conditioned and the unconditioned stimulus, cholinergic modulation in the amygdala improves the consolidation of both those stimuli during aversive learning (Malin and McGaugh, 2006). In addition, muscarinic but not nicotinic signalling are involved with olfactory fear conditioning acquisition and retrieval further on, they are also related to the specificity of learned paired cue odour and the freezing response (Kroon and Carobrez, 2009; Pavesi et al., 2013). In the amygdala, muscarinic activation improves the consolidation of contextual fear conditioning extinction (Boccia et al., 2009). Muscarinic receptors activation in the amygdala enhances contextual fear conditioning and inhibitory avoidance consolidation and, this enhancement requires functional dopaminergic receptors (Introini-Collison et al., 1996; Vazdarjanova and Mcgaugh, 1999; LaLumiere and McGaugh, 2005; Young and Thomas, 2014). Moreover, in the inhibitory avoidance paradigm muscarinic receptors are required for memory acquisition and consolidation and, the subtypes M1 and M2 receptors are the mediators of this muscarinic enhancement of memory consolidation (Barros et al., 2002; Power et al., 2003). Specific stimulation of M1 receptors in the amygdala can alone enhance FC and, this effect cannot be observed in M1 KOs. The mechanism by which the M1 receptor can enhance memory consolidation is through PLC activation with subsequent increase of IP3 levels and inhibition the M current in amygdala neurons (Young and Thomas, 2014). A crosstalk of cholinergic, dopaminergic and noradrenergic modulatory system has been proposed as key factor for aversive memory consolidation in amygdala (Lalumiere et al., 2004;

LaLumiere and McGaugh, 2005; Young and Thomas, 2014). Even though, the M1 receptor is not required for FC memory consolidation in the amygdala, muscarinic activation can enhance the consolidation for this task. This apparent non-requirement of cholinergic signalling can be explained by the presence of other neurotransmissor systems sharing the same common signalling through PLC preventing the IP3 levels to decay when muscarinic signalling is disrupted by pharmacological manipulations (Young and Thomas, 2014). This notion is reaffirmed since the concomitant blockade of the redundant signalling receptor B2 or D5 and the M1 impair FC consolidation and lead to a decay in the IP3 levels, which cannot be observed by blocking the M1 receptor alone (Young and Thomas, 2014). In the same way, disruption of cholinergic tone to the amygdala abolish memory consolidation enhancement by intramygdala infusions of NE (Power et al., 2002). In addition, modulation by histaminergic neurotransmission has been shown to have an impact in cholinergic transmition in the amygdala with possible reflexes in behaviour. The basal forebrain receives input from tuberomamilar histaminergic neurons, this histaminergic modulation in the BF is related to wakefulness state and decreased nrem (Ramesh et al., 2004; Zant et al., 2012). Further on, histaminergic modulation of the BF leads to a cortical activation dependent of cholinergic modulation (Zant et al., 2012). In the amygdala, histaminergic transmition has an effect on ACh release thought H2 and H3 receptors, endogenous histamine sustain the Ach release through H3 and diminish by H2. The blockade of these receptors disrupts fear conditioning which suggests a synergic modulation of fear conditioning by these systems (Passani et al., 2001; Cangioli et al., 2002). Moreover, histamine infusions into the BLA enhance inhibitory avoidance and, this effect is disrupted by H3 receptor blockade (Benetti and Izquierdo, 2013). Muscarinic receptors also play a role in the glucocorticoid mediated enhancement of avoidance consolidation (Power et al., 2000). Concerning the cholinergic modulation of memory by nicotinic signalling, they are required to acquire and consolidate avoidance memory (Barros et al., 2005).

3.2 Cholinergic modulation in the cortex

The participation of the NBM/SI in the processing of aversive memories by engaging the cholinergic modulation in the cortex and amygdala has been showed to be critical for different aspects of the neuronal processing. Cholinergic modulation in

the cortex seems to be more important for memory acquisition them for memory retrieval in aversive conditioning (Miranda and Bermúdez-Rattoni, 1999). Although the participation of many different cholinergic innervated cortex in aversive learning has been known, the behavioural mechanisms are still under investigation. The rACC has been implicated in aversive US processing in different tasks, the activation of muscarinic receptors improves US consolidation for a modified IA task (Malin and McGaugh, 2006). Muscarinic receptors in the insular cortex participate from acquisition and consolidation of conditioned taste aversion and inhibitory avoidance (Ferreira et al., 2002; Miranda and Bermúdez-Rattoni, 2007) Also, nicotinic receptor signalling has been demonstrated important for FC acquisition in the auditory cortex by mediating a disinhibitory mechanism in the pyramidal neurons of the L2/3 (Letzkus et al., 2011). The entorhinal cortex (EC) is involved in the acquisition of trace fear conditioning with the participation of M1 receptors (Esclassan et al., 2009). The muscarinic transmition also seems to be relevant the acquisition of latent inhibition to an irrelevant stimulus since, the blockade of muscarinic receptors in the EC during the pre-exposure of irrelevant stimuli prevents the latent inhibition in thirst-motivated conditioned emotional response. During the retrieval of avoidance memory muscarinic modulation in required in the entorhinal cortex, parietal and anterior cingulate cortex (Barros et al., 2001).

3.3 Hippocampus

Phasic cholinergic signal in the hippocampus has been related to aversive learning and, favours the CS processing in the hippocampus by inhibiting the US information coming from the entorhinal cortex to arrive the hippocampus (Malin and McGaugh, 2006; Calandreau et al., 2006; Mitsushima et al., 2013; Lovett-Barron et al., 2014). The participation of hippocampal cholinergic receptors in FC acquisition is required for contextual FC, but has less importance for FC to discrete CS (Gale et al., 2001; Rogers and Kesner, 2004). In addition, the level of MS/DB cholinergic neurons engagement during acquisition and consolidation of FC can vary depending on the CS type. The processing of context cues during FC seems to be favoured by higher Ach levels in the hippocampus, while lower ACh levels favours discrete stimulus (tone) processing leaving the contextual cues on the background (Calandreau et al., 2006). In agreement with that, animals trained in contextual FC have higher levels hippocampal ACh and, manipulations to increase hippocampal

ACh in animals trained with discrete CS (tone) diminish the CR to the CS and increase the CR to the context (Calandreau et al., 2006). The participation of cholinergic receptor also differ from encoding and retrieval, its activation can promote encoding and inhibits fear memory retrieval (Rogers and Kesner, 2004) (Fig. 3a).

Muscarinic receptors in the hippocampus are required for the fear conditioning acquisition and consolidation (Gale et al., 2001; Wallenstein and Vago, 2001). In addition, muscarinic and nicotinic cholinergic receptors are important for the persistence of avoidance memory in the hippocampus for a late consolidation phase (Parfitt et al., 2012). It has been described that muscarinic receptors participate from IA learning through increase of synapse strengthen in the hippocampus, by driven AMPA incorporation into synapses at CA1 region (Mitsushima et al., 2013). Muscarinic activation during the learning period has been demonstrated essential for excitatory synapses strengthen by enhancing the frequency and amplitude of mEPSC, while nicotinic receptors are related to inhibitory synapsis strengthen after learning by enhancing the mIPSC amplitude (Mitsushima et al., 2013). Even so Lovett-Barron (2014) have not observed alterations when nicotinic blocker were added during learning acquisition in O-LM cell response however, the reports of Mitsushima and colleagues (2013) state that nicotinic signalling can modulate mIPSC in CA1 pyramidal neurons open the possibility of post synaptic plasticity or the modulation of different GABAergic cells by nicotinic receptors in aversive conditioning (Mitsushima et al., 2013; Lovett-Barron et al., 2014).

Nicotinic receptors participate from fear conditioning and inhibitory avoidance acquisition and retrieval (Martí Barros et al., 2004). The involvement of the different subtypes of nicotinic receptors in the hippocampus apparently has different weigh for different tasks (Gould et al., 2004). For the CFC, the activation of nicotinic receptors in the dorsal hippocampus can enhance the performance with the participation β2 containing receptors with less participation of α7 receptors (Davis and Gould, 2006; Davis et al., 2007; Kenney et al., 2012b, 2012a). However, nicotinic activation in the ventral hippocampus impairs both memory acquisition and retrieval of CFC (Kenney et al., 2012b). In addition, for the IA task both α4β4 and α7 nicotinic receptors are required for the acquisition of this task and can enhance the performance (Martí Barros et al., 2004; Bitner et al., 2007; Takase et al., 2014). Nicotinic activation in the

hippocampus may enhance memory acquisition through PKA and ERK signalling by shifting their activation to late phase in consolidation. Besides, nicotinic activation induces the transcription of JNK through the increase of Creb phosphorylation binding in its promoter in a β2 nicotinic receptors activation dependent manner (Gould et al., 2014). In addition, nicotinic receptors in the hippocampus reverses FC deficits caused by NMDA antagonism suggesting a similar downstream signalling is shared by these receptors in the hippocampus (André et al., 2011).

4. CONCLUSIONS

MS/DB is activated during different physiological situations although in the occasion of the activation during memory processing or memory relevant processes were discussed here. In general, the medial septum and cholinergic cortical projections activation seem to be particularly significant for behaviour relevant stimuli processing. During the aversive conditioning the MS/DB is involved in the molecular mechanisms of plasticity and neuronal synchrony supporting the brain areas where the information is processed. New evidence demonstrated the involvement of MS/DB and NBM in the sensory processing in the hippocampus and cortex, with strong influence in GABAergic interneurons activity. This role of the MS/DB on sensory and behaviour relevant stimuli processing is particularly relevant for the processing of sensory clues during aversive tasks such as, fear conditioning and inhibitory avoidance.

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CAPÍTULO 2

RESEARCH ARTICLE

Regulation of cognitive processing by forebrain and hippocampal cholinergic tone

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Abbreviated title: Cholinergic regulation of hippocampal function

Authors: Mohammed A. Al-Onaizi[1,2#], Gustavo M. Parfitt[1,5,#], Benjamin Kolisnyk[1,4,], Clayton S.H Law[3], Monica S. Guzman [1,3] , Daniela Martí Barros[5], L. Stan Leung[3], Marco A. M. Prado[1,2,3,4*] , Vania F. Prado*[1,2,3,4]

1 Robarts Research Institute, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada, N6A5K8.

2 Department of Anatomy and Cell Biology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada, N6A5K8

3 Department of Physiology and Pharmacology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada, N6A5K8

4 Graduate Program in Neuroscience, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada, N6A5K8

5 Programa de Pós-graduação em Ciências Fisiológicas, Fisiologia Animal Comparada, Laboratório de Neurociências (FURG), Brazil.

# Equal contribution

*To whom correspondence should be addressed: Dr. Marco A. M. Prado; mprado@robarts.ca; Vania F. Prado vprado@robarts.ca

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CAPÍTULO 2

ABSTRACT

Background

Cholinergic dysfunction is thought to underlie cognitive abnormalities in distinct types of neuropsychiatric disorders, including dementia and schizophrenia. Here we tested how abnormal forebrain cholinergic activity changes information processing.

Methods

We investigated the role of forebrain and hippocampal cholinergic tone in mice by genetically-targeting the vesicular acetylcholine transporter (VAChT). We measured long-term potentiation of the synapse of the Schaffer collaterals on hippocampal CA1 neurons *in vivo* and assessed information processing by using a mouse touchscreen version of Paired Associates Learning (PAL) task, sensitive to abnormal cognition in schizophrenia and dementia. Other cognitive domains such as spatial navigation and working memory were also evaluated.

Results

Hippocampal VAChT expression is critical for acquisition of information in the mouse PAL task as performance correlated to levels of hippocampal VAChT. Accordingly, synaptic plasticity in the hippocampus *in vivo* was disturbed, but not completely abolished by decreased hippocampal cholinergic signaling. Disrupted forebrain cholinergic signaling affected working memory, a result reproduced by selectively decreasing VAChT in the hippocampus. In contrast, reference spatial memory was modestly affected, whereas reversal spatial memory was sensitive to decreased hippocampal cholinergic signaling.

Conclusions

This work provides a refined roadmap of how synaptically-secreted ACh influences distinct hippocampal–dependent behaviors. The relationship between VAChT levels and performance in PAL suggest the possibility to use the PAL task to identify individuals with cognitive dysfunction linked to hippocampal cholinergic abnormalities.

Keywords: Vesicular Acetylcholine Trasporter (VAChT); Paired Associates Learning (PAL); Long-term Potentiation (LTP); Morris Water Maze (MWM); Schizophrenia, Alzheimer's Disease (AD)

1. INTRODUCTION

Cholinergic dysfunction has been associated with cognitive abnormalities in a variety of diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and schizophrenia [6-9]. Moreover, the use of several different drugs with unwanted cholinergic blocking effects is thought to contribute to cognitive dysfunction [1-4]. Indeed, cumulative use of drugs with anticholinergic activity is associated with increased risk for dementia and Alzheimer's disease (AD) [5]. However, the relationship between cholinergic dysfunction and maintenance of cognitive abilities in these diseases is not fully understood, due to concomitant pathologies that may contribute to cognitive abnormalities [10].

Cholinergic signalling seems necessary for the regulation of glutamatergic synaptic transmission and plasticity in the cortex and hippocampus [11-15]. Time-dependent septal cholinergic activation, either by electrical stimulation or by using an optogenetic approach, allows the expression of distinct forms of hippocampal plasticity [12]. Additionally, pharmacological [16-23] and genetic studies [15, 24, 25] have shown that modulation of cholinergic receptors influence learning and memory processes. However, long-term changes in cholinergic activity, as observed in a number of neuropsychiatric diseases, are more complex to model using specific receptor knockouts, given the plethora of subtypes of muscarinic and nicotinic receptors.

One widespread alternative to mimic cholinergic dysfunction is the selective elimination of these neurons using toxins in rodents [26]. However, saporin-based toxins used to target cholinergic neurons in mice lack specificity to target small brain regions containing cholinergic neurons [27]. Moreover, elimination of neurons cannot provide insight on specific contributions of neurotransmitters in neurons that release two neurotransmitters, such as cholinergic neurons containing more than one class of neurotransmitter transporter [26, 28]. Genetic targeting of either the vesicular acetylcholine transporter (VAChT, [29]) or choline acetyltransferase (ChAT, [30]) using the Cre/lox system has provided an alternative way for investigating long-term cholinergic dysfunction [26].

The recent development of automated touchscreen behavioral testing for rodents has greatly improved the assessment of behavior.. Furthermore, as these

touchscreen tasks were designed using almost identical paradigms and methodologies used in humans, they facilitate translational studies between rodents and humans [31-35]. The paired associates learning (PAL) test in particular, has shown increased promise to detect specific cognitive alterations that are observed consistently in AD [36] and schizophrenia [37, 38]. PAL performance has been shown to correlate with severity of affective symptoms and daily functioning in schizophrenia [38]. In dementia, PAL has been shown to differentiate between middle cognitive impairment and AD [36]. Here we investigated cognitive performance in mice with deletion of VAChT, a protein required for synaptic release of ACh, in either forebrain cholinergic neurons or selectively in septohippocampal cholinergic neurons. Our experiments reveal that dysfunction in hippocampal cholinergic activity influences synaptic plasticity *in vivo* and disturbs some, but not all, hippocampal-dependent cognitive functions.

2. MATERIAL AND METHODS

Animals

Generation of VAChT$^{flox/flox}$ mice was previously described [39]. VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were generated by crossing VAChT$^{flox/flox}$ (crossed for 5 generations with C57BL/6J) with the Nkx2.1-Cre mouse line (C57BL/6J-Tg(Nkx2-1-cre)2Sand/J), purchased from The Jackson Laboratory (JAX stock no. 008661). This line has been previously used to eliminate choline acetyltransferase (ChAT) from forebrain neurons [30]. Unless otherwise stated, all control mice used for behavioral studies were VAChT$^{flox/flox}$ littermates. The reporter mouse line Nkx2.1$^{(td\text{-}Tomato)}$ was generated by crossing B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG\text{-}tdTomato)Hze}$/J mice, purchased from The Jackson Laboratory (JAX stock no. 007909) with the Nkx2.1-Cre mouse line (JAX stock no. 008661).

Animals were housed in groups of three per cage without environmental enrichment in a temperature-controlled room (12:12 light to dark cycles), and food and water were provided for ad libitum consumption for most experiments. Animals that underwent touchscreen testing were housed in pairs; food- restricted to no more than 85% of their original weight, and they were maintained at the target weight for the duration of behavioral testing. Male mice 3 month-old were used for behavioral studies. Mice were randomized for behavioral tests and the experimenter was blind to the genotype. All procedures were performed in accordance with the Canadian Council of Animal Care guidelines at the University of Western Ontario with an approved animal protocol (2008-127).

PAL task

After successfully completing the training phase, the mice were place on a PAL task (dPAL), which involves a different stimulus being presented in each trial. A trial starts in dPAL when the mouse initiates it by touching the food receptacle, which triggers the display of both S+ and S- on the screen. There were six possible trial types and three different stimuli were presented (flower, plane, and spider). Within trials, an S+ is: the flower presented in the left window, the plane in the middle window, or the spider in the right window. Thus, mice are required to learn to associate a stimulus to its correct location. A response by touching the S- resulted in a 10s time-out and the chamber light was activated for 10s, acting as an indication for an incorrect response

for the mouse. After 10s, the next initiation by the mouse was considered a correction error trial, where the same S+ and S- were presented as for the unsuccessful previous trial. The number of correction trials was not counted towards the total number of trials performed per session. An S+ response however, led to a tone, as well as the reward being dispensed in the receptacle.

Stereotaxic injections of adeno-associated virus (AAV)

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (25 mg/kg) in 0.9% sodium chloride, and 1 µl (titer of ~10^{13} GC/ml) of AAV8-GFP-Cre-GFP or control virus (AAV8-GFP, Vector BioLabs, Eagleville, PA, USA) was injected into the medial septum/vertical limb of the diagonal band (0.98 AP, 0.1 LL and 4.1 DV) of $VAChT^{flox/flox}$ mice. The injecting micropipette was inserted and left for 2 minutes to stabilize. After stabilization, a 0.2 µl per minute infusion was performed using a micropump followed by a 30 minutes rest period to allow local diffusion of the virus and avoid virus efflux. The micropipette was then slowly removed and the scalp sutured. A recovery period of 4 weeks was given before behavioral testing to allow transgene expression.

Statistical Analysis

All data are expressed as mean ± SEM. Sigmastat 3.5 software was used for statistical analysis. Comparison between two experimental groups was done with Student's t-test. When several experimental groups or treatments were analysed, two-way analysis of variance (ANOVA) or two-way ANOVA with repeated measures were used as required. When appropriate, a Bonferonni post-hoc analysis test was used.

All other methods are described in detail in supplementary information (*Immunofluorescence microscopy, Western Blotting; Electrophysiology*, *Rotarod and neuromuscular tests, Morris Water Maze, Two-Trial Morris Water Maze, Spontaneous Alternations Y-Maze, Training on the PAL task)*.

3. RESULTS

Deletion of VAChT in forebrain projection neurons.

Nkx2.1-driven Cre is expressed in forebrain cholinergic neurons as assessed using a reporter mouse line (Figure S1A and Supplementary Table 1). Immunoblot analysis shows that VAChT levels in the cortex [$t_{(4)}$=6.162, p=0.0035], hippocampus [$t_{(4)}$=4.461, p=0.0097] and striatum [$t_{(4)}$=8.625, p=0.0010] were severely diminished in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice (Figure S1B-D). In contrast, VAChT levels remained unchanged in the brainstem of VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ compared to controls [$t_{(4)}$=1.040, p=0.3571, Figure S1E]. Importantly, these mice presented no neuromuscular deficits (Figure S2A-C).

Forebrain VAChT is required for performance in the PAL task.

We tested VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice on the PAL task, which requires sophisticated processing of information in the hippocampus for proper association of images with specific locations. VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice and their matched controls were assessed on the dPAL task using an automated touchscreen system (Fig. 1A, video S3 and video 4). During the course of the 9 weeks that mice were tested on the dPAL task, we observed that control mice significantly improved their accuracy performance, while VAChT deletion mutants did not [Two-way RM ANOVA shows significant effect of weeks $F_{(8,48)}$=21.11, p<0.0001, an effect of genotype $F_{(1,6)}$=56.94, p=0.0003, and an interaction effect $F_{(8,48)}$=2.871, p=0.0074, Figure 1A] VAChT$^{flox/flox}$ mice (controls) were able to improve performance reaching an average of ~78% accuracy by week 9 (Figure 1B). In contrast, peak accuracy performance of VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice in the dPAL task was ~55%, i.e. slightly over chance (Figure 1B). Although VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were able to perform the 36 trials required in each one hour session, they failed to associate the stimulus to its correct location. Their poorer performance was also reflected in the number of correction errors performed (Figure 1C). VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice failed to decrease the number of correction errors made over the course of 9 weeks, while control mice improved the number of correction errors performed during the course of the study [Two-Way RM ANOVA shows significant effect of weeks $F_{(8,48)}$=12.05, p<0.0001, an effect of genotype $F_{(1,6)}$=39.41, p=0.0008, and an interaction effect $F_{(8,48)}$=, p=0.0306, Figure 1C]. Correct response latency was not different between the two groups over

the course of 9 weeks [Two-Way RM ANOVA shows significant effect of weeks $F_{(8,48)}$=7.508, p<0.0001, no effect of genotype $F_{(1,6)}$=2.437, p=0.1695, and no interaction $F_{(8,48)}$=1.195, p=0.3220, Figure 1D]. Furthermore, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were no different from controls when the latency to collect the reward was measured, which indicated that motivation was not a factor in their poorer performance [Two-way RM ANOVA shows a significant effect of weeks $F_{(8,48)}$=7.596, p<0.0001, no effect of genotype $F_{(1,6)}$=0.0001380, p=0.7681, and no interaction $F_{(4,48)}$=0.6061, p=0.7681 Figure 1E]. In summary, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were able to learn that they had to touch the screen when the images were shown; however, they failed drastically in making associations, that is, they were unable to assign each image to a specific position.

A potential mechanism to form associations might depend on lasting increases in synaptic strenght. To determine whether VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice have intact synaptic plasticity, we examined LTP of the synapse of the Schaffer collaterals on hippocampal CA1 neurons in anaesthetised mice *in vivo*. VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice showed decreased LTP which lasted about 90 minutes post-tetanus delivery while LTP in VAChT$^{flox/flox}$ mice was maintained for 120 minutes (Figure 2A and B). This indicated that the lack of cholinergic signalling disturbs synaptic plasticity in hippocampal CA1 area *in vivo*.

To specifically evaluate the contribution of hippocampal cholinergic tone to PAL performance, we stereotaxically injected AAV8-GFP-Cre or AAV8-GFP virus to the medial septum and vertical limb of the diagonal band (MS/VDB) of VAChT$^{flox/flox}$ mice (AAV8-GFP-Cre n= 13; AAV8-GFP n=7). Mice were trained on the dPAL task one month after viral injection. Following completion of the task, mice were sacrificed to evaluate VAChT protein levels. Given the length of the experiment (≈ 4 months), and the observation that viral injection was only partially effective to reduce hippocampal VAChT levels (Figure S4A and B), we did not exclude any mouse from the analysis, even if viral mediated recombination was not effective to eliminate the transporter. Instead, we correlated VAChT levels in the hippocampus from both AAV8-GFP-Cre and AAV8-GFP to their performance on the PAL task.

Performance on the final week of the experiment was positively correlated to VAChT protein levels in terms of response accuracy [Pearson's r=0.5208, CI=0.1015 to

0.7829, p=0.0186, Figure 3A], and negatively correlated to number of correction errors [Pearson's r=-0.6518, CI=-0.8494 to -0.2940, p=0.0018, Figure 3B]. We also evaluated the relationship between hippocampal VAChT protein levels to learning the PAL task. We calculated the rate of learning as the slope of the learning curve of both response accuracy and correction errors across all the weeks of the task. VAChT protein level was positively correlated to the rate of learning of response accuracy [Pearson's r=0.5053, CI=0.08072 to 0.7747, p=0.0231, Figure 3C], and negatively correlated to the correction error rate of learning [Pearson's r=-0.1799, CI=-0.7982 to -0.1418, p=0.0120, Figure 3D]. Importantly, VAChT protein level did not correlate to mean response latency across the task [Pearson's r=0.1349, CI=-0.3273 to 0.5450, p=0.5708, Figure 3E] or mean reward collection latency across the task [Pearson's r=-0.1799, CI=-0.5676 to 0.2731, p=0.4352, Figure 3F], suggesting that response patterns and motivation is unaltered by reduced VAChT levels. Taken together these results show that the less VAChT protein in the hippocampus the worse is the mouse performance in the dPAL task, indicating that dPAL learning is modulated by septohippocampal cholinergic signalling.

VAChT and spatial navigation

Given the strong deficit of association of the image with its correct location in the PAL task, it seemed of importance also to evaluate spatial memory in these mice. Spatial memory is widely used to assess information acquisition and storage in the hippocampus, but cholinergic dysfunction has only mild effects in the MWM in mice [27, 29]. Our data showed that spatial learning on the MWM was not completely disrupted in $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice as we observed for PAL acquisition (Figure S3A-C). On the probe trial of the MWM, both groups of mice spent significantly more time in the target quadrant compared to the opposite quadrant [Two-way ANOVA shows a significant effect of quadrant, $F_{(3,30)}=38.04$, $p<0.0001$, no effect of genotype, $F_{(1,10)}=6.346$, p=0.9937, and an interaction effect $F_{(3,30)}= 2.914$, p=0.0401, Figure S3D], post-hoc analysis revealed that both groups spent significantly more time in the target quadrant. However, $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice had significantly fewer platform crosses compared to littermate controls [$t_{(19)}=2.692$, p=0.0144, Figure S3E].

To specifically evaluate the contribution of hippocampal cholinergic tone to learning and memory performance in the spatial version of the MWM, we stereotaxically

injected AAV8-GFP-Cre (n=25) virus to the MS/VDB in another cohort of of VAChT$^{flox/flox}$ mice (Figure S4A and B). VAChT$^{flox/flox}$ mice injected with AAV8-GFP (n=14) were used as controls. AAV8-GFP-Cre injected mice that showed more than 50% of hippocampal VAChT protein levels (n=11) when compared to controls were excluded from the analysis (Fig S4D). In AAV8-GFP-Cre injected mice with reduced hippocampal VAChT levels, VAChT protein in the cortex was not changed [97% of AAV8-GFP VAChT levels, $t_{(6)}$=0.453, p=0.665, Figure S4C and D]. AAV8-GFP-Cre mediated deletion of VAChT from the medial septum did not significantly alter acquisition of the spatial version of the MWM [Latency, Two-way RM ANOVA shows an effect of days $F_{(3,39)}$=22.84, p<0.0001, no effect of Cre-virus injection $F_{(1,13)}$= 0.2228, p=0.6447, and no interaction, $F_{(3,39)}$=1.302, p=0.2876, Figure 4A]. Similar results were obtained for distance travelled [Two-way RM ANOVA shows an effect of days, $F_{(3,39)}$=23.5, p<0.0001, no effect of Cre expression $F_{(1,13)}$=0.3125, p=0.5856, and no interaction, $F_{(3,39)}$=1.329, p=0.2787, Figure 4B]. In the probe trial, mice injected with the AAV8-GFP-Cre virus did not differ from controls in terms of preference for the target quadrant [Two-way ANOVA shows a significant effect of quadrant, $F_{(3,39)}$=46.55, p<0.0001, no effect of Cre expression, $F_{(1,13)}$=1.153, p=0.3024, and no interaction $F_{(3,39)}$=0.2691, p=0.8473, Figure 4D] or platform crosses ($t_{(25)}$=0.9547, p=0.3603, Figure 4E). Taken together, these results suggest that decreased levels of hippocampal cholinergic activity do not seem to affect MWM performance.

VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were also tested on the reversal learning protocol of the MWM. During the course of 4 days, control mice significantly improved in their latency to find the hidden platform in contrast to VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice [Two-way RM ANOVA shows a significant effect of days $F_{(3,30)}$=8.802, p<0.0001, main effect of genotype $F_{(1,10)}$= 8.843, p=0.0078, and no interaction $F_{(3,30)}$=1.466, p=0.2334, Figure 5A-C). Notably, on the probe trial, control mice spent considerably more time in the target quadrant compared to the other quadrants [Two-way ANOVA shows a significant effect of quadrant, $F_{(3,30)}$=6.963, p=0.0004, no effect of genotype, $F_{(1,10)}$=1.551, p=0.9990, and an interaction effect $F_{(3,30)}$= 3.631, p=0.0168, Figure 5D], while VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice visited all quadrants almost equally. The number of platform crosses was also higher for control mice compared to VAChT mutants [$t_{(19)}$=2.606, p=0.0174, Figure 5E]. These results indicate that, different from

control mice, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were unable to extinguish the previously learned position and relearn the new position of the hidden platform.

To account for compromised striatal cholinergic signaling in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice (Figure S1D), we also tested a mouse line with selective deletion of VAChT in striatal neurons (VAChT$^{D2\text{-}Cre\text{-}flox/flox}$), but spared hippocapampal VAChT [28] in the MWM (Figure S5). Interestingly, VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice did not differ from controls (VAChT$^{flox/flox}$) in both acquisiton and reversal versions on the MWM (Figure S5 D-H). These results suggest that deficits seen in reversal learning in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice are not likely due to impaired striatal cholinergic transmission, but rather a result of hippocampal/cortical or combined cortical hippocampal dysfunction.

Selective reduction of hippocampal cholinergic tone in virus injected mice also increased latency to find the platform in the reversal learning [Two-way RM ANOVA shows an effect of days, $F_{(3,39)}=21.96$, $p<0.0001$ and a significant interaction effect, $F_{(3,39)}=7.507$, $p=0.0004$), with post-hoc analysis revealing that AAV8-GFP-Cre injected mice performed significantly worse on day 4 compared to controls (Figure 5G). During the probe trial, mice injected with AAV8-GFP-Cre virus showed significant impairments, failing to show a preference for the target quadrant [Two-way ANOVA shows a significant effect of quadrant, $F_{(3,39)}=8.759$, $p<0.0001$, no effect of Cre, $F_{(1,13)}=0.00365$, $p=0.9848$, an interaction effect $F_{(3,39)}= 5.317$, $p=0.0030$, Figure 5J]. Post-hoc analysis revealed that the AAV8-GFP-Cre mice did not prefer the target quadrant compared to the other quadrants, while the AAV8-GFP injected controls had a strong preference for the target quadrant. Furthermore, the AAV8-GFP-Cre injected mice showed a drastic decrease in the number of platform crosses ($t_{(26)}=0.9547$, $p=0.0010$, Figure 5K). These results reveal that disruption of hippocampal cholinergic tone, but not striatal or cortical cholinergic activity, compromises information processing in the MWM reversal learning.

Regulation of working memory by septohippocampal VAChT

To determine whether other cognitive domains of importance in neuropsychiatric disorders may also be regulated by synaptically-released ACh, we evaluated the performance of the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice on two measures of working memory: the working memory version of the MWM and spontaneous alternations in the Y-maze. In the working memory version of the MWM, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice failed

to improve their performance from the first to the second trial resulting in significant impairments in measures of latency savings [$t_{(12)}$=3.580, p=0.0030, Figure 6A] and distance savings [$t_{(12)}$=2.852, p=0.0127, Figure 6B], suggesting that the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice have impaired working memory. Similarly, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice revisited arms in the maze more often than controls resulting in a significant decrease in spontaneous alternations in the Y maze [$t_{(12)}$=2.674, p=0.0182, Figure 6C), suggesting that forebrain VAChT is required for normal working memory performance.

When tested on the working memory MWM test, mice with selective elimination of septohippocampal VAChT by virus injection (same cohort used in the MWM) also showed impaired latency savings ratio [$t_{(26)}$=2.847, p= 0.0111, Figure 6D] and distance savings ratio [$t_{(26)}$=2.149, p=0.0473, Figure 6E]. On the spontaneous alternations Y-maze task, AAV8-GFP-Cre injected mice showed impairments on working memory, measured as a significant decreased rate of spontaneous alternations [$t_{(26)}$=3.347, p=0.0041, Figure 6F]. It is interesting to note that working memory deficits observed for AAV8-GFP-Cre injected mice were similar to deficits observed for VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice. Taken together these results indicate that working memory is highly sensitive to hippocampal cholinergic tone.

4. DISCUSSION

Here we show that manipulation of VAChT levels in a brain-region selective manner helps to reveal the contribution of compromised hippocampal cholinergic tone for information processing. In particular, we show that hippocampal cholinergic signaling is important for the modulation of tasks that are relevant for understanding cognitive symptoms in schizophrenia and dementia, including the PAL task. Interestingly, some hippocampal-dependent tasks appear to be more sensitive to decreased cholinergic signalling than others. Our results provide a comprehensive map of cholinergic-regulated hippocampal cognitive processing that may be useful to understand similar deficits in humans with cholinergic deficiency.

Notably, we report novel data indicating the importance of cholinergic signalling in regulating the hippocampal-dependent PAL task. Clinically, the PAL task has been suggested as a potential cognitive marker of decline in psychosis [37]. Significant impairments in PAL have been observed in patients with schizophrenia with a

positive correlation between failure on the PAL task and negative symptoms [38]. Additionally, hippocampal activation during PAL has been shown to be changed in patients with mild cognitive impairment when compared to aged-matched controls [40]. Hence, PAL has also been considered a sensitive task for predicting cognitive decline in AD [36, 41].

Mouse performance in PAL, as well as in other biconditional paired-associates tasks, depends on intact hippocampal function [33, 42]. However, whether cholinergic signalling in the hippocampus is required for acquisition/recall of the task has not been clearly established. Systemic administration of donepezil, a cholinesterase inhibitor, improved PAL performance in mice, an effect that was attenuated with administration of muscarinic antagonists [42]. Similar results have been observed in monkeys where both mecamylamine (nicotinic antagonist) and scopolamine (muscarinic antagonist) induced deficits in PAL performance [43, 44]. These results suggest that cholinergic signalling might be relevant for PAL. Also, rats previously trained in PAL that received injections into the dorsal hippocampus of either scopolamine or mecamylamine and were re-tested did not show deficits in performance [33], suggesting that hippocampal cholinergic signalling might not modulate recall in this task. Our results indicate that disruption in forebrain cholinergic tone disturbs PAL learning Additionally, our data suggest that hippocampal cholinergic signalling has an important role in encoding the associations required for PAL, as performance of mice in the PAL task correlates with hippocampal VAChT protein levels in mice. Importantly, these deficits occurred in the absence of alterations in latency to touch the screen or to collect the reward, indicating that motivation was not a factor in the poorer performance of mice with lower cholinergic tone. Interestingly, mice deficient for the M1 receptor presented no differences compared to controls in their acquisition of the PAL task [45], suggesting that nicotinic and/or other muscarinic receptors might be involved in mediating learning in this hippocampal-dependent task.

The mechanisms by which ACh secretion facilitates PAL performance are not fully understood. It is possible that cholinergic tone in the hippocampus is required for specific types of synaptic plasticity. Indeed, hippocampal LTP *in vitro* is disturbed in a different mouse line lacking forebrain VAChT [29]. We corroborated this finding *in vivo* in $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice and demonstrated that in the absence of VAChT

expression, hippocampal LTP is compromised, suggesting that disturbances of synaptic plasticity might contribute to the deficit.

We showed that acquisition of the spatial version of the MWM and recall of platform location was affected in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice, while AAV8-Cre-GFP injected mice did not show any deficit in this behavioral task. In contrast to the reference memory test, both VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ and AAV8-Cre-GFP injected mice when tested in the MWM reversal-learning task presented extensive deficits, suggesting a prominent role for hippocampal cholinergic signalling in reversal learning.

Both prefrontal cortex and hippocampus have been implicated in working memory (Yoon *et al.*, 2008). A number of studies indicate that cholinergic neurotransmission is crucial for modulation of working memory in various behavioral tasks (Furey et al;, 2000, Hironaka *et al.*, 2001, Levy et al. 1991, Miyamoto *et al.*, 1987, Baxter *et al.*, 1995). Whether cholinergic modulation of working memory is dependent on ACh acting on prefrontal cortex, hippocampus or in both structures simultaneously is not known. Our results show that deficits in the working memory version of the MWM task and the Y-maze alternating task are equally severe in both forebrain VAChT mutants (VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice) and hippocampus VAChT mutants (AAV8-Cre-GFP injected mice), suggesting that hippocampal cholinergic tone is vital in regulating information processing in working memory tasks. Taking together, these results suggest that ACh may exert important roles in working memory via modulation of hippocampal function.

The present work is relevant to understand how drug-induced cholinergic dysfunction or degenerative changes in cholinergic neurons contribute to cognitive alterations in several neuropsychiatric disorders [7, 46]. Our results reveal how distinct types of hippocampal information processing are affected by hippocampal cholinergic signalling. Hippocampal cholinergic activity does not seem to be critical for spatial reference learning and memory, but has fundamental roles on working memory, reversal learning and paired-associates learning. As PAL performance is highly dependent on hippocampal cholinergic integrity, it is tempting to speculate that the PAL task could be used to identify individuals with cognitive dysfunction linked to hippocampal cholinergic abnormalities.

5.FIGURE LEGENDS

Figure 1. VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ mice display impairments in the acquisition of dPAL. (a) Image depicting a mouse performing the task, where the flower shown as the S+ and the airplane as the S-. Data for the acquisition of the dPAL task for VAChT$^{flox/flox}$ (n=7 clear squares) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (n=7 black squares) mice. Each week represents five testing sessions of 36 trials (b) Mean accuracy. (c) Mean correction errors. (d) Response latency. (e) Reward collection latency. (Data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.0001).

Figure 2. Hippocampal LTP is disrupted in forebrain-specific VAChT knockout mice *in vivo*. (a) Normalized slopes of the excitatory sink recorded at CA1 stratum radiatum (apical dendrites) of VAChT$^{flox/flox}$ (gray squares, n=5) and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ (black circles, n=6) mice. Baseline was monitored for 30 minutes prior to tetanus delivery (t=0), and post-tetanic response was monitored for 120 minutes. A 1-second 100 Hz train, delivered at 2-3 times the threshold intensity (arrow), induced higher and more prolonged potentiation in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice than VAChT$^{flox/flox}$ controls. Insets show representative current sink time response taken at 80 minutes (red traces), overlaid on the pre-tetanus baseline response (black traces), from each genotype. (b) Normalized excitatory sink slope averaged across 30 minute time intervals (mean ± SEM) in VAChT$^{flox/flox}$ and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice, with significant difference between mouse groups at 90-120 minutes ($t_{(9)}$=3.911, p=0.0036).

Figure 3. Medium septum AAV8-GFP-CRE injected mice show deficits in dPAL. (a-b) Linear regression and correlation between response accuracy (r=0.5208, p=0.0186) and correction errors (r=-0.5154, p=0.0168) on week 9 and hippocampal VAChT protein expression levels. (c-d) Linear regression and correlation between response accuracy (r=0.4460, p=0.0487) and correction errors (r=-0.1799, p=0.0120) across all the weeks of the PAL task and hippocampal VAChT protein expression levels. (e-f) The relationship between response latency (r=0.1349, p=0.5708) and reward collection latency (r=-0.1799, p=0.4352) across all the weeks of the PAL task and VAChT expression levels.

Figure 4. Performance of medium septum AAV8-GFP-CRE injected mice in the MWM. VAChT$^{flox/flox}$ injected with AAV8-GFP virus (gray squares, n=14) or AAV8-

GFP-CRE virus (black circles, n=14) were tested in the spatial paradigm of the MWM. Data average of four 90-s trials per day were plotted. (a) Latency to reach the platform, (b) Distance to reach the platform, (c) Speed to reach the platform, (d) The percentage of time spent in each quadrant of the pool measured on day 5 in a 60-s probe trial with the platform removed. (e) Number of platform crosses during the probe trial. (f) Representative path traces of two AAV8-GFP and two AAV8-GFP-CRE injected mice in the probe trial. The target quadrant is in the upper right. Data are mean ± SEM. **P*<0.05*,* ***P*<0.01. T, target; O, opposite; L, left; R, right.

Figure 5. Reversal learning is affected in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ and medium septum AAV8-GFP-CRE injected mice. VAChT$^{flox/flox}$ (gray squares, n=11), VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ (black circles, n=11) were tested in the reversal paradigm of the MWM. Data average of four 90-s trials per day were plotted. (a) Latency to reach the platform, (b) Distance to reach the platform, (c) Speed to reach the platform, (d) The percentage of time spent in each quadrant of the pool measured on day 5 in a 60-s probe trial with the platform removed. (e) Number of platform crosses during the probe trial. (f) Representative path traces for two VAChT$^{flox/flox}$ and two VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ in the probe trial. The target quadrant is in the upper left. (g-l) AAV8-GFP (gray squares, n=14) or AAV8-GFP-CRE (black circles, n=14) injected mice were tested in the reversal paradigm of the MWM. The data average four 90-s trials per day were plotted. (g) Latency to find the platform (h) Distance, (i) Speed, (j) The percentage of time spent in each quadrant of the pool was measured on day 5 in a 60-s probe trial with the platform removed. (k) Number of platform crosses during the probe trial. (l) two AAV8-GFP and two AAV8-GFP-CRE injected mice in the probe trial. The target quadrant is indicated with a T. Data are mean ± SEM. **P*<0.05*,* ***P*<0.01, ****P*<0.0001. T, target; O, opposite; L, left; R, right.

Figure 6. Working memory depends on hippocampal cholinergic tone. (a) Latency savings ratio and (b) distance savings ratio for VAChT$^{flox/flox}$ (gray, n=7) and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ (black, n=7) mice in the working memory version of the MWM. (c) Spontaneous alternations in the Y-maze for VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$. (d) Latency savings ratio and (e) distance savings ratio for AAV8-GFP (gray, n=14) and AAV8-GFP-CRE (black, n=14) mice in the working memory version of the MWM. (f) Spontaneous alternations in the Y-maze for virus injected mice. Data are mean ± SEM. **P* < 0.05, ***P* < 0.01

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8. FIGURES

Figure 1

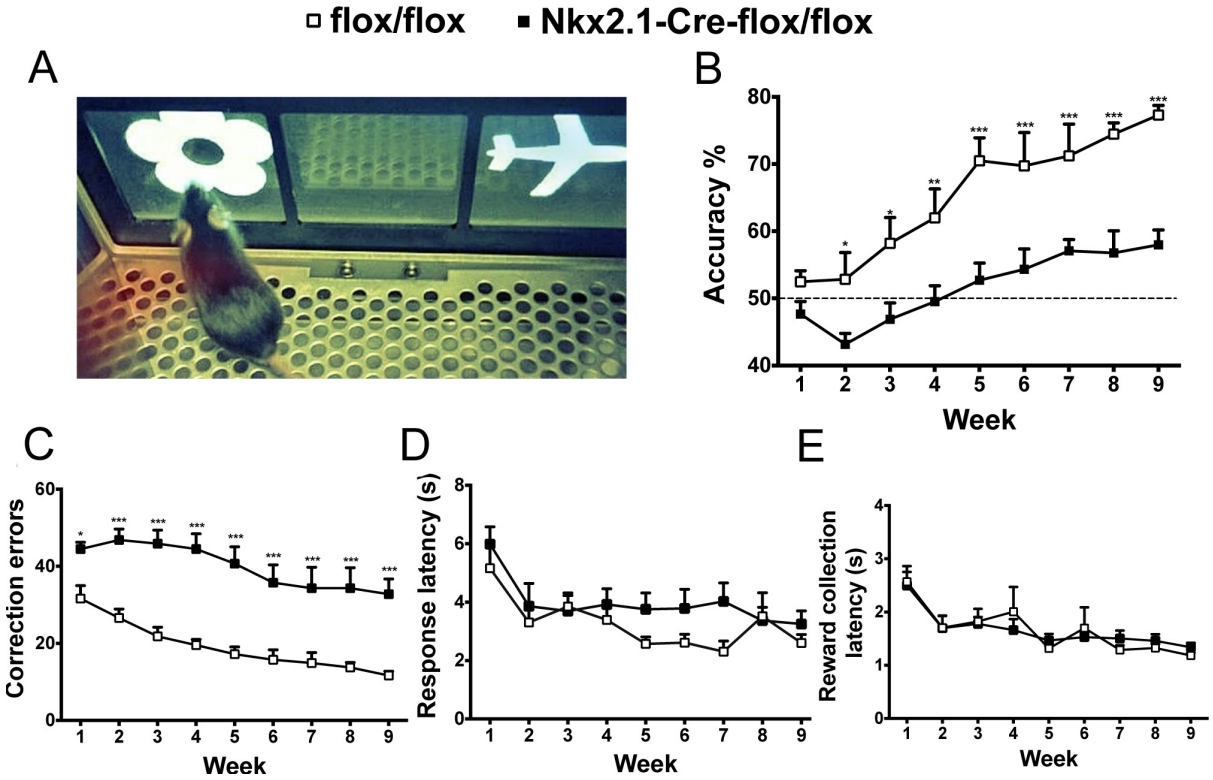


Figure 2

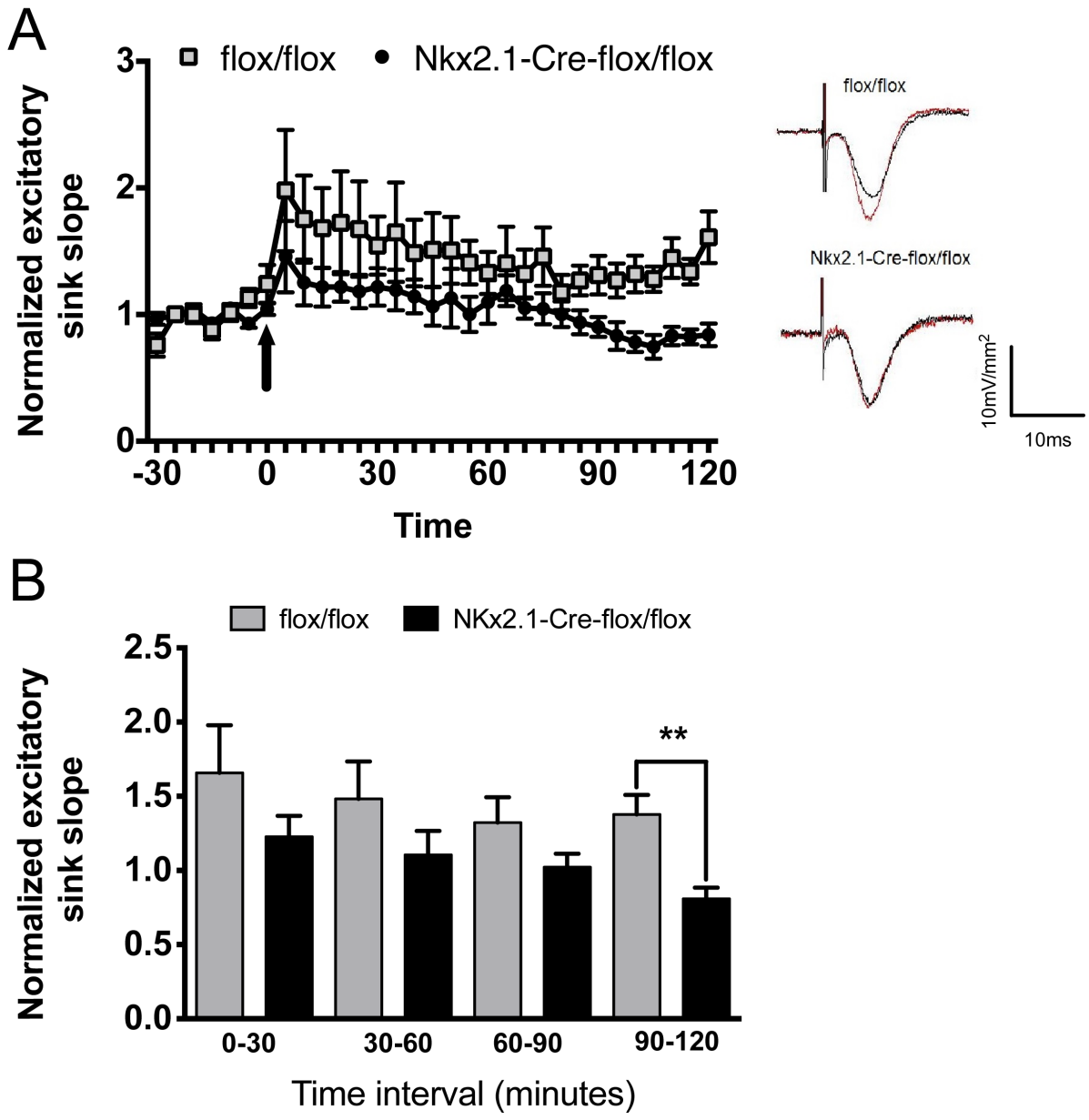

A
flox/flox
Nkx2.1-Cre-flox/flox
Normalized excitatory sink slope
Time
flox/flox
Nkx2.1-Cre-flox/flox
10mV/mm²
10ms
B
flox/flox
NKx2.1-Cre-flox/flox
Normalized excitatory sink slope
**
0-30
30-60
60-90
90-120
Time interval (minutes)

Figure 3

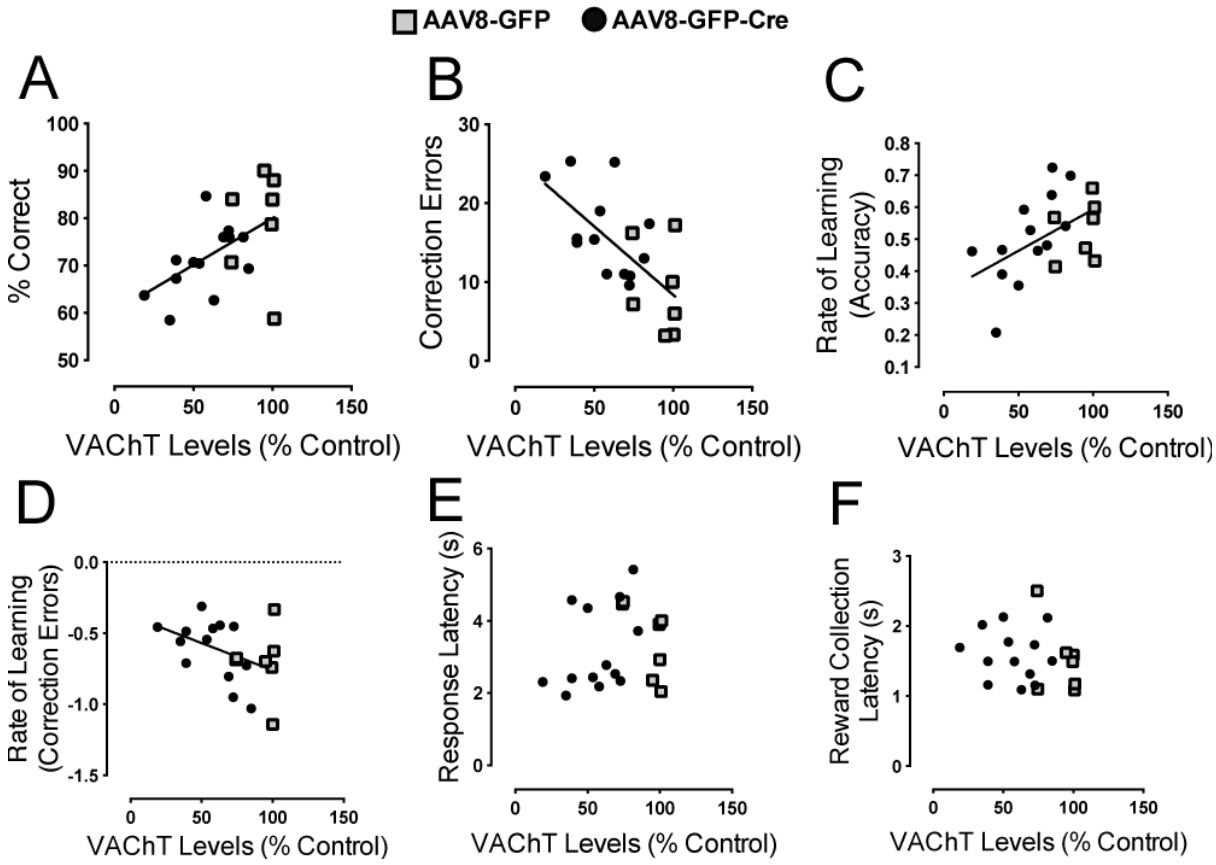

AAV8-GFP
AAV8-GFP-Cre
A
% Correct
VAChT Levels (% Control)
B
Correction Errors
VAChT Levels (% Control)
C
Rate of Learning (Accuracy)
VAChT Levels (% Control)
D
Rate of Learning (Correction Errors)
VAChT Levels (% Control)
E
Response Latency (s)
VAChT Levels (% Control)
F
Reward Collection Latency (s)
VAChT Levels (% Control)

Figure 4

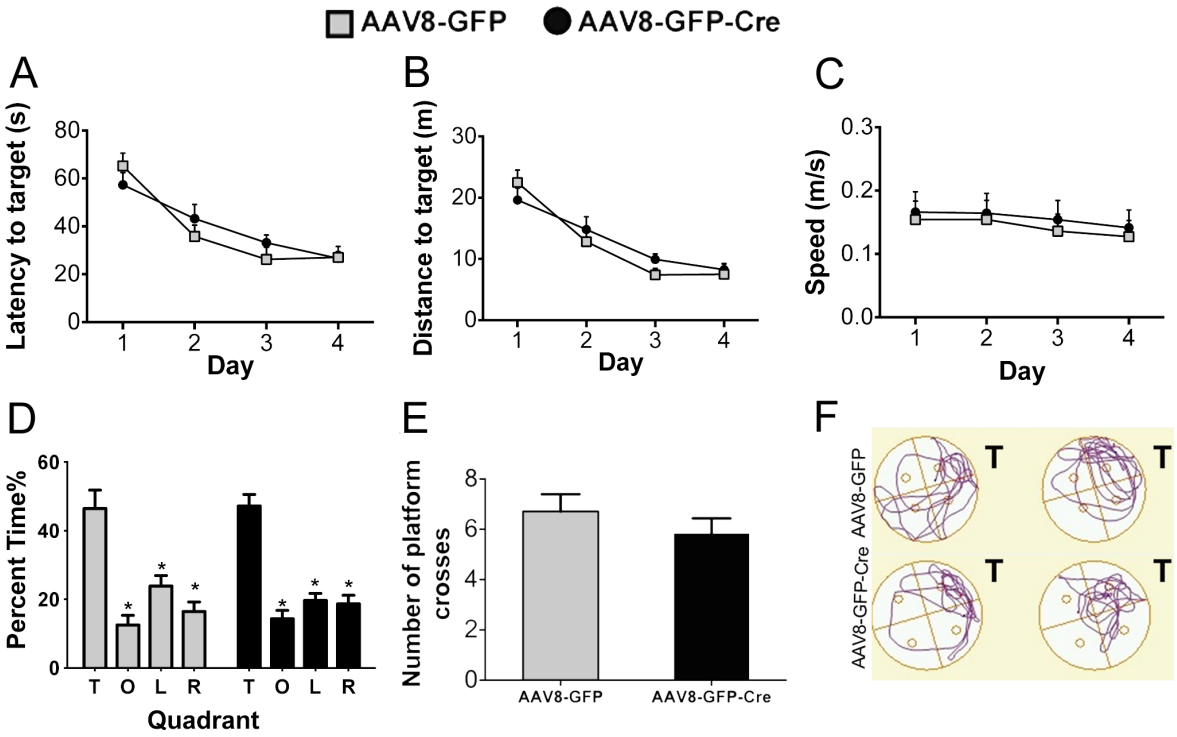

AAV8-GFP
AAV8-GFP-Cre
A
Latency to target (s)
Day
B
Distance to target (m)
Day
C
Speed (m/s)
Day
D
Percent Time%
T O L R T O L R
Quadrant
E
Number of platform crosses
AAV8-GFP
AAV8-GFP-Cre
F
AAV8-GFP
AAV8-GFP-Cre
T

Figure 5

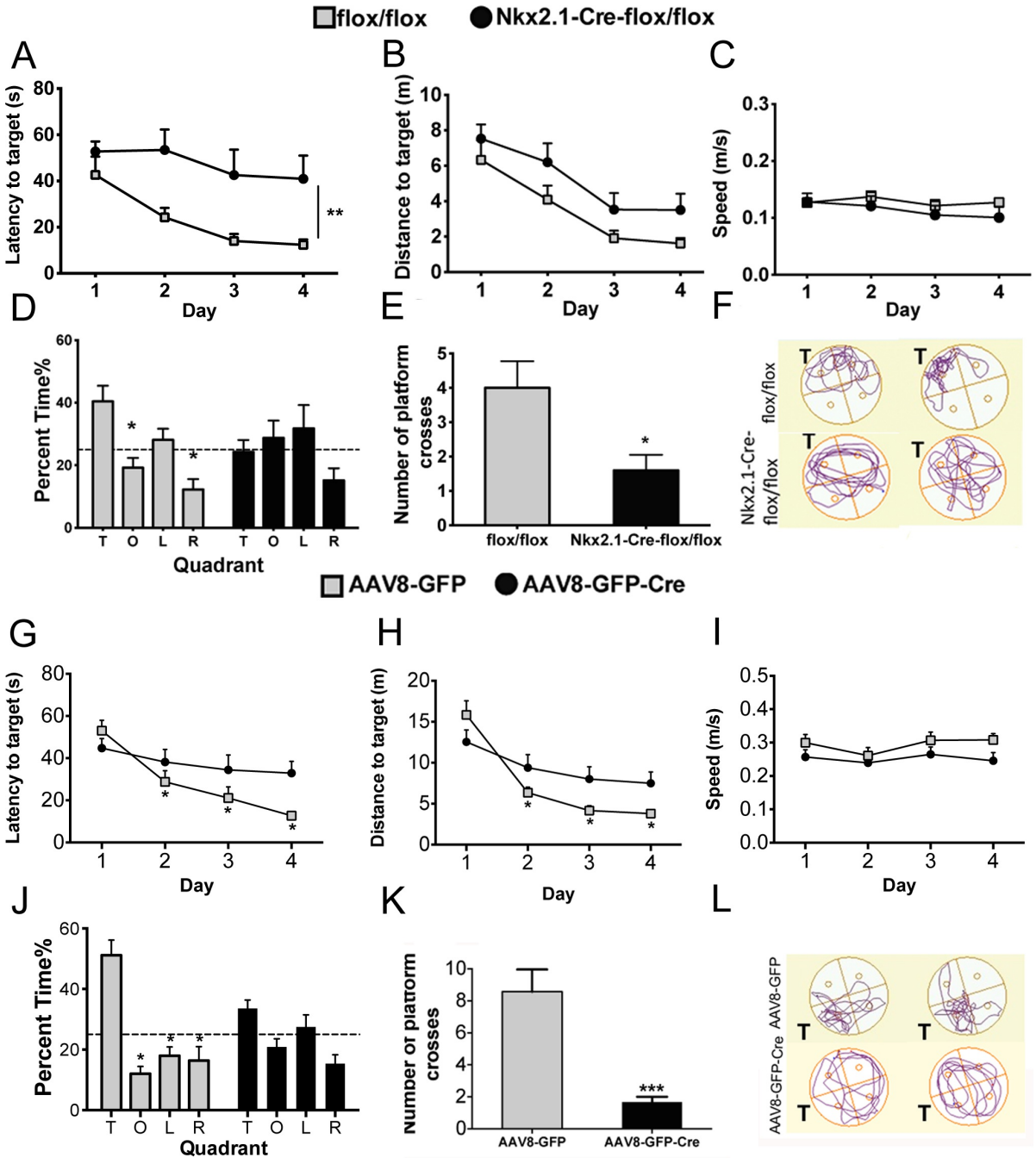
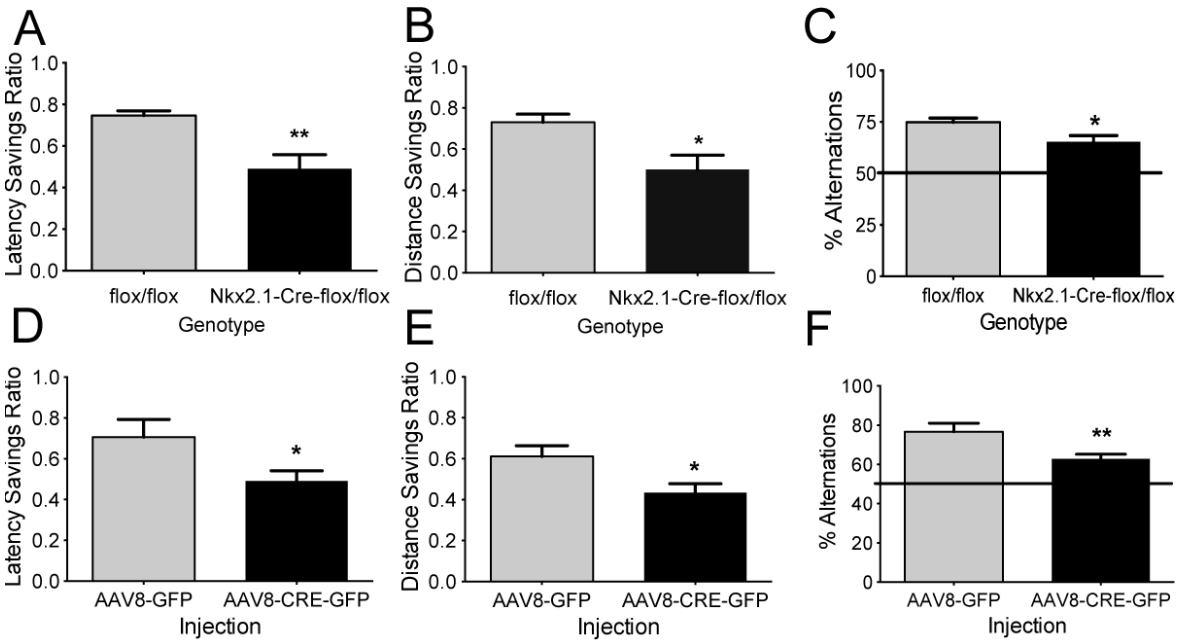

flox/flox
Nkx2.1-Cre-flox/flox
A
Latency to target (s)
Day
**
B
Distance to target (m)
Day
C
Speed (m/s)
Day
D
Percent Time%
T O L R T O L R
Quadrant
*
E
Number of platform crosses
flox/flox
Nkx2.1-Cre-flox/flox
F
T
AAV8-GFP
AAV8-GFP-Cre
G
Latency to target (s)
Day
H
Distance to target (m)
Day
I
Speed (m/s)
Day
J
Percent Time%
T O L R T O L R
Quadrant
K
Number or platform crosses
***
AAV8-GFP
AAV8-GFP-Cre
L
T

Figure 6


A
Latency Savings Ratio
1.0
0.8
0.6
0.4
0.2
0.0
**
flox/flox
Nkx2.1-Cre-flox/flox
Genotype
B
Distance Savings Ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
flox/flox
Nkx2.1-Cre-flox/flox
Genotype
C
% Alternations
100
75
50
25
0
*
flox/flox
Nkx2.1-Cre-flox/flox
Genotype
D
Latency Savings Ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
AAV8-GFP
AAV8-CRE-GFP
Injection
E
Distance Savings Ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
AAV8-GFP
AAV8-CRE-GFP
Injection
F
% Alternations
100
80
60
40
20
0
**
AAV8-GFP
AAV8-CRE-GFP
Injection

9. SUPPLEMENTAL INFORMATION

Table S1. Percentage of colocalization between cells that express CHT1 and Cre-mediated Td Tomato in different regions of the brain.

Figure S1. NKx2.1-Cre drives Cre expression in forebrain cholinergic neurons. (a) Expression of Cre detected by tdTomato fluorescence in different regions in the brain of NKx2.1-Cre; B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J mice. (Scale bar, 50μm.) (b-e) VAChT expression and quantification from Western blots in the whole cortex (b), hippocampus (c), striatum (d), brainstem (e) in VAChT$^{flox/flox}$ (gray bars) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ (black bars) mice. VAChT expression was normalized to synaptophysin (n= 3, data are mean ± SEM. ***P*<0.01).

Figure S2. Neuromuscular behavioral testing of VAChT$^{flox/flox}$ (white circles, n=6) and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ (black squares, n=6) mice. (a) Performance in the rotarod, (b) grip force and (c) wire-hang test.

Figure S3. Performance of VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice in the MWM. VAChT$^{flox/flox}$ (gray squares, n=11) and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ (black circles, n=11) mice were tested in the spatial paradigm of the MWM. The data average four 90-s trials per day were plotted. (a) Latency, (b) Distance, (c) Speed, (d) The percentage of time spent in each quadrant of the pool was measured on day 5 in a 60-s probe trial with the platform removed. (e) Number of platform crosses during the probe trial. (f) Representative path traces for two VAChT$^{flox/flox}$ and two VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice in the probe trial. The target quadrant is in the lower right. Data are mean ± SEM. **P*<0.05*,* ***P*<0.01. T, target; O, opposite; L, left; R, right.

Figure S4. Virus-induced deletion of hippocampal VAChT. (a) Immunofluorescence validation of Cre-mediated recombination in cholinergic neurons stained for ChAT in the medial septum of VAChT$^{flox/flox}$ mice (GFP represents Cre-infected neurons). (Scale bar= 200μm). (b) VAChT expression in the hippocampus of AAV-GFP injected VAChT$^{flox/flox}$ or AAV-GFP-CRE-injected VAChT$^{flox/flox}$ mice. (c) VAChT expression in the cortex of AAV-GFP-injected VAChT$^{flox/flox}$ or AAV-GFP-CRE-injected VAChT$^{flox/flox}$ mice (same cohort as b). Synaptophysin was used as loading control. (d) VAChT expression quantification in the hippocampus in all the AAV8-GFP-Cre injected mice (n=25). Animals with more

than 50% of hippocampal VAChT levels (n=11) were excluded from MWM and working memory experiments. (e) Quantification of VAChT expression from Western blots in b and c. VAChT expression was normalized to synaptophysin and normalizaed to control samples (n= 3, data are mean ± SEM. ***P*<0.01).

Figure S5. Performance of VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice in the MWM. VAChT$^{flox/flox}$ (white circles, n=9) and VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ (black squares, n=9) mice were tested in the spatial paradigm of the MWM. The data average four 90-s trials per day were plotted. (a) Latency, (b) Distance, (c) Speed, (d) The percentage of time spent in each quadrant of the pool was measured on day 5 in a 60-s probe trial with the platform removed. (e-h) Reversal testing was conducted in VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice. (e) Latency to reach the platform, (f) Distance to reach the platform, (g) Speed to reach the platform, (h) The percentage of time spent in each quadrant of the pool measured on day 5 in a 60-s probe trial with the platform removed. Data are mean ± SEM. **P*<0.05*,* ***P*<0.01. T, target; O, opposite; L, left; R, right.

Video S6. Representative media of a control mouse (VAChT$^{flox/flox}$) performing the dPAL task on the ninth week of testing.

Video S7. Representative media of a VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mouse performing the dPAL task on the ninth week of testing.

Immunoflourescence microscopy

Mice were anesthetised with ketamine (100 mg/kg) and xylazine (25 mg/kg) in 0.9% sodium chloride, and then sacrificed by transcardial perfusion: phosphate-buffered saline (PBS, pH=7.4) for 3 minutes and 4% paraformaldehyde for 5 minutes. Brains were harvested and placed in 4% paraformaldehyde in 1× PBS at 4 °C for 4 h, they were kept at 4 °C until sliced using a vibratome. Brain sections (40 µm) were prepared and free-floating sections in 1× PBS (one per well in a 24-well plate) were permeabilized with 0.4% Triton X-100 in 1× PBS for 1 h. Non-specific epitopes were blocked using a solution of 1× PBS /0.4% Triton X-100 containing 0.1% glycine (wt/vol), 0.1% lysine (wt/vol), 1% BSA (wt/vol) and 1% normal donkey serum (wt/vol). The primary antibodies used were anti-ChAT (1:200) (catalog #AB144P, Merck Millipore), and anti-Choline Transporter (CHT1; 1:200), which was kindly donated by Dr. R. Jane Rylett, University of Western Ontario, London, Ontario. The primary

antibody was incubated in blocking buffer overnight at 4 °C. Sections were then washed five times in 1× PBS/0.4% Triton X-100 (10 min each). Hoechst (2–5 μg ml−1) was diluted in blocking buffer and slices were incubated for 1 h at RT. Sections were then washed five times in 1× PBS/0.4% Triton X-100 (10 min each). Sections were mounted on slides and visualized by Zeiss LSM 510Meta (Carl Zeiss, Oberkochen, Germany) confocal system (63x objective, 488-nm Ar laser and 633-nm HeNe laser were used for excitation of fluorophores).

Western Blotting

Immuno-blotting was performed as previously described [1-3]. Antibodies used were anti-VAChT (catalog #139103; Synaptic Systems), and anti-Synaptophysin (catalog #S5768; Sigma-Aldrich).

Electrophysiology

Animals were anesthetized with urethane (1 g/kg i.p.) and placed in a stereotaxic apparatus. Atropine methyl nitrate was administered (5 mg/kg i.p.) to reduce airway secretions during stereotaxic surgery. Animal body temperature was monitored between 36.5°C and 37°C using a feedback controlled rectal thermometer and heating pad. Stimulating electrodes were placed into stratum radiatum at P 1.8, L 2.3 or P 2.5, L2.4[4] to stimulate Schaeffer collaterals projecting from CA3 to CA1[5]. A silicon probe, with 16 electrodes separated by 50 μm on a vertical shank, was placed in area CA1 at P 2.2, L 1.8. Laminar profiles of the average (4 sweeps) field excitatory postsynaptic potentials evoked by single pulse stimulation of the Schaffer collaterals at 1.5-2 x threshold stimulus intensity. Current-source density analysis using 100 μm step size was used to determine current sources and sinks. The maximal slope (of 1 ms duration) during the rising phase of the excitatory sink, at its maximum in CA1 stratum radiatum, was used for LTP assessment. After a stable baseline of the excitatory sink slope was established for 30 minutes (coefficient of variation (SEM/mean) of the sink slopes <0.05), a high-frequency tetanus (100 Hz for 1s) was delivered at 2-3 x threshold intensity, and the response was measured for 120 minutes after the tetanus. For each mouse, the slope of the excitatory sink was normalized by the average value of the baseline, and LTP across mice was averaged and reported as a multiple of the baseline slope.

Morris Water Maze

The spatial version of the MWM was performed as previously described [2, 6, 7]. Testing was performed in a 1.5-meter-diameter pool with 25°C water. A hidden platform was submerged in a constant location 1 cm below the surface of the water in one of the four arbitrarily defined quadrants, and spatial cues were distributed around the pool. Briefly, mice were given four 90-second trials for the duration of 4 days to find the hidden platform, with an ITI of 15 minutes. The animals were introduced to the pool from different locations within the pool for each trial. Mice that did not find the platform within the 90 seconds were gently guided to the platform. On the fifth day, spatial memory recall was tested by a 60-second probe trial, where the hidden platform is removed and the amount of time the animal spends in the target quadrant is calculated. To test reversal learning, the hidden platform was relocated to the opposite quadrant, where the animals were given four 90-second trials for 4 days. On the fifth day, the animals were given a 60-second probe trial. Data was analyzed using ANY-Maze video tracking software (Stoelting Co.).

Two-Trial Morris Water Maze

A task used to assess working memory was the two-trial variation of the MWM. The task was carried out using previously described protocols [2, 6]. The mice were trained on the task over the course of 5 days. During the training period, the mouse was first given a 90s trial with a 15s inter-trial-interval. Next, the mouse was given a second trial with the same platform location and starting point, this was repeated three additional times. After completing the training phase, the mouse was first given a 90s trial with a 15s inter-trial-interval. The mouse was then given a second trial with identical platform location and starting point. This was repeated with 4 unique starting location/platform location combinations a day. Mean latency and distance savings ratios were then calculated as previously described [2]. Sessions were recorded for both tests and were analyzed using the ANY-Maze video tracking software (Stoelting Co.)

Spontaneous alterations Y-maze

In order to assess working memory in the mice, we used the spontaneous alternations Y-maze as previously described [2]. Briefly, mice were placed in a

symmetrical plastic Y-maze apparatus and both the number and order of arm entries were recorded. A spontaneous alternation was defined as when the mouse visited all three of the arms in a row, without having re-visited a previous arm of the maze. Sessions were recorded and analyzed using the ANY-Maze Software.

Training on the PAL task

Prior to training, both groups of mice (3 months old) were food restricted until they reached approximately 85% of their original weight. Training of the animals to the PAL task was previously described [8]. Briefly, the training phase for the mice in the touchscreen chambers involved a habituation session, where they were placed in the chambers with the lights off for 20 minutes to habituate to the environment for 2 days. Next, the mice were put in the chamber with the same parameters as in the habituation phase, but this time a 150µl reward (strawberry milkshake; Saputo Dairy Products, Canada) was introduced in the reward receptacle. Every time the mouse attended to the reward in the reward receptacle, a tone was played. This 40 minutes training session was done for the next 2 days until mice completed 36 trials in 60 minutes.

The mice were then trained to associate the reward with a 30 second presentation of training stimuli, which varied in brightness, shape, and pattern, on one of the 3 screens. Mice were required to touch any of the screens whenever the stimulus was presented in order to receive the reward, which was paired with a tone. A new trial was automatically initiated once the mice collected the reward. This was done until the mice completed 36 trials in 60 minutes for one day. The next training phase requires the mice to touch the stimulus on the screen in order to receive the reward. This training phase also requires them to initiate a new trial by poking the reward receptacle after completing the previous trial. This was done until mice completed 30 trials in 60 minutes for one day. Next, animals were put in the last phase of the pre-training program required for the PAL task. This training phase is similar to the previous one, but if the mouse touched the incorrect screen, it was presented with a 5-second time-out. This time-out was accompanied by the presentation of a bright light in the chamber. Criterion to successfully proceed from this training phase was 23 correct responses out of 30 trials in 60 minutes for 2 consecutive days.

Rotarod and neuromuscular tests

The rotarod task was conducted as previously described [9, 10]. Forelimb and hind limb grip strength was assessed using a previously described protocol [10]. The hang-wire experiment were performed as described [11].

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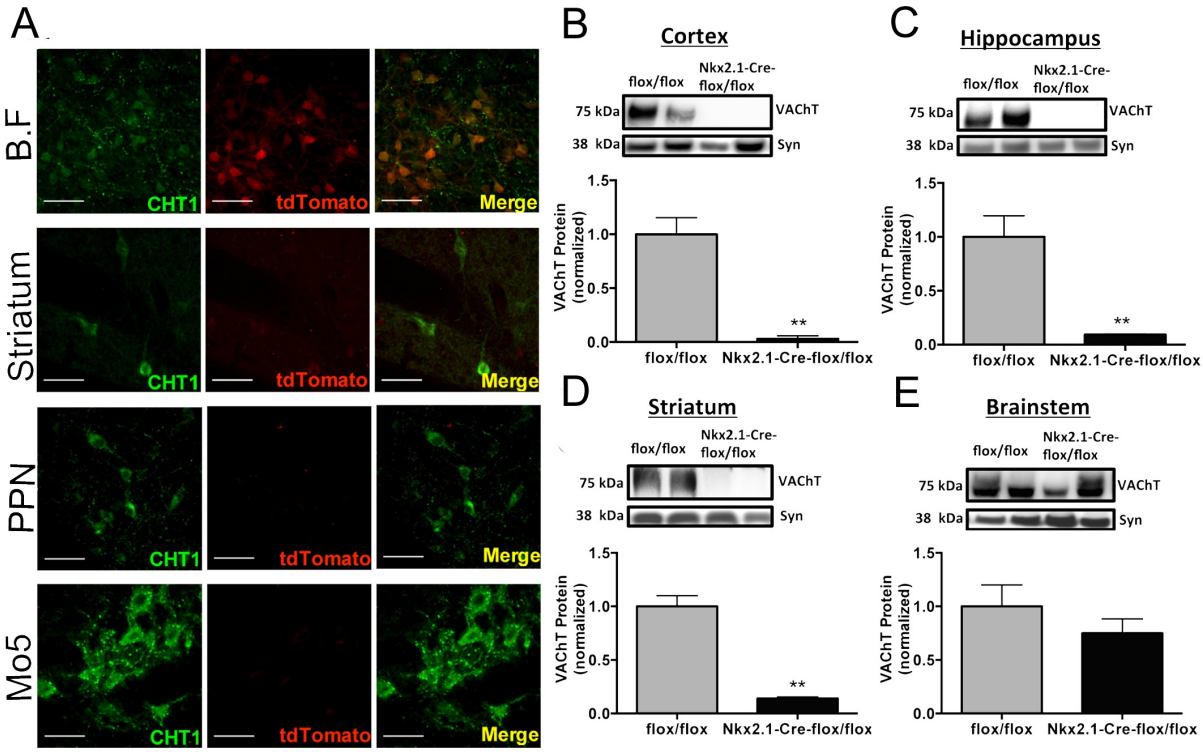
Supplemental (Figures and Table)

Table 1

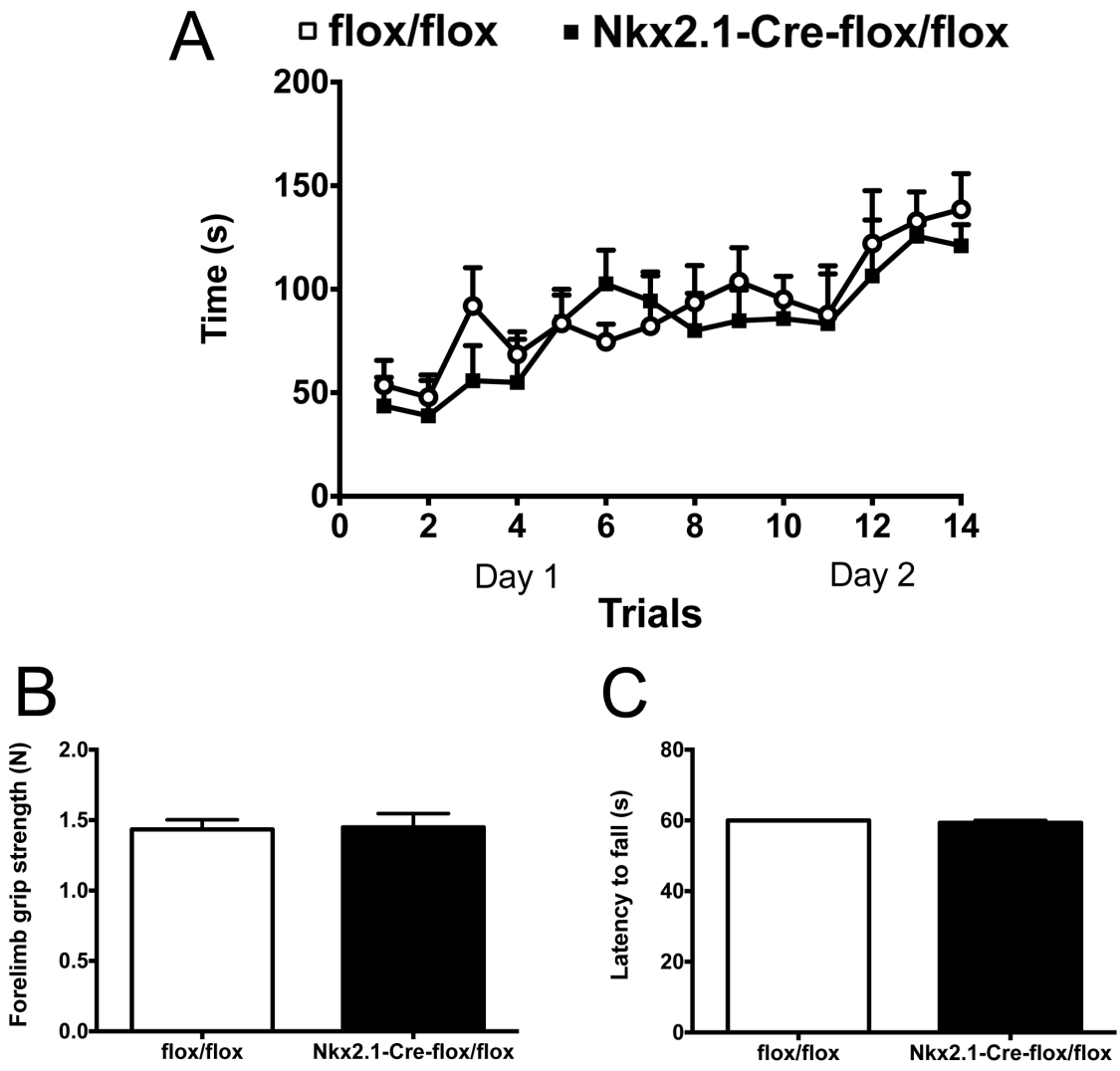
A

Brain region and mouse	Neurons immunostained for CHT1	Neurons immunostained for Td Tomato and CHT1	Colocalization %
Basal forebrain			
1	89	86	96
2	132	128	97
3	173	161	94
Striatum			
1	25	8	32
2	43	12	27
3	18	10	55
PPN			
1	44	0	0
2	86	0	0
3	27	0	0
Mo5			
1	88	0	0
2	94	0	0
3	112	0	~1

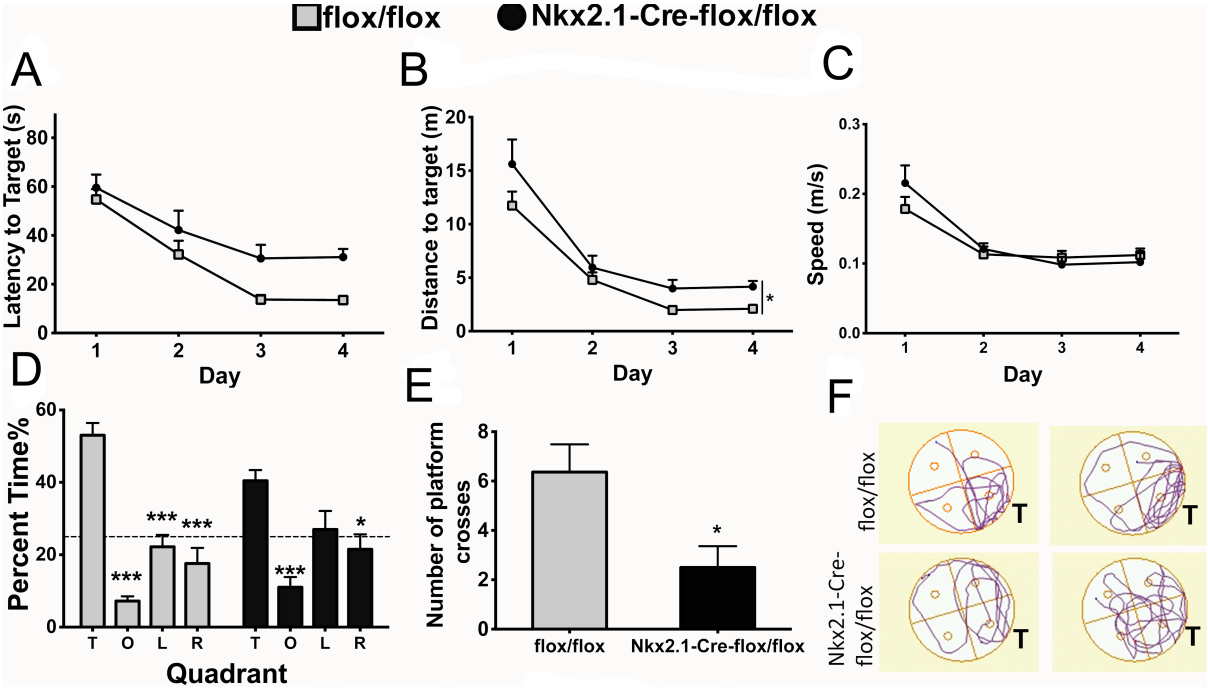
Supplemental figure 1



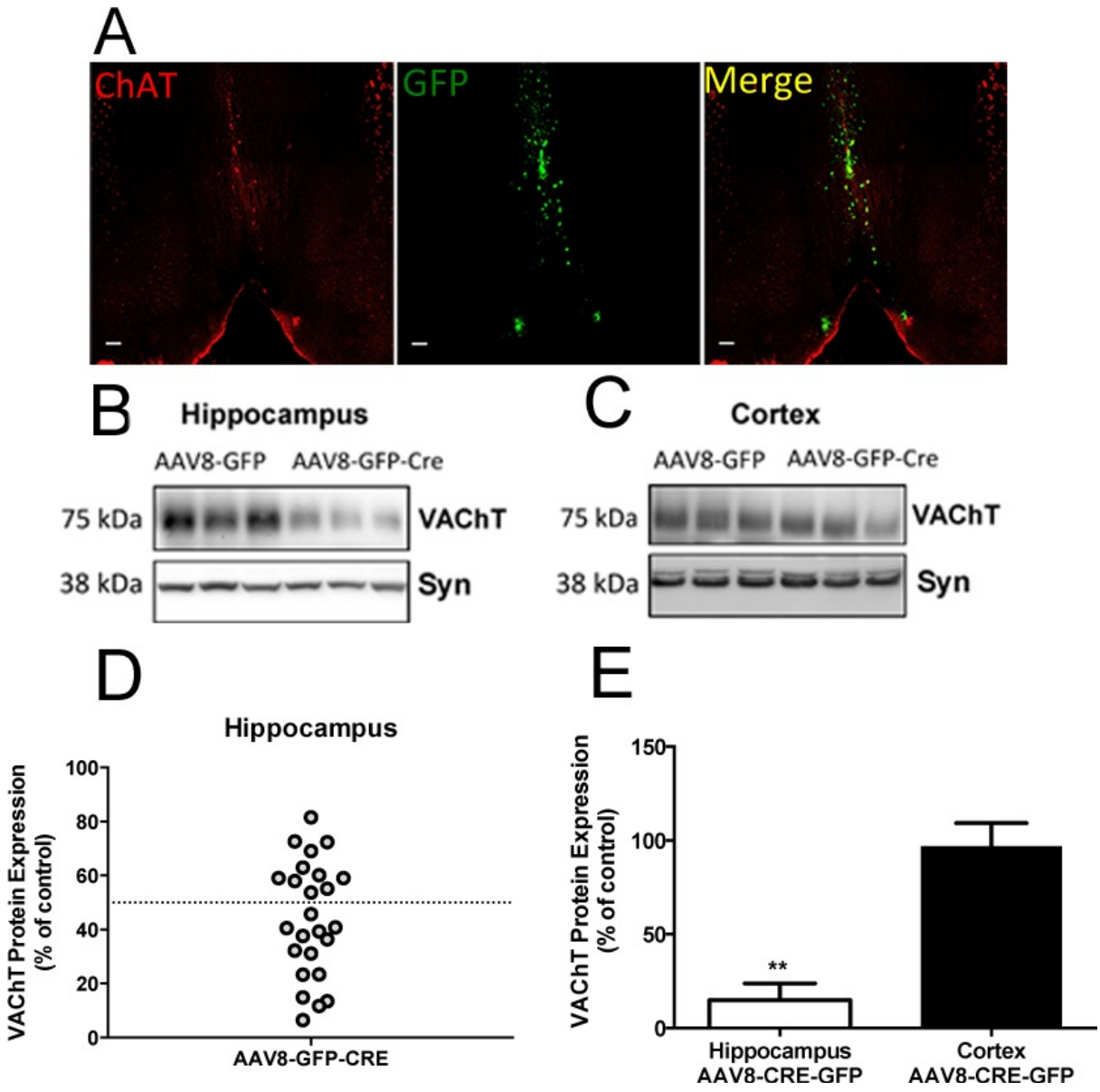
Supplemental figure 2



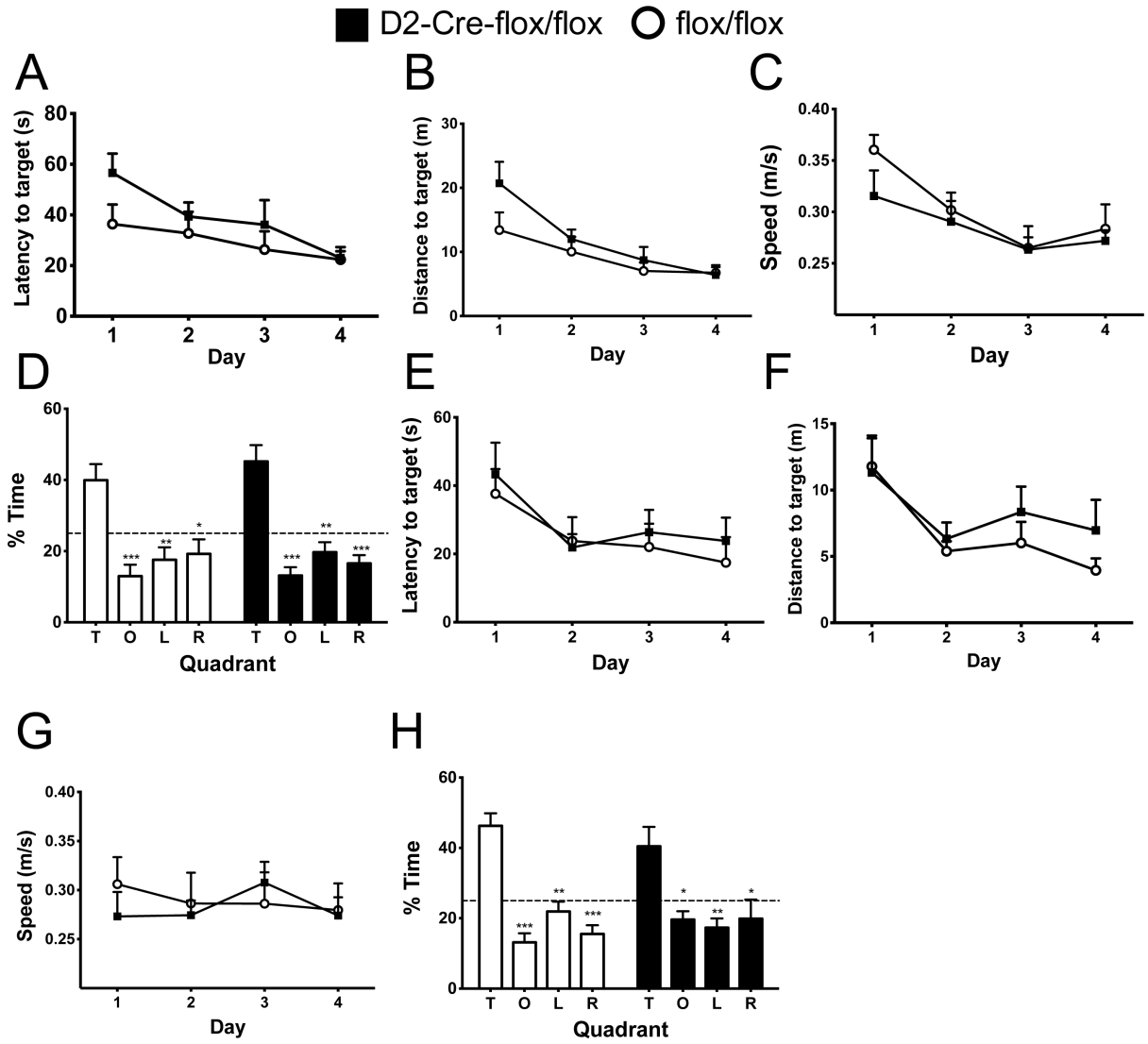
Supplemental figure 3



Supplemental figure 4



Supplemental figure 4



CAPÍTULO 3

RESEARCH ARTICLE

Regulation of locomotor activity by basal forebrain cholinergic transmission

(Este manuscrito será submetido à revista *Behavioral Neuroscience*)

Abbreviated title: Cholinergic regulation locomotor

Authors: Gustavo M. Parfitt[1,5,#], Mohammed A. Al-Onaizi[1,2#], , Benjamin Kolisnyk[1,4,], Daniela Martí Barros[5], Marco A. M. Prado[1,2,3,4*] , Vania F. Prado*[1,2,3,4]

1 Robarts Research Institute, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada, N6A5K8.

2 Department of Anatomy and Cell Biology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada, N6A5K8

3 Department of Physiology and Pharmacology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada, N6A5K8

4 Graduate Program in Neuroscience, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada, N6A5K8

5 Programa de Pós-graduação em Ciências Fisiológicas, Fisiologia Animal Comparada, Laboratório de Neurociências (FURG), Brazil.

 # Equal contribution

*To whom correspondence should be addressed: Dr. Marco A. M. Prado; mprado@robarts.ca; Vania F. Prado vprado@robarts.ca

ABSTRACT

The cholinergic basal forebrain is also involved in different aspects of cognitive function and behaviour control, including in the control of locomotion and attention. In this work, we studied the role the basal forebrain cholinergic tone in the control of locomotor activity. For this purpose, we used a VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mouse line which does not express the ACh vesicular transporter (VAChT) in the basal forebrain. To access the locomotor activity an open field coupled to an automated system was used in the locomotor analysis. In addition, the analysis of the pharmacological blocking of D2 receptors effect in the locomomor activity of the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ and the respective control VAChT $^{flox/flox}$ was also performed in the open field. To analyse the 24h locomotor activity and gross metabolism the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were submitted to metabolic chambers. Finally, the cholinergic septo-hipocampal projections control of locomotion were analysed using a seletive deletion of VAChT for the hippocampus, when an adeno-associated virus containing the transgene Cre-GFP or GFP only were in injected into the MS/DB. We observed that VAChT in the basal forebrain induces hyperactivity for a novel environment in mice. This hyperactivity can be overcome by the blocking of D2 dopaminergic receptors with the lowest dose of haloperidol. During the 24 h monitoring the locomotor activity the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ presented nocturnal hyperactivity and no gross alteration in metabolism. No alteration in the locomotor activity was found the in animals with selective deletion of VAChT in the hippocampus. In conclusion, the ACh release originated from the basal forebrain controls locomotor activity for novel environment and nocturnal activity.

Keywords: Vesicular Acetylcholine Trasporter (VAChT); Paired Associates Learning (PAL); Long-term Potentiation (LTP); Morris Water Maze (MWM); Schizophrenia, Alzheimer's Disease (AD)

1. INTRODUCTION

The hyperactive behaviour and inattention are symptoms part of the Attention-Deficit/Hyperactivity Disorder (ADHD), a disease in which the neuroanatomical basis is well characterized in humans and animals, with the prefrontal cortex playing a main role in this pathology (Davids, Zhang, Tarazi, & Baldessarini, 2003). Due to methodological impossibility, the neurochemical basis of this disease is less known in humans although strong efforts have been made to overcome the methodological limitations. Thus, animal models have been largely used to study the neurochemical basis of ADHD and, major neurotransmitter systems have been implicated in the cognitive and behavioural symptoms, as the dopaminergic and cholinergic (M E Avale et al., 2004; Gerber et al., 2001; Guzman et al., 2011; Martins-Silva et al., 2011; Martyn et al., 2012; Spielewoy et al., 2002)

The cholinergic basal forebrain contain a disperse population of cholinergic neurons present in the medial septum/diagonal band, nucleus basalis/substantia innominata and the striatal interneurons (Mesulam, Mufson, Wainer, & Levey, 1983). These aforementioned populations provide a tonic and phasic cholinergic tone to the cortex, hippocampus, amygdala and, partially to the striatum (Dautan et al., 2014; Mesulam et al., 1983). The cholinergic basal forebrain is also involved in different aspects of the cognitive function and behaviour control, including in the control of locomotion and attention (Kolisnyk, Al-Onaizi, et al., 2013; Martyn et al., 2012). Although, the role of the cholinergic system in control of locomotion have been well establish, the target region of the CNS and the cholinergic population involved in this control are still in debate.

The loss of cholinergic tone in the BF causes hyperlocomotor behaviour, whereas the loss of cholinergic tone in the cholinergic nuclei of the brain stem causes hypoactive behaviour (Patel, Rossignol, Rice, & Machold, 2012). Both muscarinic and nicotinic transmission has been implicated in the hyperlocomotor activity behaviour (Maria Elena Avale et al., 2008; Bourgeois et al., 2012; Gerber et al., 2001; Maubourguet, Lesne, Changeux, Maskos, & Faure, 2008; Miyakawa, Yamada, Duttaroy, & Wess, 2001). In addition, M1 and β2 subunit nicotinic receptors KO mice display hyperactive behaviour and, this hyperactivity is related to the loss of controls

of striatal dopaminergic transmission (Maria Elena Avale et al., 2008; Gerber et al., 2001). In addition, antipsychotics usually the class of atypical have been used in combination with psicostimulants to treat patients with ADHD in some specific conditions (Crystal, Olfson, Huang, Pincus, & Gerhard, 2009; Kronenberger et al., 2007).

In this work, we addressed the role the basal forebrain cholinergic tone in the control of locomotor activity in a novel environment and in a 24h period. To that purpose , we have used a mouse line with VAChT deletion selectively in the basal forebrain. We also challenged this hyperactive mouse line with the D2 receptor blocker haloperidol, to address a possible involvement of the dopaminergic system as part of the cause of this hyperactivity. Subsequently, we also analysed the activity for 24h in animals already habituated in the test environment. For last, we tested the participation of the septo-hippocampal cholinergic pathway as cause of the observed hyperactive in whole basal forebrain VAChT deleted mice.

2. MATERIALS AND METHODS

Animals

Animals were housed in groups of max four animals per cage without environmental enrichment in a temperature-controlled room (12:12 light to dark cycles), and food and water were provided for *ad libitum* consumption. All procedures were performed in accordance with the Canadian Council of Animal Care guidelines at the University of Western Ontario with an approved animal protocol (2008-127). The $VAChT^{flox/flox}$ line used in these experiments was previously described by (Martins-Silva et al., 2011). To generate the VAChT Knockouts (KO) directed to the basal forebrain the $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$, the $VAChT^{flox/flox}$ were crossed with a Nkx2.1-Cre mouse line (C57BL/6J-Tg(Nkx2-1-109 cre)2Sand/J), purchased from The Jackson Laboratory (JAX stock no. 008661). The $VAChT^{flox/flox}$ animals used as controls in the experiments were littermates.

Stereotaxic surgery procedure

The animals were anesthetised using a intraperitoneal injection of ketamine (100 mg/kg) and xylazine (25 mg/kg) in 0.9% sodium chloride followed by a subcutaneous injection of meloxicam (2 mg/kg). After fully anesthetised, the animals had their skin

prepared and underwent to stereotaxic surgery to inject an adeno-associed viral vector (AAV8) (AAV8, Vector BioLabs, Eagleville, PA, USA). The VAChT$^{flox/flox}$ were injected into the medial septum/diagonal band using a microinfusion pump attached to a Hamilton syringe, the system was connected to a polietilene tube coupled to a microinjection needle. During the procedure, the needle was slowly lowered to the MS/DB coordinates (0.98 AP, 0.1 LL and 4.1 DV) and, left at place for 2 min to stabilise, after the stabilization the viral vectors (AAV8-GFP or AAV8-CRE-GFP) were in injected at 0.2 µl per minute rate. After the injection, to avoid backflow, a 30 min period of rest was performed until the needle removal. To allow the viral expression and the animal to recovery a 4 weeks period was done until the start of the behavioural tests. In all the animals the VAChT protein levels analysed by western blotting in the hippocampus and, only animal with more them 50% of deletion were included in the final analysis.

Locomotor activity and haloperidol test

The system used to evaluate the locomotor activity was automated boxes (Accuscan Instruments, Inc., Columbus, OH, USA), all the procedures were performed with the lights on and between 10:00 and 16:00 h. The animals were previously habituated to the room for 20 min prior the test, for them to be placed in the boxes and allowed to explore the boxes, a novel environment for 60 min (De Castro et al., 2009). The activity was measured at 5 minutes interval and the total activity was accessed for the total of 60 min. For the haloperidol test, the animals were injected with the vehicle or haloperidol at 0.03, 0.1 or 0.3 mg/kg 30 min prior the task (Gerber et al., 2001). The same procedures above described, to evaluate locomotor activity, were performed for the haloperidol test.

Metabolic chambers to acess metabolic parameters and activity

The mice were housed individually in the metabolic chambers and monitored using Lab Animal Monitoring System (CLAMS) interfaced using Oxymax software (Columbus Instruments, Columbus, OH, USA). Before the measurements, the animals were habituated in the metabolic chambers for 16-h with water and food were provided *ad libitum* consumption and the temperature maintained at 24 ±1°C. The measurements of oxygen consumption, respiratory exchange ratio, food and

water intake, and light/dark locomotor activity were performed as described by (Guzman et al., 2013).

3. RESULTS

Disruption of cholinergic tone in the forebrain induces hyperactivity in a novel environment

To test possible effects of cholinergic signalling disruption on locomotor activity, we generate a mouse line in which the VAChT was conditionally deleted for the basal forebrain by intercrossing a VAChT Flox/flox animal with Nk-cre animal which drives the Cre expression to the basal forebrain (Al-Onaizi et al., 2015). The Deletion VAChT in cholinergic neurons causes the loss of the ability to release ACh by the terminals, resulting in lack of cholinergic tone (Guzman et al., 2011). Adult VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ animals were them submitted a locomotor test in a novel open field, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ displayed an increased locomotor activity in the first 20 minutes compared to the controls VAChT$^{flox/flox}$ mice [Two-way RM ANOVA shows a significant effect of time $F_{(11,\ 154)} = 15.07$ $p < 0.0001$; a significant effect of genotype $F_{(1,\ 14)} = 12.75$ $p = 0.0031$; and no interaction effect $F_{(11,\ 154)} = 1.293$ $p = 0.2332$, followed by Bonferroni's test $p < 0.05$]. In addition, both VAChT$^{flox/flox}$ and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ habituated in the locomotor task, as the distance had as significant decrease over time [Bonferroni's post-test $p < 0.05$]. When the total distance for the 60 min was analysed an increase in the total distance was observed in the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice [$t_{(14)}=3.389$, $p = 0.0044$].

Effect of haloperidol in the hyperactivity

To test the effects of the antipsychotic drug haloperidol on the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ hyperactive mice, we injected VAChT$^{flox/flox}$ and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ with haloperidol at different doses or the vehicle prior the test in the open field. In this experiment, we were able to reproduce our first observation of hyperactivity in the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice injected with saline since, an increase in the total distance compared the respective control was observed [Two-way ANOVA shows a significant effect of dose $F_{(3,\ 42)} = 15.58$ $p < 0.0001$; no effect of genotype $F_{(1,\ 14)} = 4.12$ $p = 0.0617$; and no interaction effect $F_{(3,\ 42)} = 2.755$ $p = 0.0543$, Bonferroni's post-test $p < 0.05$]. In addition, we observed attenuation in the hyperactivity of VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ injected with the lowest haloperidol dose, since they display a decrease total

locomotor compared to the vehicle injected VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice [Bonferroni's post-test $p < 0.01$]. Importantly, the control VAChT$^{flox/flox}$ animals did not display locomotor alterations when injected with lowest dose of haloperidol. The two higher doses of haloperidol, 0.1 and 0.3 mg/kg, also decrease the total distance in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice however, these higher doses also decreased the total distance in VAChT$^{flox/flox}$, both compared to the respective genotype injected mice [Bonferroni's post-test $p < 0.05$]. To evaluate anxiety behaviour, the time spent in the center of the open field was assessed, both genotypes spent similar amount of time in the center of the field [Two-way ANOVA shows no effect of dose $F_{(3, 42)} = 0.2076$ $p = 0.8906$; no effect of genotype $F_{(1, 14)} = 26.69$ $p = 0.6135$; and no interaction effect $F_{(3, 42)} = 0.3822$ $p = 0.7663$]. Concerning the analysis of explorative activity by rearing measurement, no alterations in the rearing levels were found between the genotypes however, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ show a decrease in the number of rearings after the treatment with the 0.3 mg/kg dose of haloperidol [Two-way RM ANOVA shows a significant effect of dose $F_{(3, 42)} = 4.272$ $p = 0.0101$; no effect of genotype $F_{(1, 14)} = 0.6191$ $p = 0.444$; and no interaction effect $F_{(3, 42)} = 0.4977$ $p = 0.6927$].

VAChT conditional KO mice display prominent nocturnal hyperactivity with no gross alteration in metabolism

To access the locomotor activity during a 24h period and gross parameters of metabolism the animals were subjected to metabolic boxes. The locomotor activity was accessed during a 24h hours period, in a habituated location, to comprehend the day and night time, during the day period no alterations were observed in the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice however, the nocturnal activity in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ was increased for the period after the lights off [Two-way RM ANOVA shows a significant effect of daytime $F_{(139, 1529)} = 4.972$ $p < 0.0001$; significant effect of genotype $F_{(1, 11)} = 5.914$ $p = 0.0333$; and significant interaction effect $F_{(139, 1529)} = 1.508$ $p = 0.0002$; followed by Bonferroni's test $p < 0.05$]. When the total activity was analysed during lights ON and lights OFF both groups had a similar locomotor activity during the day. However, in contrast to VAChT$^{flox/flox}$ mice, with the lights off VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ animals had prominent increase in locomotor activity compared to controls [Two-way RM ANOVA shows a significant effect of daytime $F_{(1, 11)} = 78.09$ $p < 0.0001$; no significant effect of genotype $F_{(1, 11)} = 4.789$ $p = 0.0511$; and significant interaction effect $F_{(1, 11)} = 5.413$ $p = 0.0401$; followed by Bonferroni's test p

< 0.05]. In the metabolic parameters analysed, the comparison of both groups did not demonstrated alterations in energy expenditure [Two-way RM ANOVA showed a significant effect of daytime $F_{(139,\ 1529)}$ = 6.490 p < 0.0001; no significant effect of genotype $F_{(1,\ 11)}$ = 0.2990 p = 0.5954; and no interaction effect $F_{(139,\ 1529)}$ = 1.125 p = 0.1615; followed by Bonferroni's test p < 0.05] or in the respiratory exchange ratio (RER) [Two-way RM ANOVA shows a significant effect of daytime $F_{(139,\ 1529)}$ = 7.213 p < 0.0001; no significant effect of genotype $F_{(1,\ 11)}$ = 0.0124 p = *0.9132*; and significant interaction effect $F_{(139,\ 1529)}$ = 2.351 p < 0.0001; followed by Bonferroni's test p < 0.05]. In addition, when the total RER was analysed for the lights ON and OFF both groups displayed similar rate [Two-way RM ANOVA shows a significant effect of daytime $F_{(1,\ 11)}$ = 5.833 p = 0.0343; no significant effect of genotype $F_{(1,\ 11)}$ = 0.02 p = 0.8887; and no interaction effect $F_{(1,\ 11)}$ = 2.698 p = *0.1287*; followed by Bonferroni's test p < 0.05]. Concerning the consuming of food and water, the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ consumed more water during the lights OFF period in comparison to the control animals [Two-way RM ANOVA shows a significant effect of daytime $F_{(144,\ 864)}$ = 647.2 p < 0.0001; no significant effect of genotype $F_{(1,\ 6)}$ = 5.076 p = 0.0652; and significant interaction effect $F_{(144,\ 864)}$ = 15.27 p < 0.0001; followed by Bonferroni's test p < 0.05].

The disruption hippocampal cholinergic tone does not affect locomotor activity for a novel environment

The VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ hyperactive mice have a decrease of cholinergic tone in cortex and hippocampus, with same levels of deletion in the striatum (Al-Onaizi et al., 2015). To test possible contribution specifically of the cholinergic septo-hippocampal pathway as the cause of the hyperactivity observed in the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$. We injected an AAV-CRE virus into the MS/DB of VAChT$^{flox/flox}$ to allow the deletion VAChT only in the MS/DB, as controls VAChT$^{flox/flox}$ mice were injected with AAV-GFP. After 4 weeks, the animals were tested in novel open field to evaluate the locomotor activity, both groups displayed similar amount of activity during the test [Two-way RM ANOVA showed no effect of the treatment $F_{(11,\ 27)}$ = 1.162 p = 0.2906; significant effect of time $F_{(11,\ 297)}$ = 11.27 p < 0.0001; and no interaction effect $F_{(11,\ 297)}$ = 1.145 p = 0.3259; followed by Bonferroni's test p < 0.05]. In addition, both groups habituate over time as they show decrease in activity compared to the first 5 min of locomotor acquisition [Bonferroni's post-test p < 0.05]. When the total 60 min

locomotor activity was analysed both groups had a similar total activity [$t_{(26)}$=1.058, p = 0.2996]. For these analyses only animals with less of 50% of VAchT expression in the hippocampus were included.

4. DISCUSSION

Chronic disruption in cholinergic tone leads to impairments of different cognitive domains such as, working memory, attention and spatial memory (Chudasama et al., 2004; Kolisnyk, Al-Onaizi, et al., 2013; Martyn et al., 2012). We observed that the chronic disruption of the cholinergic release to the hippocampus, cortex and striatum induces hyperactivity for a novel environment in mice. Further on, the disruption of cholinergic tone for these regions also induces nocturnal hyperactivity, in a non-novel environment. Interestingly, no alteration in activity were observed when the septo-hipocampal cholinergic signalling were disrupted, suggesting a possible involvement of the cortical cholinergic transition in the control of activity. Supporting this suggestion, the absence of hyperactivity in adult mice with selective deletions of VAChT, in the striatum, endorses the argument of a cholinergic cortical control of activity (Guzman et al., 2011, 2013). However, the cholinergic release from striatal interneurons seems to exert a control over activity in young mice (Guzman et al., 2013).

Our group previously reported that deletion of VAchT in the basal forebrain, including a robust deletion of in the striatum, also have hyperactive behaviour (Martins-Silva et al., 2011; Martyn et al., 2012). Similarly, acute systemic block of muscarinic receptors increase the locomotor activity in mice (Shannon & Peters, 1990; Sipos, Burchnell, & Galbicka, 1999). In accordance with the fact that disruption of cholinergic tone increase locomotion, acute or chronic manipulations to increase the levels of Ach in the CNS do not increase the spontaneous locomotion, but also do not decrease the locomotion (Farar et al., 2012; Kolisnyk, Guzman, et al., 2013; Shannon & Peters, 1990).

The attenuation $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ hyperactive behaviour in novel environments by the D2 receptor blocker haloperidol, also suggests alterations in the dopaminergic system as a possible component of the hyperactivity in these mice. The dopaminergic system has been implicated as component of hyperactivity in humans (Dougherty et al., 1999; Li, Sham, Owen, & He, 2006) and in genetic animal models

of hyperactivity (Accili et al., 1996; M E Avale et al., 2004). Loss of dopamine regulation by genetic deletion of DAT causes hyperactivity in mice (Spielewoy et al., 2002). In addition, M1 receptor KO mice are also hyperactive and lack regulation of dopamine transmission causing an increase of dopamine levels in the striatum (Gerber et al., 2001; Miyakawa et al., 2001). Mice lacking the β subunit of the nicotinic receptor also display hyperactive behaviour and lack of regulation of striatal dopamine (Maria Elena Avale et al., 2008). Altogether, point to an important cholinergic control of dopamine system with implications in locomotor activity. In addition, no anxiety related behaviours were observed in the open field and no effect of haloperidol was also found.

The increase of the locomotor activity during the night period in the $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ matches with the observation that there is general increase of ACh levels in the cortex during transition to the night period (Day, Damsma, & Fibiger, 1991; M E Jiménez-Capdeville & Dykes, 1993; M. E. Jiménez-Capdeville & Dykes, 1996). Nocturnal hyperactivity is also observed in β2 nicotinic KOs (Maria Elena Avale et al., 2008). The absence of hyperactivity during the day in the test on the metabolic chambers can possible be explained by the fact that these animal were already habituated to the box and did not have to explore a new ambient. When the parameters of metabolism as energy expendure, respiratory exchange ratio, food and water intake were evaluated no gross alterations were found indication no prominent role of basal forebrain ACh in the metabolism under regular circumstances.

As previously demonstrated by our group, animals with VAChT deletion in the striatum, and spare cholinergic transmission in the hippocampus, do not display hiperlocomotion in a novel environment whereas, the combined deletion of VAChT, in the cortex, hippocampus and striatum, induce to hyperactive behaviour (Guzman et al., 2011). Alterations in hippocampal function were reported in schizophrenia (Lodge & Grace, 2007) and Attention-Deficit/Hyperactivity Disorder (Ernst et al., 2003). In addition, both ventral and dorsal hippocampus seems to play a role in activity regulation (Bannerman et al., 1999; Sams-Dodd, Lipska, & Weinberger, 1997). However, the selective disruption of septo-hippocampal cholinergic tone did not disturb the pattern of locomotor activity to novel environments. This can point towards a more significant participation of cortical cholinergic cortical projections in

the locomotor activity control. We cannot also discard the possibility that the level of deletions, more them 50%, were not sufficient to induce the expected alterations in AAV8-CRE-GFP. In fact, the genetic manipulation to increase cholinergic tone in the hippocampus shows no alteration in locomotor behaviour but, the stimulation medial can normalize locomotion in the hiperlocomotion induced by ketamine (Ma & Leung, 2014; Mineur et al., 2013).

Taking together that fact that lack of cholinergic tone, prevenient from the basal forebrain, lead to a loss locomotor activity control during an exploratory behaviour and these mice are hyperactive during the night time, but not during the day. It points to a specific role of cholinergic tone when the general activity is increased. The increase of acetylcholine observed during arousal and/or high motivational periods, such as novel exploration, may play a role in the control of neuronal hyperactivation, maintaining the neuronal function under control (Day et al., 1991; Giovannini, Bartolini, Kopf, & Pepeu, 1998; M E Jiménez-Capdeville & Dykes, 1993; M. E. Jiménez-Capdeville & Dykes, 1996). Opposing to effects from cholinergic tone derived from the rostral brainstem, which have role in increase locomotion and/or maintain proper activity (Patel et al., 2012). It has been demonstrated that cholinergic tone regulate the amount sensorial information processed in the hippocampus and cortex by controlling Gabaergic transmission, either increasing or decreasing sensory processing which may also have implication for activity control (Letzkus et al., 2011; Lovett-Barron et al., 2014).

In conclusion, the ACh release originated from the basal forebrain controls locomotor activity for novel environment and nocturnal activity. The septo-hippocampal pathway does not seem to exert prominent control over exploratory locomotion, as observed in the AAV8-CRE-GFP mice. Taking together with precisely data from the literature showing no prominent role of cholinergic striatal interneurons, more studies in the cortical cholinergic transmission or developmental effects of cholinergic signalling are needed to clarify the mechanism of cholinergic controls o locomotion.

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6. FIGURE CAPTIONS

Figure 1. Locomotor activity for the control **VAChT$^{flox/flox}$** and **VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$** line in the open field . (A) Distance (cm) per 5 min in the open field. (B) Total activity (cm) for 60 min in the open field. (C-E) shows the total distance, center time (s) and rearing behaviour for **VAChT$^{flox/flox}$** or **VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$** treated with haloperidol (0.03, 0.1 or 0.3 mg/kg) or vehicle prior the open field test. (Data are expressed in mean ± SEM, *P<0.05, **P<0.01, vs the control genotype).

Figure 2. **VAChT$^{flox/flox}$** and **VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$** in Locomotor and metabolic parameters for a 24 h period. (A-B) Locomotor activity during light and dark period for 24 h . (C) Energy expenditure. (D) Respiratory exchange ratio (RER). (E) Total Respiratory exchange ratio (RER). (F) Water consumption and (G) Food consumption. . (Data are expressed in mean ± SEM, **P<0.01, vs the control genotype ; *P<0.05 same genotype ON vs. OFF period).

Figure 3. Locomotor activity in **VAChT$^{flox/flox}$** mice injected with AAV8-GFP or AAV8-CRE-GFP in an open field. (A) (A) Distance (cm) per 5 min in the open field. (B) Total activity (cm) for 60 min in the open field. (Data are expressed in mean ± SEM).

7. FIGURES

Figure 1

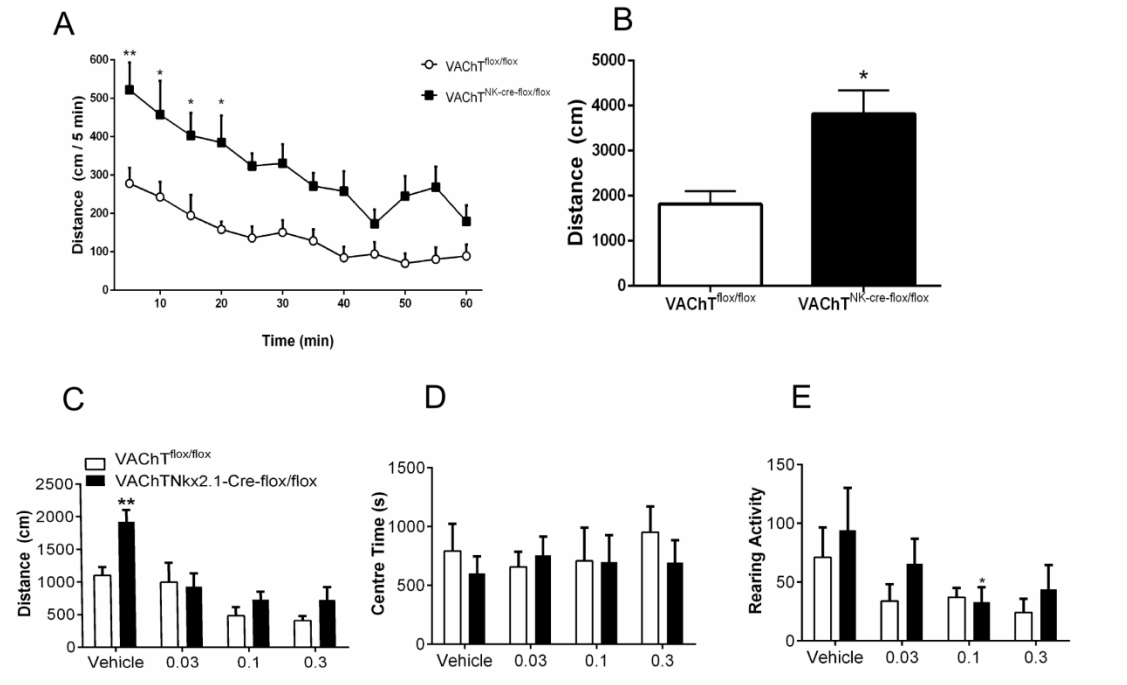
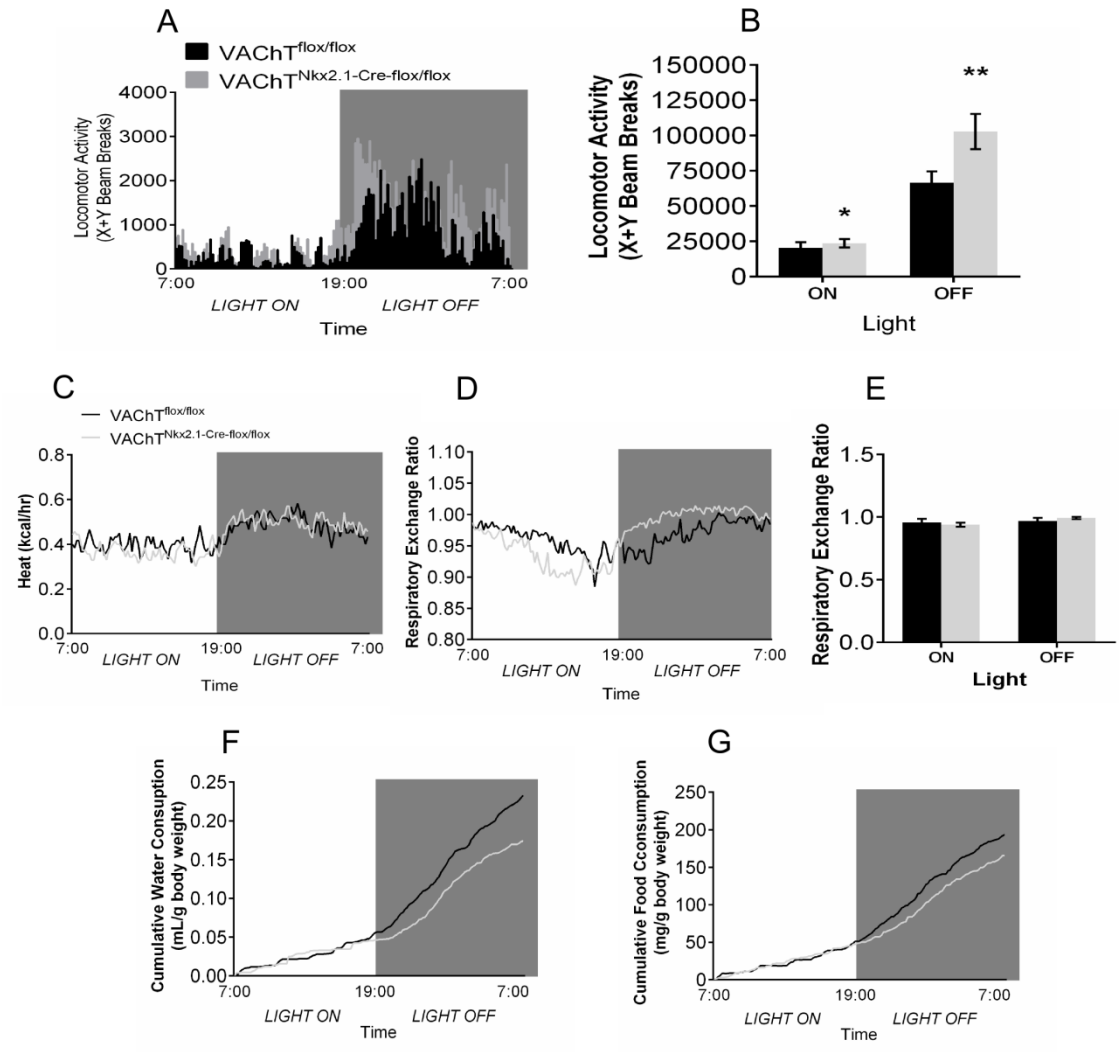
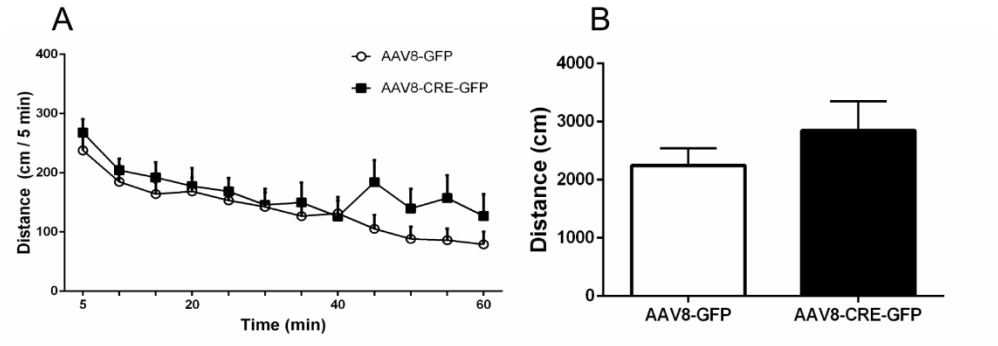


Figure 2


A
VAChT flox/flox
VAChT Nkx2.1-Cre-flox/flox
Locomotor Activity (X+Y Beam Breaks)
4000
3000
2000
1000
0
7:00
19:00
7:00
LIGHT ON
LIGHT OFF
Time
B
Locomotor Activity (X+Y Beam Breaks)
150000
125000
100000
75000
50000
25000
0
*
**
ON
OFF
Light
C
VAChT flox/flox
VAChT Nkx2.1-Cre-flox/flox
Heat (kcal/hr)
0.8
0.6
0.4
0.2
0.0
7:00
19:00
7:00
LIGHT ON
LIGHT OFF
Time
D
Respiratory Exchange Ratio
1.10
1.05
1.00
0.95
0.90
0.85
0.80
7:00
19:00
7:00
LIGHT ON
LIGHT OFF
Time
E
Respiratory Exchange Ratio
1.5
1.0
0.5
0.0
ON
OFF
Light
F
Cumulative Water Consuption (mL/g body weight)
0.25
0.20
0.15
0.10
0.05
0.00
7:00
19:00
7:00
LIGHT ON
LIGHT OFF
Time
G
Cumulative Food Cconsumption (mg/g body weight)
250
200
150
100
50
0
7:00
19:00
7:00
LIGHT ON
LIGHT OFF
Time

1

Figure 3


A
AAV8-GFP
AAV8-CRE-GFP
Distance (cm / 5 min)
400
300
200
100
0
5
20
40
60
Time (min)
B
Distance (cm)
4000
3000
2000
1000
0
AAV8-GFP
AAV8-CRE-GFP

3) CONSIDERAÇÕES FINAIS

Os principais achados desta tese são as demonstrações experimentais de que a transmissão colinérgica tônica no prosencéfalo basal (PB) participa do controle da locomoção, do aprendizado espacial na tarefa de PAL, do aprendizado reverso no tarefa de LAM além da modulação de memória de trabalho em duas diferentes tarefas.

Similarmente ao encontrado em diversos trabalhos (Berger-Sweeney et al., 1994; Lecourtier et al., 2011; Martyn et al., 2012; Murray & Fibiger, 1985) a transmissão colinérgica no PB não tem participação na aquisição da tarefa de LAM, entretanto na tarefa reversa em que os animais precisam reaprender a tarefa com a plataforma em uma nova posição, o aprendizado nestes animais encontra-se prejudicado quando a liberação de ACh é abolida no PB. Isso pode ser confirmado através da deleção da proteína VAChT na via colinérgica septo-hipocampal. Esta deleção reproduziu os achados em animais com a disrupção total da transmissão colinérgica no PB, reafirmando o papel colinérgico no aprendizado reverso. Ainda, quando seletivamente deletada no estriado proteína VAChT não causa nenhuma alteração no aprendizado normal e reverso no LAM. Isso aponta para uma participação via septo-hipocampal e/ou cortical colinérgica no processo de aprendizagem reversa.

A tarefa de PAL tem uma alto capacidade translacional e vêm sendo utilizada como adjuvante no diagnóstico de demências e da doença de Alzheimer (Nithianantharajah et al., 2013). Os animais com disrupção na transmissão colinérgica tônica, ocasionada pela deleção da proteína VAChT no PB, não conseguem adquirir a tarefa de parear a imagem correta a uma posição espacial, tendo um número de acertos raramente acima do acaso na tarefa de PAL. Isso demonstra que mesmo aprendendo a tarefa de LAM, que testa a memória espacial, estes animas apresentam déficits em tarefas em que a demanda cognitiva é superior. Similarmente, animais com a transmissão colinérgica via septo-hipocampal abolida apresentam uma correlação positiva entre os níveis de VAChT e o desempenho na tarefa.

A transmissão colinérgica no PB também participa da memória de trabalho como demonstrado pelo baixo desempenho dos animais VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ na tarefa de alternâncias espontâneas. Foi observado que roedores nesta tarefa apresentam a tendência em alternar acima de 75% das vezes em um labirinto em Y, o qual representa um ambiente novo (Dudchenko, 2004). Nos animais VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ essa frequência diminuiu significativamente, o que pode refletir em um provável prejuízo de memória de trabalho, fato similar foi encontrado na linhagem tratada com o vetor viral contendo a Cre. Para confirmar este déficit foi empregado o teste de memória de trabalho no LAM, tanto nos aninais VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ e quanto nos animais AAV8 e seus respectivos controles, e foi novamente observado prejuízos na memória de trabalho em ambos os genótipos. Essas observações demonstram que a transmissão colinérgica no hipocampo é importante para realização de tarefas em que memória de trabalho é requerida.

Finalmente, como abordado no capítulo final desta tese, foi demostrado que PB colinérgico tem uma importância crucial para o controle da atividade locomotora em ambientes novos e durante o período noturno, o período de maior atividade nos roedores. A disrupção da transmissão colinérgica no PB causa uma proeminente hiperatividade com uma possível participação do sistema dopaminérgico, desde que o bloqueio dos receptores D2 foi capaz de abolir essa hiperatividade. Além disso, a via septo-hipocampal colinérgica parece não estar envolvida no controle da atividade locomotora, pois não foram encontradas alterações nos animais em que a proteína VAChT foi seletivamente deletada no hipocampo. Esses resultados, juntamente com evidências de que a transmissão colinérgica no estriado não participa do controle da locomoção (Guzman et al., 2011), aponta para uma participação das vias colinérgicas corticais no controle da locomoção.

Futuramente, o estudo da via do NBM-córtex poderá esclarecer alguns pontos deixados em aberto nesse estudo, como por exemplo, a distinção da participação das vias colinérgicas corticais das hipocampais nas tarefas de LAM e PAL, além da memória de trabalho. Ainda como perspectivas, a utilização de modelos em que uma maior precisão na manipulação das vias colinérgicas é possível, como a utilização de técnicas de optogenética e DREADDs, que são receptores modificados ativados unicamente por drogas desenhadas, possibilitam um melhor entendimento da transmissão colinérgica no comportamento.

4) LICENÇAS E PERMISSÕES

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